

Effect of One-Lung Ventilation in Developing Postoperative Respiratory Failure after Lung Resection Surgery

By

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Abstract

Introduction: Lung surgery is a curative treatment for early-stage lung cancer.¹ This surgery is facilitated through One-Lung Ventilation (OLV), a procedure in which the operated lung is collapsed, and all mechanical ventilation is delivered to the contralateral lung.¹ Inflammation caused by OLV surgery, along with preoperative comorbidities and the extent of lung tissue resection, can contribute to postoperative respiratory failure. **This thesis aims to identify protein biomarkers that change during OLV and can identify patients at risk of developing postoperative respiratory failure.**

Method: A case-control study was conducted in patients nested within a larger prospective cohort study that recruited 556 eligible consecutive patients undergoing lung surgery with OLV.¹ A total of 41 patients were identified, with 14 patients who developed postoperative respiratory failure (cases) and 27 matched controls.¹ Cases and controls were matched in a 1:1-3 fashion based on clinical variables that present risk for respiratory failure. Bronchoalveolar lavage fluid (BALF) was collected from operated and ventilated lungs along with arterial plasma samples pre- and post-OLV.¹ Label-free untargeted proteomic screening was performed using data-independent acquisition LC-MS/MS.¹ The variance partition package was used for univariate statistical analysis; mixomics, clogitL1, and penalizedclr were used for multivariate analysis; and clusterProfiler was used for gene set enrichment analysis (GSEA) in R (significant results: $FDR \leq 0.05$, fold change ≥ 2 for BALF and ≥ 1.5 for plasma, respectively).

Results: Autotaxin (ENPP2) and proteasome activator complex subunit 2 (PSME2) proteins were notably upregulated in postoperative respiratory failure cases post- vs. pre-OLV BALF.¹ The upregulation of these proteins indicates processes involved in phospholipid metabolism and immunoproteasome.¹ Multivariate analysis revealed Annexin A1 (ANXA1) expression in matched controls, which is associated with a protective effect against acute lung injury. In plasma, Phosphatidylethanolamine-binding protein 1 (PEBP1) was upregulated in cases, suggesting systemic phospholipid metabolism.¹ Neutrophil extracellular trap formation GSEA KEGG pathways showed higher enrichment in cases than in matched controls.

Conclusion: In this nested case-control study, proteins that differ in respiratory failure cases and matched controls post- vs. pre-OLV were identified. Through further validation studies, detected proteins have the potential to identify patients at high risk for postoperative respiratory failure.¹

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Dedication

I want to dedicate this thesis to my Mom and Dad for their unwavering support throughout my entire educational journey. For the sacrifices they made to give me a better life, education and opportunities. Their hard work and perseverance are my inspiration; I cannot imagine better role models, and I am deeply honoured and proud to call them my parents. To my uncle and auntie, who supported me and believed in me every step of the way.

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List of Abbreviations

ALI – Acute Lung Injury	FDR – False Discovery Rate
ARDS - Acute Respiratory Distress Syndrome	%FEV1 – Percent Predicted Forced Expiratory Volume at 1 second
ATP – Adenosine Triphosphate	FiO2 – Fraction of Inspired Oxygen
BAL – Bronchoalveolar Lavage	FRC – Functional Residual Capacity
BALF – Bronchoalveolar Lavage Fluid	FVC – Forced Vital Capacity
BH – Benjamini-Hochberg	%FVC – Percent Predicted Forced Vital Capacity
BMI – Body Mass Index	GO – Gene Ontology
BP – Biological Process	GSEA – Gene Set Enrichment Analysis
Cat No. – Catalog Number	H ₂ O ₂ – Hydrogen Peroxide
CC – Cellular Component	HPV – Hypoxic Pulmonary Vasoconstriction
clogitL1 – Conditional Logistic Regression L1	IAA – Iodoacetamide
CO – Carbon Monoxide	ILD – Interstitial Lung Disease
CO ₂ – Carbon Dioxide	IPF – Idiopathic Pulmonary Fibrosis
COPD – Chronic Obstructive Pulmonary Disease	IV – Intravenous
COVID-19 – Coronavirus Disease of 2019	KEGG – Kyoto Encyclopedia of Genes and Genomes
CTC- Circulating Tumour Cells	LC-MS/MS – Liquid Chromatography-Tandem Mass Spectrometry
DAMP – Damage Associated Molecular Patterns	Log ₂ – Logarithm to the base 2
DDA – Data-Dependent Acquisition	LPS – Lipopolysaccharide
DIA – Data-Independent Acquisition	LUL – Left Upper Lobe
Dia-PASEF – Data-Independent Acquisition-Parallel Accumulation–Serial Fragmentation	MF – Molecular Function
DLCO – Diffusion Lung Capacity for Carbon Monoxide	MS – Mass Spectrometry
DNA – Deoxyribonucleic Acid	NET – Neutrophil Extracellular Trap
DTT – Dithiothreitol	O ₂ – Oxygen
EIT – Electrical impedance tomography	OLV – One-Lung Ventilation
	PAMP – Pathogen Associated Molecular Patterns

PaO₂ – Partial Pressure of Arterial Oxygen

PCA – Principal Component Analysis

PCV – Pressure-Controlled Ventilation

PCV-VG – Pressure-Controlled Ventilation
with Volume Guaranteed

PEEP – Positive-End Expiratory Pressure

penalizedclr – Penalized Conditional
Logistic Regression

PLS-DA – Partial Least Squares
Discriminant Analysis

PPC – Postoperative Pulmonary
Complications

ppoDLCO – postoperative predicted
Diffusion Lung Capacity for Carbon
Monoxide

ppoFEV₁ – Postoperative Predicted Forced
Expiratory Volume at 1 second

Q – Perfusion

QC – Quality Control

RF (rf) – Respiratory Failure

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

sPLS-DA – Sparse Partial Least Squares
Discriminant Analysis

TEAB – Triethylammonium bicarbonate

TLV – Two Lung Ventilation

V – Ventilation

V/Q – Ventilation/Perfusion

VATS – Video-Assisted Thoracoscopic
Surgery

VCV – Volume-Controlled Ventilation

VILI – Ventilation-Induced Lung Injury

VSN – Variance Stabilizing Normalization

V_t – Tidal Volume

List of Protein Names

ANXA1 – Annexin A1	ELP3 – Elongator complex protein 3
APOA1 – Apolipoprotein A-1	ENPP2 – Ectonucleotide
APOA2 – Apolipoprotein A-2	Pyrophosphatase/Phosphodiesterase 2 or Autotaxin
APOA4 – Apolipoprotein A-4	F11 – Coagulation factor 11
APOC3 – Apolipoprotein C-3	F9 – Coagulation factor IX
APOE – Apolipoprotein E	FABP4 – Fatty acid binding protein 4
APOM – Apolipoprotein M	GPRC5B – G-protein coupled receptor family C group 5 member B
ASC – Apoptosis Associated Speck-Like Protein Containing a Caspase Recruitment Domain	GSTO1 – Glutathione S-transferase omega-1
BCLAF1 – B-cell lymphoma 2-associated transcription factor 1	H3-3A – Histone H3
bFGF – Basic Fibroblast Growth Factor	H4C1 – H4 clustered histone 1
C1QB – Complement C1q subcomponent subunit B	HBA1 – Hemoglobin subunit Alpha 1
CA3 – Carbonic anhydrase 3	HBB – Hemoglobin subunit Beta
CCL15 – C-C motif chemokine 15	HMGB1 – High-mobility group box 1
citH3 – Citrullinated H3	HSPA1A – Heat Shock 70 kDa Protein 1A
CPB1 – Carboxypeptidase B1	IFN- γ – Interferon-gamma
CPB2 – Carboxypeptidase B2	IGFBP1 – Insulin Growth factor binding protein 1
CPE – Carboxypeptidase E	IGKV2-24 – Immunoglobulin Kappa Variable 2-24
CPN2 – Carboxypeptidase N subunit 2	IL-17D – Interleukin-17D
CSTB – Cystatin-B	IL-18 – Interleukin-18
CXCL17 – C-X-C motif chemokine 17	IL-1R1 – Interleukin-1 Receptor Type 1
CYB5A – Cytochrome b5a	IL-1RA – Interleukin-1 Receptor Antagonist
DES – Desmin	IL-1 β – Interleukin-1 beta
DMBT1 – Deleted In Malignant Brain Tumors 1	IL-23 – Interleukin-23
ECHS1 – Enoyl-CoA Hydratase, Mitochondrial	IL-6 – Interleukin-6
	IL-8 – Interleukin-8

ILF3 – Interleukin enhancer-binding factor 3	PRB4 – Basic salivary proline-rich protein 4
ITIH1 – Inter-alpha-trypsin inhibitor heavy chain H1	PRR15 – Proline-rich protein 15
ITIH2 – Inter-alpha-trypsin inhibitor heavy chain H2	PSME2 – Proteasome activator complex subunit 2
ITIH3 – Inter-alpha-trypsin inhibitor heavy chain H3	PTPRZ1 – Protein Tyrosine Phosphatase Receptor Zeta 1
ITIH4 – Inter-alpha-trypsin inhibitor heavy chain H4	RAB5B – Ras-related protein Rab-5B
JAG1 – Protein jagged-1	RAGE – Receptor for Advanced Glycation End-Products
KRT18 – Keratin, type II cytoskeletal 18	SAA4 – Serum amyloid A-4
KRT19 – Keratin, type II cytoskeletal 19	SAP/APCS – Serum Amyloid P-component
KRT7 – Keratin, type II cytoskeletal 7	SCPEP1 – Serine carboxypeptidase 1
KRT75 – Keratin, type II cytoskeletal 75	SERPINA6 – Corticosteroid-binding globulin
LGALS1 – Galectin-1	SERPINF2 – Alpha-2-antiplasmin
LMNA – Prelamin-A/C	SFTPD – Pulmonary surfactant-associated protein D
MET – Hepatocyte growth factor receptor	SLC31A1 – High affinity copper uptake protein 1
MIA – Melanoma-derived growth regulatory protein	SLC5A8 – Sodium-Coupled Monocarboxylate Transporter 1
MT1G – Metallothionein-1G	SRGN – Serglycin
MyD88 – Myeloid Differentiation Primary Response 88	THBS1 – Thrombospondin 1
NF- κ B – Nuclear factor kappa-light-chain-enhancer of activated B cells	TLR – Toll-Like Receptor
NLRP3 – NOD-like receptor family pyrin domain-containing 3	TNF – Tumour Necrosis Factor receptor-1
P2X – Purinergic Receptor P2X	TNFr-1 – Tumour Necrosis Factor receptor-1
PEBP1 – Phosphatidylethanolamine binding protein 1	TNF- α – Tumor necrosis factor-alpha
PEBP4 – Phosphatidylethanolamine-binding protein 4	TPI1 – Triosephosphate Isomerase 1
PRB1 – Basic salivary proline-rich protein 1	TPM1 – Tropomyosin alpha-1 chain
PRB3 – Basic salivary proline-rich protein 3	TPM2 – Tropomyosin alpha-2 chain
	TRPV4 – Transient receptor potential vanilloid 4

TSLP – Thymic Stromal Lymphopoietin

VIM – Vimentin

UGDH – UDP-glucose 6-dehydrogenase

Chapter I: Introduction

Lung cancer is one of the most common cancer types with the highest cancer-related mortality in Canada and globally.²⁻⁴ Lung surgery is considered a curative treatment for lung cancer.^{1,5,6} This high incidence of lung cancer indicates the need for safer and less invasive treatments. A main reason for the demand for safer curative treatments is the substantial rate of complications that arise after lung surgery.^{5,7-9} The incidence rate for postoperative complications after lung surgery is highly variable, ranging from 9% to 53.4%.^{5,9} These complications not only contribute to mortality but also contribute to increased length of stay and healthcare-related costs.^{10,11}

Among the many possible postoperative complications, postoperative pulmonary complications (PPCs) tend to occur more commonly and confer worse survival in patients undergoing lung surgery compared to other surgery types.⁸ According to the Ottawa Thoracic Mortality and Morbidity index, PPCs include atelectasis, pneumonia, acute respiratory distress syndrome (ARDS), and ventilator support >48 hours.¹²

Among these complications, pneumonia has the highest incidence,¹³⁻¹⁶ while ARDS and postoperative respiratory failure lead to high mortality rates of up to 40 to 60%.^{17,18} Atelectasis or lung regional collapse represents a slightly lower incidence compared to pneumonia.¹⁶ PPCs after lung surgery are not limited to the complications mentioned above. Other sources indicate that pleural effusion, pneumothorax, bronchospasm, and pulmonary embolus are postoperative pulmonary and pleural complications that can arise after lung surgery.¹⁹⁻²¹ Moreover, the high severity of any of these complications can lead to increased mortality.

Understanding the factors contributing to PPCs requires examining how lung surgery is performed. One-Lung Ventilation (OLV) facilitates lung surgery by collapsing the operated lung and providing gaseous exchange through the contralateral lung using a mechanical ventilator. Even though OLV provides the necessary ventilation during lung surgery, it can contribute to the development of PPCs by causing injury to the single ventilated lung. Thus, preoperative lung comorbidities coupled with OLV-induced lung injury and the extent of tissue resection during surgery can contribute to the occurrence of PPCs.

To understand the mechanisms by which OLV can lead to one of the most severe PPCs, postoperative respiratory failure, we set out to screen proteins in bronchoalveolar lavage fluid (BALF) and plasma from respiratory failure patients pre- and post-OLV, and from matched control patients. Protein screening was performed using label-free mass spectrometry for both BALF and plasma samples. Proteome-wide screening enables the identification of novel biomarkers of lung injury and respiratory failure, and patients who may benefit from anti-inflammatory medications as soon as intraoperatively.

This thesis is organized in a manner to provide background of literature surrounding OLV-surgery, the rationale and methodology employed to achieve objectives, and the results of proteomic analysis of BALF from ventilated and operated lungs, and arterial plasma post-vs-pre-OLV supplemented by a discussion of findings, strengths and limitations of this study.

Chapter II: Background

Overview of Respiratory Physiology

Under natural conditions, the work of breathing is performed by respiratory muscles, which are under nervous system control. Ventilation is achieved by creating a negative pressure that allows the lungs to be filled with a volume of air. During negative-pressure breathing, inspiration is achieved by contraction of the diaphragm and the chest wall, allowing the lungs to expand. Although the elastic recoil of the lungs counteracts the air pressure going into the lungs, the increase in lung volume due to expansion decreases pressure, allowing the lungs to be filled with air. On the contrary, expiration is achieved by the relaxation of the diaphragm and the chest wall, allowing the lungs to retract. The elastic recoil of the lungs allows for expiration to occur passively as the diaphragm and chest wall relax. This recoil would decrease lung volume and increase alveolar pressure, allowing air to be released. Thus, alveolar pressure can be expressed relative to atmospheric pressure to illustrate the dynamics of normal airflow. This intrinsic elasticity of the lungs arises from elastin and collagen that make up the extracellular matrix of the lungs.

To allow gas exchange in the alveoli while avoiding alveolar collapse, alveolar type II cells secrete a fluid made from phospholipids called surfactant to reduce surface tension. This reduction in surface tension increases lung compliance; allowing easier alveolar expansion during inspiration. It also allows effective inflation of smaller alveoli relative to larger inflated alveoli while preventing alveolar collapse during exhalation. Surfactant dysfunction is implicated in acute respiratory distress syndrome (ARDS).²²

However, ventilation during lung surgery would require cessation of muscular activity to prevent respiratory effort while the patient is intubated. All respiratory effort would be provided by the mechanical ventilator. Thus, the ventilator uses a positive-pressure breathing mechanism to deliver pressurized air into the lungs during inspiration. Expiration is achieved by lowering the pressure in the lungs to Positive-End Expiratory Pressure (PEEP) levels, along with the elastic recoil of the lungs. In comparison to negative-pressure breathing, positive-pressure breathing during mechanical ventilation requires determining the volumes and pressures to be delivered to

the lungs. In the context of OLV, all ventilation is delivered to the “one lung” being ventilated; hence, these parameters need to be adjusted to account for ventilation of that lung.

Under normal conditions, the volume of air exchanged during resting breathing, known as tidal volume, in an adult is about 500 ml.²³ The volume of air remaining after tidal volume (Vt) in the lungs makes up the functional residual capacity (FRC). Total lung capacity involves is the total maximum volume of air in the lungs. This volume combines residual volume which is the volume of air remaining in the lungs that cannot be exchanged and vital capacity which is the volume of air that can forcibly be exchanged. Vital capacity involves the maximum amount of air that can be forcibly inspired (inspiratory reserve volume), forcibly expired which is the expiratory reserve volume and tidal volume. Thus, the FRC is what remains after tidal volume as it combines expiratory reserve volume and residual volume.

The combined volume in the alveoli is about 3000 ml.²³ However, not all ventilated air volume is used for gas exchange. The air volume not used for gas exchange is referred to as dead space.²⁴ The lung can be divided anatomically into conducting and respiratory zones. The conducting zone is not involved in gas exchange and hence represents the anatomical aspect of dead space.²⁴ On the other hand, the respiratory zone represents the regions of the lung involved in gas exchange. However, there remains dead space for which air that is ventilated is not exchanged in the respiratory zone. The combined effect of the dead space in both conducting and respiratory zones is considered physiological dead space.²⁴

Contraction and relaxation of the diaphragm and accessory respiratory muscles allow airflow into the lungs and generate a pressure differential within the lungs. This relative pressure differential, along with the partial pressure of O₂ and CO₂, would allow gaseous exchange between the atmosphere and the lungs.²³ The elastic recoil, which contributes to the lungs' tendency to collapse, counteracts the expansion of the lung space during inspiration and promotes lung contraction during expiration.²³ The chest wall exerts its own recoil in opposition to the elastic recoil of the lungs, balancing out to prevent the lung from collapsing at the end of expiration and maintaining FRC.²³

Lung compliance determines the extent to which the lung can expand, allowing the lung volume to increase with changes in pressure.²³ Lung resistance, on the other hand, opposes airflow through the airways in the lungs.²⁵ Lung compliance depends on the lung's structural

elements, which can be altered in disease contexts.²⁵ Lung stiffness becomes even more important during OLV, where some patients undergoing surgery may have either too compliant or too stiff lungs, making it difficult to ventilate. Increased lung compliance would allow disproportionate ventilation, with some high-compliance regions receiving higher tidal volumes, leading to regional overdistention.²⁶ Decreased lung compliance would make it difficult to ventilate the lung, as a greater pressure change is required to achieve the same level of ventilation. This difficulty in ventilating fibrotic lung spaces can worsen with time.²⁶ This is because fibrotic lungs require a greater pressure change to achieve inflation due to increased lung stiffness. This makes it easier for stress and strain injuries to develop in the lungs from both increased pressure and regional overdistention.²⁶

To achieve gas exchange from ventilation, O_2 and CO_2 need to cross the alveolar-capillary membrane through simple diffusion. However, the partial pressure of oxygen in the alveoli depends on the fraction of inspired oxygen (FiO_2), the air pressure provided (atmospheric pressure in natural ventilation), and the CO_2 levels produced by the body.²⁵ Even with high partial pressure of oxygen, the diffusion distance between alveoli and capillaries affects the amount of oxygen entering pulmonary circulation.²⁵ A higher diffusion distance lowers gas diffusion across the alveolar capillary membrane. This distance can increase further with age. Furthermore, the thin alveolar-capillary membrane can exhibit structural alterations due to increased fibrosis.

The concentration of oxygen in the atmosphere is 21%, which is the FiO_2 under natural breathing. Under mechanical ventilation conditions, FiO_2 and driving pressure are altered. FiO_2 is higher than 21% in most cases, being above 50%.²⁷ Increasing FiO_2 can increase alveolar oxygen partial pressure and compensate for diminished oxygenation during ventilation without requiring high tidal volumes or driving pressures. However, FiO_2 close to 100% used to compensate for hypoxemia can lead to increased risk for impairment in oxygenation after lung surgery.²⁷

To achieve effective gas exchange, minute ventilation must match the rate of cardiopulmonary blood flow.²⁵ Minute ventilation (V) must be matched to perfusion (Q), hence V/Q match.²⁵ Ventilation depends on the ease of getting air to the alveoli through the airways, which can be affected by pulmonary function, the ability of the alveoli to collapse and expand in

response to pressure gradients, compliance, and adequate surfactant function.²⁵ Perfusion can be affected by cardiac output and by blood flow through the pulmonary vessels.²⁵ If stroke volume and/or heart rate are diminished, cardiac output to the lungs can be lowered as a consequence.²⁵ However, pulmonary vessels can also adjust to changing cardiac output.²³

Pulmonary arteries alter vascular resistance due to smooth muscle contraction drastically when ventilation is reduced or halted, thereby redirecting blood flow to well-ventilated areas of the lung.²⁵ This mechanism, called hypoxic pulmonary vasoconstriction, ensures efficient blood flow to ventilated areas of the lung, thereby maximizing oxygenation.²⁵ Now, this physiological mechanism becomes more important in conditions where ventilation drops in some regions of the lungs. This drop in ventilation under spontaneous breathing conditions can be due to airway and alveolar inflammation and surfactant dysfunction, which can diminish ventilation. Thus, blood flow to these poorly ventilated alveoli can be redirected to regions that are ventilated. Pathologic resistance in the pulmonary arteries can cause difficulty in lung perfusion. One of these conditions is pulmonary hypertension, which is characterized by increased pulmonary vascular resistance.²³

The Role of Spirometry in Respiratory Pathophysiology

One of the most common reasons for lung surgery is lung resection to treat lung cancer in patients. In this patient cohort, the impact of smoking and other lung comorbidities, such as Chronic Obstructive Pulmonary Disease (COPD), Interstitial Lung Disease (ILD), and Idiopathic Pulmonary Fibrosis (IPF), can affect patients' risk for PPCs. Spirometry measurements (spirometry) are an important diagnostic tool for assessing a patient's lung function before surgery. Additionally, lung resection surgery can further reduce lung function, depending on the extent of resection. Thus, it is crucial to predict postoperative lung function to assess the impact of surgery on lung function.

From a spirogram, three important lung function measurements can be interpreted, amongst others. One of them is forced expiratory volume in 1 second (FEV1), which describes the amount of air forcefully exhaled in one second after a full inhalation.²⁵ The other is forced vital capacity (FVC), the extent of air expired forcibly after a full inspiration.²⁵ The last one being FEV1/FVC. These values describe a person's lung function and can be affected by age, height, biological sex, smoking history, and lung diseases.²⁵ By dividing a patient's FEV1 by the

average FEV1 of a normal population with the same age, height, biological sex, and ethnicity, a predicted %FEV1 can be calculated.²⁵ The same calculation can be performed to generate a predicted %FVC as well. An FEV1/FVC less than 70 indicates obstructive lung diseases with % predicted FEV1 < 70% would indicate moderate to very severe obstructive lung disease based on decrements from this value.²⁸ An FEV1/FVC greater than 0.70 and % predicted FVC less than 80% would indicate restrictive lung disease.²⁸

Another lung function marker is DLCO, which measures the diffusion capacity of carbon monoxide from the alveoli to the bloodstream.²⁹ Carbon monoxide (CO) is preferred over oxygen due to its higher affinity for hemoglobin.²⁹ This measurement allows evaluation of the patient's gas exchange capabilities across the alveolar-capillary membrane. Impairments in gas exchange can manifest as reduced DLCO, indicating a risk of pulmonary complications after lung surgery. % predicted DLCO value less than 75% would indicate a patient's reduced gas exchange capability and presence of disease.²⁹ However, lower DLCO regardless of cutoffs could indicate worse prognosis. Lower DLCO can be associated with, among other conditions, interstitial lung disease, emphysema, and pulmonary fibrosis.²⁹ Additionally, it can also flag conditions that affect blood flow and the amount of free hemoglobin available, such as pulmonary hypertension and other "pulmonary vascular diseases, anemia, hepatopulmonary syndrome, and an increased carboxyhemoglobin (COHb)." ²⁹ for which otherwise would not be noticed based on % predicted FEV1 and FEV1/FVC.

Introduction to Respiratory Failure

One of the most severe postoperative pulmonary complications is respiratory failure. It is a heterogeneous condition characterized by low oxygen saturation, described as hypoxemia, which is typically <90% O₂ saturation. This hypoxemia is treated with mechanical ventilation to allow the underlying cause of respiratory failure to resolve while providing gas exchange. The causes of respiratory failure can be heterogeneous, but a common feature across these underlying conditions is poor delivery of oxygen into the bloodstream. One cause of these effects could be poor ventilation, which can impair oxygen delivery to the pulmonary vasculature. Lung conditions, such as pulmonary edema, pneumonia, atelectasis, and ARDS, can impair ventilation. These conditions can result in hypoxic respiratory failure that leads to hypoxemia systemically.

Another aspect could be due to occlusion of the pulmonary vessels due to pulmonary embolism or cardiogenic pulmonary edema from heart failure. Both instances are non-pulmonary conditions that can lead to respiratory failure. However, these conditions may not be associated with ventilation but rather with perfusion. Outside the heart and the lungs, neurological conditions such as Guillain-Barré Syndrome can decrease the work of breathing, thereby causing respiratory failure.³⁰

As mentioned earlier, ARDS is one of the common PPCs that lead to respiratory failure. It is an inflammatory condition characterized by a PaO₂/FiO₂ of less than 300; hence, an inability to deliver oxygen into the bloodstream. This condition is characterized by an intense pro-inflammatory cytokine storm that can lead to further lung damage and impair gas exchange. Calfee et al. described, in a landmark study, two subphenotypes of ARDS.³¹ One that leads to severe inflammation with upregulation of interleukin (IL)-8, IL-6, and tumour necrosis factor receptor-1 (TNFr-1) cytokine and another described by a less severe inflammatory profile.^{31,32}

Pulmonary Risk Factors for Postoperative Pulmonary Complications

Another important aspect of lung surgery and its contribution to PPCs is the preoperative risk factors and comorbidities associated with the patient population. Preoperative risk factors for PPCs are assessed based on their impact on patients' lung function and on intraoperative inflammation and wound healing. This is largely due to further diminishment of lung function after a greater extent of lung resection and the impact of surgery and OLV that contributes to lung injury.

In terms of preoperative risk factors, one consideration is the patient's functional residual capacity (FRC), which indicates the volume of air leftover in the lungs after tidal breathing. This value can be reduced by pressure from the abdomen, which pushes into the diaphragm; obesity is one factor that contributes to this.³³ Increased FRC observed in COPD patients can also be associated with lung hyperinflation due to gas trapping and expiratory air flow limitation.³⁴

Another factor is the patient's age at the time of surgery, with older patients having a reduced ability to handle physiological stress.³⁵ Additionally, an increase in age is also associated with decreased lung function specifically in %FEV₁, FVC and peak expiratory flow rate, which can further increase the risk for lung resection.^{36,37}

Focusing on metrics that indicate breathing and oxygenation, percent predicted %FEV1, and DLCO are important parameters in assessing lung function for surgery.³⁸ Consideration of these values concerns the flow of air through the lungs and the capacity to exchange gases. These values can also be affected by age and exposure to damage to the lung, one of which is smoking history. Smoking not only affects lung function but is also associated with increased PPCs³⁹. Yamamichi et al. show that stopping smoking at least 2 months before lung surgery can decrease the risk for PPCs.³⁹

Thus, age, BMI, smoking history, pulmonary function assessed by predicted %FEV1, and diffusion capacity testing assessed by DLCO are important factors to consider when assessing risk for lung resection. A combination of these factors, along with other lung function and physiological metrics, can also provide much broader insight into risk for PPCs. Additionally, postoperative extrapolation of lung function and gas exchange values, such as postoperative predicted FEV1 (ppoFEV1) and postoperative predicted DLCO (ppoDLCO), can predict how these values change after surgery.³⁸

Deviations in some of these markers, such as pulmonary function and DLCO, from normal can be either caused or exacerbated by preexisting lung comorbidities, such as COPD, ILD, and IPF.^{40,41} Additionally, chronic alcohol consumption, diabetes, and high BMI can contribute to PPCs as risk factors by affecting immune response to intraoperative inflammation, the establishment and management of ventilation, and wound healing after lung resection.^{40,42}

Identifying the interplay between preoperative risk factors and postoperative complications is important because it underpins that some patients undergoing lung resection surgery are often in a compromised lung physiological state, independent of lung cancer for which surgery is needed. This further emphasizes the need to reduce the incidence of these complications after lung surgery.

In addition to preoperative risk factors and the extent of lung resection, this need is also driven by the role of intraoperative interventions in developing PPCs, such as OLV, necessary to perform lung surgery under general anesthesia. OLV used to facilitate lung surgery is an understudied major contributor to PPCs.^{43–48} Prior to and after OLV, Two-Lung Ventilation (TLV) is established and is essential for OLV initiation and cessation. Although switching back to TLV can occur during OLV surgery, OLV lasts longer than TLV, spanning the entire surgical

duration. Thus, all potentially harmful mechanical ventilation forces are applied to the “one-lung” ventilated throughout surgery.

One-Lung Ventilation Physiology and Pathophysiology

During OLV surgery, the patient's respiratory drive is pharmacologically inhibited to allow air delivery from a ventilator to both lungs. Induction of general anesthesia and inhibition of normal respiratory drive lower the FRC of the patient.^{49–52} This FRC reduction is attributed to the loss of diaphragmatic and accessory muscle activity, which alters the natural stability of elastic recoil and the chest wall.^{49–52} Thus, the ventilated lung is dependent on positive pressure and tidal volume from the ventilator to remain above residual volume.⁴⁹

Intraoperative ventilation is established once the patient has been sedated and intubated with an endotracheal tube. Lung isolation is done by using a double-lumen tube or a bronchial blocker, with the former used more commonly. In cases of difficult airway or pediatric cases, a bronchial blocker may be preferable for lung isolation.^{53–56} The correct placement of a double-lumen tube or bronchial blocker is confirmed using a bronchoscope.⁵⁶ An OLV setup for lung surgery using a double-lumen tube is shown in Figure 1 below.

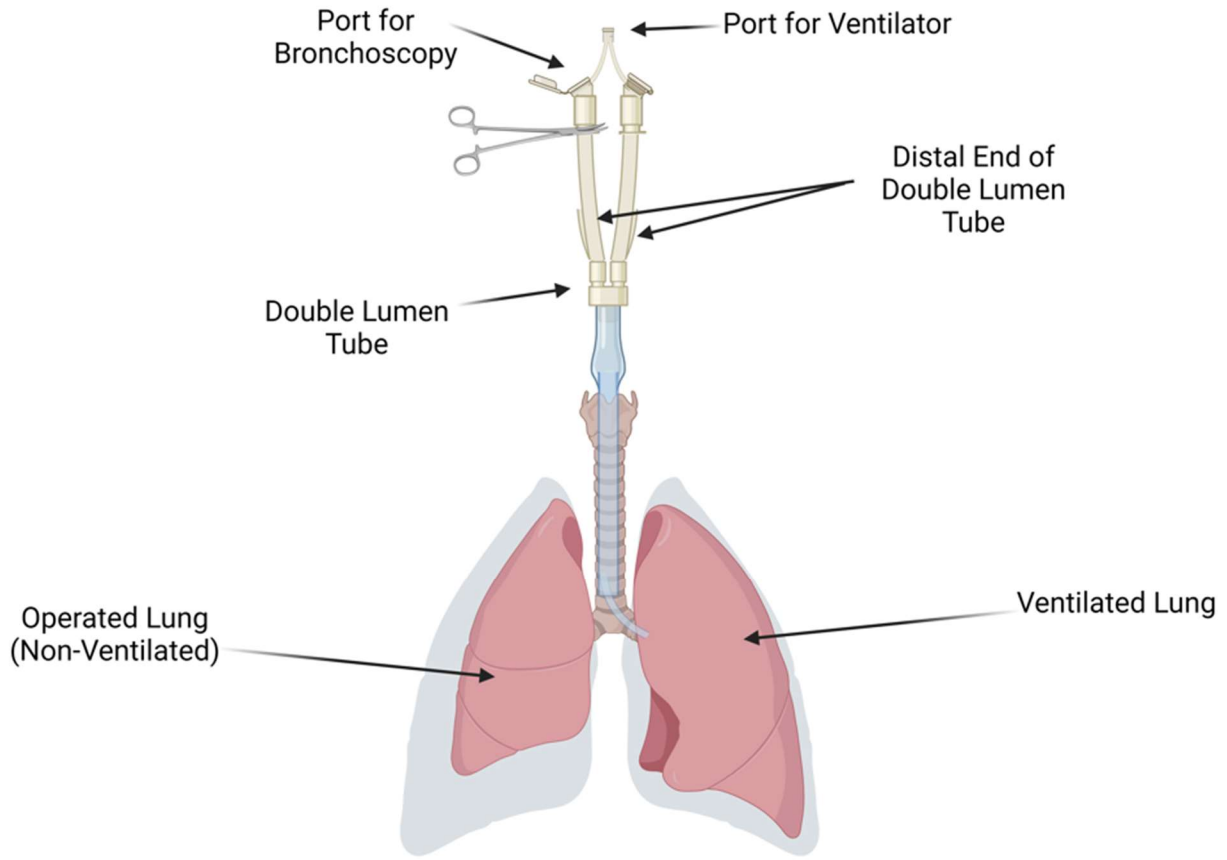


Figure 1. OLV setup prior to lung surgery. The operated lung is collapsed by clamping the corresponding side of the double-lumen tube while delivering mechanical ventilation to the ventilated lung.

During OLV, the operated lung is collapsed to facilitate surgery; therefore, it is not ventilated for the duration of the procedure. Due to hypoxic pulmonary vasoconstriction, blood flow will be redirected to the ventilated lung.⁴⁹ Thus, the operated lung will be ischemic throughout the duration of OLV and will experience decreased blood flow relative to the ventilated lung. In addition, the lateral decubitus positioning allows for increased blood flow to the ventilated lung due to gravity supplementing HPV redirected blood flow.⁵¹

Although HPV maintains adequate perfusion to ventilated regions, V/Q mismatch can occur in areas of variable ventilation, ranging from increased or decreased ventilation and collapsed alveoli, which do not match subsequent perfusion.⁵⁷ HPV can further be affected by pulmonary vascular resistance and choice of anesthetic – intravenous or inhalation – with the latter attributed to HPV inhibition.⁵¹ Thus, V/Q mismatch due to these factors can lead to

hypoxemia.^{51,57} Hypoxemia during OLV can be mitigated by increasing oxygenation if the issue arises from the ventilation side.

During air delivery from a mechanical ventilator, the ventilator controls respiratory rate, airway pressure, inspiratory flow rate or time, tidal volume, and fraction of inspired oxygen.⁵⁸ Tidal volume in mechanical ventilation is adjusted based on the patient's predicted body weight.⁵⁸ Whether the ventilator controls tidal volume or airway pressure depends on the ventilation mode.⁵⁸ In volume-controlled ventilation (VCV), the tidal volume, inspiratory flow rate and respiratory rate are set.⁵⁸ This allows a set delivery of air volume to the lung at a controlled rate per breath. Additionally, controlling the respiratory rate allows for the number of breaths delivered per minute.⁵⁸ In pressure-controlled ventilation (PCV), the inspiratory air pressure is controlled along with the inspiratory time.⁵⁸ Thus, controlled air pressure is delivered over a specified inspiratory time. In both ventilation delivery methods, the fraction of inspired oxygen and pressure of air left in the lungs after expiration, PEEP, are adjusted.⁵⁸ These variables control the oxygen concentration and prevent alveolar collapse after expiration, respectively.⁵⁸

Regarding which ventilation is beneficial during OLV, the consensus remains unclear. A randomized controlled trial by X.-F. Li, L. Jin et al. showed that there was no difference among pressure-controlled, volume-controlled, and pressure-controlled with volume guaranteed (PCV-VG) ventilation modes regarding PPCs, PPC severity grade within 7 days, or between 7 and 30 days.⁴³ PCV-VG offers a guaranteed tidal volume that starts at a lower airway pressure and adjusts to lung compliance the patient in subsequent breaths.⁵⁹⁻⁶¹ Other studies have corroborated this result by examining outcomes such as PPCs and oxygenation.⁶²⁻⁶⁵ Another group compared VCV with PCV-VG and found higher peak inspiratory pressure in the VCV group, with reduced neutrophil elastase in PCV-VG as a surrogate marker of neutrophil infiltration and inflammation.⁶⁶ However, this group looked at an older population with lower lung function.⁶⁶ With regard to respiratory failure, the choice of ventilator mode, whether PCV or VCV, was not seen as beneficial with the existing randomized controlled trial literature.⁶⁷ Thus, the line of evidence regarding ventilator mode choice remains conflicting. However, accounting for the ranges of tidal volume, peak inspiratory pressure, and PEEP used has been shown to reduce PPCs rather than ventilator modes.⁶⁸

Adjusting ventilator values of tidal volumes, airway pressure, PEEP and FiO₂ is performed to ensure sufficient gas exchange without causing damage to the lungs. Historically, higher tidal volumes have been proposed to prevent alveolar collapse (atelectasis) and mitigate hypoxia from hypoventilation.^{69,70} However, using high tidal volumes can cause alveolar overdistention, an injury mechanism known as volutrauma.^{49,51,70} Lower tidal volumes have been proposed as lung-protective and to minimize volutrauma.^{49,51,70} The consideration of a tidal volume that is lung-protective in the TLV context may not be the same as in OLV. A lung-protective ventilation regimen distributed to both lungs is not the same as in one-lung ventilation, due to fewer alveolar units recruited in the latter. This disparity arises especially from studies that examine intraoperative ventilation but do not specify whether it involves one or both lungs.^{69,70} The collapse of the operated lung and the lateral decubitus positioning of the patient during surgery alter the inherent physiological properties of the lung due to hypoxic pulmonary vasoconstriction and gravitational impact.⁴⁹ Moreover, the single ventilated lung responsible for gas exchange may be more prone to lung injury from volutrauma and a lack of PEEP during OLV than from lower tidal volumes and PEEP application.^{71,72}

PEEP is a pressure of air applied at end-expiration to prevent alveolar collapse, offer better oxygenation and compensate for decreased FRC.⁷³ A systematic review and meta-analysis from our lab has shown that alveolar recruitment maneuvers and PEEP improve PaO₂.⁷⁴ Alveolar recruitment maneuvers reopen collapsed alveoli by briefly delivering high transpulmonary pressures.⁷⁵ However, increased PEEP and a short-term increase in transpulmonary pressures may subject the lungs to barotrauma or lung injury due to high pressures.

One of the largest randomized controlled trials that examined lung recruitment combined with PEEP titration found an increase in mortality from all causes with this approach, referred to as the “open-lung approach,” compared to low PEEP.^{74,76,77} Although this trial was conducted in ARDS patients in a critical care setting, it underscores that mitigating atelectasis can come at the cost of barotrauma. Most notably, the secondary outcome of this trial, barotrauma within 7 days, was significantly higher in the PEEP titration with lung recruitment group than in the low PEEP group.⁷⁷

Another important variable used to improve oxygenation, while preventing volutrauma and barotrauma, is increasing the fraction of inspired oxygen (FiO₂). This maneuver creates

higher oxygen levels in the alveoli and was presumed to be compensatory for hypoxemia during OLV.⁵¹ However, an observational study has shown not only that high FiO₂ was used in the majority of the patients enrolled in their study but also that it was associated with the occurrence of PPCs.⁴⁸ Their results showed no significant difference in tidal volume, and PEEP applied between their PPC and control group as well.⁴⁸ In another cohort study in a critical care setting, the authors looked at systemic hyperoxemia with PaO₂ greater than 200 to be linked with mortality, and the odds were greater if the hyperoxemia exposure was for a day.⁷⁸ Even outside OLV-lung surgery, patients exposed to systemic hyperoxemia during cardiopulmonary bypass cardiac surgery were associated with higher PPC occurrence.⁷⁹ The mechanisms by which supplemental oxygen can lead to lung injury and PPCs are linked to oxidative stress.⁸⁰

Inflammatory Response to Injurious OLV

During OLV, injurious mechanical ventilation strategies used to mitigate hypoxemia and improve gaseous exchange can cause lung injury. However, the direct link between ventilation strategies and lung injury is appreciated by biotrauma. Biotrauma is the body's response to injurious ventilation strategies and describes lung tissue damage driven by a hyperactivated inflammatory response.⁸¹

The lungs have a natural mechanism for responding to pathogens and damage. One of the major executors of the innate immune response in the lungs is alveolar macrophages, which reside in the alveolar space and are sensitive to threats from pathogens.^{82–88} Upon sensing a threat, alveolar macrophages in the alveoli are activated, phagocytose nearby pathogens, and secrete cytokines and chemokines to recruit leukocytes.^{82,89–94}

Following lung injury, the alveolar-capillary membrane becomes more permeable, allowing neutrophils, short-lived, rapidly responding immune cells, to be recruited into the alveoli.^{95,96} Neutrophils have the capability to undergo phagocytosis and also release web-like DNA structures called neutrophil extracellular traps and proteases to trap and destroy pathogens.^{97–99} This function of neutrophils involves removing pathogens, such as bacteria; however, a careful balance between protective and excessive neutrophil activation is needed to avoid further lung injury in addition to the primary insult.^{99,100} In lung conditions, clinical patient variables such as age and smoking history can affect this balance.^{99,101,102}

In the context of ventilation-induced lung injury, one proposed mechanism of inflammatory response is the toll-like receptors (TLRs) signalling. TLRs are known pattern recognition receptors by which damage- and/or pathogen-associated molecular patterns (DAMPs and PAMPs) bind and activate.^{103,104} Various distinct TLRs can be activated during mechanical ventilation.^{103–106} These activated TLRs lead to NF- κ B translocation through the MyD88 pathway.^{104–106} NF- κ B activation further leads to cytokine production, such as IL-6, IL-8, and tumour necrosis factor alpha (TNF- α), which coordinate leukocyte recruitment and immune response activation.¹⁰⁶

Another mechanism of ventilation-induced lung injury involves the NLRP3 inflammasome pathway. This pathway is activated by DAMPs such as ATP, activated by the P2X receptor, HMGB1, heat shock proteins and other molecules that are detected by TLRs and receptor for advanced glycation end-products (RAGEs).^{107–109} NF- κ B activation can produce NLRP3, which assembles into the NLRP3 inflammasome along with caspase-1 (which in turn is cleaved from pro-caspase-1 by NLRP3) and ASC.^{109–112} In OLV context, activation of NLRP3 inflammasome can be dependent on ventilation strategies with lung-protective ventilation reducing its activation.¹¹³

The formation of this inflammasome can cleave pro-IL1 β and pro-IL18 zymogens into IL-1 β and IL-18, which can be released to further activate immune response.^{109,112} IL-1 β production and release can further amplify immune response activation by binding to IL-1 receptor type 1 (IL-1R1) and activating NF- κ B through MyD88.¹¹⁴ Activation of NF- κ B in OLV context of ventilation-induced lung injury has been confirmed by multiple studies.^{115,116}

A more recently appreciated role of mechanosensitive channels, Piezo, describes mechanisms of mechanotransduction in the lungs, which are relevant in ventilation parameters that cause lung stretch.¹¹⁷ Piezo channels allow calcium influx and subsequent NF- κ B activation.^{118–120} Another mechanosensitive channel facilitating calcium entry is transient receptor potential vanilloid 4 (TRPV4).¹²¹ TRPV4 has been shown to increase vascular permeability in response to barotrauma.¹²¹ Further describing the biological mechanisms of high tidal volume and pressure ventilation-induced lung injury.

To prevent hypoxemia, hyperoxia is another mechanism used, in addition to high tidal volumes and pressures. However, hyperoxia can cause and contribute to lung injury by damaging

the alveolar-capillary membrane and leading to fluid and protein leakage into the alveoli.^{122–130} Excess oxygen generated by high FiO₂ during hyperoxia can produce reactive oxygen species (ROS), leading to necrosis and apoptosis.^{123,124,126–130} Reactive oxygen species generated by hyperoxia can also cause DNA damage.^{125,127,130–134} An understudied topic regarding hyperoxia is the mechanism of lipid peroxidation induced by hyperoxia exposure. Brain studies of hyperoxia exposure have shown results of increased lipid peroxidation with hyperoxia, both *in vivo* and *in vitro*.^{135,136} Fessel et al. have shown that hyperoxia causes lipid peroxidation by measuring isofurans with a linear relationship between FiO₂ and isofurans formation.⁸⁰ A more definitive result in the context of hyperoxia during OLV has come from Olivant Fisher et al., who have shown an increase in myeloperoxidase and protein carbonyls due to hyperoxia in a pediatric porcine OLV model.¹²²

In clinical OLV-surgery context, hyperoxia, volutrauma and barotrauma may occur simultaneously depending on the demands for ventilation and oxygenation. In those cases, OLV-induced lung injury may not be strictly from volutrauma, barotrauma, atelectrauma or hyperoxia but a combination of multiple of these insults as shown in Figure 2.

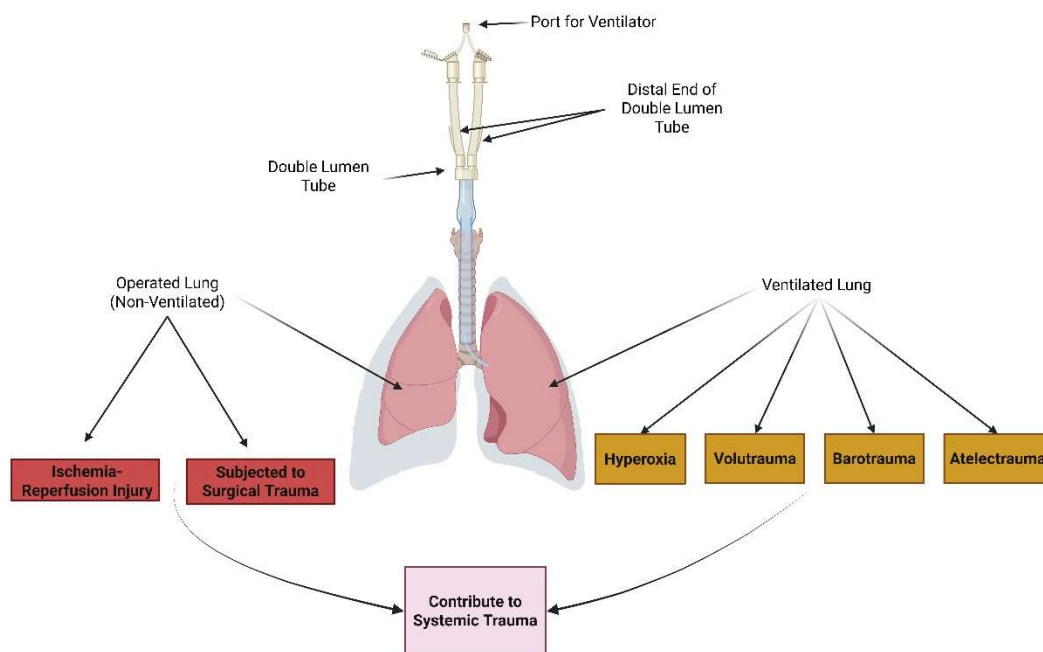


Figure 2. Mechanisms of OLV-surgery induced lung injury and their contribution to systemic trauma.

In a study of combined volutrauma and hyperoxia, the combination of these effects led to greater neutrophil recruitment to the alveoli and pulmonary edema.¹³⁷ Another study examined hyperoxia and volutrauma but did not find a similar effect on neutrophil recruitment when hyperoxia was included along with volutrauma compared with volutrauma alone.¹³⁸ However, this study defined hyperoxia as 50% FiO₂, whereas the former study defined it as 100%.^{137,138} In OLV studies, normoxia is typically defined as 50%, which creates ambiguity when some consider 50% to be hyperoxia, since atmospheric oxygen is 21%.

Oxidative injury in OLV context does not stop with hyperoxia exposure. In fact, the operated lung, which is not only subjected to surgical manipulation, is also subjected to oxidative injury by virtue of being collapsed. This oxidative injury comes from ischemia-reperfusion injury caused by the collapse of the operated lung to facilitate surgery.⁴⁹ The redirection of perfusion to ventilated areas of the lung causes ischemia, and re-expansion of this collapsed lung can cause oxidative stress due to reperfusion after a period of ischemia.⁴⁹

Oxidative injury occurs during periods of ischemia and reperfusion.^{139–141} This phenomenon occurs because O₂ radicals are generated from oxygen introduced after a period of ischemia during reperfusion.^{139–141} Thus, the operated lung may exhibit an inflammatory response not only due to surgical impact but also ischemia-reperfusion injury.¹⁴² One aspect of ischemia-reperfusion injury is the shedding of the endothelial glycocalyx, which affects vascular integrity.^{143,144} However, the choice of anesthetics can affect endothelial glycocalyx shedding, with sevoflurane having a protective effect.^{143,145,146}

Biomarker Discovery for Respiratory Failure

PPCs occur after surgery and there remains an unmet need to identify dynamic biomarkers capturing preoperative risk and response to injury especially for the most severe of PPCs which is respiratory failure.¹⁴⁷ A proteomic approach is an exciting option as it has the ability to identify multiple proteins either through a targeted or untargeted approach. Untargeted discovery-based mass spectrometry (MS) proteomics approach has the ability to identify novel biomarkers without preselection bias.¹⁴⁸ ELISA or western blot approaches come with a preselection bias – investigating a single biomarker or multiple biomarkers of interest. Mass spectrometry proteomics addresses this drawback with its ability to identify novel proteins that may have a diagnostic or therapeutic potential.¹⁴⁸

There are two types of mass spectrometry proteomics approaches: bottom-up and top-down. Bottom-up proteomics involves breaking down of proteins into peptides and performing mass spectrometry offering high-throughput analysis.^{149–151} Top-down proteomics involves analysing intact proteins with mass spectrometry allowing for identification of proteoforms.^{151–155}

Bottom-up proteomics involves breaking up proteins into peptides, separated using chromatography or electrophoresis techniques, and detection using mass spectrometry.¹⁵¹ Breaking up proteins into peptides requires denaturing the tertiary structure of proteins using detergents such as SDS.^{151,156} The disulfide bridges composed of cysteines are reduced with chemicals such as dithiothreitol (DTT) to break them and alkylated with chemicals such as iodoacetamide (IAA) to ensure they don't form again.¹⁵¹ Digestive enzymes such as trypsin are used to turn the denatured, reduced and alkylated proteins into peptides.¹⁵¹ Chromatography or electrophoresis techniques are then used to separate peptides based on their adherence to stationary and mobile phases in chromatography or based on their charge or isoelectric point in electrophoresis.¹⁵⁶ These processes allow for MS to receive peptides sequentially based on their separation.

Detection of whole peptide ion is coupled by adding another peptide fragmentation to generate peptide fragment ions, thus making up MS/MS. The mass-to-charge ratio of these ions are used to identify the peptide amino acid sequence.^{151,156} MS/MS detection can be further classified into data-dependent acquisition (DDA) and data-independent acquisition (DIA).¹⁵¹ DDA utilizes the top n peptides within a smaller mass-to-charge window for subsequent fragmentation.¹⁵¹ DIA preselects peptides within wider mass-to-charge windows for subsequent fragmentation rather than top n peptides.^{151,157} DIA can be further supplemented by DDA-generated libraries from pooled sample peptide digests. Inclusion of DDA-generated spectral library increases the resolution of DIA proteomics to achieve robust sensitivity.¹⁵⁸ The MS/MS identified peptides are then used to identify proteins using a proteomics database such as Uniprot.¹⁵⁹ Identified proteins are then assessed for differential expression between conditions to understand proteins associated with a certain condition and biological pathways involved.¹⁶⁰

Lung Injury in the Context of Lung Cancer

Even though OLV-induced lung injury, extent of lung resection, operative trauma, and preoperative lung function and comorbidities are contributors to acute lung injury after lung surgery, another contributor is cancer treatment – related lung injury.¹⁶¹ This type of lung injury occurs in the context of pre-operative or neoadjuvant cancer therapies that can be either for lung cancer as a primary or metastasis of another primary cancer to the lungs. Another consideration is whether the treatment is localized to the lungs or administered systemically. In the latter context, the lungs constitute the pulmonary circulation, which is intertwined with the systemic circulation, providing gas exchange.¹⁶¹ Hence, systemic administration of toxic treatments can affect the lungs. This impact can be described clinically due to a reduction in DLCO after chemotherapy, which can increase the risk for PPCs.^{162–164} Systemic therapies such as chemotherapy and immunotherapy may cause pneumonitis which can increase the risk for respiratory failure when used in the neoadjuvant setting.¹⁶⁵ The impact of cancer-related treatments can affect the lungs through inflammation and oxidative stress pathways and can contribute to the risk for acute lung injury.¹⁶¹

In line with lung surgery treatment for lung cancer, minimizing the incidence of PPCs is not only about limiting mortality and morbidity but also about preventing the risk for cancer recurrence from infectious PPCs. Various studies have described a link between infectious postoperative complications and cancer recurrence in different cancer types.^{14,166–170} Cools-Lartigue and Spicer et al. have shown that neutrophil extracellular traps released from neutrophils to immobilize and destroy pathogens in a septic model can promote metastasis by trapping circulating tumour cells (CTCs).¹⁶⁶ CTC-NET interaction promotes CTC escape out of the lung, survival in circulation and extravasation to a metastatic site which otherwise CTCs may suffer from a decrease in metastatic potential. Thus, preventing the occurrence of infectious pro-inflammatory PPCs that yield in increased neutrophil recruitment and NETosis as soon as intraoperatively may potentially prevent lung cancer recurrence through CTC-NET interactions. This goes hand-in-hand with decreasing mortality and morbidity after lung surgery as well.

Chapter III: Rationale

In this thesis, I propose a mechanism to understand the link between OLV and postoperative respiratory failure by comparing those who developed this condition with those who did not. I hypothesize that OLV-surgery induced lung injury can contribute to the development of respiratory failure after lung surgery. As mentioned earlier, preoperative lung conditions and comorbidities can pose a risk for postoperative respiratory failure. By matching patients who developed respiratory failure with those who did not based on variables that can contribute to this risk, I propose to effectively isolate the impact of OLV-surgery and identify biomarkers of lung injury and respiratory failure. Furthermore, a nested case-control design is well-suited for conditions such as postoperative respiratory failure, which have low incidence, are difficult to find matched control patients for retrospective analysis and are costly to perform multiple simultaneous biomarker screening.¹⁷¹⁻¹⁷⁴

Proteins are among the responders to and mediators of acute lung injury. These proteins can be secreted from the lung parenchyma and airways which can then be captured in BALF. This sampling method provides a direct mechanism for studying proteomic profiles of patients with lung injury. The other sampling method involves plasma isolation from arterial blood collection to investigate proteomic changes in patients. This approach allows for an understanding of how local injury can translate into a systemic condition. Furthermore, plasma sampling provides a convenient, non-invasive means to assess prognostic protein biomarkers of postoperative respiratory failure. Thus, I have set out to investigate the differential expression of protein biomarkers:

1. Changing before and after OLV in patients who developed postoperative respiratory failure and matched control patients.
2. Distinctive to the ventilated and operated lungs that are exposed to OLV and lung surgery, respectively.
3. Detectable before and after OLV in plasma samples and associated with OLV-surgery exposure.

Our lab's prior published work has shown detectable changes in cytokines in ventilated lung BALF and arterial plasma samples, pre- and post-OLV, in postoperative respiratory failure patients and matched controls.¹⁷⁵ IL-1RA, IL-23, and IL-6 were found to be different between

cases and matched controls, whereas IL-17D, IL-23, TSLP, and bFGF changed across pre- and post-OLV timepoints in ventilated lung BALF.¹⁷⁵ In plasma samples, IL-6, IL-1 β , and IFN- γ were changed post-vs-pre-OLV, while IL-1 β was significant across the interaction effect in respiratory failure status and OLV timepoint.¹⁷⁵

This study laid the foundation for the current investigation by measuring inflammation-associated cytokines and chemokines at the intraoperative stage.¹⁷⁵ Nevertheless, it was limited to the 55 proteins measured, and identification of novel protein biomarkers was needed.¹⁷⁵ A mass spectrometry proteomics approach was opted to fill this gap in prior research. In collaboration with the Manitoba Center for Proteomics and Systems Biology, we implemented a state-of-the-art data-independent acquisition (DIA) mass spectrometry approach that uses identified peptides within specified mass-to-charge windows to improve detection of low-abundance proteins. The analytical approach that follows has been shown to enable proteome screening even with low sample volumes.¹⁷⁶

BALF samples have been analyzed by mass spectrometry, and the results have been reported in various studies.^{177,178} Mass spectrometry plasma proteomics has been shown to characterize the differential effects of mechanical ventilation and to predict survival in COVID-19 patients, some of whom may develop respiratory failure.^{179,180} Although targeted proteomics may offer greater precision and accuracy, the specificity offered by DIA mass spectrometry is high and offers greater protein quantification depth. In the context of this study, untargeted discovery proteomics benefits in terms of identifying novel protein biomarkers with greater depth in an unbiased manner.¹⁸¹

The purpose of performing untargeted proteomics is two-fold. One is to identify novel biomarkers of lung injury and postoperative respiratory failure that can be detected intraoperatively in the two specimen types. The second is to identify therapeutic targets that can attenuate respiratory failure by administering prophylactics before its onset.

Chapter IV: Materials and Methods

Patient recruitment and eligibility criteria

Patients undergoing lung surgery at the Health Sciences Center Thoracic Surgery department were recruited into a large cohort study based on their ability to make informed consent. The OLV study was approved by the Health Research Ethics Board (HREB), the provincial health research ethics board for Manitoba, under the identifier HS22088 (H2018:334). At the time this proteomics study was initiated, 566 patients had been recruited into this large cohort study. At this time, more than 600 patients have been recruited into the study, with ongoing recruitment. Patients enrolled in this study had lung resection, such as wedge resection, lobectomy, segmentectomy, and pneumonectomy, with various modifications. Some surgeries had the removal of two lobes, such as bilobectomy or removal of a lobe along with a wedge or segmental resection. The vast majority of surgeries were video-assisted thoracoscopic surgeries (VATS), with some being open or thoracotomy and some being converted from video-assisted to open during the procedure. More recent cases recruited involved robotic-assisted thoracic surgeries.

Preoperatively, patients' age at surgery, biological sex, weight (kilograms), height (meters), BMI, smoking status and pack-years, and pulmonary function values, including %FEV1, DLCO, and %FEV1/FVC, were recorded. Comorbidities were also included, such as cardiac, vascular, cerebrovascular, pulmonary, connective tissue, gastrointestinal, hepatic, renal, oncological and hematological comorbidities. With respect to oncological comorbidities, since most patients in this study are undergoing lung surgery for lung cancer treatment – cancer treatments, recurrence outcomes, and pathological staging are recorded as well. Once the patient is in the operating room, their oxygen saturation on room air is recorded. Additionally, for most patients in this study, ventilation parameters are recorded using the iXTrend software.

Sample Collection and Processing for Biobank

Intraoperatively, the patient is sedated, and intubation is performed using an endotracheal tube. A double-lumen tube is placed to allow lung isolation for the operation and ventilation. Two-lung ventilation is established while inhibiting the patient's spontaneous breathing activity, so that the ventilator controls breath delivery. Right before one-lung ventilation is initiated,

BALF and arterial plasma samples are collected simultaneously. BALF is collected using a flexible fiberoptic bronchoscope through the endotracheal tube and double-lumen tube. This is performed by instilling approximately 30 ml of sterile warm saline through the bronchoscope and collecting the fluid from each lung. Depending on the volume of BALF retrieved, the volume of saline instilled may vary from 30 ml, as lung conditions may affect BALF recovery.

The lavage fluid is then collected in two 15 ml Falcon tubes for each lung lavaged. To ensure consistency in sampling, bronchoalveolar lavage is performed on contralateral lobes. For instance, if the left upper lobe is resected, then BAL sampling would be done on the left lower lobe from the operated lung and right lower lobe from the ventilated lung. If right lower lobe is resected, then operated lung BALF will be collected from the right middle lobe while ventilated lung BALF will be collected from the lingula. BALF collection using this procedure is performed on both the operated and ventilated lungs. For BALF samples included in this project, only one patient (OLV 299), a matched control, did not have a pre-OLV BALF specimen collected from the operated lung. Since we are comparing post-vs-pre-OLV protein abundances separately for the ventilated and operated lungs, we decided to keep this patient for the ventilated lung comparison but not for the operated lung comparison.

Whole blood is collected via an established arterial line for the surgical procedure in a 6 ml EDTA tube. However, since blood is collected from systemic circulation, there is no separation between operated and ventilated lung specimens. After lung surgery is performed and ventilation is switched to two-lung ventilation, BALF and plasma samples are collected immediately, in a manner similar to the pre-OLV time point. All samples are placed on ice until surgery is completed, and then are ready for processing. The sample collection, steroid administration and dosage, and TLV-OLV start and end times are also recorded in clinical report forms (attached in Appendix).

After specimens were collected in the operating room, they were processed by centrifuging BALF samples at 3500 rpm for 5 min and plasma samples at 5000 rpm for 10 min at 4 °C. Arterial plasma samples take priority in sample processing to avoid hemolysis. The supernatant from the centrifuged samples was aliquoted into 1.5 ml sterile Eppendorf tubes and stored in a -80 °C freezer. The pellet collected from the BALF samples was stored in a separate

1.5 ml Eppendorf tube, whereas white and red blood cell pellets from arterial plasma were discarded.

Nested Case-Control Patient Selection and Matching Strategy

Postoperatively, patients' pulmonary (including days on ICU ventilator), pleural, anastomotic, cardiac, renal, gastrointestinal, neurological, and wound complications were recorded. Long-term patient outcomes were also recorded in the context of early cancer recurrence within 6 months, 1 year and 2 years for patients who underwent lung surgery for lung cancer treatment. Additionally, patient mortality was also recorded. Patients who developed post-operative respiratory failure were considered the main outcome for proteomics assessment. Similar to a prior study from our lab¹⁷⁵, we considered respiratory failure as mechanical ventilation dependence 48 hours after lung surgery, mechanical ventilation dependence after post-operative day one, or home oxygen dependence.

Patients recruited from December 17, 2018, to April 02, 2024, were considered for proteomics analysis. Patients who developed respiratory failure after lung surgery, which is attributed to OLV surgery, were identified. Additionally, patients who developed high-grade pulmonary complications such as pneumonia, ARDS and atelectasis were considered as respiratory failure cases. Patients with other complications, such as cardiac and pleural complications, for instance, in addition to respiratory failure, were also considered as cases. Patients who developed respiratory failure after surgery due to other reasons, such as aspiration, excessive anesthetic effect, and hemorrhage, were excluded. Additionally, a lack of inventory for respiratory failure patient biospecimens would also exclude them from downstream analysis.

We identified 14 patients who developed postoperative respiratory failure. For these 14 patients, matched controls were identified in the OLV database at that time based on close values for surgery type and resection extent, biological sex, age, DLCO, FEV1, FEV1/FVC, BMI, smoking status, and pack-years. This approach aimed to get similar values for these variables between cases and matched controls. However, when matching on all parameters was difficult, similarity based on preoperative predicted lung function and the extent of tissue resection was prioritized. In extreme situations where matching cases and controls was difficult, a matched control with a higher risk of developing respiratory failure, based on the matching variables, was used. Thus, a matched control with worse lung function and a greater extent of tissue resection

was considered a better control for these variables. Additionally, some patients considered as matched controls have developed pulmonary, pleural, cardiac, and renal complications after lung surgery. They were considered matched controls, despite these complications, as they did not experience respiratory failure. Hence, 27 matched controls were generated and matched to respiratory failure cases in 3:1, 2:1, and 1:1 fashion. Figure 3 shown below displays the postoperative respiratory failure and matched control patient IDs. Control patients well-matched to multiple cases are shown in Figure 3 as duplicate controls. However, these controls that were matched to the most optimal case were considered as primary controls and to any other optimal cases were considered as duplicate controls.

Proteomics Case-Control Design

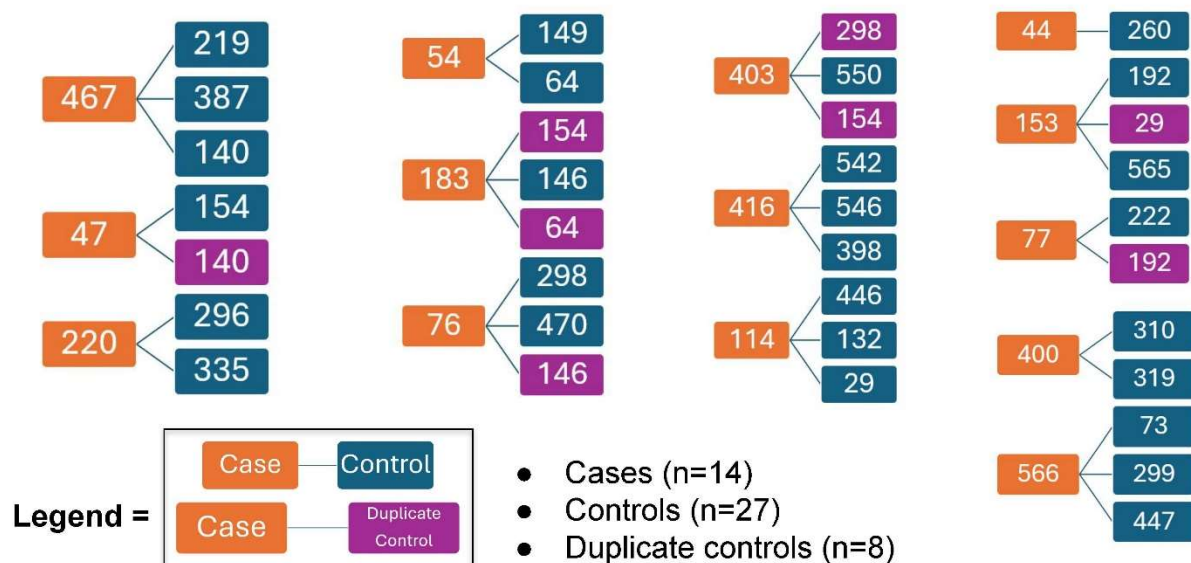


Figure 3. Proteomics nested case – matched control design from a large cohort study. Postoperative respiratory failure (in orange) and matched control (in dark blue) patient IDs that were matched based on preoperative comorbidities and extent of lung resection. Control patients that serve as optimal matched controls for multiple cases were considered as duplicate controls (in purple).

In matched-control samples, patients with BALF and plasma samples were included, and those with missing samples were excluded. However, for one patient, ID 299, the BALF operated lung pre-OLV specimen was missing. The patient was included in the study as a matched control because the ventilated lung BALF and plasma samples were considered more

important than those from the operated lung. For the bioinformatic analyses of BALF from the operated lung, the patient was excluded as a matched control.

Proteomics Sample Conditions and Preparation

In terms of sample quality, BALF samples showed notable differences in mucus and blood content. Regarding blood contamination, 22 operated lung post-OLV BALF samples showed noticeable contamination. This could be attributed to the impact of surgical resection. There were 7 and 5 operated and ventilated lung BALF pre-OLV samples, respectively, with noticeable blood contamination. In ventilated lung post-OLV samples, 11 (11/27; 40.74%) samples showed blood contamination, with 4 of 14 (28.57%) respiratory failure cases and 7 of 27 (25.93%) matched controls showing blood contamination. Interestingly, in both the operated and ventilated lung post-OLV BALF, a higher number of samples showed blood contamination. Nevertheless, it would be impossible to rule out whether the blood contamination in the ventilated lung post-OLV was due to alveolar hemorrhage, lung injury or cutting off small vessels during flexible bronchoscopic bronchoalveolar lavage. Further, the presence of blood and mucus was assessed by eye and is not a precise or accurate metric of blood contamination, other than to inform downstream sample processing. There were roughly 28 samples with mucous present, which may reflect some degree of airway irritation and inflammation, as well as smoke exposure.

BALF Sample Preparation

For individual sample analysis, samples were thawed on ice horizontally and had protease inhibitor cocktail (ThermoScientific, Catalog Number (Cat No.) 87785) added for a final conc of 1X. Samples were then vortexed for 10 min and centrifuged for 10 min at 15000 x g. Micro-BCA assay was done using Micro BCA™ Protein Assay Kit (ThermoScientific, Cat No. 23235) as per manufacturer protocol to determine protein concentration.

15 µg of protein from each sample was transferred to a new vial and had 20% SDS and 1M TRIS, pH 8.5 added to them for a final concentration of 3.1% and 77 mM, respectively. All samples then underwent boiling at 90 °C for 5 min, centrifuging at 2500 x g for 1 min, moderate strength vortexing for 10 min, and centrifuging for 1 min at 2500 x g. Reduction of disulfide bonds was performed by adding 10 mM dithiothreitol (DTT) final concentration and incubation at 57 °C for 30 min. Alkylation of free Cys residues was performed by adding 50 mM

Iodoacetamide (IAA) final concentration and incubation at room temp in the dark for 45 min. Quenching was performed with 17 mM DTT final concentration and incubation at room temperature for 10 min.

For purification and digestion, S-trap micro spin columns (Protifi, Cat No. C02-micro-80) were used for purification and digestion by following manufacturer protocol starting at the acidification of the sample all the way to elution. Volume modifications were done as per instructed in manufacturer protocol Appendix B. The TEAB in the binding wash solution was substituted with HEPES, pH 8.0 at the same concentration. The TEAB in the digestion buffer was substituted with HEPES, pH 8.0 at the same concentration. Digestion was carried out at a 1:10 enzyme to protein ratio with a 16-hour incubation at 37 °C. The three elution steps were done at 4000 x g for 1 min instead of 10000 x g. Peptide concentration was determined using the Pierce Quantitative Fluorometric Peptide Assay (Fisher Scientific, Cat No. 23290). 200 ng of sample was loaded into Evotip Pure (Evosep, Cat No. EV2011) using manufacturer protocol for MS submission and analysis.

Spectral library was created using a 112 µg pool from the remaining digests by taking an equal amount of material from each sample. High pH fractionation was performed on 66.8µg of the digest pool (40 fractions done with concatenation, 30sec per fraction). Fractions were lyophilized and 200 ng of each fraction was loaded into Evotip Pure (Evosep, cat no. EV2011) using manufacturer protocol for MS submission and analysis. Library was generated by searching the DDA-derived data against a human Swissprot database.

Plasma Sample Preparation

For individual plasma sample analysis, samples were thawed on ice horizontally and had protease inhibitor cocktail (ThermoScientific, Catalog Number (Cat No.) 87785) for a final concentration of 1X. Samples were then vortexed for 10 min and centrifuged for 10 min at 15000 x g. Highly abundant plasma protein depletion was carried out as per manufacturer protocol but using a 1:30 (v/v) ratio of sample to High-Select™ Top14 Abundant Protein Depletion Resin (ThermoScientific, Cat No.A36372) instead. Bradford assay was done using Pierce™ Detergent Compatible Bradford Assay Kit (ThermoScientific, Cat No.23246) as per manufacturer protocol to determine protein concentration. 17.4 ug of protein from each sample was transferred to a new

vial and had 20% SDS and 1M TRIS, pH 8.5 added to them for a final concentration of 3.1% and 77 mM, respectively.

Disulfide bond reduction was done by adding 10 mM dithiothreitol (DTT) final concentration and incubation at 57 °C for 30min. Alkylation of free Cys residues was performed by adding 50 mM Iodoacetamide (IAA) and incubation at room temp in the dark for 45 min. For purification and digestion, S-trap micro spin column (Protifi, Cat No. C02-micro-80) was used by following manufacturer protocol starting from the acidification of the sample all the way to elution. Volume modifications were done as per instructions in manufacturer protocol Appendix B. Triethylammonium bicarbonate (TEAB) in the binding wash solution was substituted with HEPES buffer, pH 8.0 at the same concentration. The TEAB in the digestion buffer was substituted with HEPES buffer, pH 8.5 at the same concentration. Digestion was carried out at a 1:10 enzyme to protein ratio with a 16-hour incubation at 37 °C. The three elution steps were done at 4000 x g for 1 min instead of 10000 x g. Pierce Quantitative Fluorometric Peptide Assay (Fisher Scientific, Cat No. 23290) was used to determine peptide concentration. 200 ng of sample was loaded into Evotip Pure (EvoSep, Cat No. EV2011) using manufacturer protocol for MS submission and analysis.

Spectral library was created using a 112 µg pool from the remaining digests by taking an equal amount of material from each sample. High pH fractionation was performed on the digest pool (40 fractions done with concatenation, 30sec per fraction). 200 ng per fraction was loaded into Evotip Pure (EvoSep, cat no. EV2011) as per manufacturer protocol for MS submission and analysis. Library was generated by searching the DDA-derived data against a human Swissprot database.

BALF Mass Spectrometry Protocol

An equivalent of 200 ng of peptides was analyzed by nanoflow LC-MS/MS using an EvoSep One coupled to a TimsTOF Pro2 working in dia-PASEF (parallel accumulation-serial fragmentation) mode.¹⁸² An EvoSep EV1137 column was used to separate peptides (1.5 µm particle, 150 µm ID x 15 cm, EvoSep) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using the pre-configured 30 samples per day gradient and flowrate. Mass and inverse reduced ion mobility ($1/K_0$) values were calibrated using a doped filter prepared from commercially obtained calibrants (m/z 622, 922, 1222) according to

the manufacturer's instructions.¹⁸² The source parameters were set as follows: 1650 V for emitter voltage, 3.0 L/min for dry gas and 200 °C for dry temperature.

The dia-PASEF method was enhanced using py_diAID¹⁸³ over an m/z range from 350 to 1300. Variable isolation window widths split along two ion mobility ranges adjusted based on precursor intensity were used for each dia-PASEF scan. One dia-PASEF cycle consisted of 17 scans and the cycle time was 1.91 sec. As similarly described in Neale et al. "the ion mobility was scanned from 0.7 to 1.50 V*s/cm² at a ramp time of 100 ms while ramp and accumulation times are locked to 100% duty cycle. The collision energy was ramped linearly as a function of the mobility from 59 eV at $1/K_0 = 1.6 \text{ Vs cm}^{-2}$ to 20 eV at $1/K_0 = 0.6 \text{ Vs cm}^{-2}$ ".¹⁸²

Plasma Mass Spectrometry Protocol

An equivalent of 200 ng of peptides was analyzed by nanoflow LC-MS/MS using an EvoSep One coupled to a TimsTOF Pro2 working in dia-PASEF (parallel accumulation-serial fragmentation) mode.¹⁸² An EvoSep EV1137 column was used to separate peptides (1.5 μm particle, 150 μm ID x 15 cm, EvoSep) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using the pre-configured 30 samples per day gradient and flowrate. Mass and inverse reduced ion mobility ($1/K_0$) values were calibrated using a doped filter prepared from commercially obtained calibrants (m/z 622, 922, 1222) according to the manufacturer's instructions.¹⁸² The source parameters were set as follows: 1600 V for emitter voltage, 3.0 L/min for dry gas and 200 °C for dry temperature.

The dia-PASEF method was refined using py_diAID¹⁸³ over an m/z range from 300 – 1200. Variable isolation window widths split along two ion mobility ranges adjusted based on precursor intensity were used for each dia-PASEF scan. One dia-PASEF cycle consisted of eight scans and the cycle time was 0.95 sec. As similarly described in Neale et al. "the ion mobility was scanned from 0.6 to 1.45 V*s/cm² at a ramp time of 100 ms while ramp and accumulation times are locked to 100% duty cycle. The collision energy was ramped linearly as a function of the mobility from 59eV at $1/K_0 = 1.6 \text{ Vs cm}^{-2}$ to 20 eV at $1/K_0 = 0.6 \text{ Vs cm}^{-2}$ ".¹⁸²

Spectral Library Mass Spectrometry Protocol

Per high-pH fraction, an equivalent of 200 ng of peptides was analyzed by nanoflow LC-MS/MS using an EvoSep One coupled to a TimsTOF Pro2 working in PASEF (parallel accumulation-serial fragmentation) mode.¹⁸² An EvoSep EV1137 column was used to separate

peptides (1.5 μm particle, 150 μm ID x 15 cm, EvoSep) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using the pre-configured 30 samples per day gradient and flowrate. Mass and inverse reduced ion mobility ($1/K_0$) values were calibrated using a doped filter prepared from commercially obtained calibrants (m/z 622, 922, 1222) according to the manufacturer's instructions. The 20 μm nanoflow emitter was charged at 1650V and the TOF analyzer is set to a scan range of 100 – 1700 m/z .

The overall acquisition cycle of 0.74 sec comprised of one complete TIMS-MS survey scan and six PASEF MS/MS scans (standard polygon filter was used to exclude singly charged ions). Ion mobility was surveyed across a range of 0.7 – 1.5 $\text{V}\cdot\text{s}/\text{cm}^2$ using a 100ms ramp time. Both ramp and accumulation times were configured to preserve a 100% duty cycle. Precursor ions were selected with an intensity threshold of 1000 counts and dynamic exclusion equaled at 0.4 min besides low-abundance precursors which were repeatedly targeted for fragmentation until a cumulative intensity of 12500 counts was achieved. The energy of collision energy was boosted linearly using mobility as a function from 59 eV at $1/K_0 = 1.6 \text{ Vs cm}^{-2}$ to 20 eV at $1/K_0 = 0.6 \text{ Vs cm}^{-2}$.

BALF Mass Spectral Data Processing

Using FragPipe v22.0 (with MSFragger v4.1, Philosopher v5.1.1), we searched the data-dependent acquisition (DDA) 2D-LC-MS/MS data to generate a BALF spectral library with 6882 protein isoforms and 127,392 precursors using the 2D-LC-MS/MS DDA. MSFragger searches were performed against a target-decoy UniProt database (downloaded in April 2025) using 20 and 15 ppm precursor and fragment mass tolerances, respectively, and trypsin with up to 2 missed cleavages as enzyme. Cysteine carbamidomethylation was configured as a fixed modification. Methionine oxidation and N-terminal acetylation were variable modifications. Peptide-spectrum matches were rescored with Percolator using target-decoy competition, and peptide and protein-level identifications were filtered to a 1% false discovery rate (FDR) using Philosopher.

DIA-NN (v1.9) was used to process the data independent acquisition (DIA) data, using the abovementioned project-specific spectral library. The UniProt human database was used for protein annotation, with tryptic specificity allowing one missed cleavage, fixed cysteine carbamidomethylation, and variable N-terminal methionine excision. Mass accuracy was

optimized individually for each run (average precursor ≈ 12 ppm, MS1 ≈ 18 ppm), and peptide and protein identifications were filtered to a 1% FDR using DIA-NN's built-in target-decoy strategy.

Quantification in DIA-NN was performed using fragment ion intensities from the matched precursors across all DIA runs. The software applied retention time alignment and normalization to construct precursor- and protein-level expression matrices, ensuring consistent quantitation across samples under 1% precursor and protein group FDR control.

Plasma Mass Spectral Data Processing

Using FragPipe v20.0 (with MSFragger v3.8 and Philosopher v5.0.0), 2D-LC-MS/MS DDA data were searched to generate a plasma spectral library containing 2,356 protein groups and 45,083 precursors. Searches were performed against a target-decoy reviewed human UniProt database using 20 ppm precursor and fragment mass tolerances, strict trypsin specificity with up to two missed cleavages, and fixed cysteine carbamidomethylation. Oxidation of methionine, protein N-terminal acetylation, and N/Q deamidation were allowed as variable modifications. PSMs were rescored using Percolator, and peptide- and protein-level identifications were filtered to 1% FDR using Philosopher.

DIA data were analyzed using DIA-NN v1.9 with the plasma spectral library. The library was reannotated against reviewed human UniProt supplemented with cRAP contaminants. Tryptic peptides of 7–35 amino acids, precursor charge states 1–4, and up to one missed cleavage were considered; fixed cysteine carbamidomethylation. Mass accuracy and scan windows were optimized independently for each run.

Following the first-pass analysis, DIA-NN generated a study-specific library, which was used for second-pass reanalysis of all 89 DIA runs. Final averaged mass-accuracy settings were approximately 12 ppm for precursor ions and 17 ppm for MS1. The DIA-NN output matrix contained 1,468 protein groups, and precursor- and protein-level quantitative matrices were generated under 1% precursor and protein-group FDR control.

Data Processing for Statistical Analysis

A few samples had to be redone on a second day; to assess system reproducibility, a set of 5 samples was remeasured that same day (labelled as redo samples). In total, 185 runs were acquired, 17 of which were QC pools. First, using Perseus for data analysis, we confirmed that across all samples, using log2-transformation, z-scoring, and unsupervised clustering (all samples) and PCA (only redo and QC-assigned samples), the redo samples are very close to the original ones. Since the original and redo samples are very close, averages for these redo samples and their respective originally failed samples were used for downstream analysis.

Downstream data analysis was performed in the R 4.5.1 (Great Square Root) environment using scripts written with the following packages: DEP2,¹⁶⁰ ComplexHeatmap,^{184,185} mixomics,¹⁸⁶ VariancePartition,¹⁸⁷ ClusterProfiler,^{188–191} enrichplot,¹⁹² igraph,¹⁹³ penalizedclr,¹⁹⁴ clogitL1,¹⁹⁵ EnhancedVolcano,¹⁹⁶ and DOSE.¹⁹⁷

Potentially contaminant proteins were removed by filtering out protein names with “CRAP” referenced in the CRAPome repository.¹⁹⁸ This method was applied to both BALF and plasma data. For BALF samples, QC pools were removed from the statistical comparison, yielding 168 runs. Afterwards, ventilated lung and operated lung BALF runs were separated to allow separate comparisons of ventilated lung post-vs-pre OLV and operated lung post-vs-pre OLV. This yielded 87 ventilated lung BALF runs, out of which 5 were redo runs. Since averages between original and redo samples were considered, this yielded 82 runs (41 pre- and 41 post-OLV).

For the operated lung analysis, the pre-OLV BALF specimen was missing for one patient (ID: 299); therefore, the corresponding post-OLV BALF run was removed from downstream analysis to ensure consistency in the differential expression comparison post-vs-pre-OLV. This removal did not affect the case-control matching design, since there were two other matched controls with complete sample inventories for the case. This yielded 80 operated lung BALF runs (40 pre- and 40 post-OLV) samples kept for downstream analysis.

The BALF and plasma runs were integrated into our experimental design using a metadata table containing SampleID, Condition, rf (respiratory failure status: case or control), timepoint (pre- or post-OLV), BioReplicate, and strata (matched-pair identifier) for each run.

Downstream analysis following this step is summarized in Figure 4. To allow statistical comparison of timepoints for postoperative respiratory failure status (rf), a Condition variable was generated using the interaction $rf * timepoint$. Thus, yielding four conditions: Cases_Post_OLV, Cases_Pre_OLV, Controls_Post_OLV, and Controls_Pre_OLV.

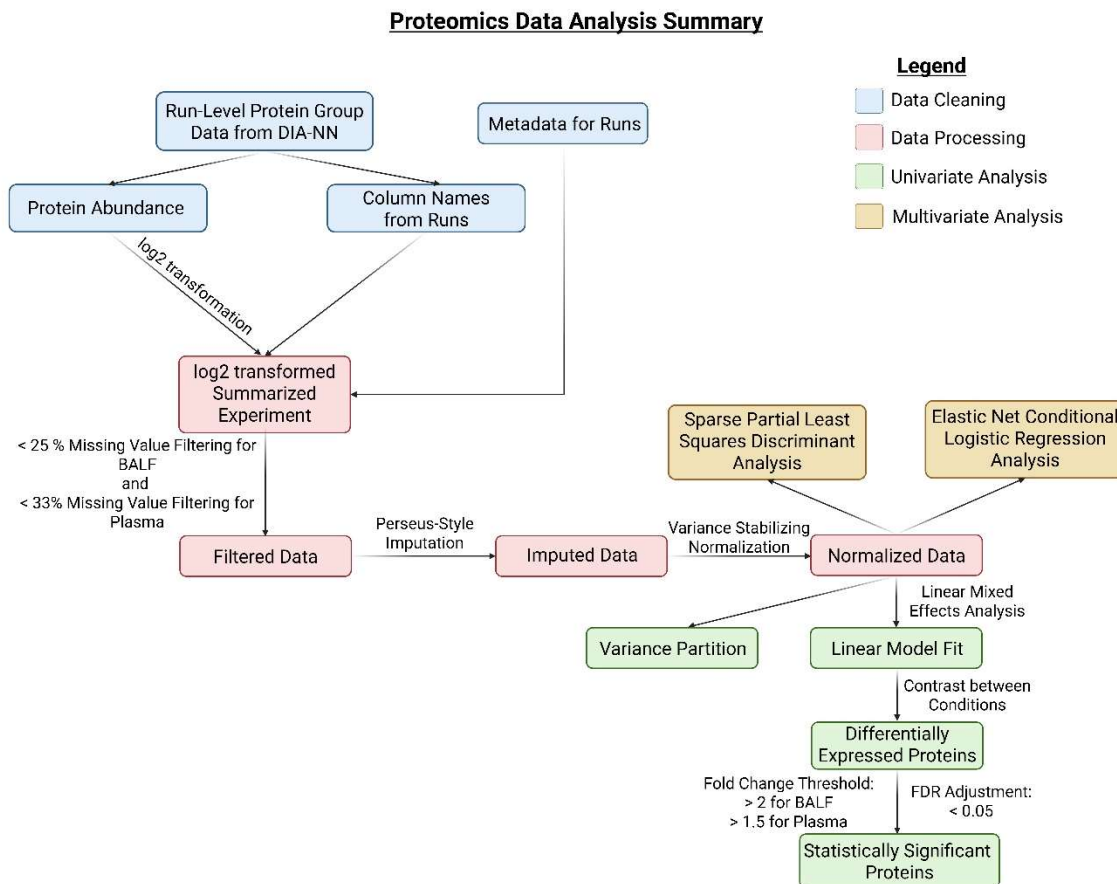


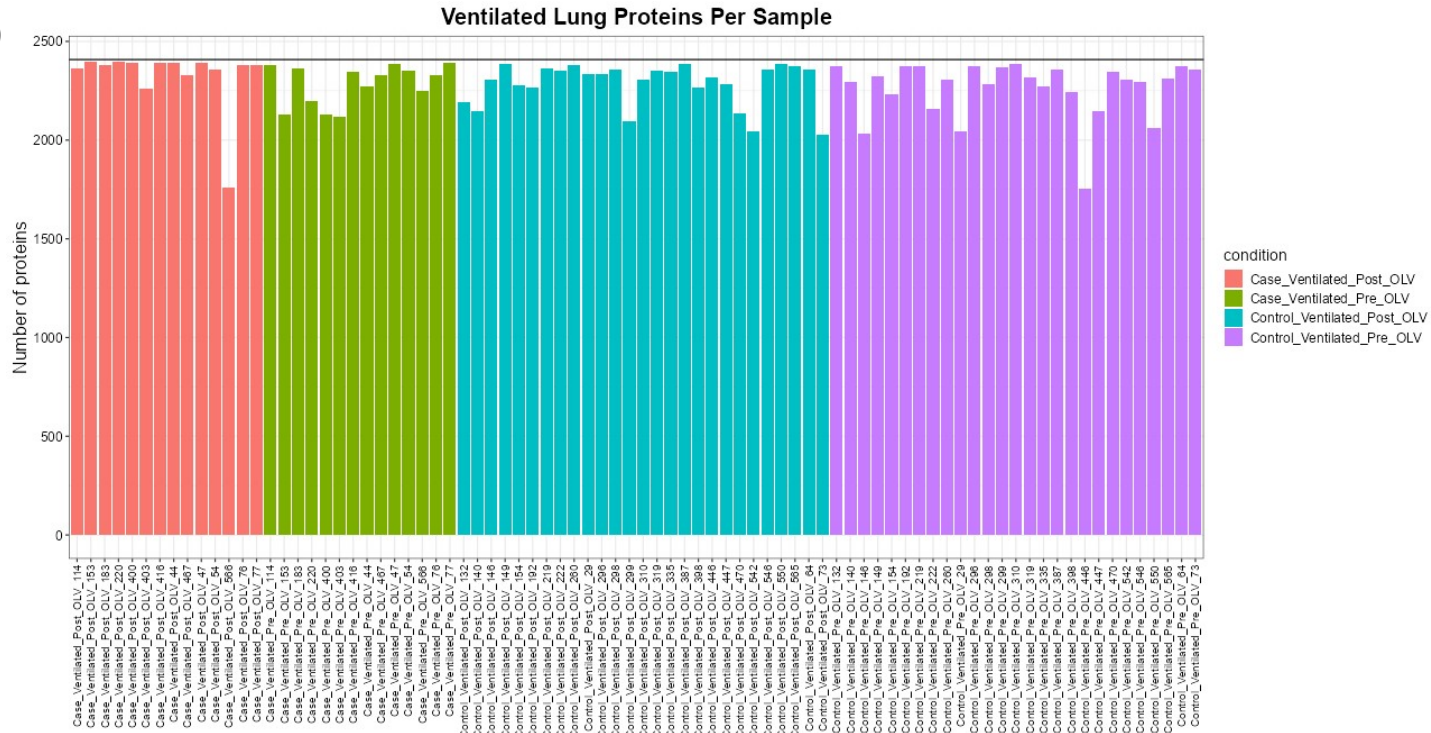
Figure 4. Proteomics Data Analysis Pipeline. Created in <https://BioRender.com>.

Due to ambiguous protein inference, multiple protein groups referenced to a single gene: i.e. P07437;P68371;Q13885;Q9BVA1 protein group referred to TUBB;TUBB2A;TUBB2B;TUBB4B gene names. This yielded the same gene name (TUBB) for multiple protein groups. DEP2 package performs analysis using gene names. Hence, to avoid the same gene name from having multiple protein abundance measurements, duplicate gene names were converted into unique gene names by adding a numeric identifier after a decimal point for each duplicate gene: i.e. TUBB, TUBB.1, TUBB.2 etc. To retain consistent proteins across all 82 samples (80 for operated lung BALF), we filtered proteins that are present in more than 75% of

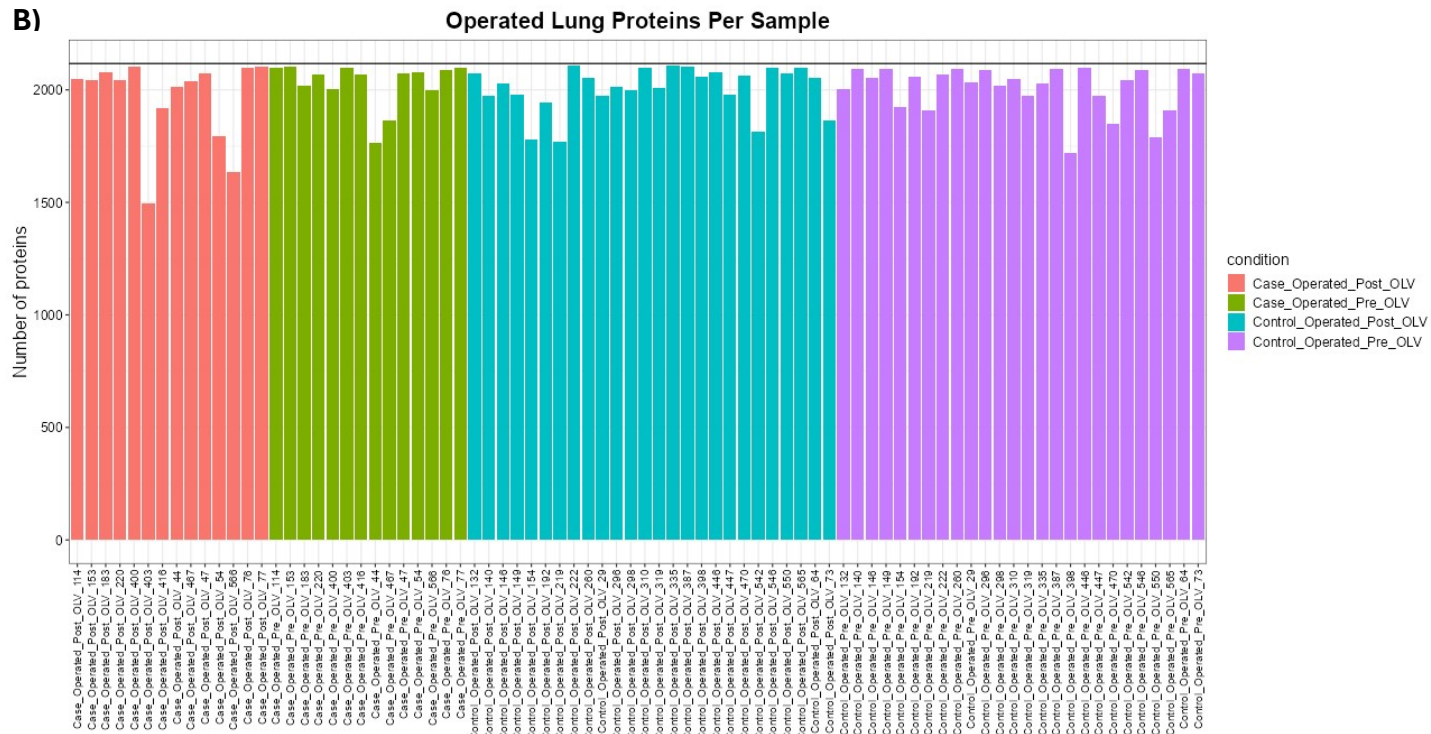
samples in at least one out of the four conditions. This missing value filtration approach was intended to keep proteins that may be missing in some of the conditions as those proteins may be missing due to biological implications. If they had more than 75% presence in at least one of the conditions, then their missingness in other conditions may not be at random and may be biologically relevant. The identified protein overlaps before and after applying this filtration approach are shown in Supplementary Figures 1A-B, 1D-E, and 1G-H.

Furthermore, to ensure that protein features are not determined to be present when comparing across different conditions based on largely imputed values, we kept proteins quantified in more than 75% of all samples after applying the previous condition-based missing value filtration approach. The above-mentioned 75% presence threshold was applied to BALF samples, whereas 67% presence threshold was applied to plasma samples. A higher presence threshold was used for BALF samples due to increased heterogeneity in BALF sampling compared to plasma. The resulting protein identification overlap is shown in Supplementary Figures 1C, 1F, and 1I. The number of proteins quantified after our above-mentioned filtration steps across all samples is shown in Figure 5A-C below. To ensure proteins that could be biologically relevant are not penalized by our second filtration approach, we have analyzed the proteins yielded by the first filtration step but were discarded by the second filtration step for statistical significance. Those proteins are shown in Supplementary Table 2.

A)



B)



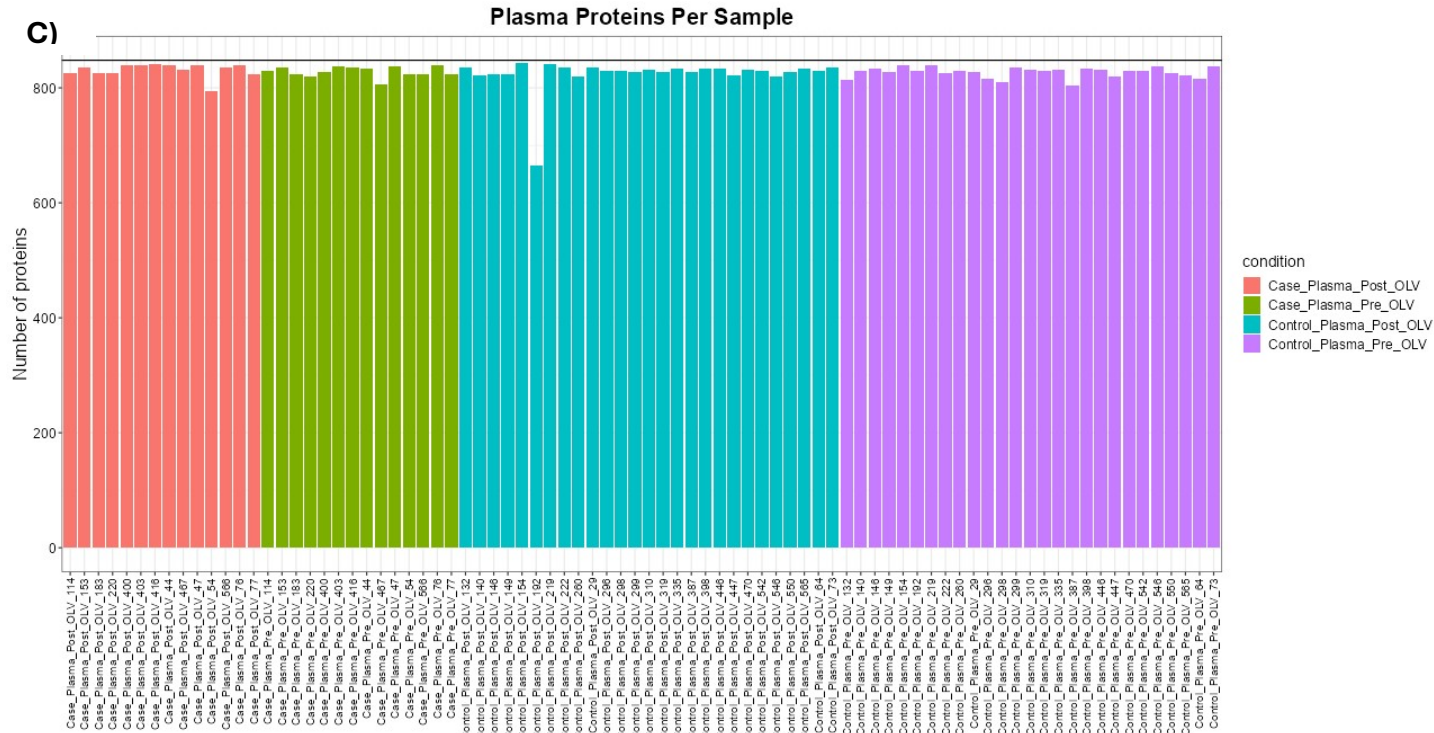


Figure 5A-C. Number of proteins found in each sample after applying missing value filtration. **A)** ventilated lung BALF, **B)** operated lung BALF, and **C)** plasma proteome.

In the ventilated lung BALF, Case ID 566 post-OLV and Control ID 446 pre-OLV yielded fewer than 2,000 proteins. In the operated lung BALF, Case ID 403 post-OLV yielded fewer than 1500 proteins and Case ID 566 post-OLV yielded just above 1500 proteins. These two samples had the fewest proteins identified among the rest. In the plasma samples, Control ID 192 post-OLV had the fewest proteins identified, with just above 600.

The log₂ intensities of the proteins relative to density and cumulative fraction of missing values of both lung BALFs and plasma samples are shown in Supplementary Figures 2A-C. The density vs log₂ intensity distributions indicate that the highest densities of missing values had lower log₂ intensities compared to non-missing values. This behaviour is further substantiated by the shift in log₂ intensity of missing values toward the left and of non-missing values toward the right when plotted against the cumulative fraction. Hence, the missing protein values are due to a lower protein signal.

The remaining missing values were imputed with the default settings used in the Perseus software.¹⁹⁹ This imputation approach replaces missing values with “random numbers drawn

from a normal distribution of 1.8 standard deviation down shift and with a width of 0.3 of each sample” as described in the missing value imputation options by Nesvilab.²⁰⁰ This missing value imputation technique is suitable for proteins missing not at random by replacing them with imputed values near lower limit of detection. The impact of various imputation strategies is shown in Figure 6A-C below.

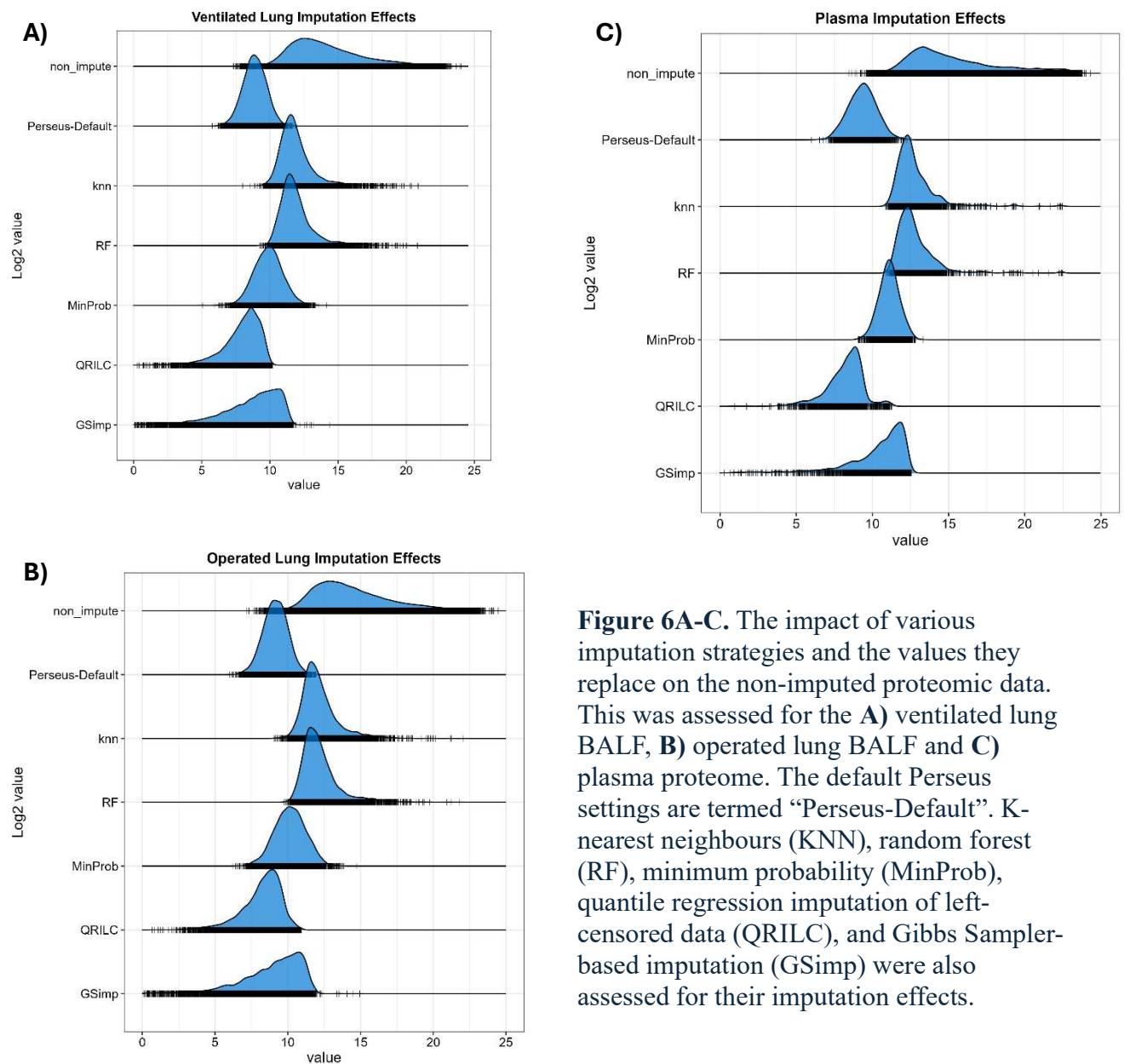
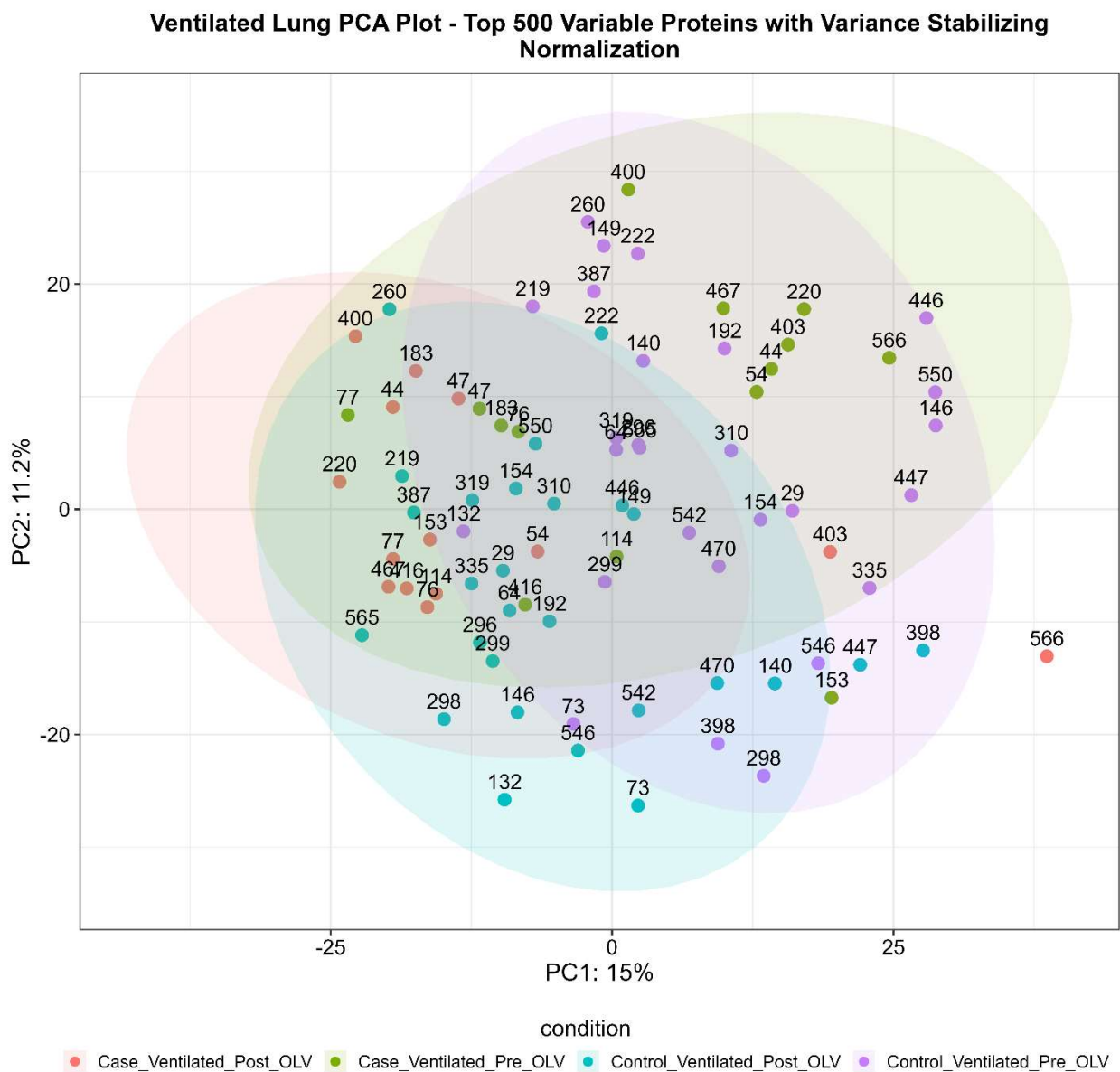


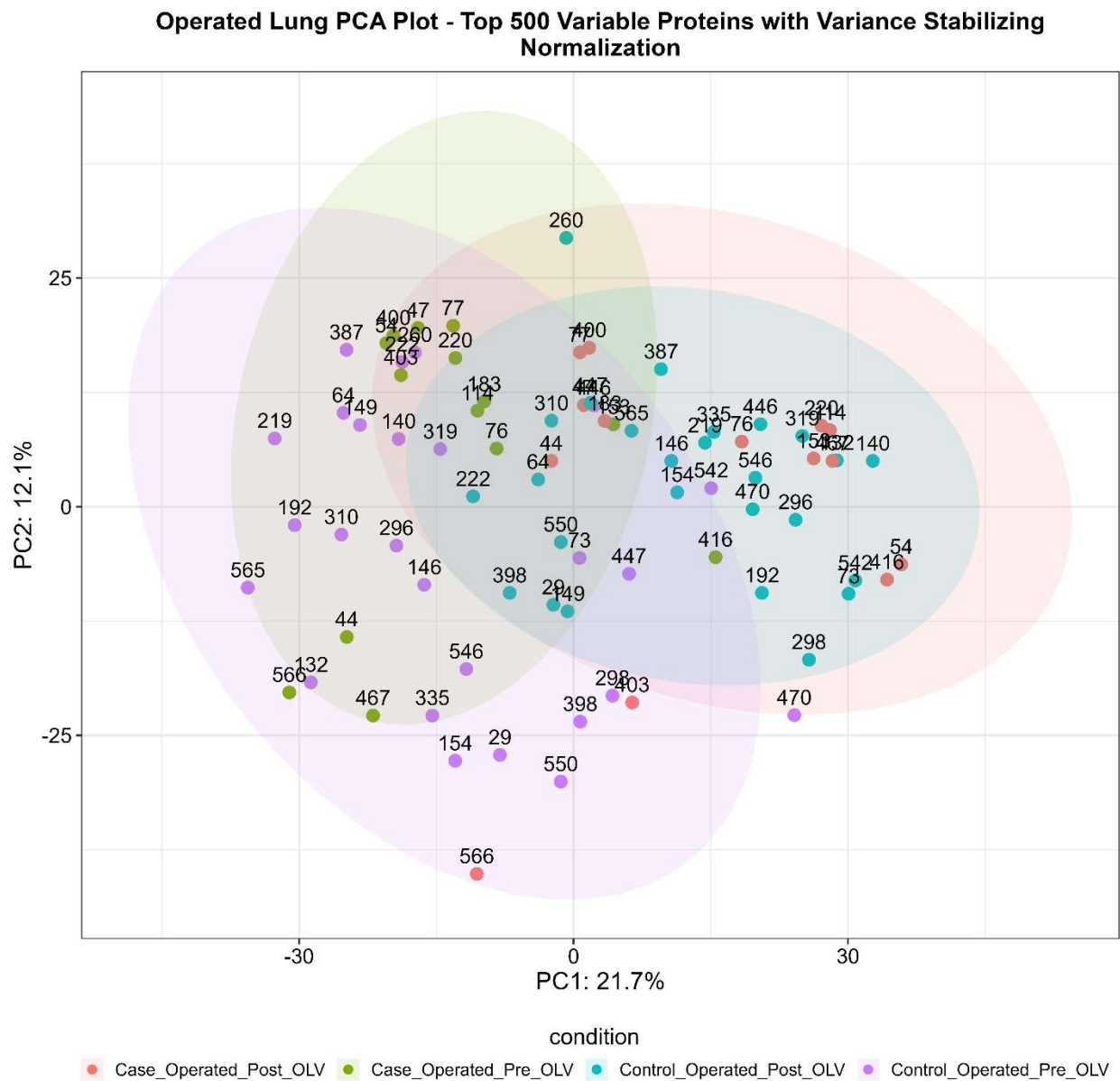
Figure 6A-C. The impact of various imputation strategies and the values they replace on the non-imputed proteomic data. This was assessed for the **A)** ventilated lung BALF, **B)** operated lung BALF and **C)** plasma proteome. The default Perseus settings are termed “Perseus-Default”. K-nearest neighbours (KNN), random forest (RF), minimum probability (MinProb), quantile regression imputation of left-censored data (QRILC), and Gibbs Sampler-based imputation (GSimp) were also assessed for their imputation effects.

The resulting imputed data were normalized using the variance stabilizing normalization approach offered through the DEP2 package. This approach equalizes the variance across

features so that higher protein expression does not lead to higher variance.²⁰¹ The impact of applying Variance Stabilizing Normalization (VSN) is visualized through Principal Component Analysis (PCA). The PCA of the top 500 most variable proteins in the ventilated and operated lung and plasma proteome before applying VSN is shown in Supplementary Figures 3A-C. The PCA after applying VSN in the ventilated and operated lung and plasma proteome is shown through the PCA in Figures 7A-C.



B)



C)

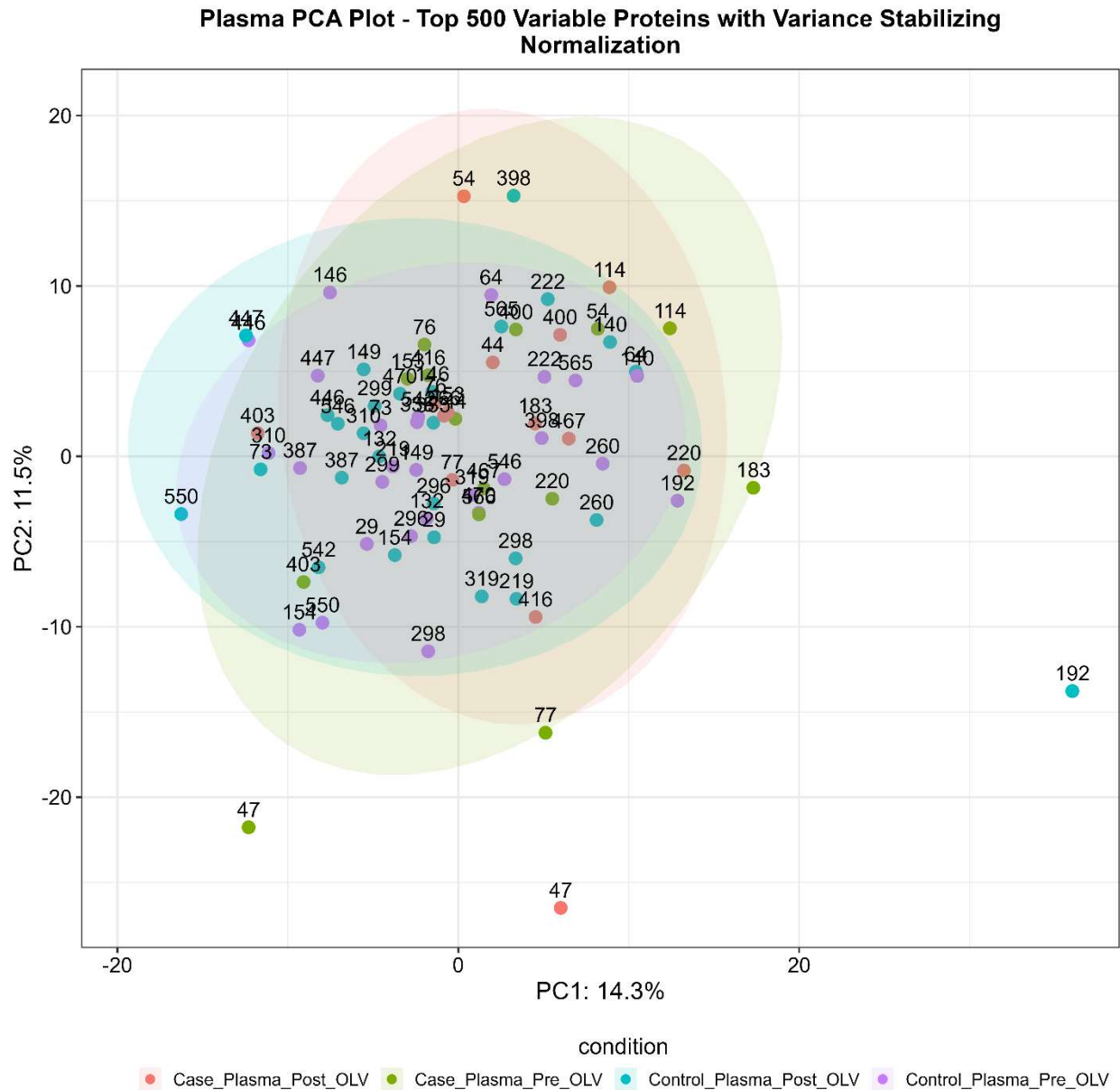


Figure 7A-C. Principal Component Analysis displaying the variation in pre- and post-OLV proteomes after applying variance stabilizing normalization. **A)** ventilated lung BALF, **B)** operated lung BALF and **C)** plasma proteome.

Differential Protein Expression Analysis

After applying variance stabilizing normalization, the normalized proteomics dataframe was extracted from the DEP2 SummarizedExperiment object. This extracted dataframe was passed to the variancePartition package to quantify the variation in proteomic data due to

multiple factors¹⁸⁷. The following formula was used to fit the variance partition model: Protein Intensity $\sim (1|\text{condition}) + \text{Age} + (1|\text{Sex}) + \text{BMI} + (1|\text{strata}) + (1|\text{replicate}) + (1|\text{strata:replicate})$. In this formula, condition, sex, BMI, strata representing the case-control matched pairs, replicate representing the patient ID assigned to each individual or biological replicate, and the interaction term between strata and replicate were all set as random effects.

This formula was implemented on metadata yielding fourteen case-control matched pairs (strata) and forty-one unique patient IDs (replicates), each represented twice to account for the repeated-measures design in the OLV timepoint variable. Age, sex, and BMI were included in this model because they were not well-matched, as shown in Figure 8. FEV1/FVC and smoking status were not accounted for in this model because there were missing values on the patient charts for these variables.

Differential expression analysis was performed at the protein level using the differential analysis for repeated measures designs (dream) function, which is part of the variancePartition package.²⁰² The following formula was used to fit the dream model: Protein Intensity $\sim 0 + \text{condition} + \text{Age} + (1|\text{Sex}) + \text{BMI} + (1|\text{strata/replicate})$. In this model, the strata and replicate interaction term was included to account for the matched case-control design with repeated measures at OLV beginning and end.

To compare between post-vs-pre-OLV timepoints in respiratory failure cases and matched controls, the makeContrastsDream function was used. Three contrasts were generated: Case Post-OLV – Case Pre-OLV, Control Post-OLV – Control Pre-OLV, and (Case Post-OLV – Case Pre-OLV) – (Control Post-OLV – Control Pre-OLV). These contrasts were made using the dream model formula. The differential protein expression was generated by combining the dream formula with the three contrasts. This differential protein expression was supplemented by the Kenward–Roger hypothesis test to increase statistical power due to the small sample size of respiratory failure individuals.^{202,203} Even though our differential expression analysis worked for most proteins, a few failed to converge due to negative eigenvalues. The proteins that failed model fitting using Dream are listed in Supplementary Table 1.

Statistical significance for differential expression was determined using Benjamini-Hochberg (BH) adjusted p-values < 0.05 and fold change ≥ 2 for the ventilated and operated lung BAL proteome. A fold change of two was set arbitrarily due to the local impact of lung injury.

Hence, larger differences are expected to be observed post-vs-pre-OLV BALF sample comparisons. A fold-change threshold of two, however, may not capture the impact of proteins that may have large biological impact with small fold changes. To account for this a separate comparison post-vs-pre-OLV was performed in BALF samples with a fold-change threshold of 1.5. The statistically significant proteins with this threshold that were not present in a fold-change threshold of 2 are included in Supplementary Table 2.

The plasma differential protein expression statistical significance threshold was changed from that of the BALF proteome by lowering the fold-change threshold to 1.5. This was done to account for the distal impact of OLV surgery on the systemic profile. The BALF sample from the operated and ventilated lung is closer to the OLV surgery impact, whereas plasma sampling via the patients' arterial line is farther from this impact. Differential protein expression across all three contrasts was visualized using a volcano plot from the package EnhancedVolcano.¹⁹⁶

The sample-specific differential protein expression was visualized across all four conditions, respiratory failure cases, and matched controls using clustered and unclustered heatmaps. The ComplexHeatmap package was used to generate heatmaps.^{184,185} To show differential expression in either respiratory failure case or matched control samples, the top 25 most significantly differentially expressed proteins were z-score normalized and visualized in heatmaps. Heatmap row and column clustering was performed using the "ward.D2" method and Euclidean distances. Clustered heatmaps used both row- and column-wise clustering, whereas unclustered heatmaps used only row-wise clustering.

On the differentially expressed proteins from case post-vs-pre-OLV and control post-vs-pre-OLV comparisons, the gene symbols were converted to their respective Uniprot IDs for gene set enrichment analysis (GSEA) using the clusterprofiler package.^{188–191} The UniProt IDs were converted to Entrez IDs using the human genome-wide annotation "org.Hs.eg.db". Distinct Entrez IDs from entire dataset of proteins along with their foldchanges (post-vs-pre-OLV) in respiratory failure cases and matched controls were used for GSEA. GSEA was performed to identify enriched gene sets for gene ontology (GO) terms in biological processes (BP), molecular function (MF), and cellular component (CC).^{204,205} Additionally, GSEA was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG)^{206–208} and Reactome^{209,210} databases. For all GSEA analyses, the minimum gene set size was 5, and the maximum was 800. The number of

permutations was set to 10,000. A statistical significance threshold of adjusted $p < 0.05$ was set, and p-values were adjusted using Benjamini-Hochberg (BH) false discovery rate (FDR) control.

Multivariate Analyses based on Normalized Protein Data

Since we are measuring thousands of proteins across about 40 or 41 patients' samples repeatedly before and after OLV, we anticipated collinearity among the measured proteins, which can be better explained by dimensionality reduction techniques such as Partial Least Squares Discriminant Analysis (PLS-DA). PLS-DA transforms highly correlated variables into latent components that explain variation across the conditions of interest, making it a supervised approach compared to PCA. Furthermore, we implemented sparse feature selection in our PLS-DA by selecting proteins that best discriminate between conditions and then applying PLS-DA. This approach was made possible by using the mixOmics package.¹⁸⁶ The VSN normalized proteome data were passed to mixOmics for sPLS-DA analysis, accounting for repeated measurements pre- and post-OLV. The data were split 50/30 for feature selection. This method was implemented in the ventilated and operated lung and the plasma proteome.

Our sPLS-DA analysis did not take into account the nested case-control design and could be driven by post-vs-pre-OLV changes. Hence, to identify proteins that can discriminate between cases and controls while taking into account the nested case-control design. An elastic net conditional logistic regression model was fit using the clogitL1 package¹⁹⁵ across alpha values from 1 to 10 on the post-vs-pre-OLV ratios of log2 transformed and VSN normalized proteomic data. Each model fit was 10-fold cross-validated. The model with alpha and lambda values that yielded the minimum cross-validated deviance plus one standard error, from clogitL1, was used to fit the final model with the penalizedclr package. In the final model, the proteomic covariates were standardized in penalizedclr. Stability selection was performed using the same alpha and lambda inputs, and a selection probability threshold (π_{thr}) of 0.55 – 0.9 was set. Stability selection runs the elastic net analysis on subsamples ($B = 100$) from the overall sample size and identifies how frequently each variable is selected (selection probability, π_{thr}).²¹¹ The top 15 non-zero regression coefficients were plotted to visualize the association of the top 15 selected proteins with either postoperative respiratory failure cases or matched controls.

Chapter V: Results

Clinical Variables of Case and Control Groups

Respiratory failure cases and matched controls were matched on variables that reflect preoperative risk based on preoperative lung function and intraoperative risk based on the extent of tissue resected. The differences between cases and matched controls based on these variables are shown in Table 1. Some preoperative variables that could indicate increased risk of respiratory failure or worse postoperative outcomes, such as lower mean DLCO, FEV1, and FEV1/FVC, and higher mean BMI and percentage of current smokers, were more common in respiratory failure cases. To further understand and visualize differences between cases and controls based on matched covariates, a love plot (Figure 8) was prepared to assess covariate balance. However, there was no statistically significant difference between these variables and the intraoperative variables that were matched on. Nevertheless, age, sex and BMI were adjusted for in our analysis. Smoking history and FEV1/FVC were not adjusted for due to missing values for some patients.

To visualize the patient metadata distribution in a PCA taking into account the matched categorical and continuous variables, we performed a factor analysis of mixed data (FAMD) shown in Figure 9. Across the x-component (dimension 1), there was no variation explained by respiratory failure status, whereas, in the y-component (dimension 2), there was a small variation accounted by respiratory failure status.

Table 1. Differences in matched covariates across postoperative respiratory failure and matched control groups

Characteristic	Postoperative Respiratory Failure Status		p-value ²
	Case N = 14 ¹	Control N = 27 ¹	
Surgery Type			0.7
Open	5 (36%)	7 (26%)	
VATS	9 (64%)	20 (74%)	
Resection Extent			>0.9
Bilobectomy	0 (0%)	3 (11%)	
Bilobectomy + Wedge	1 (7%)	1 (4%)	
Lobectomy	8 (57%)	14 (52%)	
Lobectomy + Wedge	0 (0%)	1 (4%)	
LUL Lobectomy	4 (29%)	6 (22%)	
Wedge	1 (7%)	2 (7%)	
Sex			0.2
Female	4 (29%)	14 (52%)	
Male	10 (71%)	13 (48%)	
Age (years)	68.4 (8.2)	71.8 (7.5)	0.2
DLCO (%)	62.9 (16.0)	63.3 (12.3)	0.8
FEV1 (%)	72.0 (13.6)	75.0 (14.6)	0.4
FEV1/FVC (%)	78.8 (15.8)	86.1 (13.2)	0.12
Unknown	0	1	
BMI	29.1 (6.8)	26.7 (5.6)	0.3
Smoking Status			0.2
Current	9 (69%)	10 (37%)	
Nonsmoker	0 (0%)	2 (7%)	
Quit	4 (31%)	15 (56%)	
Unknown	1	0	
Smoking Pack-Years	38.1 (18.3)	37.5 (22.5)	0.8

¹n (%); Mean (SD)

²Fisher's exact test; Pearson's Chi-squared test; Wilcoxon rank sum test

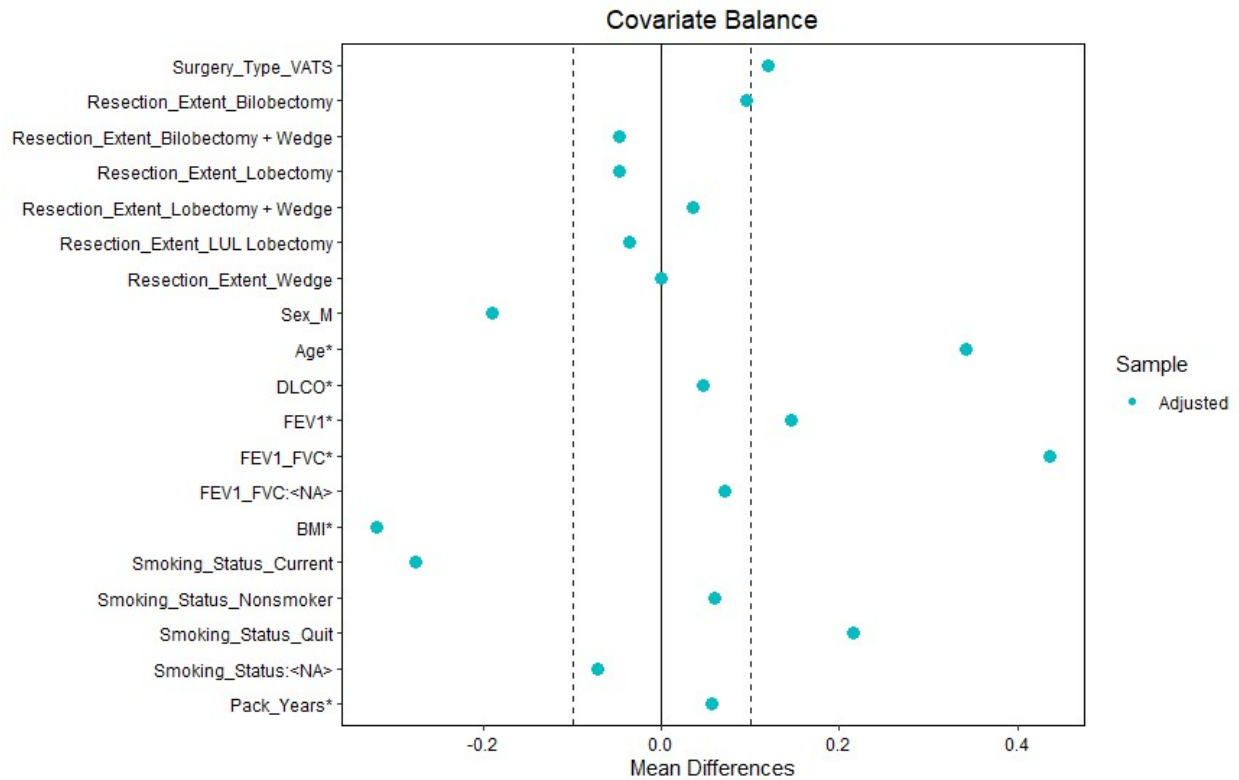


Figure 8. Love plot visualizing the mean differences in matching variables used between respiratory failure cases and matched controls. Continuous variables were standardized (*) to allow comparison between the two groups.

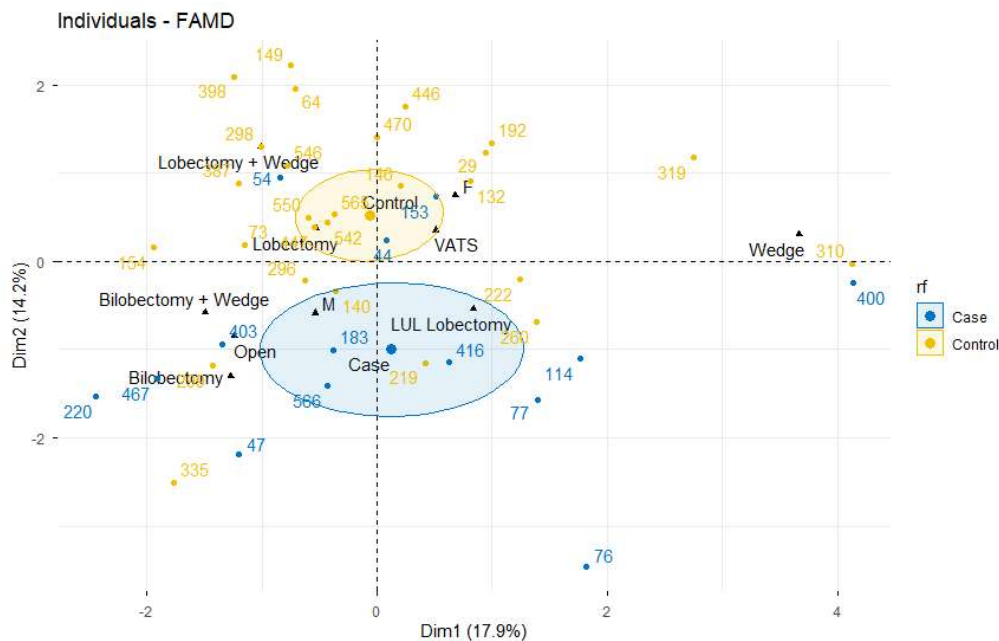


Figure 9. Factor analysis of mixed data performed on patient metadata used for case-control matching.

Ventilated Lung Proteome Analysis

Variance Partition Analysis

Variance partition analysis was performed to quantify multiple sources of variation by using a linear mixed effects model.¹⁸⁷ Thus, the impact of each variable included in the formula: $\text{Intensity} \sim (1|\text{condition}) + \text{Age} + (1|\text{Sex}) + \text{BMI} + (1|\text{strata}) + (1|\text{replicate}) + (1|\text{strata:replicate})$ on protein intensity or abundance was calculated as variance explained percentage. The percentage of variance explained by each of these variables: condition (case post-OLV, case pre-OLV, control post-OLV, and control pre-OLV), age, sex, BMI, strata (matched pair ID for each case and control), replicate (patient ID), and strata and replicate interaction, is shown in Figure 10 below.

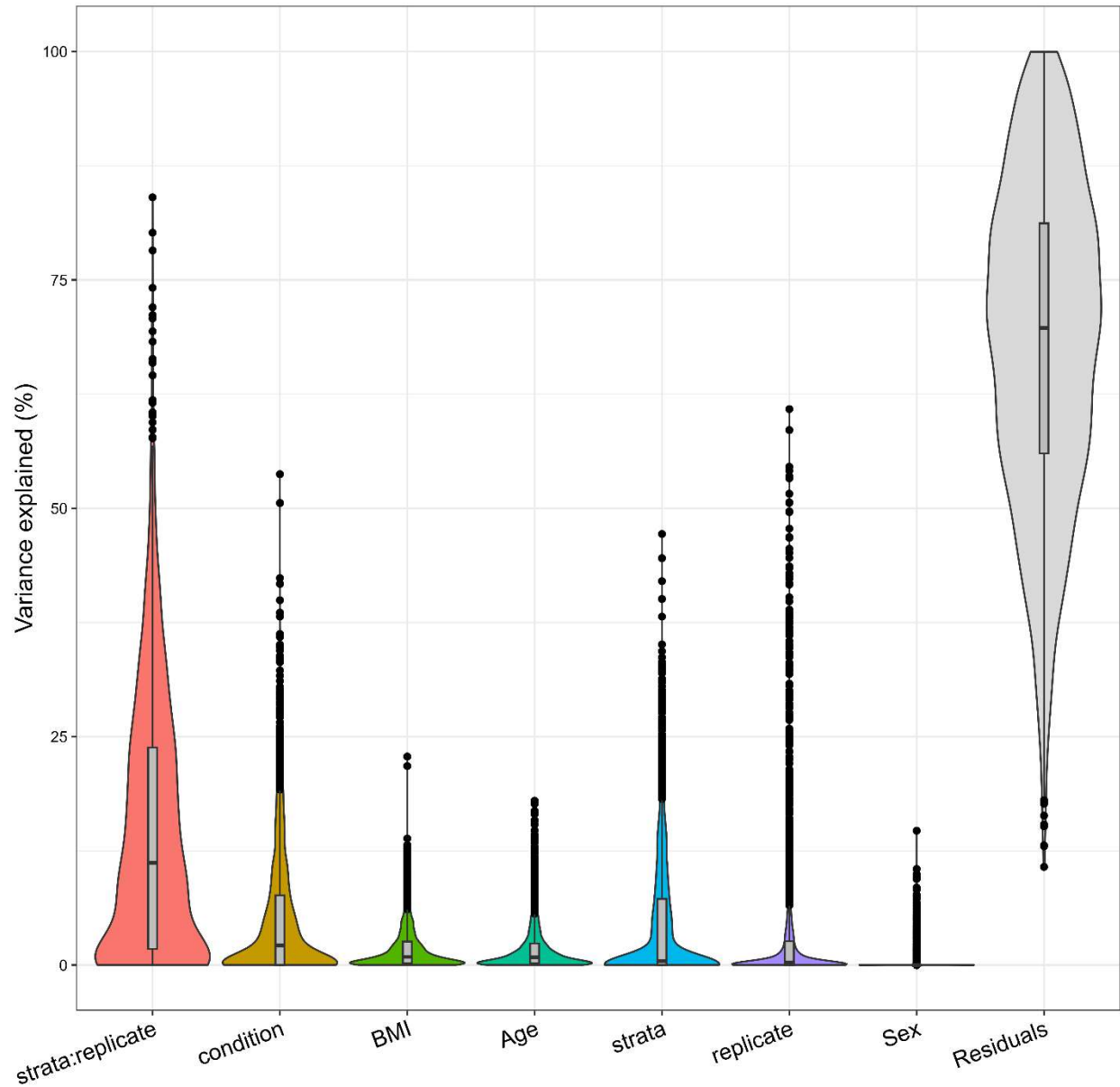


Figure 10. Percentage of variance explained by each covariate in the variance partition model in the ventilated lung BALF proteome. Each dot represents a protein described by variance explained values.

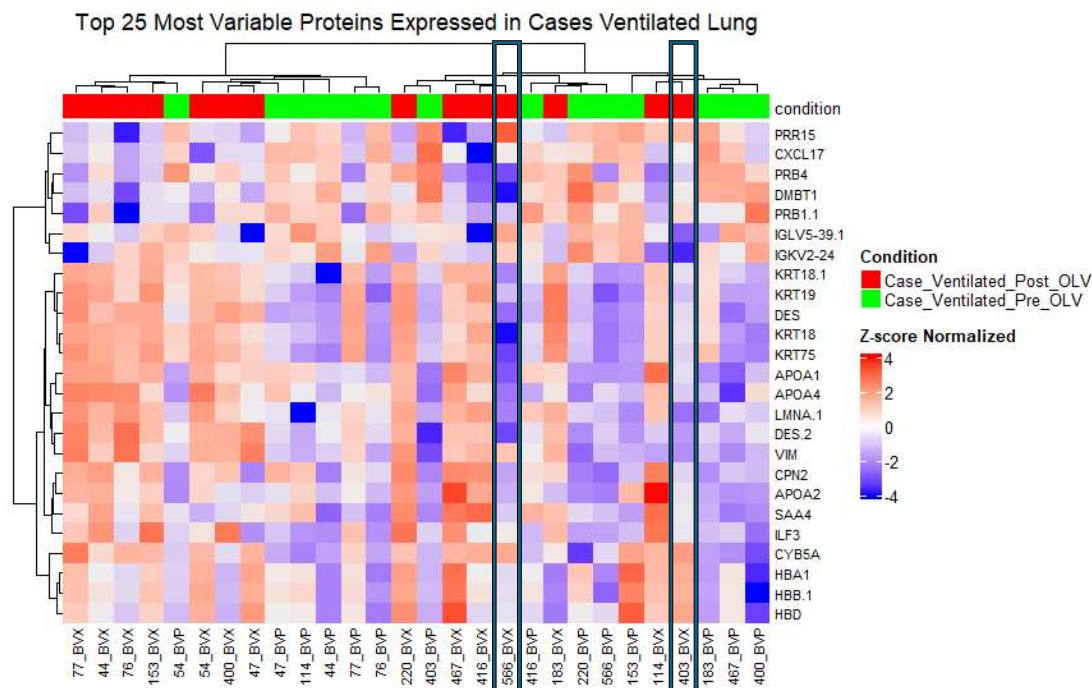
Among the variables included in the model, the strata (matched pairs) and replicate (patient ID) interaction term accounts for the greatest percentage of variance. This interaction term accounts for both case-control matched pairs design along with the repeated measures design. Thus, within the linear model each patient is measured twice and is nested within a matched pair. Hence, patient 467 and patient 219 belong to the same matched pair while

accounting for repeated measurements (pre- and post-OLV) for both patients. This ensures that each repeated patient ID is not going assumed to be independent. Further, the postoperative respiratory failure status and OLV timepoint interaction term described by “condition” accounts for the second most variation in this model. BMI and age show the third- and fourth-highest variance percentages, and these parameters were poorly matched between respiratory failure cases and their matched controls. Strata and replicate variables tend to have much lower contributions to the variance percentage, while biological sex has a negligible contribution. The residuals of the variance partition model account for the highest variance percentage. Thus, the majority of the factors contributing to the variance in ventilated lung BALF proteome intensity were not accounted for in our model. These factors could be OLV duration, use of intercoastal vs IV steroids including dose, differences in ventilation parameters such as Vt, PEEP, PIP, and FiO₂.

Differential Protein Expression Presented in Heatmaps

The differential expression of the top 25 differentially expressed proteins across all samples was visualized with clustered and unclustered heatmaps for cases and control conditions post-vs-pre-OLV. The clustered heatmap in Figure 11A shows respiratory failure case samples whose protein expression profiles group similarly based on z-score patterns across pre- and post-OLV samples. Looking at the unclustered heatmap in Figure 11B below, we observed a more consistent expression profile across conditions. However, we can further appreciate this when comparing this heatmap with its clustered version (Figure 11A), as some samples show inconsistent directionality in protein expression. Two of these samples are post-OLV samples for patients 566 and 403, ventilated lung. These samples show a strikingly opposite relationship relative to other ventilated lung post-OLV samples. 566 post-OLV had the lowest number of proteins detected and was an outlier in PCA. 403 post-OLV had slightly lower number of proteins detected compared to other post-OLV respiratory failure samples as well but not to the same level of 566.

A)



B)

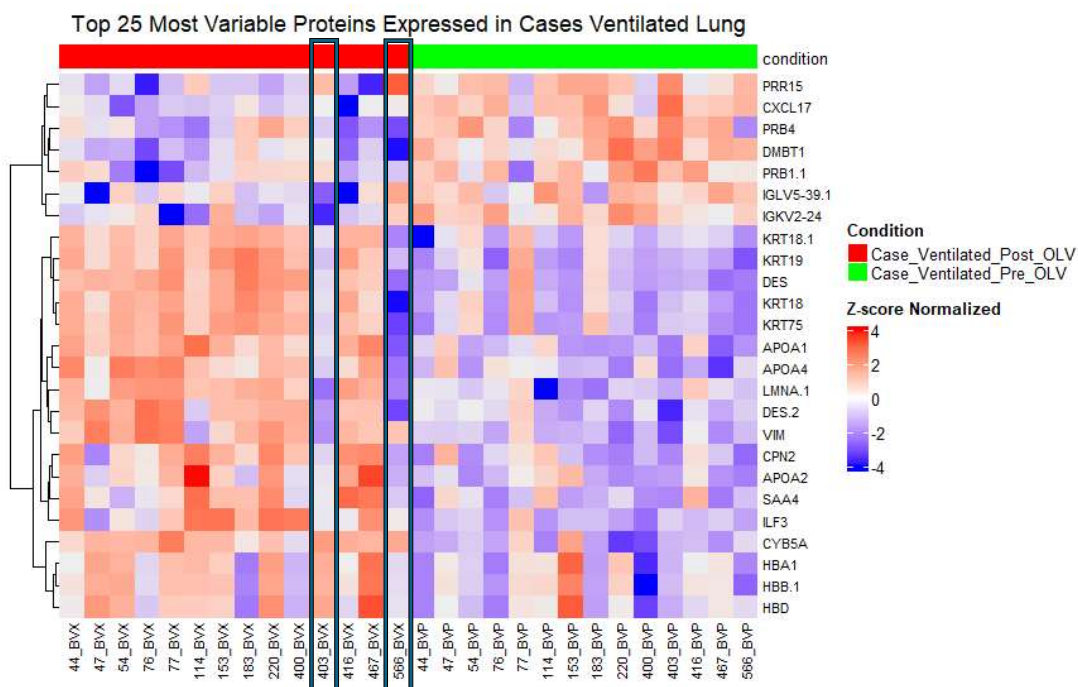
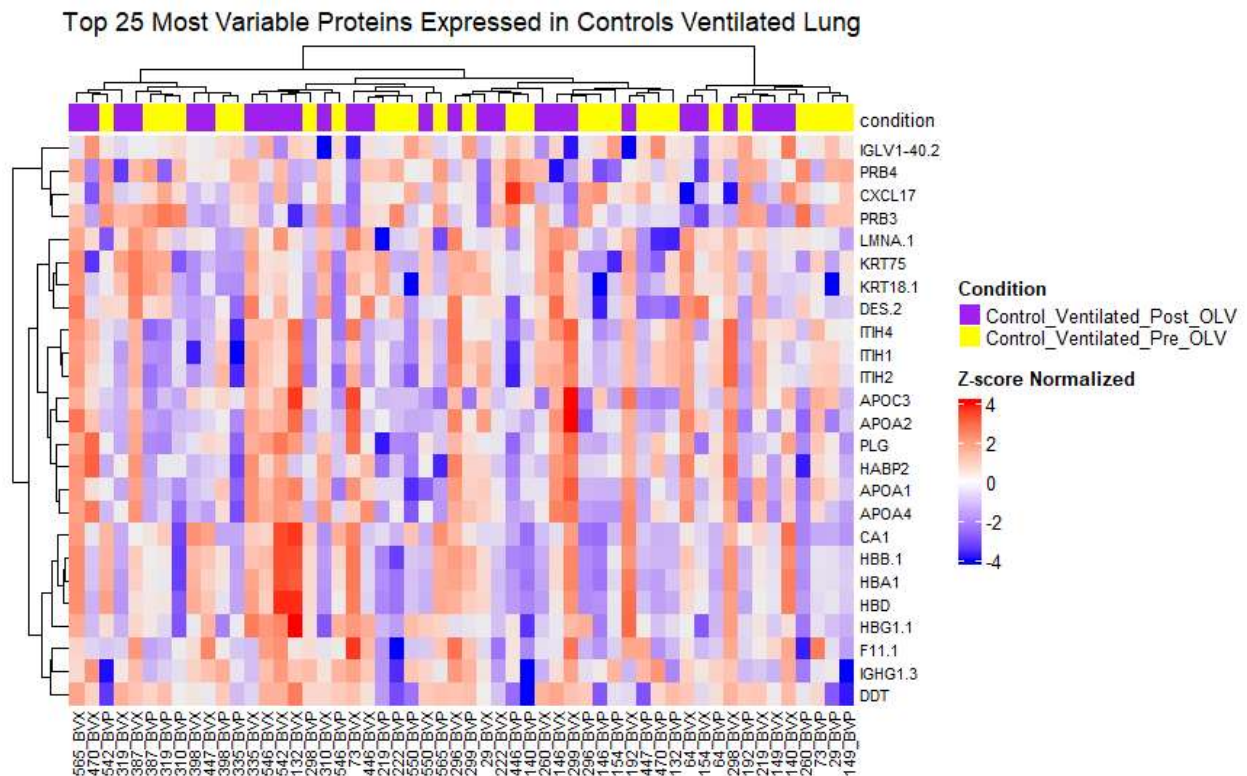


Figure 11A-B. Heatmap of the top 25 differentially expressed proteins in respiratory failure ventilated lung BALF samples at pre- and post-OLV timepoints. **A)** clustered heatmap and **B)** unclustered heatmap. Post-OLV samples 403 and 566 were highlighted as they had opposing pattern of protein expression relative to other post-OLV samples.

Proteins such as prelamin-A/C (LMNA), Cytochrome B5A (CYB5A), and Vimentin (VIM) are consistently upregulated across all post-OLV samples in respiratory failure cases. Whereas DMBT1 and CXCL17 proteins tend to be downregulated. Lamin A/C proteins are intermediate filament proteins which make up the nuclear lamina.²¹² Studies have shown that LMNA proteins have been implicated in idiopathic pulmonary fibrosis and ventilation-induced lung injury.^{213,214} Another BALF proteomic study has shown CYB5A as potential early biomarkers of ALI in a mouse model of *S. aureus* exposure.²¹⁵ Both of these biomarkers have shown overexpression in our unclustered heatmap in respiratory failure cases post-vs-pre OLV, Figure 11B. Looking at downregulated proteins, DMBT1 has been associated with surface tension^{216,217} and its downregulation may impair surfactant surface tension maintenance. CXCL17 is a chemokine known to attract macrophages in the lung.²¹⁸

Based on the top 25 differentially expressed proteins across the two timepoints (post- and pre-OLV) in matched control samples, no clustering pattern between timepoints was observed. In fact, compared with the unclustered heatmap, post-OLV samples from patients 149 and 64 appear to cluster more closely with their pre-OLV counterparts.

A)



B)

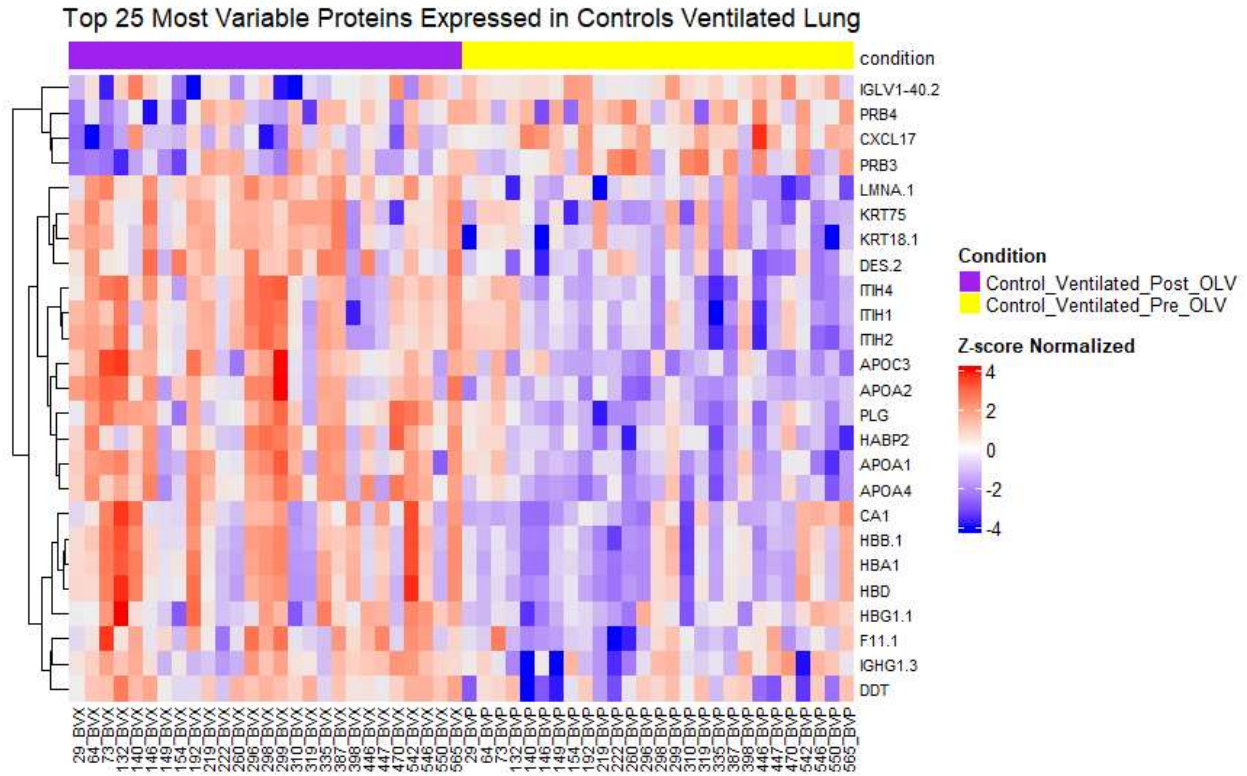


Figure 12A-B. Heatmap of the top 25 differentially expressed proteins in matched control ventilated lung BALF samples at pre- and post-OLV timepoints. **A)** clustered heatmap and **B)** unclustered heatmap.

As in respiratory failure cases, matched control post- and pre-OLV samples show upregulation of LMNA and downregulation of CXCL17, as shown in the unclustered heatmap in Figure 12B. Keratin 18 (KRT18) and Desmin (DES) protein are also upregulated in respiratory failure cases. ITIH1, 2, 3 and 4 proteins tended to be upregulated in matched control post-OLV samples and ranked among the top 25 differentially expressed proteins in matched control samples relative to respiratory failure cases. Furthermore, CCL15, a chemoattractant cytokine for leukocytes, is downregulated in post-OLV vs. pre-OLV in matched control samples.

Differential Protein Expression Presented in Volcano Plots and Gene Set Enrichment Analysis

In the case post-vs-pre-OLV timepoint contrast, there were 148 differentially expressed proteins with foldchange ≥ 2 that reached statistical significance. Out of these proteins, 73 were upregulated proteins, and 75 were downregulated. There were 102 upregulated and 160

downregulated proteins with foldchange ≥ 1.5 shown in Supplementary Table 3. Some of the top upregulated proteins, based on foldchange ≥ 2 , include KRT18, DES, interleukin enhancer-binding factor 3 (ILF3), apolipoprotein A-2 (APOA2), LMNA, carboxypeptidase N subunit 2 (CPN2), vimentin (VIM), keratin 75 (KRT75), serum amyloid A-4 (SAA4), apolipoprotein A-1 (APOA1), CYB5A and keratin 19 (KRT19). The names of these proteins, as well as their functions, were obtained from UniprotKB.¹⁵⁹ The majority of these proteins, especially keratins, lamin, desmin, and vimentin, are cytoskeletal proteins. The apolipoproteins are involved in lipid transport and metabolism. Hence, indicating that the majority of the top upregulated proteins are involved in cytoskeletal proteins being released into the extracellular space and lipid transport and metabolism.

In the 75 downregulated proteins, some of the top downregulated proteins include: immunoglobulin kappa variable 2-24 (IGKV2-24), C-X-C motif chemokine 17 (CXCL17), basic salivary proline-rich protein 1 (PRB1), deleted in malignant brain tumors 1 (DMBT1), proline-rich protein 15 (PRR15), basic salivary proline-rich protein 4 (PRB4), basic salivary proline-rich protein 3 (PRB3), G-protein coupled receptor family C group 5 member B (GPRC5B), and melanoma-derived growth regulatory protein (MIA).¹⁵⁹ The majority of the top downregulated proteins, based on log2 fold change, were the basic proline-rich proteins found in saliva and immunoglobulins. However, CXCL17 and GPRC5B are both involved in mediating inflammatory response in macrophages.^{218,219}

The differentially expressed proteins between cases post-vs-pre-OLV timepoints are summarized in the volcano plot shown in Figure 13A. Using foldchanges generated from the linear mixed effects model for the ventilated lung BALF proteome (2393 proteins), GSEA was performed to identify pathways and components. Figure 13B shows the GO Molecular Function pathways related to DNA binding, membrane transport and cytoskeleton structural constituent. Figure 13C shows the REACTOME pathways which indicate histone and chromatin modifications such as HDACs deacetylate histones and HATs acetylate histones. Similarly, the GO Biological Processes enriched pathways show that the majority of the biological processes enriched involve platelet formation through megakaryocyte differentiation and extracellular DNA. Each of these processes may be involved in wound healing or clotting, and DNA release due to neutrophil extracellular trap formation during NETosis or potential nuclear DNA release

through cell damage.²²⁰ The GSEA cellular components enriched are shown in Supplementary Figure 4A.

Complementing our GO biological and molecular function-enriched pathways, KEGG pathways enriched in respiratory failure cases post-vs-pre-OLV (in Figure 13D) included complement and coagulation cascades, and neutrophil extracellular trap formation, most notably. Enrichment of these pathways further supplements the hypothesis that coagulation and extracellular DNA release as a result of NETosis are processes that occur in post-vs-pre-OLV. Furthermore, *S. aureus* infection and systemic lupus erythematosus interactions are further enriched, further highlighting inflammatory processes similar to those observed in responses to infection and autoimmune conditions.

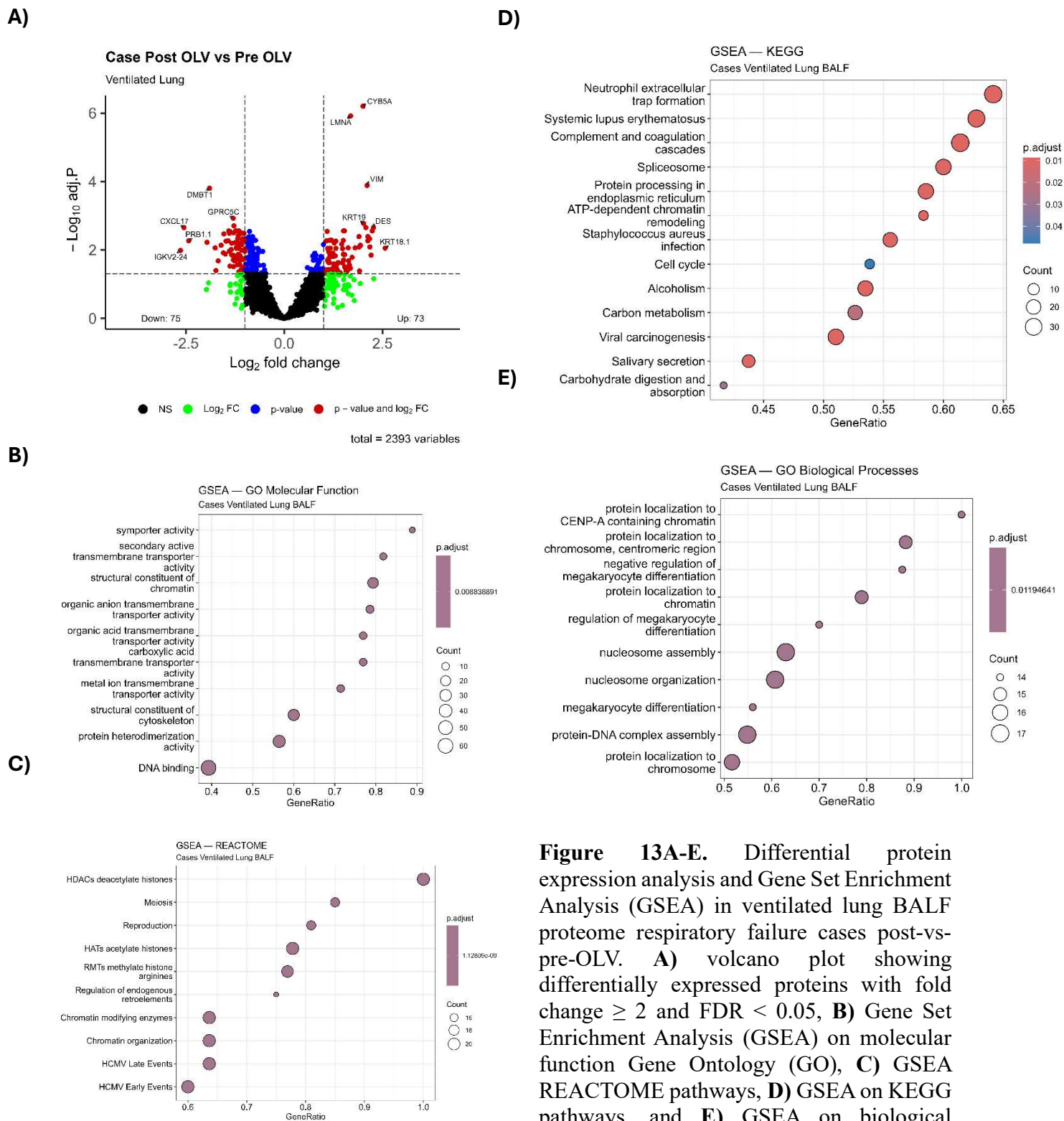


Figure 13A-E. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in ventilated lung BALF proteome respiratory failure cases post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** Gene Set Enrichment Analysis (GSEA) on molecular function Gene Ontology (GO), **C)** GSEA REACTOME pathways, **D)** GSEA on KEGG pathways, and **E)** GSEA on biological processes GO all with FDR < 0.05 .

In the matched controls, the post-vs-pre-OLV time point showed 110 proteins significantly upregulated and 58 downregulated, yielding 168 differentially expressed proteins as shown in Figure 14A. The top upregulated proteins include hemoglobin subunit delta (HBD), apolipoprotein A-4 (APOA4), keratin 18 (KRT18), prelamin-A/C (LMNA), hemoglobin subunit alpha (HBA1), apolipoprotein A-2 (APOA2 and C3), keratin 75 (KRT75), Inter-alpha-trypsin inhibitor heavy chain 2 and 4 (ITIH2 and ITIH4) and coagulation factor 11 (F11). Aside from the hemoglobin proteins, ITIH2/4 and F11, majority of the processes involving most of the proteins are related to cytoskeleton, and lipid metabolism which are also upregulated in cases post-vs-pre-OLV timepoint.

In terms of downregulated proteins, CXCL17, PRB3 and 4, serine carboxypeptidase 1 (SCPEP1), DMBT1, UDP-glucose 6-dehydrogenase (UGDH), CCL15, and pulmonary surfactant-associated protein D (SFTPD) are some of the most downregulated proteins. Most notably, SCPEP1 has been implicated in oxidative stress in the context of IPF in basal cells.²²¹ Furthermore, surfactant-associated protein D inhibition was shown to drive pro-inflammatory processes following systemic infection.²²² Surfactant treatment can reduce these pro-inflammatory processes.²²³

Regarding GSEA results, molecular functions such as nucleic acid, oxygen and haptoglobin, and heat shock protein binding were notably enriched as shown in Figure 14B. The corresponding cellular components enriched are shown in Supplementary Figure 4B. In KEGG pathways (Figure 14D), complement and coagulation cascades, neutrophil extracellular trap formation, *S. Aureus* infection, and systemic lupus erythematosus were notably enriched in matched controls as well as respiratory failure cases. Thus, indicating that the same inflammatory pathways may be happening in the matched controls as well as respiratory failure cases. Protein localization to chromatin and megakaryocyte differentiation biological processes were also notably enriched in matched controls (Figure 14E). In REACTOME pathways (Figure 14C), similar pathways were enriched between matched controls and respiratory failure cases with regulation of RNA expression pathway being enriched in matched controls.

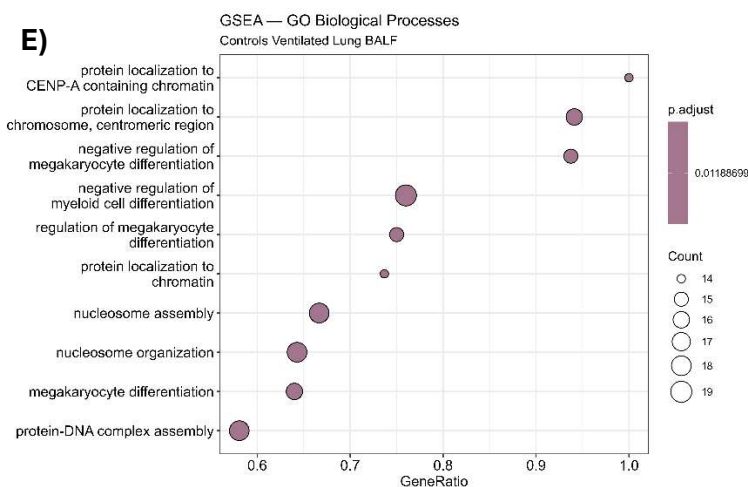
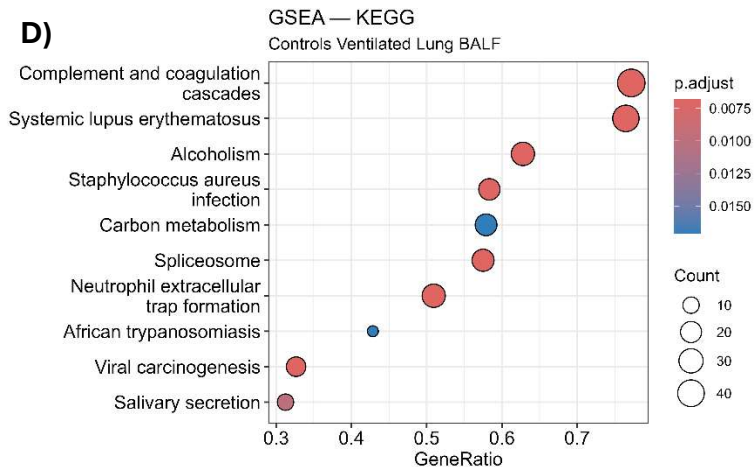
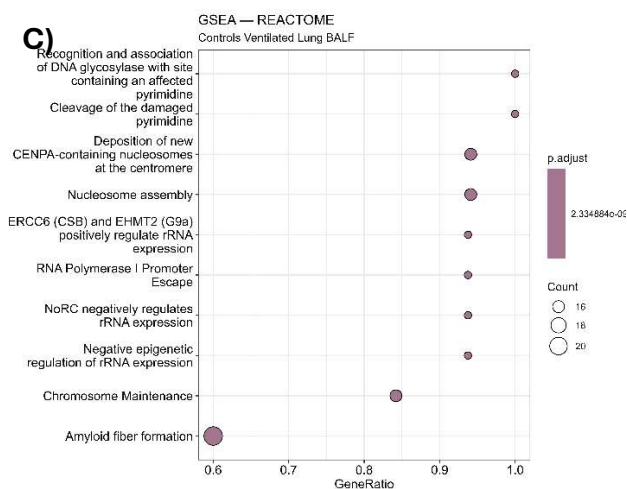
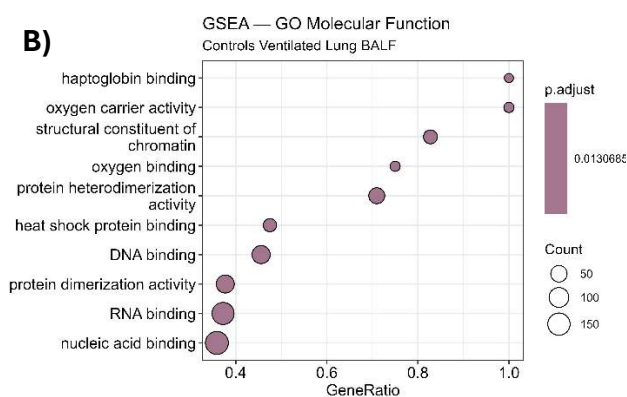
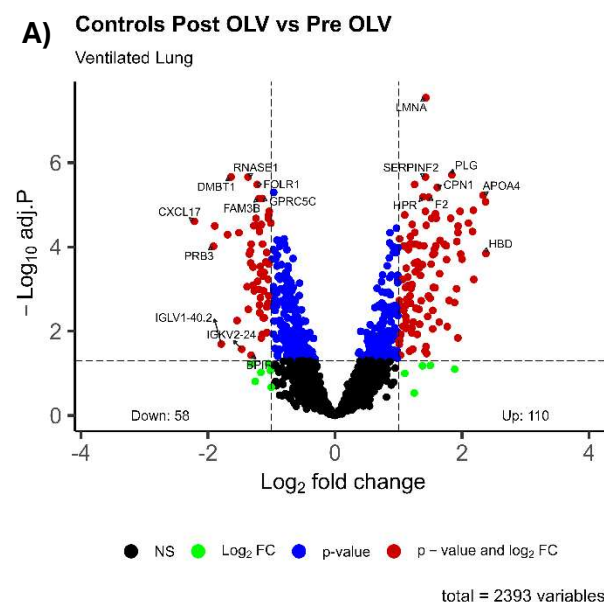


Figure 14A-E. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in ventilated lung BALF proteome matched controls post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** Gene Set Enrichment Analysis (GSEA) on molecular function Gene Ontology (GO), **C)** GSEA REACTOME pathways, **D)** GSEA on KEGG pathways, and **E)** GSEA on biological processes GO all with FDR < 0.05 .

To identify proteins that may be associated with respiratory failure, proteins that differ between respiratory failure cases and matched controls post-vs-pre-OLV based on their log2 vsn-normalized abundance are shown in Figure 15 below. Proteins such as PSME2, ENPP2, ILF3, PDIA6 and SLC5A8 are statistically significant with foldchange ≥ 2 in respiratory failure cases post-vs-pre-OLV while ILF3.1, and SFTPD was statistically significant in matched controls post-vs-pre-OLV. Notably, ILF3 was upregulated in respiratory failure cases while ILF3;STRBP (ILF3.1) protein group was upregulated in matched controls. ANXA1, PLTP. And GYG1 were not statistically significant in either of the comparisons.

Potentially respiratory failure associated proteins

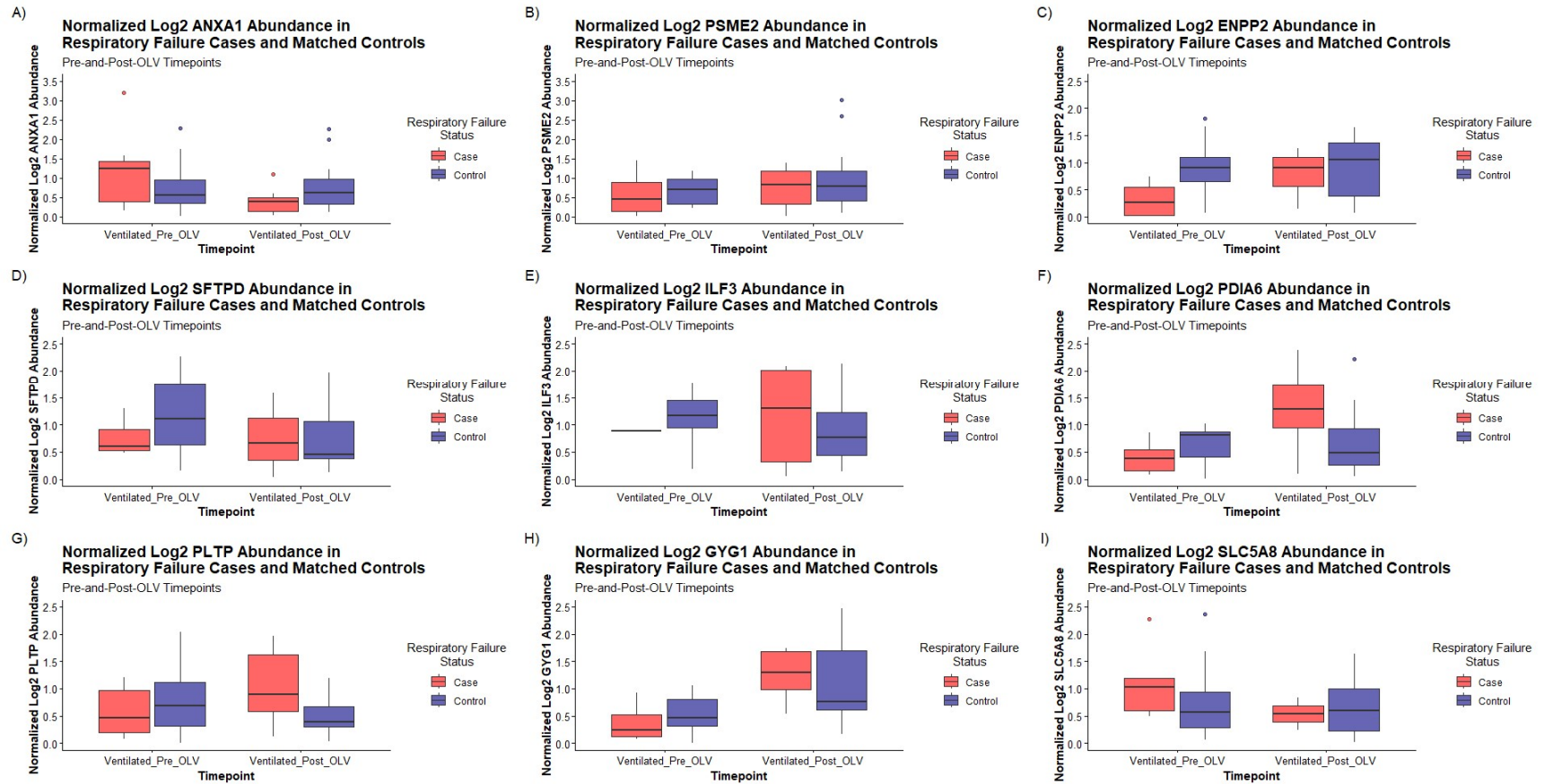


Figure 15. VSN-normalized log2 abundances for ventilated lung BALF proteins that could potentially be associated with respiratory failure risk and candidates for validation studies. Ventilated lung BALF protein abundances were plotted for pre- and post-OLV timepoints based on respiratory failure status which includes respiratory failure cases and matched controls.

Other proteins that showed differential expression post-vs-pre-OLV could potentially be associated with ventilation. Their role in development of postoperative respiratory failure may not be determined solely based on the directionality of their expression. These proteins are shown below in Figure 16. LMNA, CYB5A, VIM, DES, APOA4, and SERPINI1 were both statistically significant with foldchange ≥ 2 in both respiratory failure cases and matched controls. BCLAF1 was only statistically significant in respiratory failure cases.

Potentially ventilation-related proteins

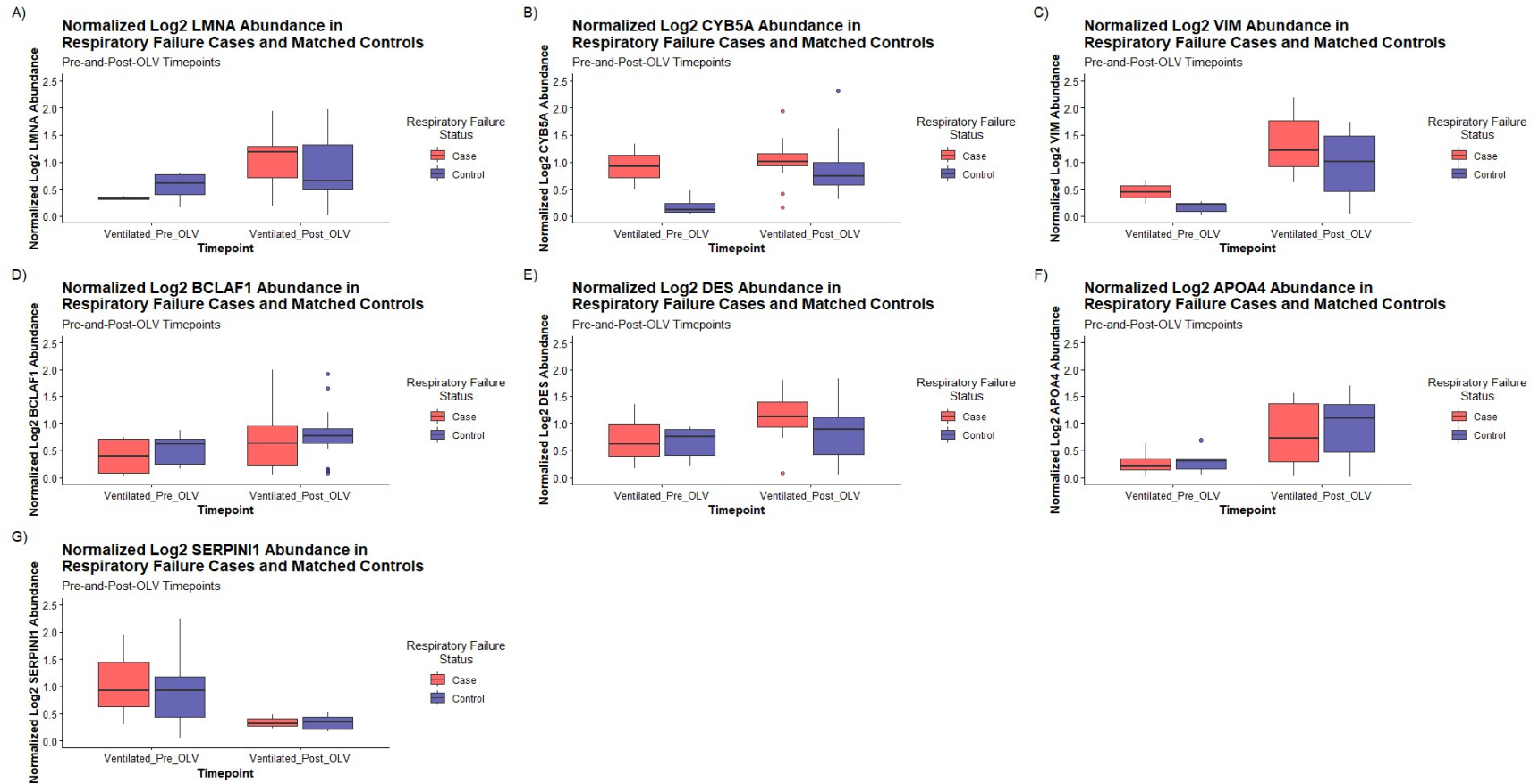


Figure 16. VSN-normalized log₂ abundances for ventilated lung BALF proteins that whose differential expression could be associated with ventilation. Ventilated lung BALF protein abundances were plotted for pre- and post-OLV timepoints based on respiratory failure.

One of the KEGG pathways enriched in GSEA in both respiratory failure and matched controls is neutrophil extracellular trap formation. Proteins that may be associated with neutrophil degranulation, and neutrophil extracellular trap formation are shown in Figure 17. ITGB2, RAC2, ITGAM, H4C1, H3-3A.1 were statistically significant with foldchange ≥ 2 in respiratory failure cases, whereas H4C1, and ITGAM were in matched controls.

Neutrophil extracellular trap formation and degranulation related proteins

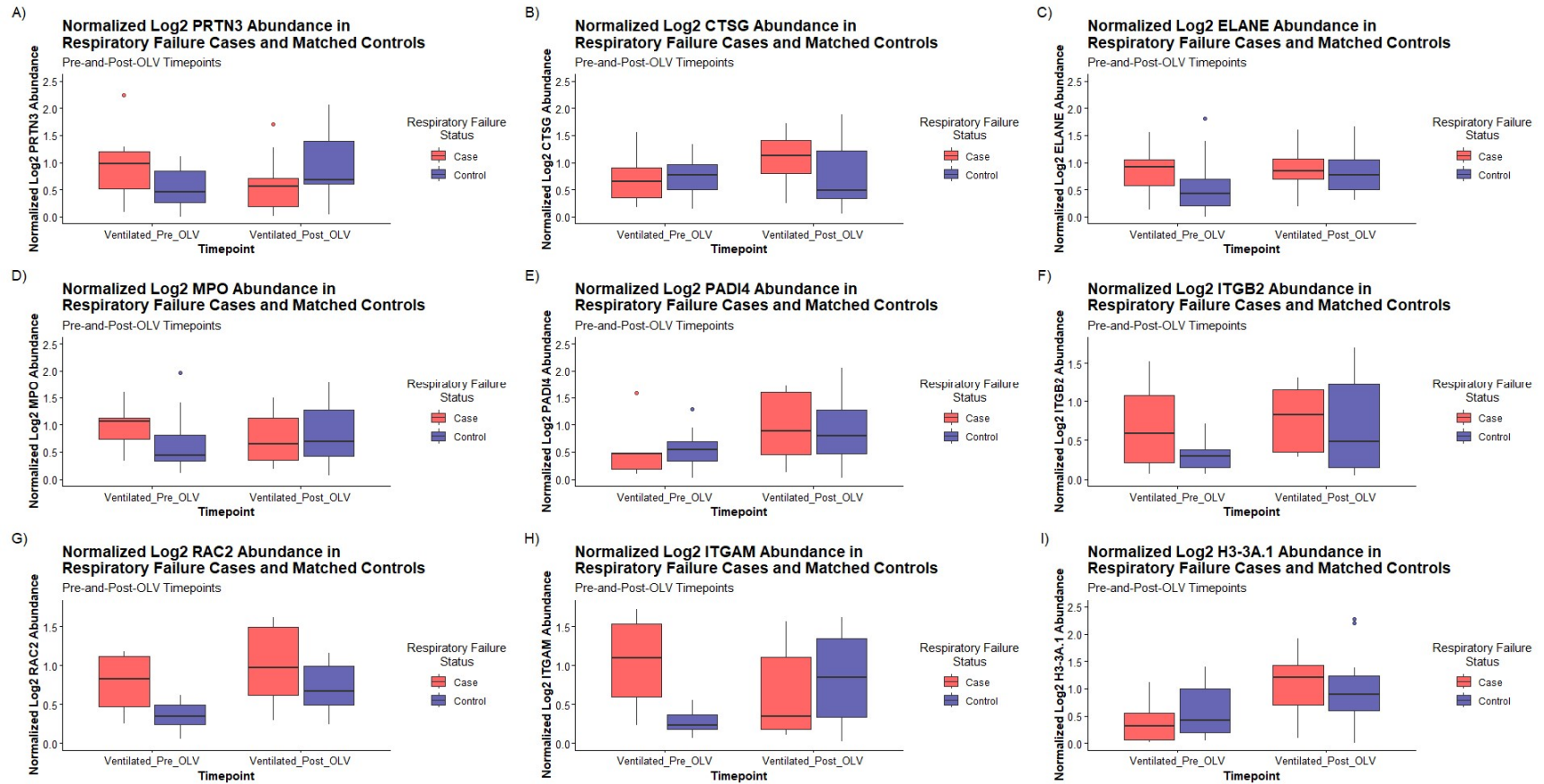


Figure 17. VSN-normalized log2 abundances for ventilated lung BALF proteins associated with neutrophil degranulation and extracellular traps. Ventilated lung BALF protein abundances were plotted for pre- and post-OLV timepoints based on respiratory failure.

With regards to inflammation, cytokines, chemokines and select proteins that are associated with cytokines and inflammation are shown in Figure 18. CCL15, and CXCL17 were downregulated in both respiratory failure cases and matched controls post-vs-pre-OLV with foldchange and statistical significance thresholds. CXCL10 was downregulated in respiratory failure cases, while IL6ST was downregulated in matched controls while meeting thresholds. CXCL1, CXCL6, IL1RAP, CRP and MAPK1 did not reach statistical significance.

Detected cytokines, chemokines and inflammation-related proteins

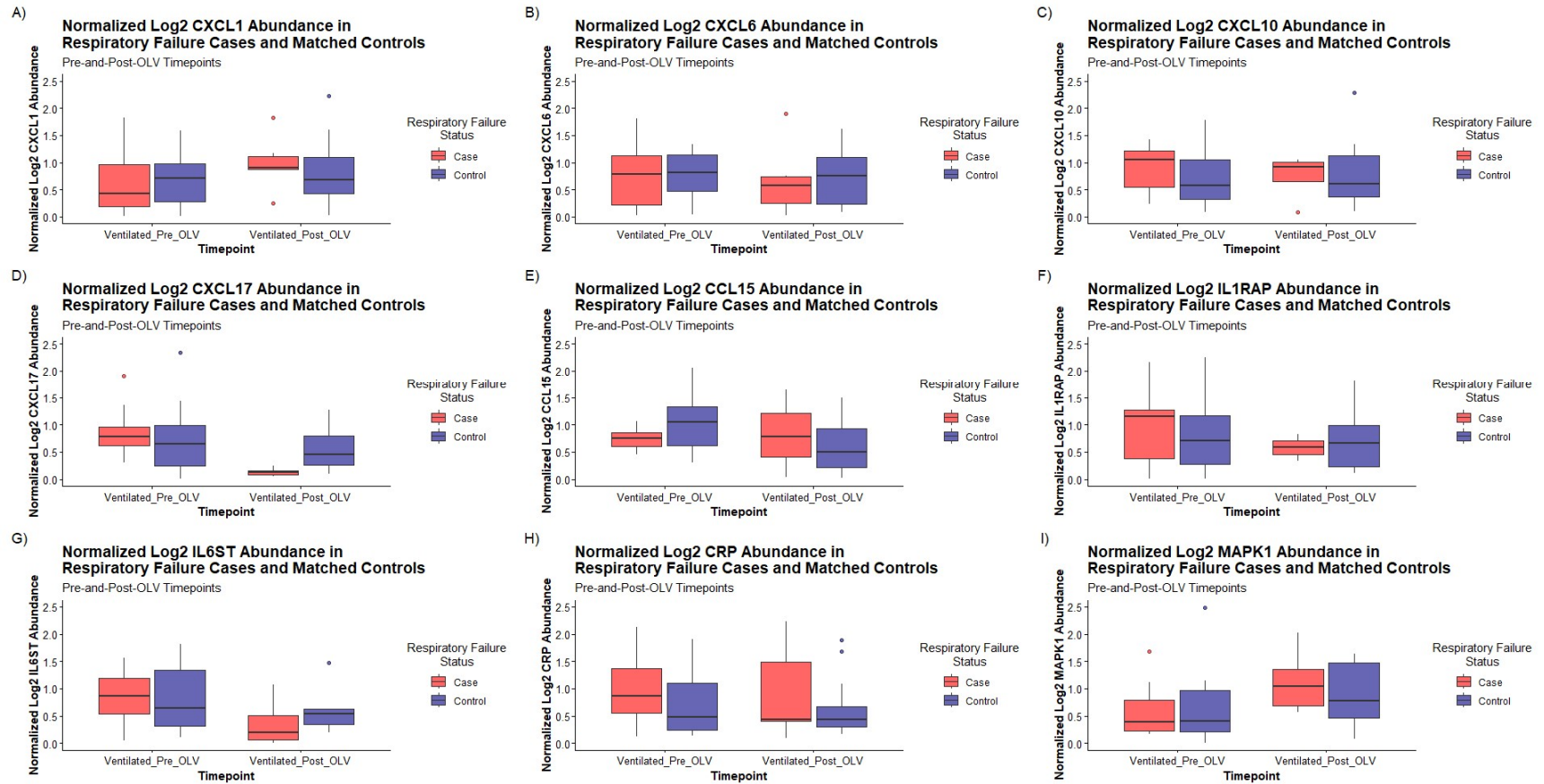


Figure 18. VSN-normalized log2 abundances for ventilated lung BALF cytokines, chemokines and inflammation-related proteins. Ventilated lung BALF protein abundances were plotted for pre- and post-OLV timepoints based on respiratory failure.

Multivariate sPLS-DA and Elastic Net Conditional Logistic Regression Results

So far, our analysis has focused on differences between pre-OLV and post-OLV timepoints in respiratory failure cases and matched controls. To better understand the proteins that can discriminate the four conditions combined, we implemented a sparse partial least squares discriminant analysis (sPLS-DA). The results from this analysis are plotted in Figure 19.

As shown in Figure 19, the horizontal axis, represented by component 1, shows differences between pre-OLV and post-OLV timepoints. Although the timepoints are not plotted in a paired manner similar to how the protein measurements were repeated, there may be a third component on the z-axis that can account for that, which is not included on the 2D plane. Nevertheless, the variation observed in the y-axis aligns with differences between respiratory failure cases and matched controls. The variation observed in component 1 is closely associated with the OLV time point, whereas that in component 2 is more closely aligned with respiratory failure status. Moreover, the variation observed in component 1 is larger than that of component 2. Thus, indicating a much larger variation in proteomic data, potentially due to OLV rather than respiratory failure status. It is important to note that the variation observed in both components is small. Further supplementing our observation that the small variation is accounted for by respiratory failure and timepoint variables in our variance partition analysis in Figure 10.

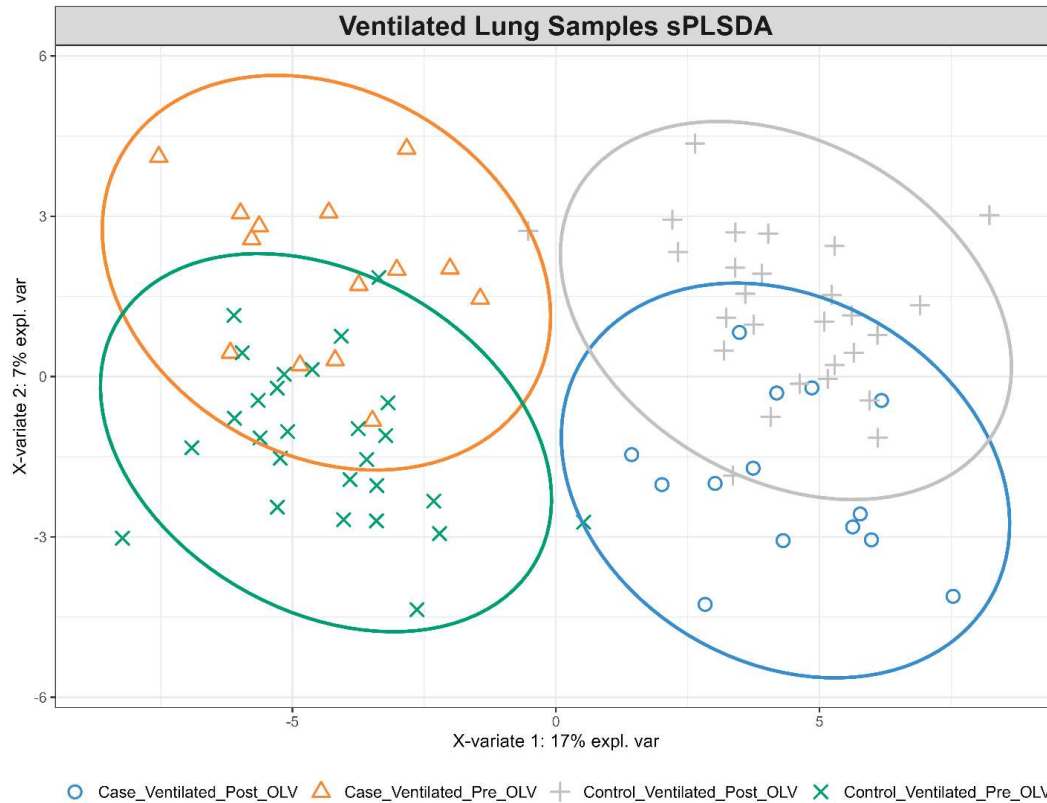
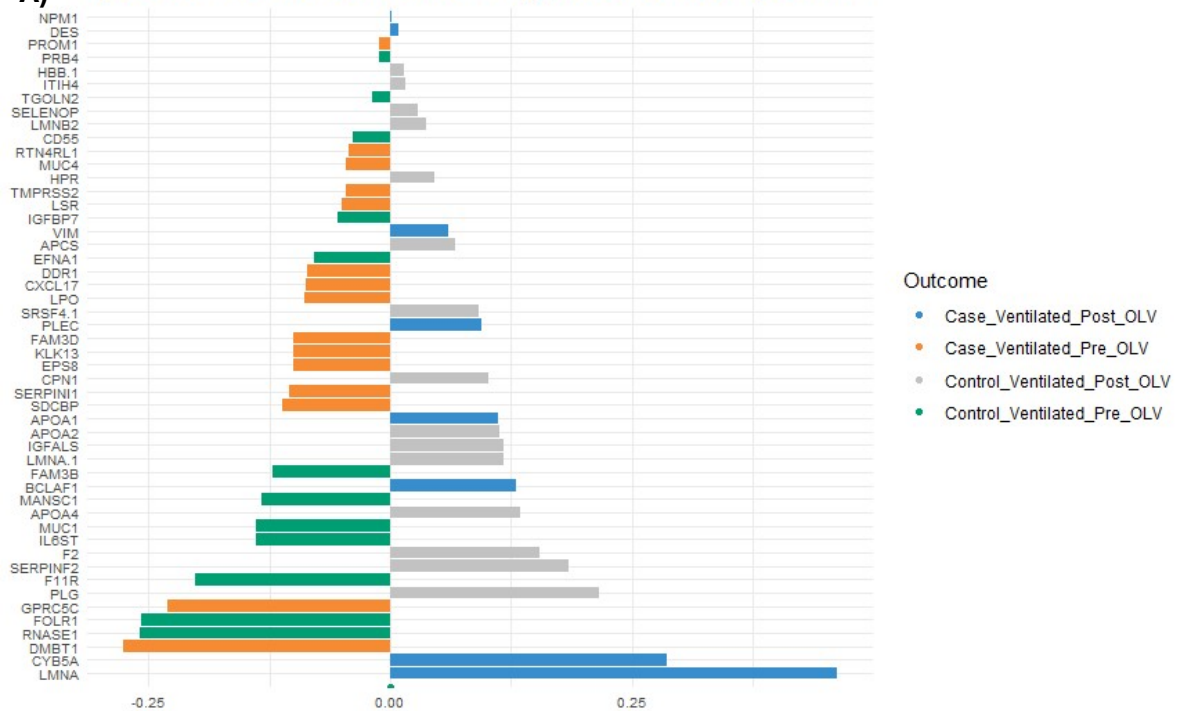


Figure 19. Sparse PLS-DA plot for ventilated lung BALF proteome showing the variation explained by components 1 and 2.

The respective loading plots for each of the horizontal (component 1) and vertical (component 2) components are presented in Figure 20A and 20B. In Figure 20A, LMNA and CYB5A contribute most to the variation observed in component 1, as confirmed by our differential expression analysis post-vs-pre-OLV.²¹² On the other hand, DMBT1 and GPRC5C contribute to this variation but in the opposite direction of post-vs-pre-OLV similar to differential expression analysis results.

In Figure 20B, PSME2 contributes the most to the variation observed in component 2 due to respiratory failure cases post-OLV. On the other hand, ANXA1 contributes to variation in this component in matched controls post-OLV. SLC5A8 tends to have variation in the opposite direction in respiratory failure cases post-vs-pre-OLV. Component 2 explains the variation between respiratory failure cases and matched controls. Hence, these proteins may explain drivers of respiratory failure as early as intraoperatively, although the overall variation explained by this component is small.

A) Sparse PLSDA of Ventilated Lung based on Component 1



B) Sparse PLSDA of Ventilated Lung based on Component 2

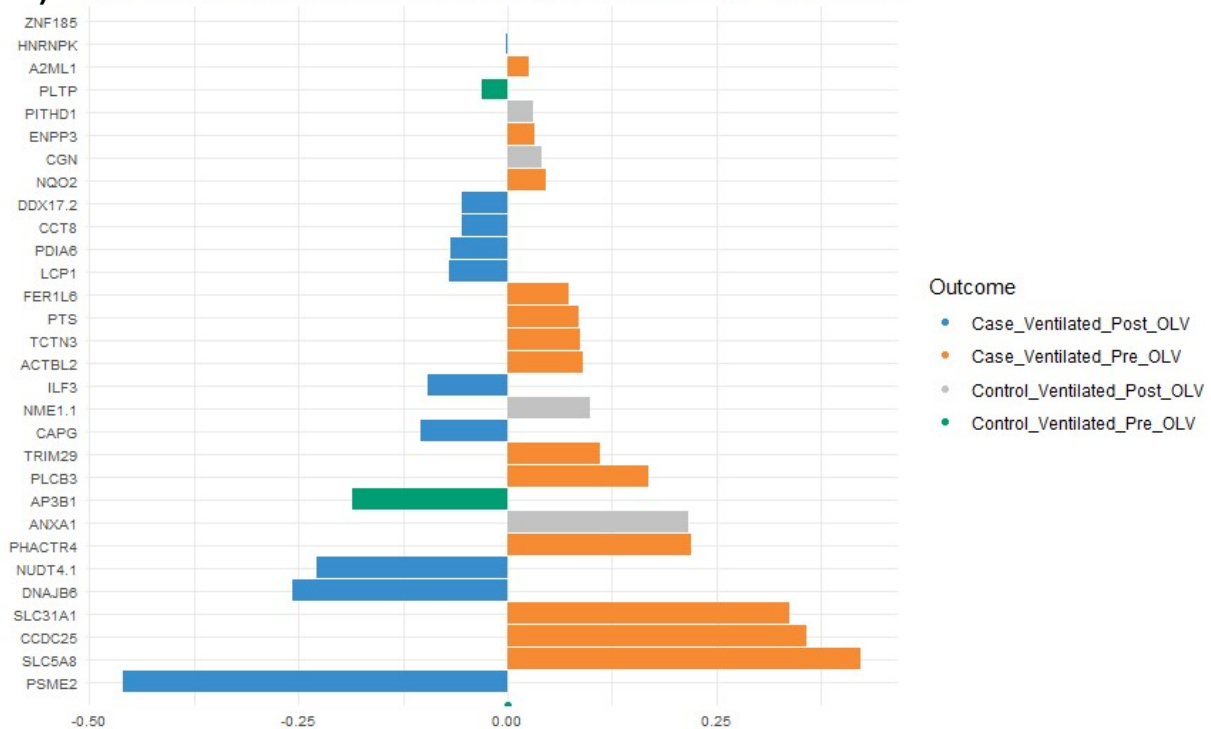


Figure 20A-B. Sparse PLS-DA plot showing the proteins that varied in ventilated lung BALF proteome. **A)** component 1 and **B)** component 2 of sparse PLS-DA.

Based on our matched case-control design, we implemented an elastic net penalized conditional logistic regression analysis based on the differences in protein abundance post-vs-pre-OLV. The results shown in Figure 21 show the top 15 proteins selected by a penalty value that yielded a minimal cross-validated deviance. The abundances of these top 15 select proteins is shown in Supplementary Figure 5A-B. In this model, notable proteins, including CYB5A, PSME2, ILF3, and ENPP2, had higher regression coefficients, indicating a higher risk of postoperative respiratory failure. On the other hand, ANXA1 had the lowest regression coefficients, indicating a trend towards matched controls. These proteins were also described in our sPLS-DA analysis, further confirming their association with their respective conditions. Out of these proteins, SLC5A8 had the highest probability for stability selection ($\pi_{thr} = 0.645$) whereas PSME2 had $\pi_{thr} = 0.555$. Both SLC5A8 and PSME2 met recommended thresholds for selection probability. CYB5A and SLC31A1, on the other hand, had a probability for stability selection above 0.5 but below lower limit of threshold.

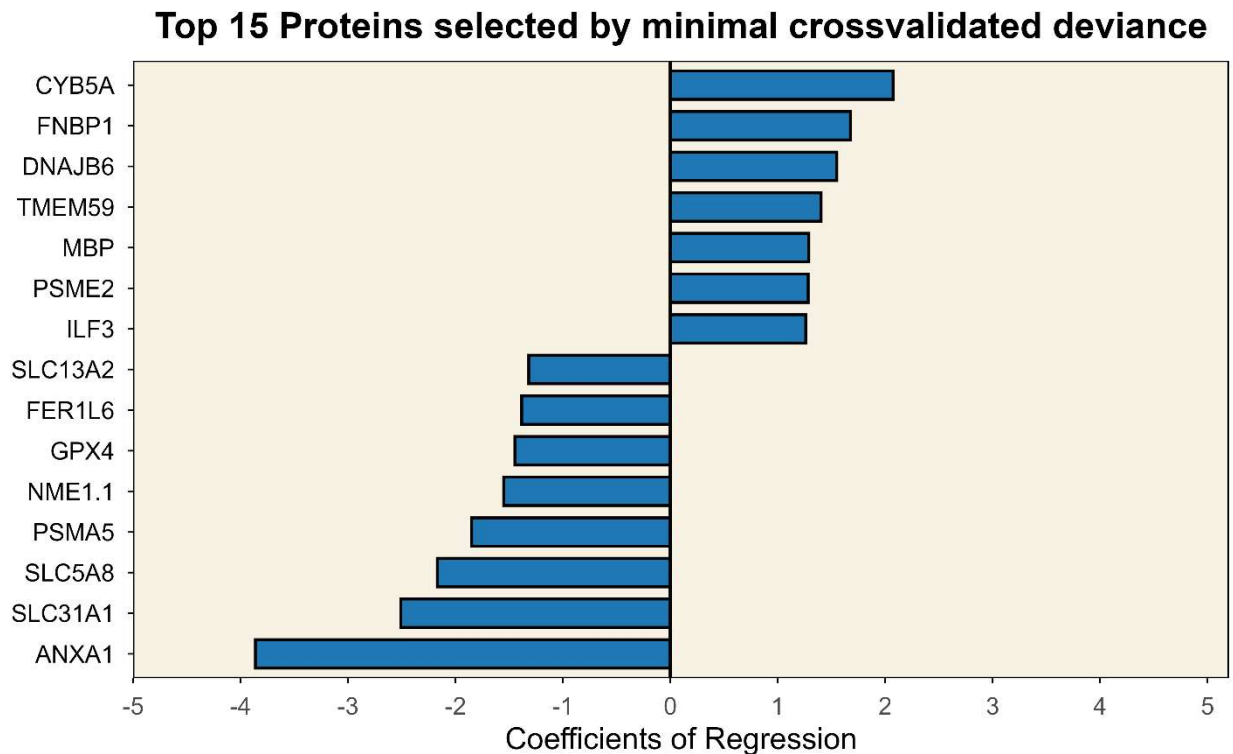


Figure 21. Top 15 elastic net conditional logistic regression coefficients for proteins in ventilated lung BALF selected by a model with minimal cross-validated deviance.

Operated Lung Proteome Analysis

Variance Partition Analysis

In the operated lung BALF proteomics data, variance partitioning results revealed that the condition variable accounted for a higher percentage of variance explained than the other variables in the model. Next, the individual heterogeneity described by the replicate variable accounted for a higher percentage of variance, followed by age and BMI. The matched-pair grouping by strata and biological sex explained near-negligible variance in the model. Outside the modelled variables, the vast majority of the variance explained was accounted for by residuals, which the model did not account for. The results of our variance partition model are shown in Figure 22 below.

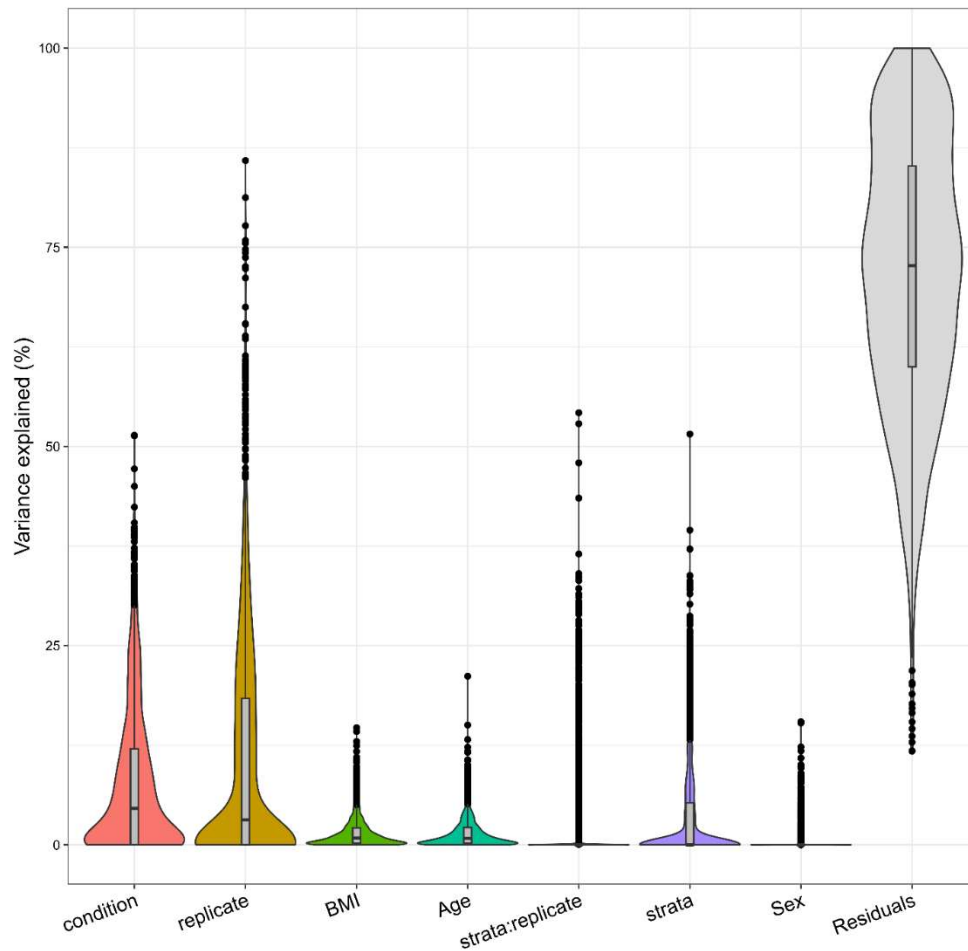


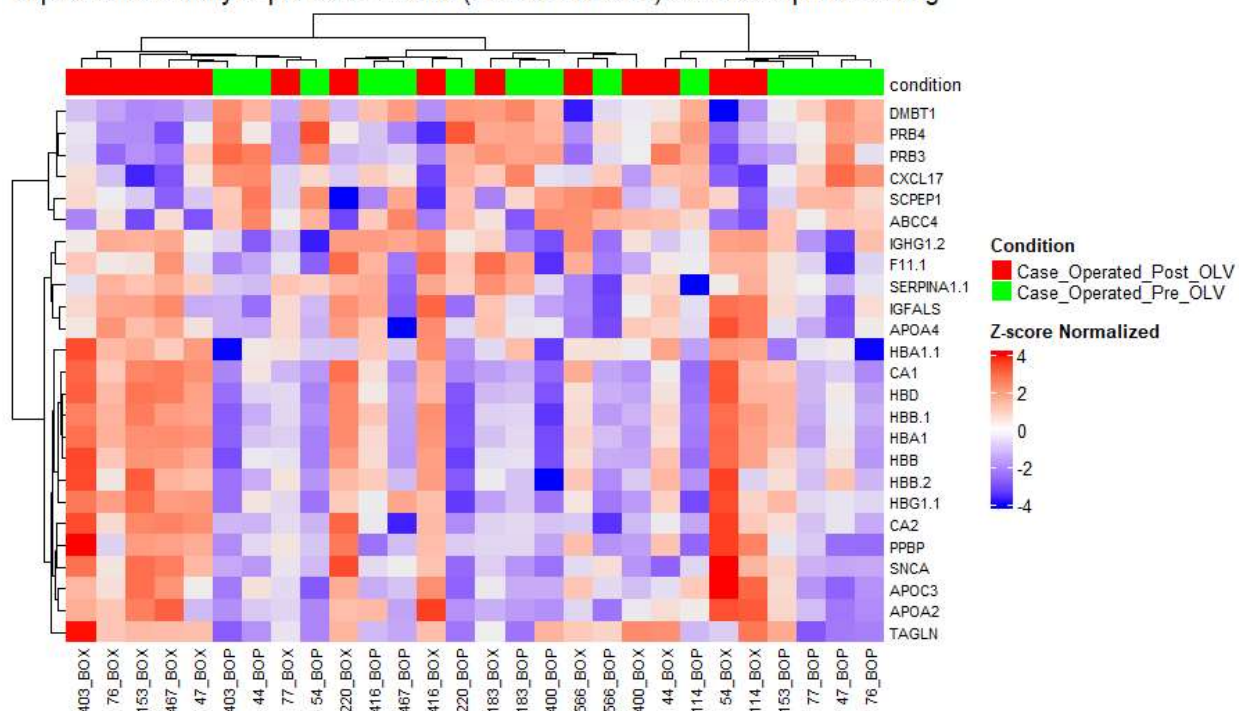
Figure 22. Percentage of variance explained by each covariate in the variance partition model in the operated lung BALF proteome.

Differential Protein Expression Presented in Heatmaps

From figures 23A and B, the respiratory failure cases' operated lung samples show a similar pattern among the top 25 differentially expressed proteins; however, post-OLV samples, such as IDs 77, 400, and 566, show a different trend of upregulation compared to other post-OLV case samples—additionally, IDs 44 and 183 show upregulation of proteins that tended to be downregulated at post-OLV cases. At the pre-OLV time point, ID 416 appears to be similar to post-OLV samples compared to other pre-OLV cases.

A)

Top 25 Differentially Expressed Proteins (Post vs Pre OLV) in Cases Operated Lung



B)

Top 25 Differentially Expressed Proteins (Post vs Pre OLV) in Cases Operated Lung

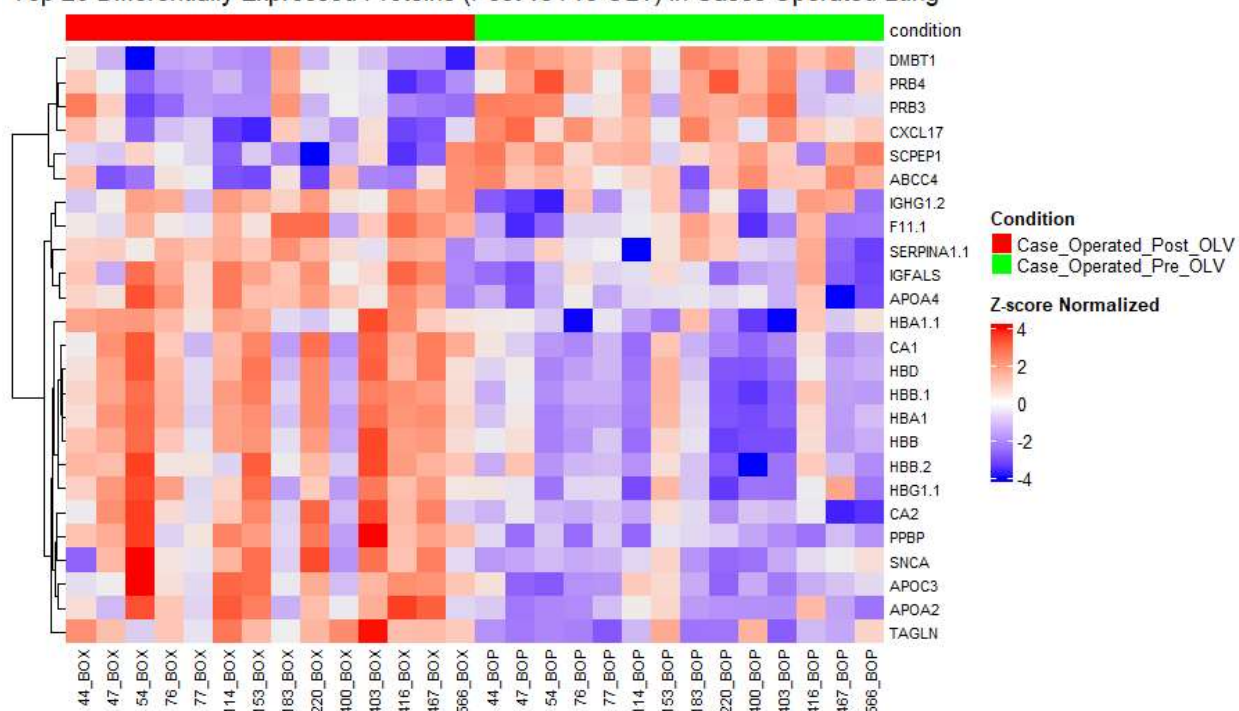
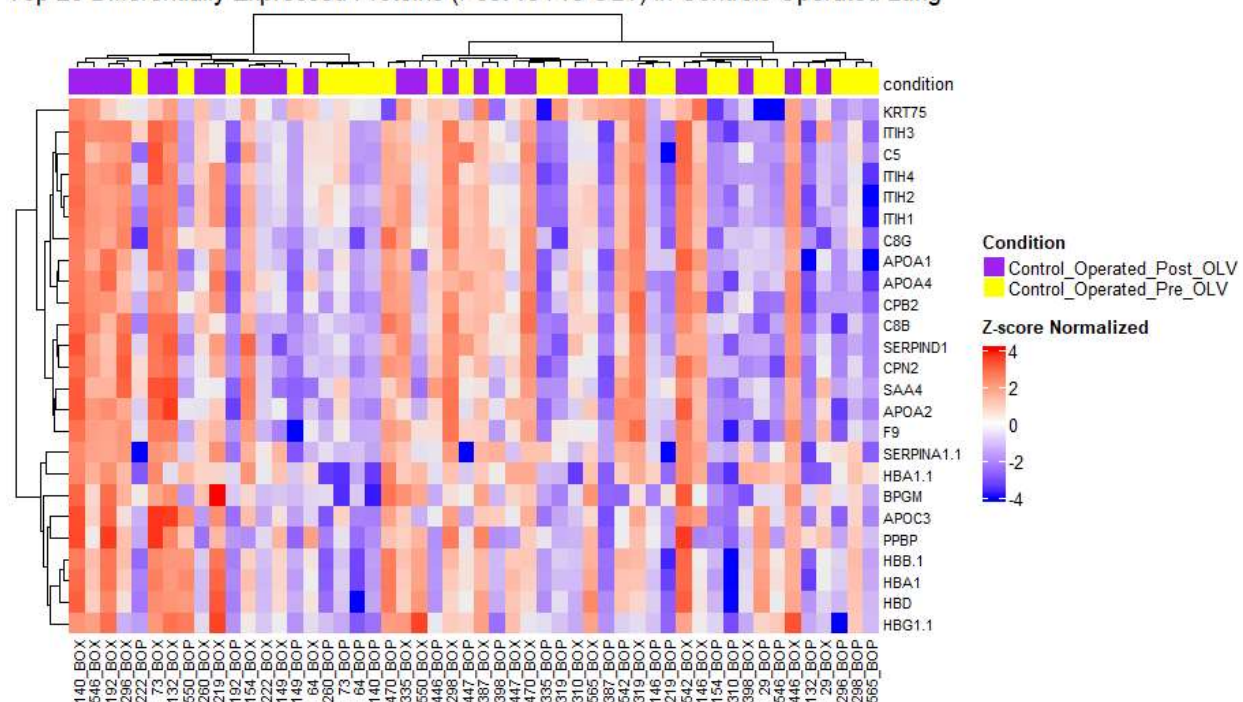


Figure 23A-B. Heatmap of the top 25 differentially expressed proteins in respiratory failure operated lung BALF samples at pre- and post-OLV timepoints. **A)** clustered heatmap and **B)** unclustered heatmap.

Similarly, in matched controls, 398, 446, 447, 470, and 542 pre-OLV samples showed an opposite pattern to the rest of the pre-OLV samples, with mostly downregulated genes being upregulated in these samples. At the post-OLV time point, samples such as 222 and 398, amongst others, had a different pattern with downregulation of upregulated proteins. The corresponding heatmaps for matched controls are shown in Figures 24A and B.

A) Top 25 Differentially Expressed Proteins (Post vs Pre OLV) in Controls Operated Lung



B) Top 25 Differentially Expressed Proteins (Post vs Pre OLV) in Controls Operated Lung

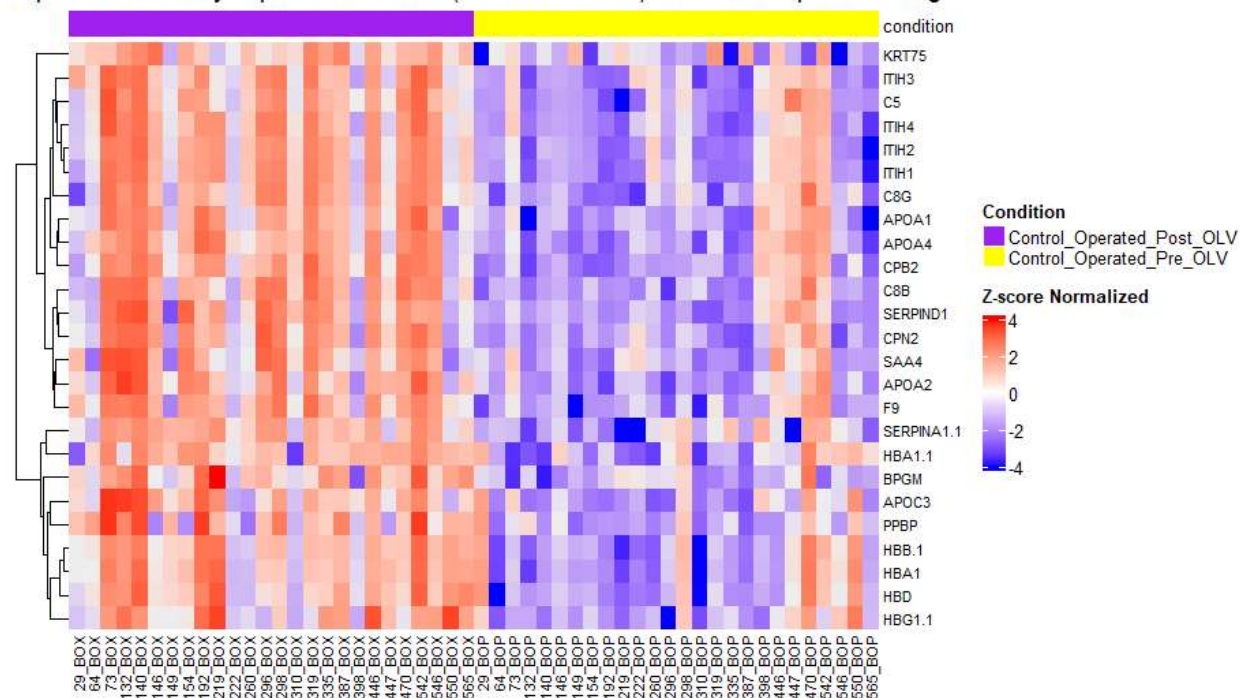


Figure 24A-B. Heatmap of the top 25 differentially expressed proteins in matched control operated lung BALF samples at pre- and post-OLV timepoints. **A)** clustered heatmap and **B)** unclustered heatmap.

Differential Protein Expression Presented in Volcano Plots and Gene Set Enrichment Analysis

As shown in Figure 25A, in the operated lung, 355 proteins were differentially expressed in respiratory failure cases post-OLV vs. pre-OLV. 204 proteins were upregulated while 151 proteins were downregulated – yielding more differentially expressed proteins compared to the ventilated lung. Similar to the ventilated lung post-vs-pre-OLV, CYB5A, LMNA, VIM, CXCL17, DMBT1, APOA1/2, CPN2, SAA4, DES, and MIA were also differentially expressed with statistical significance post-vs-pre-OLV in the operated lung proteome. However, notably expressed proteins such as PSME2 and ILF3 in the ventilated lung cases were not differentially expressed in the operated lung cases.

Hemoglobin subunit proteins, such as HBA1, HBB, and HBD, along with apolipoproteins, such as APOA1/2/4, APOE, APOM, and APOC3, TPM1/2, and inter-alpha-trypsin inhibitor heavy chain proteins, ITIH1-4, were upregulated. In terms of downregulated proteins, podocalyxin was notably downregulated, and it has been described to promote epithelial-mesenchymal transition, thereby increasing metastatic potential in cancer.^{224,225} This may be potentially associated with the resected tumour from the operated lung for respiratory failure cases with a pathologically confirmed lung cancer diagnosis.

Based on the differentially expressed proteins in the operated lung proteome of respiratory failure cases, we performed gene set enrichment analysis. Figure 25E shows the Gene Ontology (GO) biological processes enriched in respiratory failure cases. Biological processes such as coagulation, wound healing, and complement activation are expected to occur due to the impact of the surgery on the operated lung. In addition to those processes, neutral lipid metabolic process, and protein localization to centromeric region of chromosome were enriched. Other Gene Ontologies, such as molecular function and cellular compartment, were analyzed. In this comparison, ribonucleotide (GTP) binding and modifications to this binding were the most enriched molecular function terms, with hydrolase and antioxidant activities also enriched. As for cellular components shown in Supplementary Figures 4C, the majority of the enriched terms involved CENP-A containing nucleosome, platelet alpha granule, blood microparticles, collagen-containing extracellular matrix, heterotrimeric G-protein complex, and protein DNA

components. Blood microparticles, and extracellular matrix components align with potential blood contamination released into the bronchoalveolar space during surgical manipulation.

Our GSEA KEGG analysis (Figure 25D) also revealed a similar pattern of complement and coagulation cascades, neutrophil extracellular trap formation, cholesterol metabolism, and *S. Aureus* infection pathway. These pathways can be involved in clotting and wound healing, and a pro-inflammatory signalling response in the operated lung. Interestingly, prostate cancer was enriched which may potentially have something to do with cancer pathways being enriched in the operated lung. Similarly, GSEA REACTOME pathway enrichment in Figure 25C also revealed HDACs deacetylate histones, RMTs methylate histone arginines, complement and clotting processes, with potential for red blood cell infiltration into the BALF supernatant.

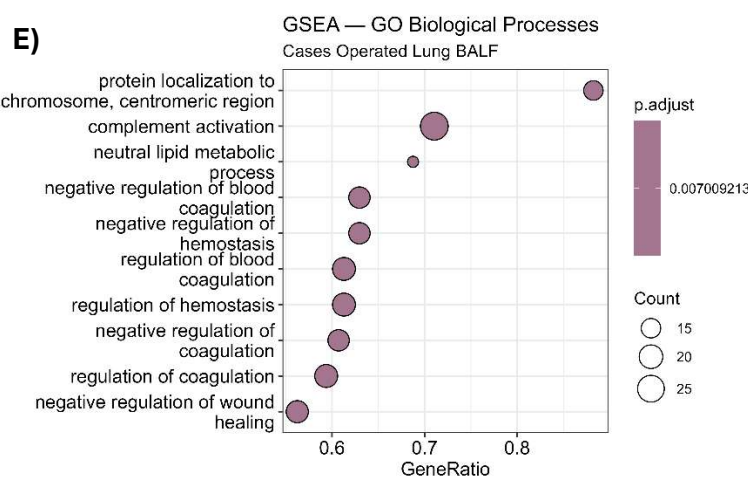
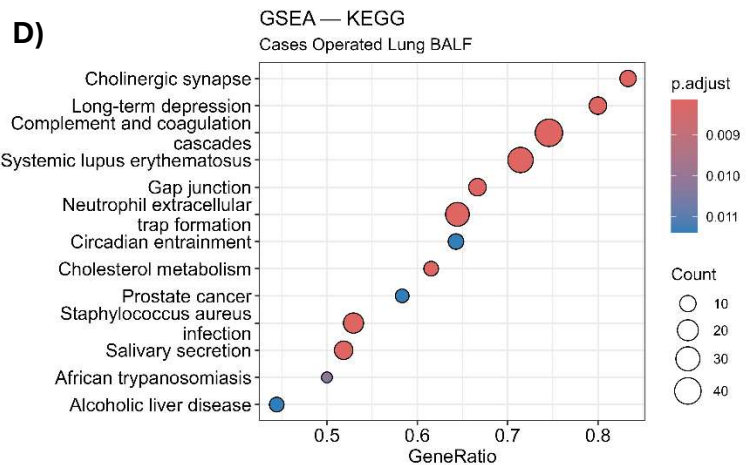
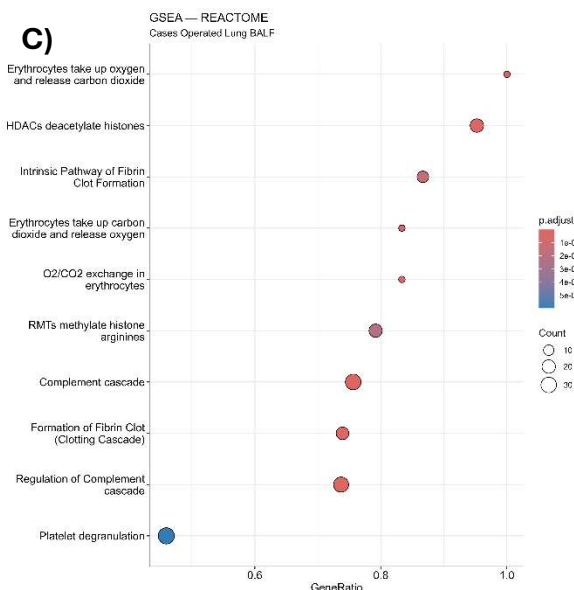
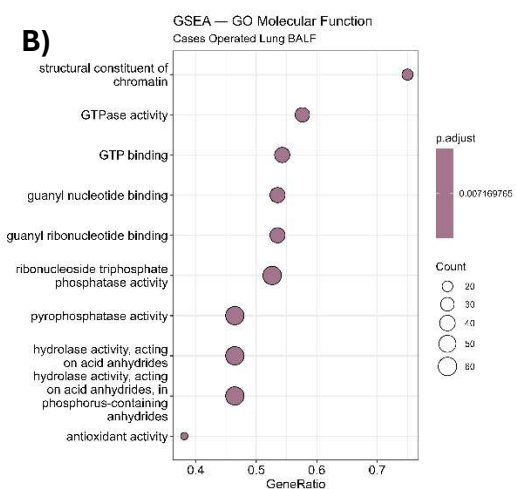
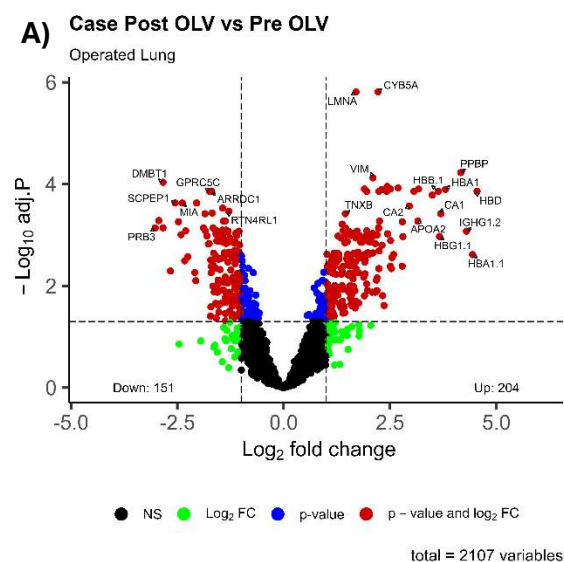


Figure 25A-E. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in operated lung BALF proteome respiratory failure cases post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** GSEA on molecular function Gene Ontology (GO), **C)** GSEA REACTOME pathways, **D)** GSEA on KEGG pathways, and **E)** GSEA on biological processes GO all with FDR < 0.05 .

In matched controls, the operated lung BALF proteome post-vs.-pre-OLV showed differential expression of 368 proteins, with 232 upregulated and 136 downregulated. Proteins differentially expressed in respiratory failure cases were also found to be differentially abundant in matched controls. Similarly, CYB5A, LMNA, VIM, CXCL17, DMBT1, APOA1/2, CPN2, SAA4, DES, and MIA proteins were also found to be differentially expressed in matched control operated lung BALF proteome as they were in the ventilated lung BALF. However, the Elongator complex protein 3 (ELP3) protein appears to be significantly downregulated in matched controls relative to cases in the operated lung BALF. Figure 26A shows a volcano plot of the differentially expressed proteins post-vs-pre-OLV.

Enrichment analysis using GSEA of the matched control operated lung proteome showed enrichment of GO biological processes such as protein-lipid complex, classical complement pathway, humoral immune response mediated by B cells, and small molecule biosynthetic process. As for GO molecular functions, oxidoreductase and peptidase activity were enriched in matched controls. The GO molecular functions and biological processes for matched controls operated lung proteome are shown in Figures 26B and C, respectively.

The cellular component gene ontologies also showed lipoprotein activity, blood microparticles due to possible blood contamination in BALF supernatant, and platelet alpha granule being enriched. The GO cellular component for matched controls operated lung proteome is shown in Supplementary Figures 4D. KEGG GSEA enriched terms in matched controls were similar to those in cases involving inflammatory processes, complement and coagulation cascades, with additional enrichment for the glycosaminoglycan degradation and neuroactive ligand-receptor interaction KEGG pathways. The REACTOME-enriched terms involved complement cascade and clotting processes, DNA damage checkpoint, chylomicron remodeling, and retinoid metabolism. The KEGG and REACTOME GSEA dotplots are shown in Figures 26D and E, respectively.

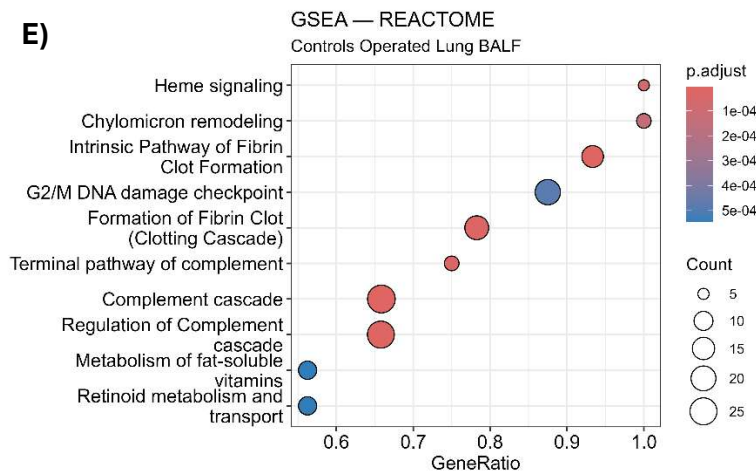
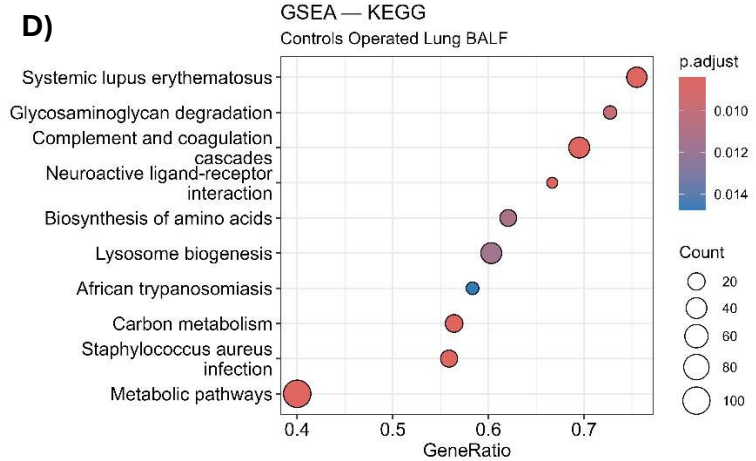
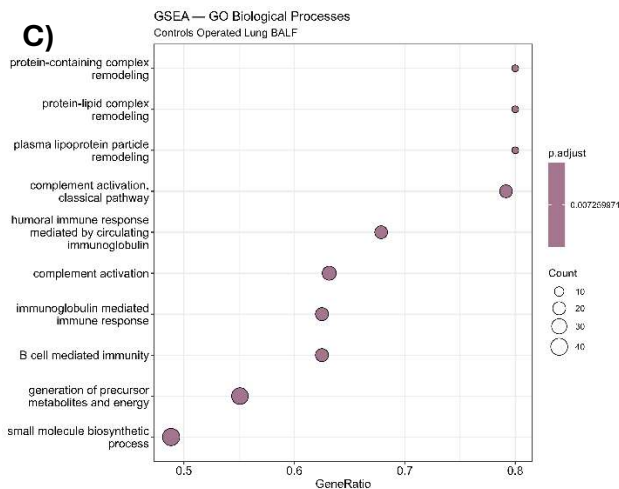
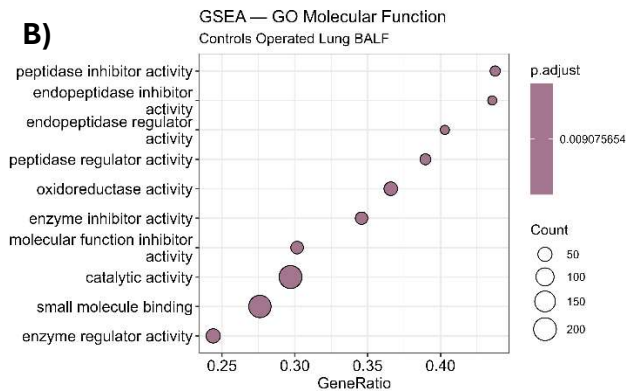
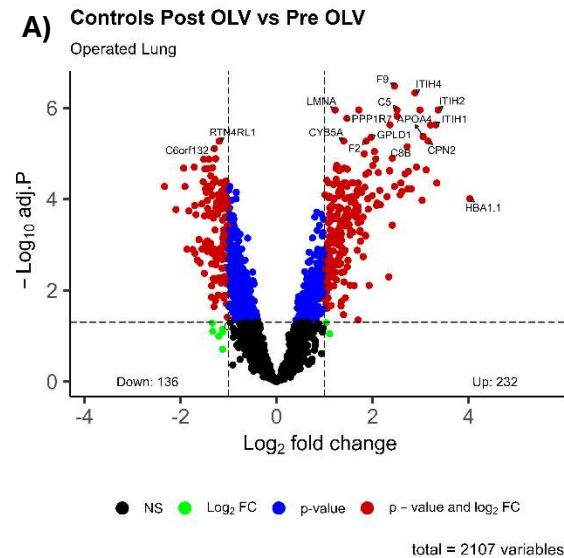


Figure 26A-E. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in operated lung BALF proteome matched controls post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** GSEA on molecular function Gene Ontology (GO), **C)** GSEA on biological processes GO, **D)** GSEA on KEGG pathways, and **E)** GSEA REACTOME pathways all with FDR < 0.05 .

Multivariate sPLS-DA and Elastic Net Conditional Logistic Regression Results

To understand the variability in the BALF proteome of the operated lung across conditions, we implemented an sPLS-DA similar to that used for the ventilated lung BALF analysis. The sPLS-DA plot showing the top two components that describe the variability in our operated lung BALF proteomics data is shown in Figure 27.

Most notably, both components in the operated lung BALF sPLS-DA had greater variation explained than in the ventilated lung BALF sPLS-DA. Both components in this sPLS-DA follow a similar trend to the ventilated lung, with component 1 on the x-axis showing variability across timepoints and component 2 on the y-axis showing variability across respiratory failure status. There could be a third component along the z-axis that might better explain the variability across timepoints, with correct pairings between timepoints and respiratory failure status. Nevertheless, component 1 shows greater variability than component 2 across matched control samples pre-OLV and respiratory failure case samples post-OLV, and across pre-OLV cases and matched controls post-OLV. Component 2, which explains variability across respiratory failure status, had lower variability explained, indicating less change in the proteome with regard to respiratory failure in the operated lung BALF, similar to the ventilated lung BALF.

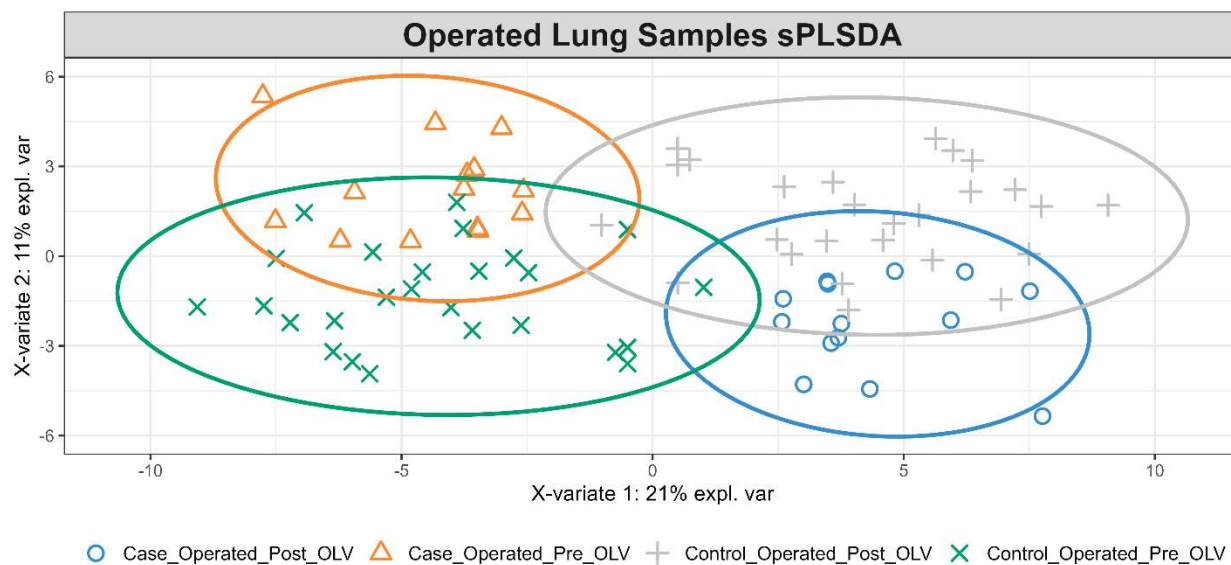
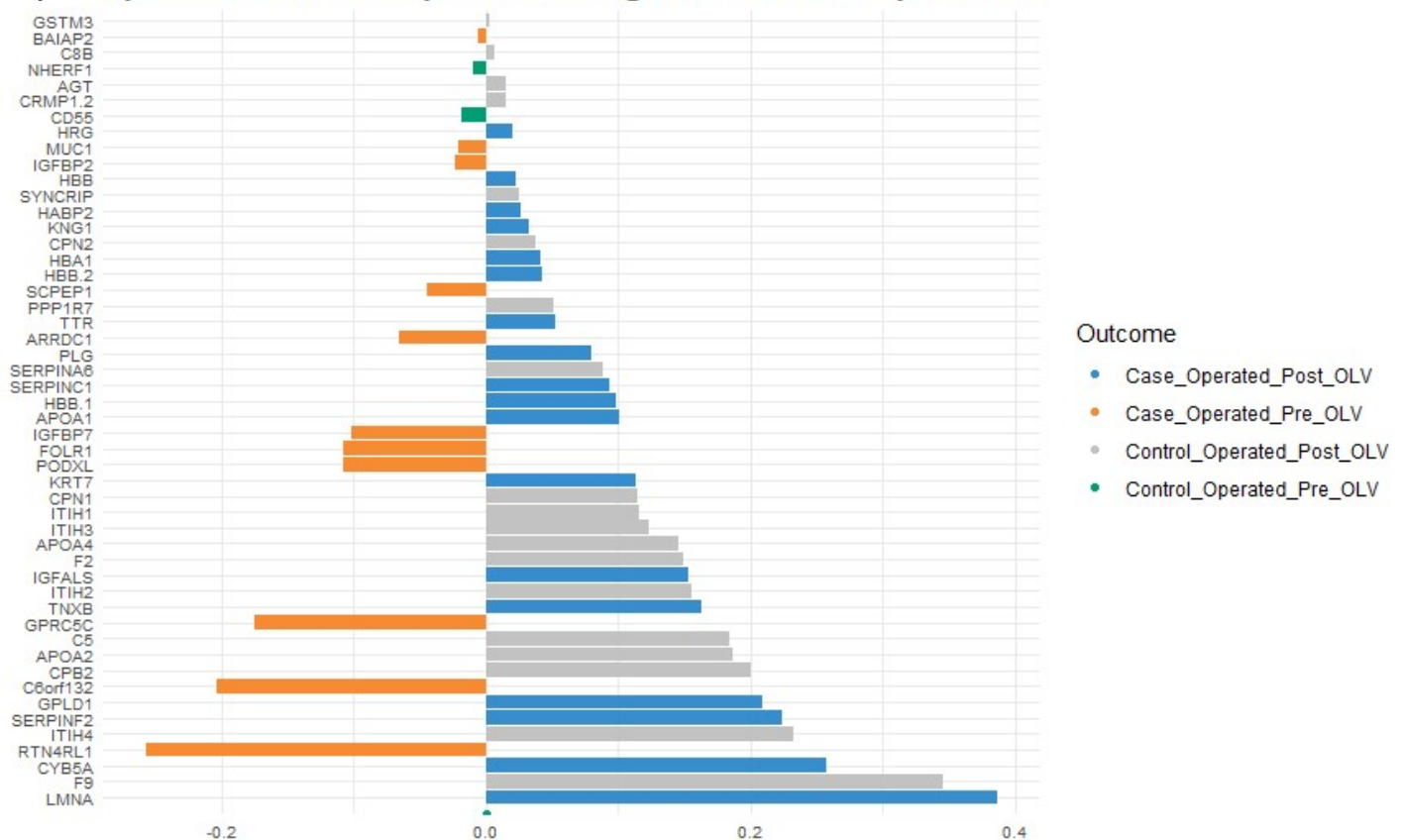


Figure 27. Sparse PLS-DA plot for operated lung BALF proteome showing the variation explained by components 1 and 2.

Proteins that contributed to the variation explained in component 1 and component 2 of the operated lung BALF sPLS-DA are shown in Figures 28A and B. The proteomic data from component 1 show that LMNA, CYB5A, and SERPINF2 contributed to respiratory failure cases post-OLV, while Coagulation factor IX (F9), ITIH4, and Carboxypeptidase B2 (CPB2) contributed to matched controls post-OLV in the operated lung BALF. In component 2, TPM1 and TPM2 were ELP3 proteins that showed a trend toward respiratory failure cases post-OLV, whereas the Carboxypeptidase E (CPE) protein showed the opposite trend.

A) Sparse PLSDA of Operated Lung based on Component 1



B) Sparse PLSDA of Operated Lung based on Component 2

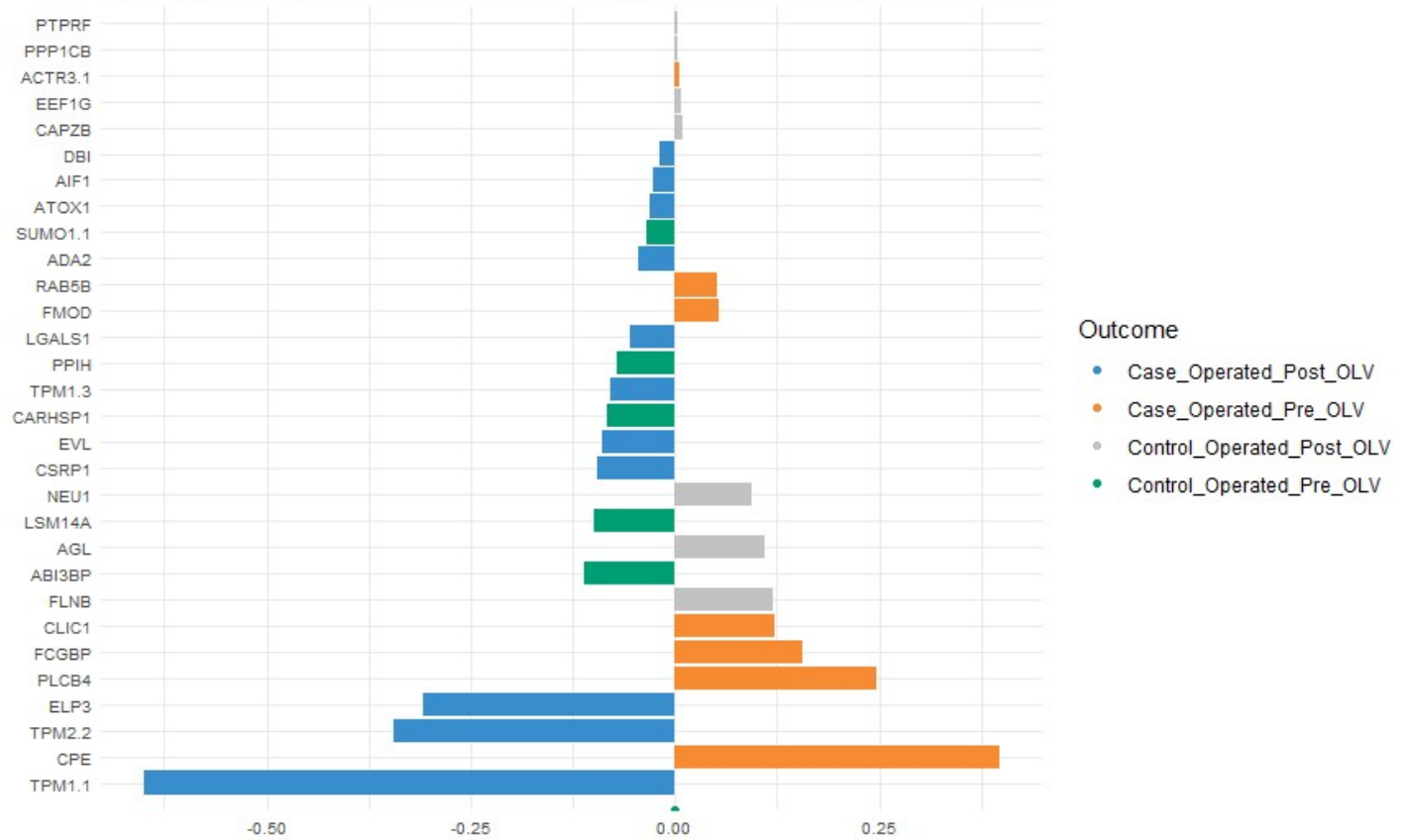


Figure 28A-B. Sparse PLS-DA plot showing the proteins that varied in operated lung BALF proteome. **A)** component 1 and **B)** component 2 of sparse PLS-DA.

To identify proteins that can distinguish between cases and controls in the operated lung proteome, we implemented an elastic net conditional logistic regression model, which identified 130 proteins based on post-vs-pre-OLV differences. TPM1/2, Glutathione S-transferase omega-1 (GSTO1), and LMNA proteins had some of the highest coefficients of regression towards cases, while Hepatocyte growth factor receptor (MET) and Ras-related protein Rab-5B (RAB5B) had the lowest coefficients towards matched controls. The elastic net conditional logistic regression for the operated lung proteome is shown in Figure 29. TPM1, TPM2, ELP3, and CPE proteins passed the probability for stability selection threshold.

Top 15 Proteins selected by minimal crossvalidated deviance

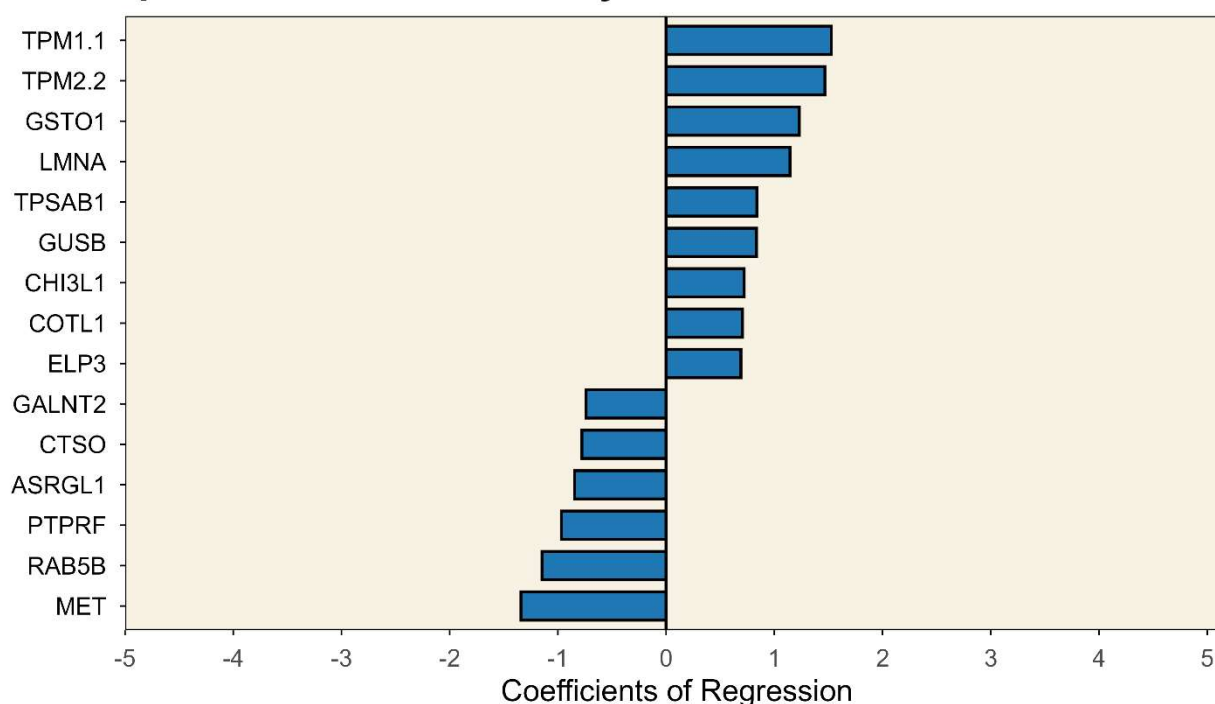


Figure 29. Top 15 elastic net conditional logistic regression coefficients for proteins in operated lung BALF selected by a model with minimal cross-validated deviance.

Comparisons Across Ventilated and Operated Lung BALF Differentially Expressed Proteins

Majority of the comparisons performed thus far involved post-vs-pre-OLV differences in respiratory failure cases and matched controls. These comparisons were performed separately in the ventilated and operated lung BALF samples. To understand whether certain proteins are differentially expressed in both the operated and ventilated lung BALFs, we compared proteins found in both compartments in respiratory failure cases and matched controls. In cases, we found 84 differentially expressed proteins in both operated and ventilated lung BALFs of respiratory failure cases. These proteins are shown in Table 2 below.

Table 2. Proteins differentially expressed in both operated and ventilated lung BALFs of respiratory failure cases.

Protein	Ventilated Lung BALF logFC	Operated Lung BALF logFC	Protein	Ventilated Lung BALF logFC	Operated Lung BALF logFC
DES	2.273	1.881	CPE	-1.021	-1.042
ILF3	2.242	1.390	TTN	-1.057	-1.108
CPN2	2.215	2.193	PROM1	-1.058	-1.603
SAA4	2.174	2.083	CRISP3	-1.062	-1.264
LMNA.1	2.132	2.240	FAM3D	-1.064	-1.241
APOA2	2.130	3.154	LTF	-1.083	-1.049
VIM	2.110	2.100	EFNA1	-1.084	-1.663
APOA1	2.078	2.444	TCN1	-1.087	-1.309
CYB5A	2.008	2.219	ACE	-1.090	-1.174
DES.2	1.943	1.980	HMGNI	-1.106	-1.214
HBD	1.916	4.546	PRR4	-1.130	-1.375
APOA4	1.908	2.802	SLC5A1	-1.155	-1.681
KRT18	1.905	1.394	FOLR1	-1.155	-1.660
HBB.1	1.804	3.638	FRZB	-1.169	-1.216
HBA1	1.727	3.805	PODXL	-1.177	-1.885
LMNA	1.692	1.699	KLK13	-1.181	-1.083
SERPIND1	1.687	2.327	IGFBP7	-1.183	-1.489
HABP2	1.640	2.153	MUC16	-1.187	-2.047
APOC3	1.632	3.493	AMY1A.1	-1.191	-1.149
HBB	1.628	3.065	CALML3	-1.225	-1.167
ITIH2	1.604	2.197	MUC4	-1.236	-1.751
PGLYRP2	1.571	2.237	TNFRSF19	-1.254	-1.524
KRT7	1.533	1.444	PRH1	-1.272	-2.304
CPB2	1.512	2.196	CCL15	-1.272	-1.541
F12	1.508	2.028	SERPINI1	-1.288	-1.439
HRG	1.351	2.425	ZG16B	-1.293	-1.771
ENPP2	1.334	1.521	GPRC5C	-1.297	-1.738
ITIH4	1.325	2.060	BTN2A1	-1.307	-1.010
PLG	1.316	2.252	RPL29	-1.337	-1.317
IGFALS	1.314	2.692	AMY1A	-1.350	-1.339
LUM	1.300	2.233	PRB3.1	-1.383	-2.327
C5	1.290	1.900	MMP7	-1.385	-1.558
F2	1.274	1.779	LPO	-1.396	-1.841
SERPINA6	1.243	1.532	SMIM5	-1.397	-1.706
PNP	1.242	1.177	RTN4RL1	-1.413	-1.289
SYNCRIP	1.133	1.154	MIA	-1.607	-2.383
ARPC5	1.120	1.199	PRB3	-1.680	-3.019
APCS	1.105	2.506	PRB4	-1.758	-2.837
LMNB2	1.075	1.022	DMBT1	-1.899	-2.837
GOLM1	-1.004	-1.330	PRR15	-1.965	-1.587
CEL	-1.016	-1.003	PRB1.1	-2.433	-2.411
MUC1	-1.016	-1.590	CXCL17	-2.551	-2.935

Out of the proteins differentially expressed post-vs-pre-OLV in operated and ventilated lungs of respiratory failure cases, there were some proteins whose upregulation was higher in the operated lung compared to the ventilated lung. Some of those proteins include HBD, APOA4, HBB.1, HBA1, SERPIND1, APOC3, HBB, ITIH2, PGLYRP2, HRG, IGFALS, LUM, and C5. Proteins such as KRT18, DES, ILF3, and KRT7 had higher upregulation in the ventilated lung BALF compared to the operated lung BALF. In the opposite direction, proteins such as PROM1, EFNA1, PODXL, SCL5A1, MUC16, FOLR1, PRH1, PRB3.1, MIA, PRB3 and 4, and DMBT1 had higher downregulation in the operated lung relative to the ventilated lung BALF. However, PRR15, RTN4RL1, BTN2A1, and CALML3 were more downregulated in the ventilated lung compared to the operated lung BALF.

In matched controls, there were 129 differentially expressed proteins detected in both operated lung and ventilated lung BALFs. These proteins along with their log2 foldchanges are shown in Table 3. HBD, APOA1/A2/A4/C3, HBA1, KRT75, ITIH1/H2/H3/H4, HBB, HBB.1, C5, CPN2, FETUB, SERPINA6/D1, TAGLN and C8A had higher upregulation in the operated lung compared to the ventilated lung BALF in matched controls. On the other hand, LMNA.1, F11.1, SNCA, FGB, LMNA, A1BG, IDH2 and GANAB had higher log2 foldchange in the ventilated lung BALF. In terms of downregulated proteins, MUC1/16, TNFRSF19, PRR15, PODXL, IDFBP7, SFTPD, PRB3.1, CCL15, and UGDH had lower log2 foldchange in the operated lung compared to the ventilated lung BALF. However, LPO, TTN, PRB3/4 and CXCL17 had lower log2 foldchange in the ventilated lung BALF.

Table 3. Proteins differentially expressed in both operated and ventilated lung BALFs of matched controls.

Protein	Ventilated Lung BALF logFC	Operated Lung BALF logFC	Protein	Ventilated Lung BALF logFC	Operated Lung BALF logFC
HBD	2.377	3.337	A1BG	1.225	1.163
LMNA.1	2.371	1.330	C7	1.217	1.673
APOA4	2.332	3.052	APCS	1.215	1.484
KRT18.1	2.186	2.302	MAT2A	1.212	1.474
APOA2	2.179	3.204	GPI	1.212	1.473
APOC3	2.166	2.589	IDH2	1.211	1.018
HBA1	2.101	2.912	TKT	1.201	1.234
KRT75	2.001	2.729	GBE1	1.190	1.481
ITIH2	1.979	3.366	F13B	1.181	1.218
ITIH4	1.969	2.884	PPBP	1.173	2.576
HBB.1	1.939	2.744	TTR	1.156	1.359
F11.1	1.935	1.805	A2M	1.149	1.680
IGHG1.3	1.935	1.928	PRMT1	1.145	1.299
HABP2	1.918	2.120	KRT7	1.122	1.306
CA1	1.884	2.409	C1R	1.121	1.703
PLG	1.840	1.917	ITGAM	1.117	1.086
APOA1	1.822	2.413	A2M.1	1.112	1.621
ITIH1	1.798	3.316	LMNB2	1.099	1.008
HBG1.1	1.768	3.031	ALAD	1.083	1.336
DES.2	1.757	1.542	TAGLN	1.070	1.746
DES	1.752	1.993	LDHA	1.037	1.328
CPB2	1.722	2.510	BLVRB	1.029	1.380
HBB	1.655	2.356	GNPDA1	1.017	1.827
SNCA	1.647	1.532	PNP	1.014	1.705
CPN1	1.611	2.038	SYNCRIP	1.013	1.187
F11	1.594	1.604	MANSC1	-1.009	-1.056
HRG	1.589	1.659	MT2A	-1.013	-1.020
FGB	1.581	1.086	IL6ST	-1.035	-1.002
ITIH3	1.575	2.986	MUC16	-1.041	-1.315
F12	1.567	1.628	TGOLN2	-1.044	-1.247
C1S	1.565	2.005	MUC1	-1.050	-1.514
C5	1.542	2.508	SIRPA.1	-1.054	-1.213
CPN2	1.539	3.155	TNFRSF19	-1.091	-1.345
FGG	1.524	1.072	RUFY1	-1.128	-1.108
KRT18	1.514	1.701	ICAM1	-1.133	-1.118
SAA4	1.498	2.676	RTN4RL1	-1.141	-1.192
F2	1.476	1.869	PRR15	-1.141	-2.332
CA2	1.472	1.682	GPRC5C	-1.145	-1.405
FETUB	1.460	2.092	KLK13	-1.158	-1.145
KLKB1	1.436	1.568	AMY1A.1	-1.171	-1.354
HBB.2	1.435	2.149	PODXL	-1.174	-1.712
LMNA	1.431	1.221	PEBP4	-1.174	-1.197
SERPINF2	1.425	1.713	IGHV5-10- 1.1	-1.184	-1.088

IGFALS	1.424	2.067	IGFBP7	-1.188	-1.420
LUM	1.400	1.837	FAM3B	-1.198	-1.101
HPR	1.389	1.773	IGHV2-5	-1.218	-1.278
KRT19	1.376	1.651	FOLR1	-1.225	-1.491
PDXK	1.357	1.665	EFNA1	-1.244	-1.446
SERPINA6	1.346	2.361	ALDH7A1	-1.272	-1.399
KRT17.1	1.333	1.683	LPO	-1.279	-1.049
ARPC5	1.329	1.102	MIA	-1.288	-1.368
VIM	1.325	1.400	TTN	-1.320	-1.248
C8A	1.322	2.356	SMIM5	-1.328	-1.336
GPLD1	1.306	1.970	AMY1A	-1.332	-1.480
C4A	1.303	1.741	SFTPD	-1.364	-1.616
SERPIND1	1.295	3.115	RNASE1	-1.369	-1.336
MDH2	1.273	1.353	PRB3.1	-1.384	-1.648
GANAB	1.270	1.108	CCL15	-1.511	-1.933
AHSG	1.266	1.430	UGDH	-1.541	-1.827
KNB1	1.257	1.467	DMBT1	-1.636	-1.705
CYB5A	1.255	1.401	SCPEP1	-1.690	-1.907
AGT	1.254	1.859	PRB4	-1.893	-1.745
RBP4	1.247	1.348	PRB3	-1.912	-1.865
C4BPA	1.232	1.433	CXCL17	-2.214	-2.093
GPI.1	1.225	1.851			

Although there were proteins differentially expressed in both the operated and ventilated lung BALFs, there were also 103 proteins differentially expressed uniquely in the ventilated lung BALF of respiratory failure cases (64) and matched controls (39). These proteins are shown in Table 4. Some of the proteins upregulated in respiratory failure cases ventilated lung BALF include H4C1, ITGAM, RAC2, ITGB2, PSME2, BCLAF1 and MACROH2A1. Notably in matched controls, PADI4 and H4C1 were also upregulated.

Table 4. Proteins differentially expressed only in the ventilated lung BALF post-vs-pre-OLV in respiratory failure cases and matched controls.

Case Proteins	logFC	Case Proteins	logFC	Control Proteins	logFC
KRT18.1	2.569	IGLV3-10.1	-1.100	DDT	1.914
KRT75	2.182	CADM1	-1.122	TPM3.1	1.642
KRT19	2.024	CRISP2	-1.126	ILF3.1	1.549
MSN.1	1.660	EFNB3	-1.129	PADI4	1.540
H4C1	1.572	LCN1.1	-1.153	SET	1.443
TFRC	1.538	ADGRF5	-1.156	RCN1	1.419
DEFA1.1	1.537	IGHV4-28	-1.169	SRSF7	1.267
SFN.2	1.512	RNASE1	-1.176	FGA	1.187
SRSF7	1.504	TCTN3	-1.178	HINT1	1.182
ITGAM	1.485	CXCL10	-1.186	CTCF	1.156
RAC2	1.485	CSF1	-1.192	RCC1	1.131
KRT8	1.465	SLC23A1	-1.212	GMFG	1.121
H3-3A.1	1.423	NEBL	-1.219	HNRNPA1.2	1.112
C9	1.376	SLC5A8	-1.225	SAE1	1.083
GMFG	1.355	A2ML1	-1.258	NCL	1.063
ECHS1	1.324	SLC13A2	-1.278	H4C1	1.032
SAE1	1.273	ALDH7A1	-1.291	ANP32A	1.027
GMFB	1.270	TNFAIP8L1	-1.337	HGFAC	1.019
TUBB	1.242	GPRC5B	-1.425	GCLM	1.013
ITGB2	1.241	PYM1	-1.481	SRSF2.1	1.007
U2AF2	1.200	CHI3L2	-1.501	TNFRSF14	-1.041
PSME2	1.192	IGKV1-16.1	-1.534	B3GNT7	-1.043
RBM8A	1.173	SPATA33	-1.547	CPM	-1.052
NCL	1.167	IGLV5-39.1	-1.737	IGLV3-25	-1.071
MACROH2A1	1.164	IGKV2-24	-2.641	MBP	-1.075
PDIA6	1.154			NBL1	-1.077
LMNB1	1.153			FABP6	-1.114
PSPC1	1.134			GPRC5A	-1.115
EPCAM	1.134			IGLV3-10.1	-1.122
HNRNPR.1	1.102			MARCO	-1.159
SFPQ	1.096			SERPINI1	-1.171
BCLAF1	1.094			CHI3L2	-1.189
YWHAB.1	1.053			ZG16B	-1.192
TWF2	1.032			CST6	-1.251
BBS2	-1.003			CSF1	-1.290
MANSC1	-1.005			BPIFB2	-1.322
NEGR1	-1.022			PRH1	-1.347
ATP12A.1	-1.024			IGKV2-24	-1.469
PROM2	-1.058			IGLV1-40.2	-1.790

Plasma Proteome Analysis

Variance Partition Analysis

In the plasma proteomics analysis, we implemented a variance partitioning approach similar to that used for ventilated and operated lung BALF proteomics data. As shown in Figure 30, a strikingly high percentage of the variation was explained by the matched pairs and the individual interaction, describing individual heterogeneity nested within the matched pairs. However, little to no variation was explained by the interaction between respiratory failure status and OLV timepoint. This implies that the systemic impact of OLV and the ability to distinguish patients at risk of developing respiratory failure within the plasma proteome are negligible. BMI and age explained only a small percentage of the variation, but the variable with the least variation explained by the proteomics data was biological sex. Similar to the BALF proteomics variance partition, a large percentage of the variance was not explained by the model, which is described as the residuals.

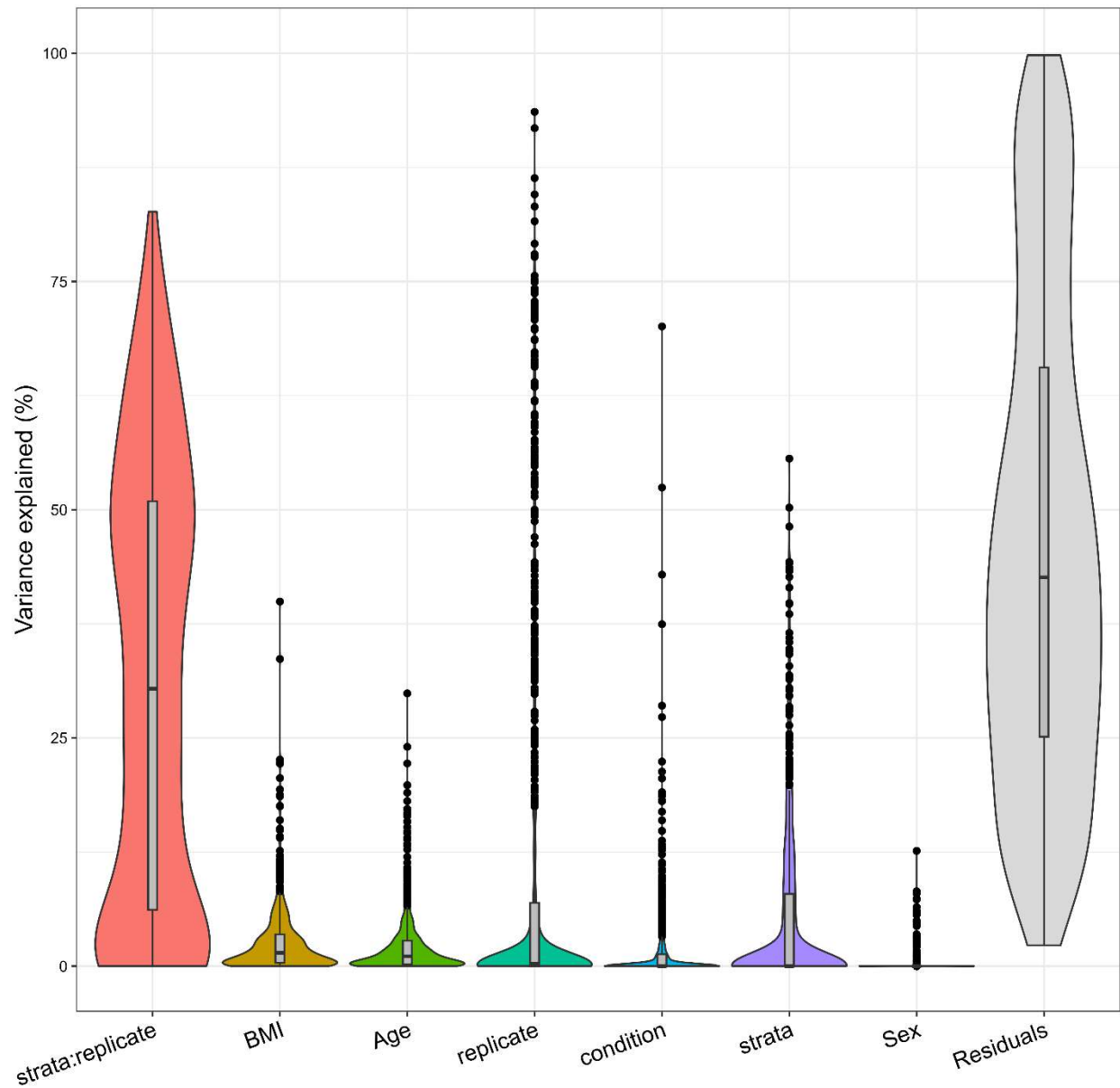


Figure 30. Percentage of variance explained by each covariate in the variance partition model in the plasma proteome.

Differential Protein Expression Presented in Heatmaps

To visualize the pattern of protein expression in plasma samples, a heatmap, with and without clustering, was generated for the significantly variable plasma proteins across all conditions. Figures 31A and B show the clustered and unclustered heatmaps for all conditions.

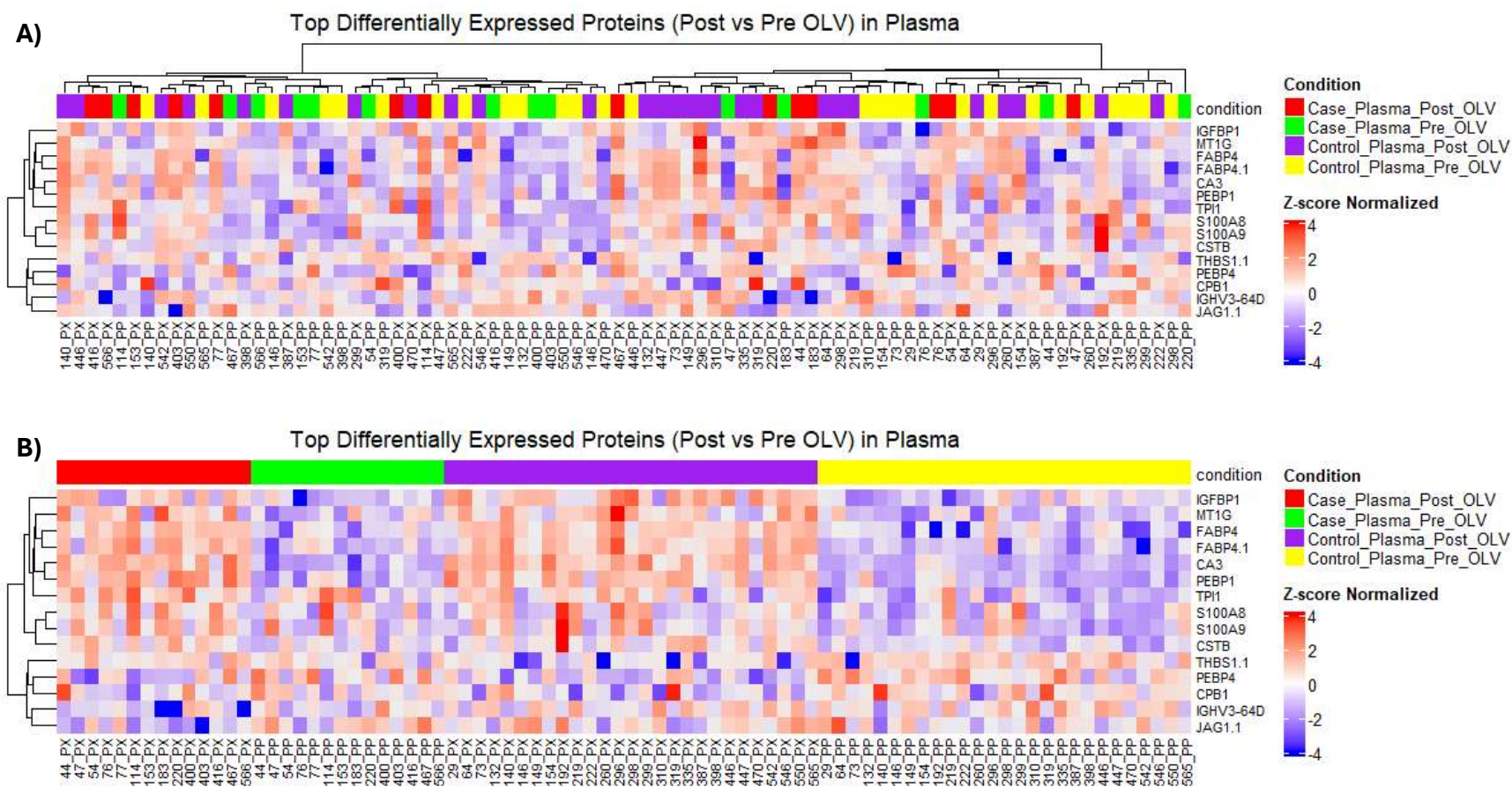


Figure 31A-B. Heatmap of all differentially expressed proteins in plasma samples at pre- and post-OLV timepoints. **A)** clustered heatmap and **B)** unclustered heatmap.

From these heatmaps, the s100A8/9 proteins show modest upregulation across the board in cases post-OLV, with an intense upregulation in 192 post-OLV and a slightly intense increase in 296 and 299 post-OLV matched controls. In pre-OLV, there was a more pronounced pattern of upregulation, even at baseline, in these proteins in respiratory failure cases compared to matched controls. The pattern of differential expression of these proteins may be an important marker of perioperative inflammation in OLV context. Phosphatidylethanolamine-binding protein 4 (PEBP4) was upregulated pre-OLV in matched controls and to a lesser but similar extent in cases, but not in post-OLV samples. Protein jagged-1 (JAG1), on the other hand, showed greater downregulation in post-OLV cases, especially in OLV ID 403, compared with matched controls.

Differential Protein Expression Presented in Volcano Plots and Gene Set Enrichment Analysis

Looking at differentially expressed proteins in the plasma proteome post-vs-pre-OLV, there were eight differentially expressed proteins in respiratory failure cases and twelve in matched controls as shown in Figure 32A-B. Of the eight proteins in cases, six were upregulated, and two were downregulated. The upregulated proteins included carbonic anhydrase 3 (CA3), Phosphatidylethanolamine Binding Protein 1 (PEBP1), Fatty Acid Binding Protein 4 (FABP4), Insulin Growth Factor Binding Protein 1 (IGFBP1), and metallothionein-1G (MT1G), whereas the downregulated proteins included JAG1 and IGHV3-64D.

In terms of enriched pathways, GSEA REACTOME pathways such as keratinization, cornified envelope formation, RHO GTPase effectors, metabolism, and cellular responses to stimuli were enriched as shown in Figure 32B. BRAF and RAF1 fusions, oncogenic MAPK signaling, and RAS mutant signaling pathways were also enriched which may indicate cancer- or cancer treatment-related pathway enrichment. Carbon metabolism and cornified envelope formation KEGG pathways were enriched in Figure 32C. Biological processes such as cytoskeletal organization, pyridine nucleotide and nicotinamide metabolic processes, keratinocyte and epidermal cell differentiation were in enriched. GSEA enriched biological processes are shown in Figure 32D.

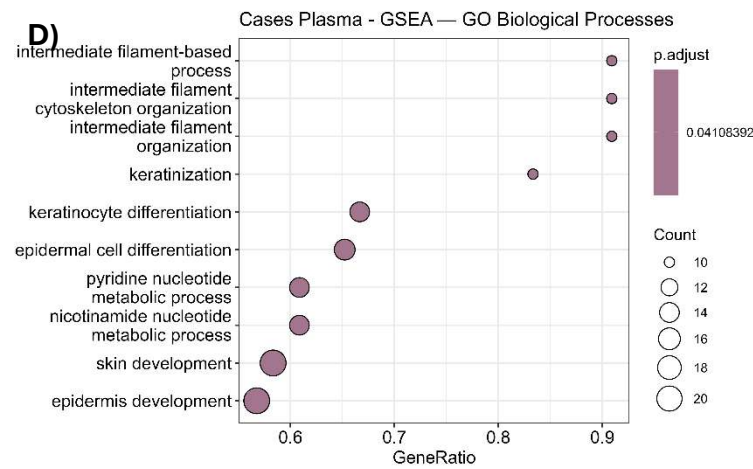
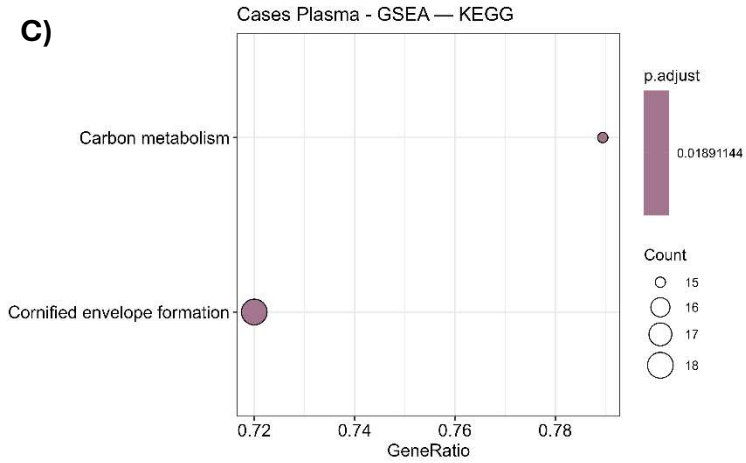
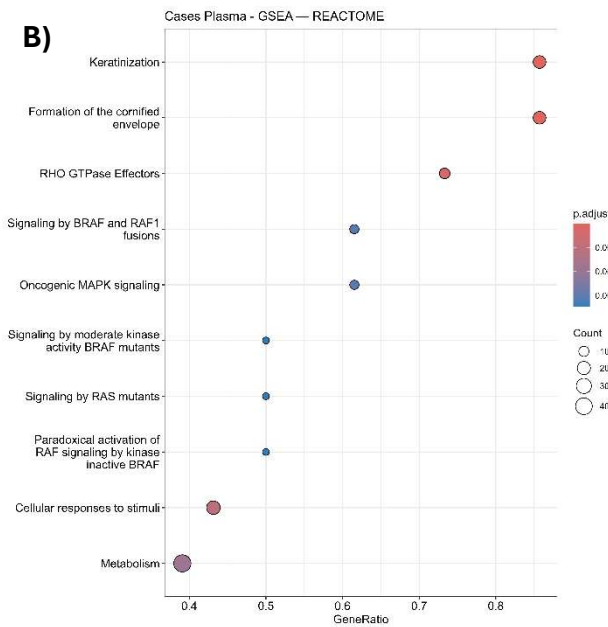
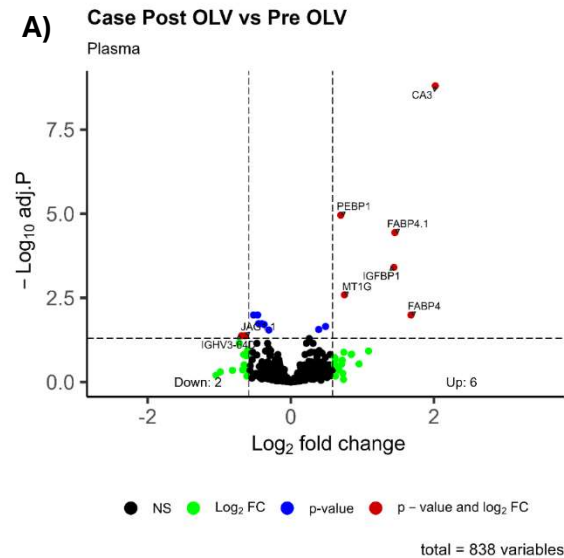


Figure 32A-D. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in plasma proteome respiratory failure cases post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** GSEA on REACTOME pathways, **C)** GSEA KEGG pathways, and **D)** GSEA on Gene Ontology (GO) biological processes, all with FDR < 0.05 .

In matched controls, CA3, IGFBP1, FABP4, and MT1G are upregulated as in respiratory failure cases. However, PEBP4, Carboxypeptidase B1 (CPB1), and Thrombospondin 1 (THBS1) were uniquely downregulated in matched controls, while s100A8/9 proteins, Cystatin-B (CSTB), and triosephosphate isomerase (TPI1) were uniquely upregulated in matched controls. Thus, making PEPB1 upregulated and JAG1 and IGHV3-64D downregulated only in respiratory failure cases. S100A8/A9 upregulation promotes leukocyte recruitment and cytokine release and modulates inflammation. Its upregulation in matched controls is paradoxical, as these patients did not develop inflammatory responses driving respiratory failure.²²⁶ Furthermore, downregulation of PEBP4 may worsen acute lung injury response after LPS instillation, as shown in PEBP4 knockout mice subjected to LPS-acute lung injury.²²⁷ Downregulation of this protein, similar to the upregulation of s100A8/9 proteins, is contradictory to the absence of respiratory failure.

In matched controls GSEA, molecular functions such as ATP hydrolysis, hydrolase activity, monocarboxylic acid, fatty acid and anion binding were enriched as shown in Figure 33B. In Figure 33C, biological processes such as ATP metabolic process, and nucleotide-related metabolic processes were enriched. KEGG pathways related to glucose metabolism such as glucagon signaling pathway and glycolysis and gluconeogenesis pathways were enriched, with carbon and lipid pathways being enriched as well. Infectious disease, stress and stimuli cellular responses, metabolism and keratinization REACTOME pathways were enriched in matched controls plasma proteome. KEGG and REACTOME GSEA pathways are shown in Figure 33 D and E.

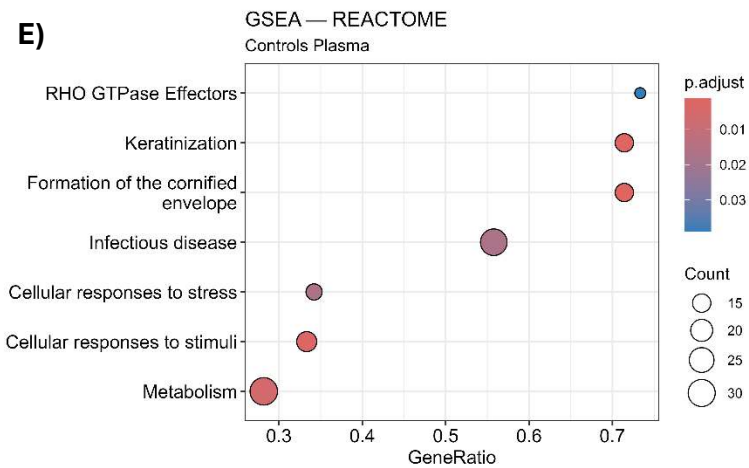
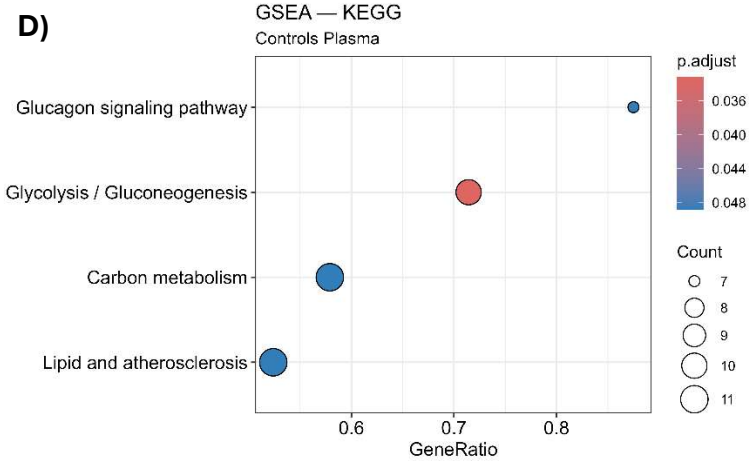
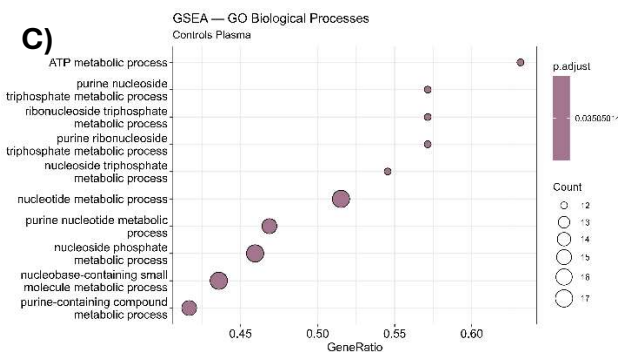
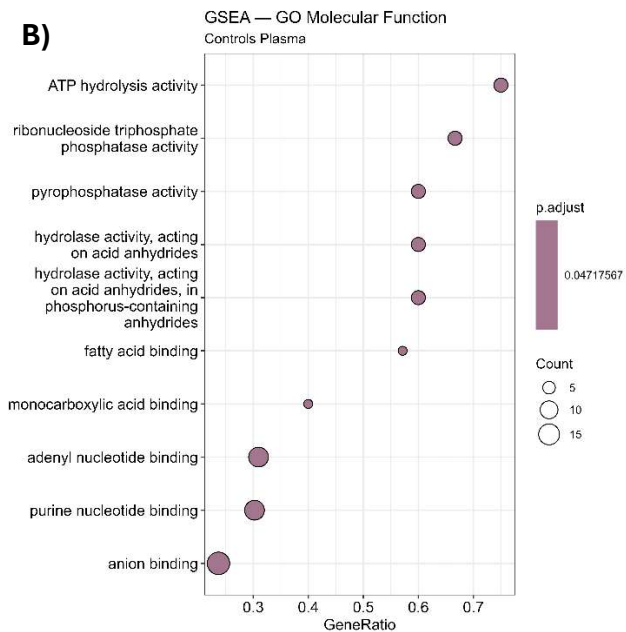
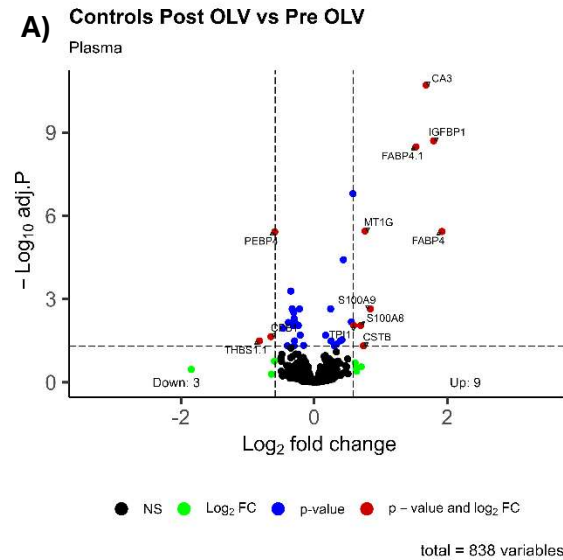


Figure 33A-E. Differential protein expression analysis and Gene Set Enrichment Analysis (GSEA) in plasma proteome matched controls post-vs-pre-OLV. **A)** volcano plot showing differentially expressed proteins with fold change ≥ 2 and FDR < 0.05 , **B)** GSEA on REACTOME pathways, **C)** GSEA KEGG pathways, and **D)** GSEA on Gene Ontology (GO) biological processes, all with FDR < 0.05 .

Multivariate sPLS-DA and Elastic Net Conditional Logistic Regression Results

Sparse PLS-DA comparison of plasma proteomics data across all four conditions yielded a small percentage of variation in both the first and second sPLS-DA components. Component one across OLV timepoints showed a higher variance percentage than component two, which showed variation across respiratory failure status. The sPLS-DA plasma samples trend is shown in Figure 34.

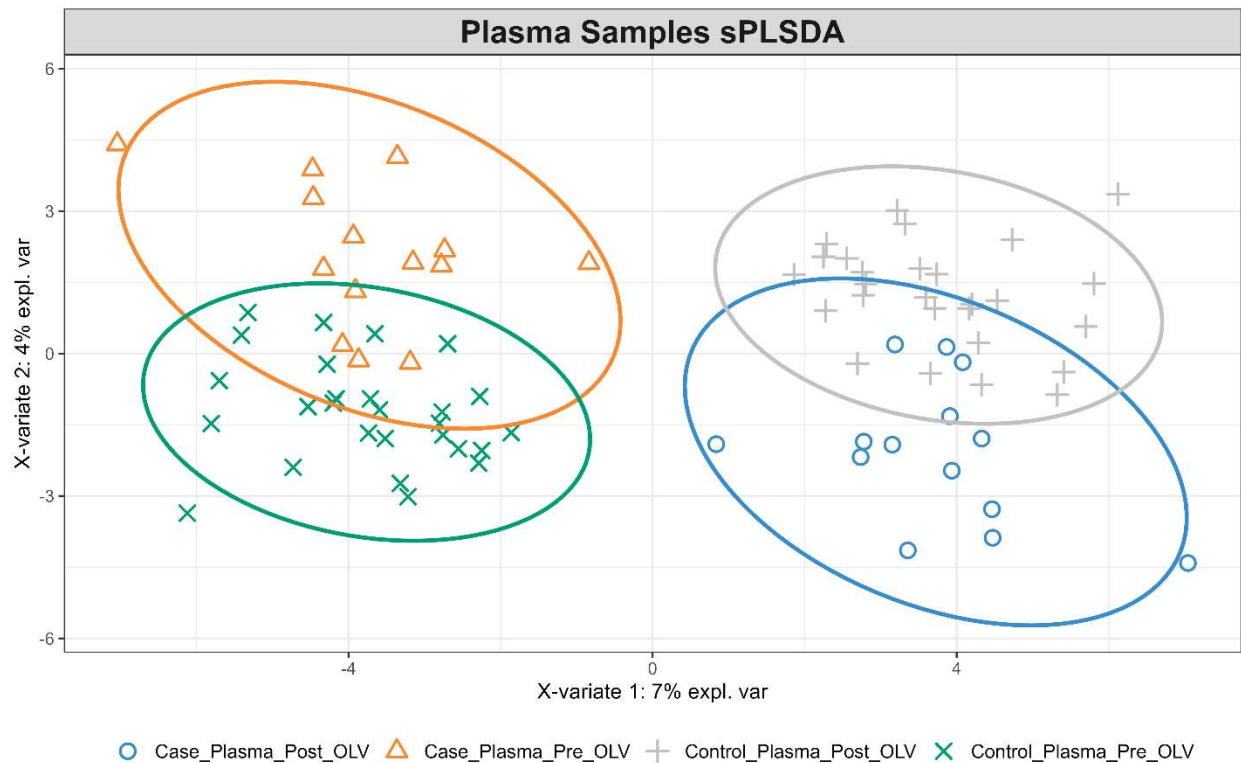
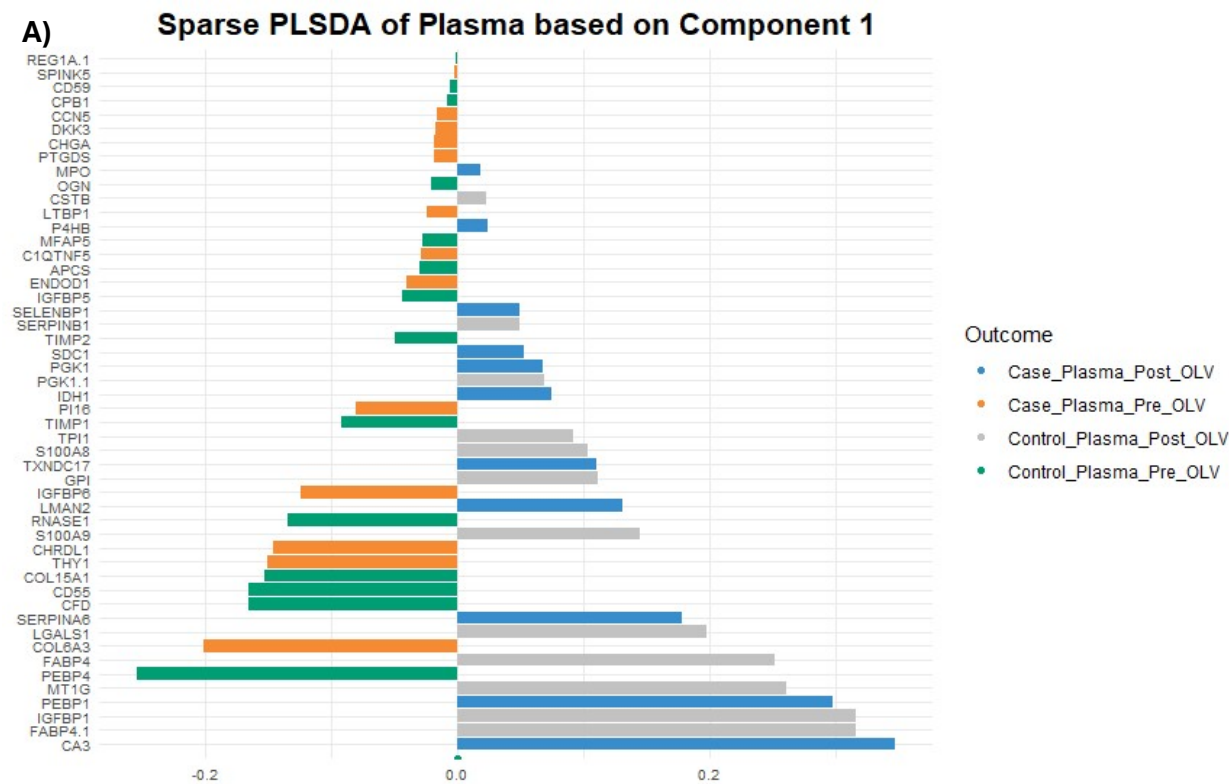


Figure 34. Sparse PLS-DA plot for plasma proteome showing the variation explained by components 1 and 2.

Aside from the differentially expressed proteins, SERPINA6 contributed towards cases post-OLV, while Galectin-1 (LGALS1) had an increase towards matched controls post-OLV in component 1. Corticosteroid-binding globulin (SERPINA6) is a protein involved in corticosteroid transport in the bloodstream, and LGALS1 was shown to promote immune cell apoptosis.^{228–230} The contribution of plasma proteins in component 1 and 2 is shown in Figures 35A and B. In component 2, Heat Shock 70 kDa Protein 1A (HSPA1A) and Serglycin (SRGN) contributed towards cases post-OLV. HSPA1A is a ubiquitous protein involved in cellular

hemostasis, stress protection, and lipid interactions 193.²³¹ SRGN, on the other hand, is associated with neutrophil elastase organization, a key protease involved in NETosis.²³²



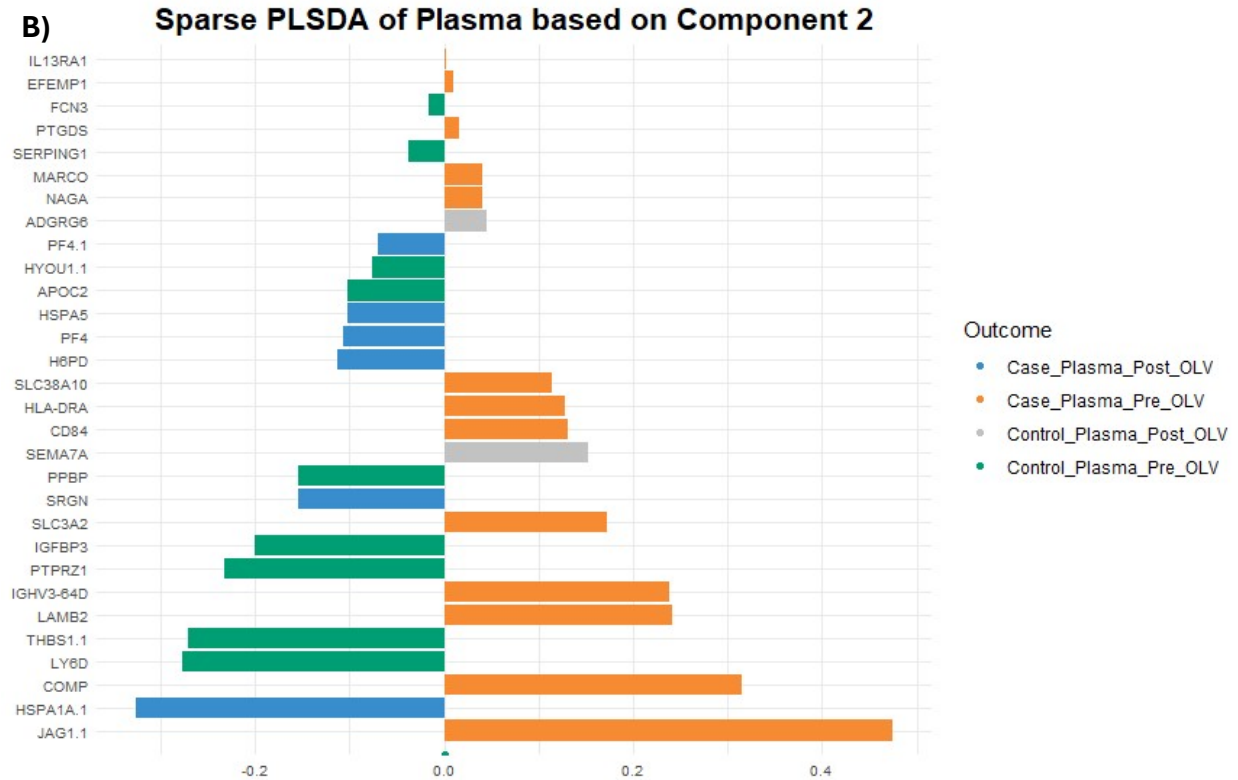


Figure 35. Sparse PLS-DA plot showing the proteins that varied in plasma proteome. **A)** component 1 and **B)** component 2 of sparse PLS-DA.

We implemented an elastic net conditional logistic regression on plasma proteomics data. The results are shown in Figure 36. From this analysis, serum amyloid P-component (SAP/APCS), Protein Tyrosine Phosphatase Receptor Zeta 1 (PTPRZ1), and Complement C1q subcomponent subunit B (C1QB) showed a stronger association with respiratory failure. SAP has been reported to have a protective effect against fibrosis and neutrophil recruitment to the lungs.²³³ C1QB may indicate complement activation through the lectin-complement pathway.²³⁴ None of the proteins selected by the elastic net model passed the stability selection probability threshold. However, PTPRZ1 protein had a probability value, $\pi_{thr} = 0.53$.

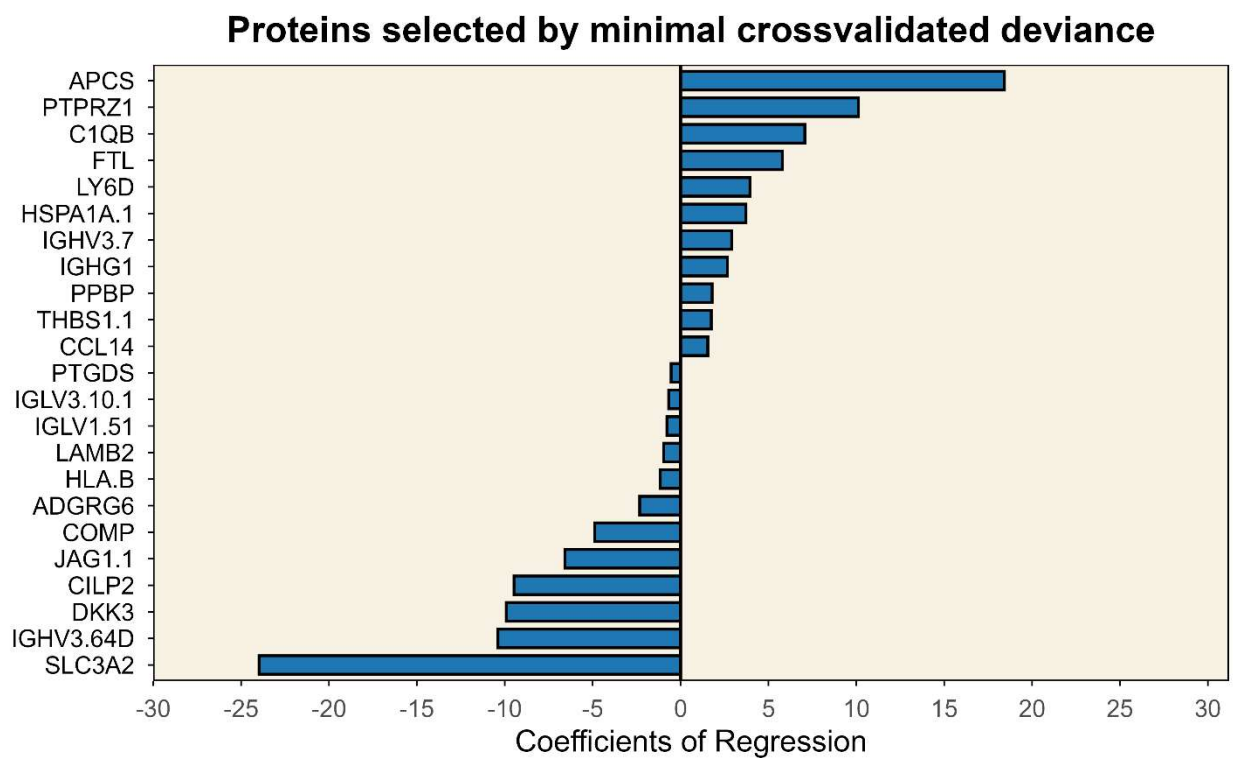


Figure 36. Elastic net conditional logistic regression coefficients for proteins in plasma proteome selected by a model with minimal cross-validated deviance.

Chapter VI: Discussion

In this study, our goal was to identify patients undergoing lung surgery who developed postoperative pulmonary complications (PPCs). Preoperative comorbidities and surgical factors are prognostic for PPCs. However, an additional factor concerns the ventilated lung during surgery. OLV enables lung surgery by facilitating gaseous exchange in the non-operated lung, while the operated lung is collapsed during the procedure. Because all mechanical ventilation is delivered to the single ventilated lung via OLV, the patient's respiratory demands are met by this lung. Hence, when the patient's respiratory demands rise, they are met by increasing the ventilator's volume, pressure, and/or oxygen concentration to the sole ventilated lung. These injurious strategies can exacerbate already existing risks to PPCs from preoperative comorbidities and the extent of lung tissue removed. Thus, to identify patients at risk of developing PPCs and to offer therapeutic interventions as early as intraoperatively, we performed an untargeted proteomic biomarker screening of patients who developed the most severe PPC, which is respiratory failure.

We performed a nested case-matched control study within a large cohort that enrolled consecutive consenting patients undergoing lung surgery, for whom BALF and arterial plasma samples were collected. These sampling approaches offer an insight into the local and systemic injury patterns, respectively. Fourteen respiratory failure patients were matched with twenty-seven matched controls on preoperative comorbidities and extent of lung tissue resection. This design was implemented to isolate the impact of OLV on the development of respiratory failure.

Due to the heterogeneous nature of respiratory failure, more patients enrolled in the large cohort study had developed postoperative respiratory failure than the 14 included in this nested case-control study. Thus, we screened patients and included only those who had developed postoperative respiratory failure attributable to OLV-surgery. For instance, patients who developed respiratory failure because of situations that occurred in the surgical intensive care unit, not related to their surgery, such as due to aspiration or hemorrhage, were not included. This process was reviewed by a thoracic surgeon who is also the principal investigator of this study. This approach ensures that patients who underwent proteomic analysis truly developed the outcome as a result of the OLV-surgery intervention.

In terms of results, one of the most highly upregulated proteins post-vs-pre-OLV is LMNA. This protein is upregulated in BALF from operated and ventilated lungs in both respiratory failure cases and matched controls. In respiratory failure cases, LMNA was slightly upregulated post-vs-pre-OLV in the operated lung BALF relative to ventilated lung BALF (logFC: 1.699 vs 1.692), whereas in matched controls it was more upregulated in the ventilated lung compared to the operated lung BALF (logFC: 1.431 vs. 1.221). However, in both the operated and ventilated lung BALFs, LMNA showed a higher log2 fold change in respiratory failure cases post-OLV-vs-pre-OLV, compared with matched controls. In a mouse model of barotrauma and in mechanically ventilated patients, López-Alonso et al. reported that Lamin-A was significantly upregulated in both contexts, but not in ARDS patients.²¹⁴ They further elucidate that the LAMIN-A-to-B ratio can model nuclear compliance in pulmonary epithelial cells.²¹⁴ The upregulation of this protein in both lungs could be due to ventilation, as both lungs receive TLV, while the ventilated lung receives both OLV and TLV. Its higher abundance in patients with respiratory failure compared with matched controls may reflect the greater susceptibility of patients' lungs to ventilation-induced injury. However, this requires further validation in animal studies with healthy lungs and disease model lungs subjected to various injurious ventilation strategies.

Another protein found upregulated in operated and ventilated lung BALF in both respiratory failure cases and matched controls is ITIH4. In respiratory failure cases, this protein was highly upregulated in the operated lung BALF compared to ventilated lung BALF (logFC: 2.060 vs. 1.325). Similarly, in matched controls, it was highly upregulated in the operated lung compared to the ventilated lung (logFC: 2.884 vs. 1.969). However, this protein was highly abundant in matched controls post-vs-pre-OLV in both operated and ventilated lung BALF proteomes compared to respiratory failure cases. ITIH4 was upregulated in response to IL-6 and LPS administration in the liver epithelial cell line.²³⁵ However, its role in response to acute trauma in the context of acute ischemic stroke and acute lung injury due to particulate matter (in mice) is proposed to be anti-inflammatory.^{236,237} ITIH4 administration in mice also decreased 8-isoprostane levels, a marker of oxidative stress, after iron-particulate matter exposure.²³⁷ Thus, ITIH4 may play an anti-inflammatory role in both cases and controls; however, matched controls tend to have a higher abundance of this protein and may potentially benefit from this upregulation.

A protein highly upregulated in respiratory failure cases is PSME2. Sarma et al. reported that PSME2 was found to be upregulated in hyperinflammatory vs hypoinflammatory non-COVID ARDS patients in bulk transcriptomics analysis of tracheal aspirate samples.³² Out of the RNA transcripts that were upregulated in their analysis with more than a two-fold change, PSME2, ECHS1, KRT7, HBA1, and HBB were also upregulated as proteins in our respiratory failure post-vs-pre-OLV analysis.³² On the other hand, lactoperoxidase and MANSC1 proteins were downregulated by a double-fold change in both our datasets.³² Most notably, the downregulation of lactoperoxidase, which is an enzyme that is associated with the regulation of cellular H₂O₂, may indicate loss of protection from hyperoxic lung injury, which gives rise to H₂O₂.^{32,238,239} Although our study lacks results from specimens collected after the onset of respiratory failure, those of Sarma et al. provide complementary validation. Their results coincide with our findings that proteins detected in non-COVID ARDS patients with hyperinflammatory phenotype were also detected in our study, as soon as intraoperatively, before respiratory failure onset.³²

Another important molecule associated with matched controls but not with respiratory failure cases was ANXA1. Although this protein was not found to be significant due to not meeting the statistical threshold, it has been implicated in mitigating ventilator-induced lung injury (VILI) and ARDS.^{240–243} In addition to VILI, it has been shown to ameliorate ischemia-reperfusion injury.²⁴⁴ A more interesting aspect of this protein is that its release can be induced by dexamethasone^{245,246}, which is a glucocorticosteroid administered as an anti-inflammatory therapeutic in OLV surgery. The reason it is upregulated in matched controls but not in respiratory failure cases may potentially be that dexamethasone is unable to induce ANXA1 release in the latter group. This behaviour may also be relevant in the context of ARDS, where patients in the early phase of ARDS may benefit from dexamethasone treatment.²⁴⁷

Another interesting marker upregulated in respiratory failure cases is Histone H3 (H3-3A). H3 is a protein wrapped around DNA, and its presence in BALF may indicate possible NET formation. Citrullinated H3 (citH3) has been shown as a candidate marker of NETosis.^{248–250} Although at this point in our study, it is not possible to identify whether H3-3A is citH3, it warrants further investigation with citH3-specific assays. If neutrophil degranulation and NETosis are confirmed with such assays, then preventing excess activation of these processes

can be possible with inhibitor treatments.²⁵¹ Further, H4 Clustered histone 1 (H4C1) is also upregulated in cases and matched controls, but the log2 fold change was greater in cases than in matched controls.

In terms of enriched pathways, Wen et al. has observed fibrinolysis, negative regulation of endopeptidase activity, and cellular protein metabolic process GO biological processes along with complement and coagulation cascade, neutrophil extracellular trap formation and neuroactive ligand-receptor interaction KEGG pathways which are processes and pathways enriched in our study as well.²⁵² Their study was in an animal model with a similar experimental approach of performing proteomics screening of BAL fluid in mechanically ventilated mice.²⁵² Furthermore, they observed significant upregulation of ANXA1 amongst other proteins at day-8 of mechanical ventilation which they also argue may mitigate ventilation-induced lung injury.²⁵² Although their study involves long-term mechanically ventilated mice²⁵², it has direct implications for our study such that we observed similar mechanisms of lung injury in short-term OLV.

In the operated lung proteome, the condition variable, describing case or control and pre-or-post-OLV interaction effect, accounted for the highest percentage of variance explained, aside from residuals. Indicating that the impact of the operation is high post-vs-pre-OLV in the operated lung. Whereas in the ventilated lung, the condition variable had the second-highest percentage of variance explained. This effect could be explained by the increased number of differentially expressed proteins in the operated lung and higher degree of differential expression (fold change). However, whether this effect has to do with determining respiratory failure remains up to the changes in proteins that contribute to respiratory failure in ventilated and operated lung BALFs. Thus, validation studies are required to confirm proteins contributing to respiratory failure and comparing their expression in the operated lung and ventilated lung. The other explanation is that these proteins have more to do with ventilation. Some of the proteins differentially expressed in the ventilated lung BALF were also differentially expressed in the operated lung BALF during OLV. CYB5A, LMNA, VIM, CXCL17, DMBT1, APOA1/2, CPN2, SAA4, DES, MIA and ITIH proteins were found in both contexts. This could be due to both lungs being ventilated in TLV setup before OLV and after OLV or due to intermittent ventilation

of the operated lung during surgery. Thus, these proteins may potentially be ventilation responsive.

Additionally, the operated lung BALF analysis identified the most differentially expressed proteins, as shown in the volcano plots and elastic net conditional logistic regression analysis. This could be attributed to the sheer impact of the operation post-vs-pre-OLV and, to some extent, contamination with plasma proteins. Although the extent of tissue resection was matched between cases and matched controls, any aspect of surgical manipulation may change due to the unexpected events during surgery.

Another explanation is that the pathways leading to the release of these proteins into the bronchoalveolar space are activated in both operation and ventilation settings. In the GSEA of the operated lung proteome, GO terms and pathways indicative of oxygen carrier activity and hemoglobin-related pathways were enriched. These pathways may be enriched due to potential blood contamination in the bronchoalveolar space during lung surgery when dissecting pulmonary vessels and stapling airways.

Another aspect of the operated lung is that it may contain a tumour for which lung resection is performed. Hence, the tumour and preoperative local therapies may affect the BALF proteome of the operated lung and how it responds to OLV-surgery. Thus, the variation observed between respiratory failure cases and matched controls may not be entirely due to respiratory failure risk.

Regarding the plasma proteome, the variation explained by respiratory failure status and OLV timepoint interaction was very small. This indicates that our ability to detect the impact of OLV and proteins that could identify risk for respiratory failure was low. Furthermore, there were far fewer proteins that changed significantly during OLV. This phenomenon could be explained by the lesser systemic impact relative to the local impact of lung injury. Additionally, the plasma proteome may be responsive to the impact of the operation, ventilation, any systemic cancer therapy burden and any antibiotic, anesthetic and analgesic drugs used. Thus, changes in plasma proteome post-vs-pre-OLV may not be solely due to the impact of OLV.

Nevertheless, plasma sampling offers prognostic utility, ease of access through the arterial line already established for surgery, and the ability to measure proteins with low sample

volumes. Detection of respiratory failure at the intraoperative stage in plasma can offer significant benefit by predicting patients at risk and candidates for prophylactic medication. Relative to BALF sampling, which can be considered invasive outside the lung surgery context, blood sampling is routinely performed by anaesthesiologists for blood gas measurements. Thus, performing an assay to detect candidate prognostic proteins can have a significant clinical impact. Additionally, many assays have been developed and validated to efficiently measure proteins in plasma samples. The ability to optimize similar assays to detect prognostic plasma proteins has tremendous potential. For plasma analysis in our OLV proteomics study, it could be beneficial to perform discovery-based proteomics panels that are more sensitive to low-abundance proteins, which may be involved in acute lung injury and the development of postoperative respiratory failure. Our current plasma screening could serve as a springboard for such assays in the future.

In addition to volutrauma, barotrauma, hyperoxia and atelectrauma, ventilator-induced lung injury may manifest as biotrauma, which is the lung injury caused by inflammation due to ventilation. The biotrauma hypothesis further links that inflammation in and of itself can exacerbate lung injury. Lex and Uhlig et al. make an important distinction between “weak” inflammation that can cause respiratory failure in conjunction with other contributing factors and a “strong” inflammation that is enough to cause respiratory failure on its own.²⁵³ Nevertheless, these types of models can be represented in animal models; in clinical studies, lung comorbidities can intertwine with ventilation-induced inflammation to cause lung failure. In patients with good pulmonary function, as denoted by high % predicted FEV1, there was no difference in BALF cytokine in ventilated lungs between lung protective vs conventional tidal volume usage while controlling for PEEP and FiO₂, as described by Fiorelli et al.²⁵⁴ Thus, preoperative lung function may also dictate the inflammatory response to VILI.

In terms of preoperative lung function, a point of controversy is the use of race to adjust for predicted %FEV1 and %FVC values. Race correction for pulmonary function measurement comes from a racially biased perspective that can potentially mask disease, contribute to disparities in healthcare access, and provide no improvement clinically compared to unadjusted values.^{255–258} The 2023 official American Thoracic Society statement recommended replacing race-adjusted pulmonary function formulas with race-neutral alternatives.²⁵⁹

The impact of race-neutral and race-adjusted spirometry measurements on lung surgery lies in eligibility and risk assessment. Using race-neutral measurements lowered both the % predicted FEV1 preoperative and postoperative measurements compared to using race-adjusted measurements in African American patients.²⁶⁰ The implications include an increased risk of lung surgery due to lower % predicted FEV1, which can affect surgeons' decision-making regarding eligibility.²⁶⁰ However, surgical eligibility is based on DLCO, smoking history, arterial blood gases (PaO₂ and PaCO₂), and VO₂ max, which are not race-dependent and were not evaluated in this study.²⁶⁰ Thus, future investigations should be supplemented with unbiased anatomic and physiological indicators of lung function and disease to advance race-neutral spirometry assessments cautiously in lung surgery eligibility assessment.

In lung surgery, the removal of lung tissue can, in fact, further diminish lung function after surgery. Lex and Uhlig noted that even in a murine model, dexamethasone treatment was not able to reverse ventilator-induced inflammation along with neutrophil infiltration.²⁵³ In our investigation, we observed that ANXA1 was upregulated in matched controls but not in respiratory failure cases, as determined by multivariate analyses. Even though ANXA1 did not reach statistical significance in univariate analysis, it may explain biologically why corticosteroid treatment did neither reverse inflammation nor mitigate respiratory failure, as both groups were treated with dexamethasone.^{245,246} Although there are multiple intersecting factors, such as oxidative stress, ventilator damage, and alveolar-capillary barrier dysfunction, during OLV surgery that can contribute to respiratory failure other than inflammation, one hypothesis could be that there may be degradation of ANXA1, requiring further investigation.²⁶¹

Another insight from Lex and Uhlig is that in a murine model, respiratory failure was induced by barotrauma at more than 27 cm H₂O, and a study from our lab examining ventilator data from patients in the OLV cohort found that more than 25 cm H₂O was associated with PPC incidence.^{253,262} This study further found that, among the 225 patients in the OLV cohort, lung-protective ventilation strategies were violated, with protective tidal volume and plateau pressure being most violated.²⁶² Thus, these two studies emphasize that OLV lung-protective ventilation protocols are not always followed, and that dexamethasone administration may not always reverse inflammation due to ventilation-induced lung injury.^{253,262}

Corticosteroids and statins are options in modulating inflammation. Although dexamethasone is used routinely in thoracic surgeries and across the OLV cohort, there still remains an occurrence of PPCs. With statins, the NHLBI ARDS network had investigated the role of rosuvastatin as a therapeutic option for sepsis-ARDS, and it was not associated with better outcomes and, in fact, may contribute to hepatic and renal toxicity.²⁶³ Simvastatin is another statin which was investigated to lower the incidence of PPCs after OLV surgery, but was not able to reduce PPC incidence.²⁶⁴

A randomized controlled trial by Theroux et al. has shown that in pediatric OLV, methylprednisolone has been shown to lower inflammation, as described by IL-6 and IL-10 measurements.²⁶⁵ Pediatric patients may not have the same preoperative lung damage as adult patients and may have better lung function relative to their weight and height. Hence, the inflammatory response of pediatric patients may not be the same as that of older adult patients, who may have an exacerbated inflammatory response due to pre-existing damage to the lungs, coupled with lung injury during OLV.²⁶⁶ Nevertheless, low-dose corticosteroid treatments tend to be well-tolerated in the ARDS context²⁶⁷ and may be preferable to statins. Additionally, low-dose infrequent administration of these drugs may not have the feared effect of suppressing immune response to infections, which is observed when used to treat chronic inflammatory lung conditions.²⁶⁸

In addition to these classes of drugs, dexmedetomidine has been shown to present as an anti-inflammatory analgesic that could mitigate the various mechanisms of lung injury occurring during OLV surgery.^{269–271} The choice of anesthetic also has an impact on inflammatory response, with inhalational sevoflurane having an anti-inflammatory effect and lower PPC incidence compared to IV propofol.^{44,272}

Limitations

Our study comes with limitations ranging from case-control design, matching across variables, missing sample, sample quality, and data analysis. Our case control design involved 14 patients who developed respiratory failure after lung surgery, and 27 matched controls. Although having the 27 matched controls allowed for better matching across confounding variables, there were nearly twice as many controls for every case. Thus, having more controls than cases can cause an imbalanced design especially when handling missing values for proteomic data.

Typically, proteins that are consistently found in most of the samples can pass the missing value quality control check. In this case, since there were more controls than cases this may bias towards proteins detected in matched controls compared to cases. We accounted for this bias by first handling missing values based on proteins being present in at least 75% of samples in each condition (case pre-OLV, case post-OLV, control pre-OLV, and control post-OLV). However, this might result in some proteins being differentially expressed due to the impact of imputation. To avoid this, we applied a second missing value filtration step which retained only proteins found in at least 75% of all samples. With this second step, it is possible to miss biologically relevant proteins that are present in one condition but not in the other. Thus, we have included proteins that gain statistical significance when applying only the first missing value filtration step in Supplementary Table 2.

To understand the implications of the missing value filtration approaches used on each sample, the number of proteins per sample was shown in Figure 5A-C previously. In the ventilated lung, after our missing value filtration approach, more than 2,000 proteins were quantified in almost all ventilated lung BALF samples, except for two samples. The first sample is from a patient who developed postoperative respiratory failure, taken at the post-OLV time point. The second sample is from a patient who did not develop post-op respiratory failure, taken at the pre-OLV timepoint. There was no notable blood or mucous presence in these two samples.

The sample for patient ID 566 had more than 1 mL of BALF supernatant for proteomic analysis. In contrast, the sample for patient ID 446 had an 800 μ L aliquot, a relatively low volume. Additionally, the patients from whom these samples were taken did not have notable comorbidities attributable to lung extra-bronchoalveolar protein content, other than type 2 diabetes with nephrolithiasis in the former and coronary artery disease in the latter. Patient ID 403 and 566 post-OLV operated lung BALF samples also had some of the lowest numbers of proteins identified. In plasma samples, 192 post-OLV had the fewest proteins identified. Patient ID 403 had congestive heart failure and underwent coronary artery bypass grafting, whereas 192 had renal cystitis and other renal comorbidities along with aortic valve stenosis. None of which would be associated with decreased protein in BALF.

The decrease in protein yield in patient 566 post-OLV could have been timepoint-specific, as the affected samples were from both the operated and ventilated lungs. This could be

attributed to potentially low protein recovery in BALFs post-OLV during bronchoscopy or sample processing after collection. The decrease in the number of proteins quantified in the remaining samples could be attributed to experimental procedures rather than having biological significance. It is possible that some proteins were lost during protein purification and digestion. At this step, detergents and salts incompatible with chromatography and mass spectrometry are removed, and proteins are digested into peptides using trypsin, which are then eluted for subsequent desalting. If peptides are left in the digestion and purification column for too long without a buffer or solution, they could dry out in the column. Hence, it makes it harder to elute them from the column, reducing protein recovery. In addition, it could also be attributed to variation in protein recovery during bronchoscopy. These factors could contribute to the lower protein recovery in these two samples.

At the analytical step, we implemented a linear mixed effects model to account for the variables that were not well-matched and the within-patient pairing (repeated measures) and between-patient pairing with a nested case-matched control design. By treating each replicate as a random effect within its matched pair, we can mitigate the effects of covariates that may confound our proteomic results while accounting for repeated measurements, such as pre- and post-OLV.

Additionally, age, sex and BMI were not well-matched in our case-control matching process. There were fewer number of female patients who developed respiratory failure after surgery compared to matched controls. Case-control designs are typically matched by sex, and this is a limitation of our study. Finding matched controls for these imbalanced confounding variables was difficult, we prioritized finding matched controls for variables that directly affect pulmonary function and gas exchange capacity after surgery such as extent of lung tissue removed during surgery, DLCO, and %FEV1. Nevertheless, this imbalance in age, sex and BMI may affect our downstream results. Thus, we set continuous variables as fixed effects and categorical variables as random effects to isolate changes post-vs-pre-OLV due to the interaction between respiratory failure status and specimen collection timepoint. Future investigations using this nested case control design should match for age, sex and BMI along with resection extent, PFTs and smoking history including packyears.

Although we had consistent sample collection for arterial plasma for most patients recruited in the OLV large cohort, inconsistencies exist in BALF collections due to clinical considerations for some patients. Out of the 41 patients, pre-OLV operated lung BALF for patient ID 299 was missing. However, as this patient was one of three matched controls for ID 566 (postoperative respiratory failure case), we considered including it regardless. Thus, the operated lung BALF proteomic data may not be directly comparable to the ventilated lung BALF due to this missing sample. Since the analysis focused on post-vs-pre-OLV in each lung BALF and case 566 has two other matched controls (i.e 73 and 447), we considered disregarding ID 299 in the operated lung BALF post-vs-pre-OLV analysis and the matched-pair category remained the same. But it was included in the ventilated lung BALF post-vs-pre-OLV analysis.

The BALF and arterial plasma samples used for proteomic screening were processed as soon as possible after retrieval from the operating room. However, the time from sample retrieval to processing varies across samples. All samples were kept on ice (or refrigerated for longer periods) with blood samples placed in EDTA tubes to prevent coagulation. This may present as a variation in individual samples, regardless of the batch in which they were processed for mass spectrometry proteomics. However, the effect of blood sample storage and delays prior to processing has lesser impact on mass spectrometry analysis which uses peptides, compared to ELISA analyses which require intact protein.²⁷³ Regardless, this variation can be accounted for by including the patient replicate variable as a random effect in our linear mixed-effects model. Pre- and post-OLV specimens were processed by the same person using the same protocol as soon as it was possible after OR retrieval. Hence, the sample variability introduced at these timepoints is reduced. The samples were stored at -80 °C to ensure cryopreservation until mass spectrometry processing.

Despite our efforts to use linear mixed-effects modelling across filtered and normalized proteins, some proteins failed to converge due to negative eigenvalues. Thus, we report in Supplementary Table 1 a list of proteins that failed in our model and that were not used for downstream analysis. The majority of the proteins, however, were successfully modelled using the linear mixed effects model. Using this model, we were able to identify differences post-vs-pre-OLV for cases and matched controls, at best.

Aside from statistical comparisons, we used heatmaps to identify clustering based on top 25 differentially expressed proteins taken from the statistically significant differentially expressed proteins. However, we did not observe distinct clustering based on the top 25 differentially expressed proteins clustered using the ward D2 and Euclidean methods for rows and columns, respectively. The unclustered heatmaps, on the other hand, showed better clustering based on conditions. This behavior may indicate that either the choice of top 25 differentially expressed proteins or the clustering method used did not separate the conditions properly. This warrants performing other clustering methods, adjusting the choice of top n proteins and/or using a different method of determining the top n proteins.

To achieve our aim of identifying biomarkers that can discriminate between cases and controls, we opted for a repeated-measures sparse PLS-DA approach. This approach can identify components that explain the most variation in our data in a supervised manner. The first component would explain the most variation, with subsequent components following in a decreasing order of variation explained in our proteomics data. The top 2 components we identified aligned with the OLV timepoint and the respiratory failure status. However, the variation explained by these two components is very small. Thus, we identified only a handful of biomarkers that distinguished cases from controls. Another drawback of this approach is that our sPLS-DA does not account for the between-patient pairing introduced by our nested case-control design.

To address this drawback, we implemented an elastic net penalized conditional logistic regression based on the post-vs-pre-OLV difference of each protein. This method would utilize conditional logistic regression's ability to handle nested case-control designs. Elastic net penalization would squeeze out the most informative proteins from the regression model. However, there would not be a way to determine statistical significance. We thereby implemented stability selection on the penalized conditional logistic regression model to determine the biomarkers that meet the specified selection probability threshold. For the ventilated lung BALF proteome, Sodium-Coupled Monocarboxylate Transporter 1 (SLC5A8), and PSME2; for the operated lung BALF proteome: TPM1, TPM2, ELP3, and CPE, and for the plasma proteome, no protein passed the selection probability threshold. However, PTPRZ1 was close to reaching the selection probability threshold in the plasma proteome.

For the elastic net conditional logistic regression model, there were too many proteins measured, with over 2,000 in BALF and over 800 in plasma. These proteins were undermined by the small sample size in this study, especially in the respiratory failure group. Even though elastic net penalization solves this problem, it still carries the risk of overfitting.

Chapter VII: Conclusion

This thesis described proteomic screening approach to identify biomarkers of postoperative respiratory failure from BALF and arterial plasma samples collected before and after lung surgery. By utilizing a nested case control design from a large cohort study, patients who developed postoperative respiratory failure were matched with matched controls who did not develop this outcome. These two groups were matched on variables that pose risk for postoperative respiratory failure. Thereby, isolating the impact of OLV to be examined. This method allowed comparison of differentially expressed proteins post-vs-pre-OLV in the ventilated and operated lung BALF, and arterial plasma samples.

From the differentially expressed proteins, various inflammatory pathways were enriched in both respiratory failure cases and matched controls. This phenomenon warrants further investigation to identify the responses of patients, who are preoperatively primed to develop respiratory failure, and to OLV-induced lung injury. As there may be biological differences in OLV-injury response between respiratory failure and matched control patients.

Utilizing multivariate analyses that take into account the repeated measures and case-control matching design allowed for identification of unique protein biomarkers. These biomarkers have been investigated in the literature to have had a role in respiratory failure. These proteins could undergo various validation studies both *in vitro* and *in vivo* to confirm their role in development of postoperative respiratory failure and mitigating this outcome as soon as during lung surgery.

Future Directions

Unbiased proteomics screening of patient BALF and plasma samples requires validation to ensure reproducibility of the proteins identified as significantly expressed. In a prior study, our lab has implemented a cytokine/chemokine Meso Scale Discovery multiplex ELISA approach to test inflammatory biomarkers from a preselected panel.¹⁷⁵ We intend to apply either the same approach with the Meso Scale Discovery targeted panel or a targeted 96-well biomarker panel from the Olink Proteomics Platform. The latter offers superior sensitivity for low-abundance protein detection and works with small sample volumes, making it an ideal experimental validation platform that complements our mass spectrometry-based screening.^{274,275} These

approaches will allow us to confirm mass spectrometry-differentially expressed proteins within the same patient group using another proteomics panel. We also have the capability to perform ELISA assays for biomarkers of clinical and biological interest, such as ANXA1 and PSME2, to confirm their presence using a more sensitive assay platform. These validation assays can be used to generate hypotheses for downstream clinical applications and biological impact.

The current analysis has focused on post-vs-pre-OLV comparisons to assess the impact of OLV-surgery in developing postoperative respiratory failure. However, there may be differences in the pre-OLV proteomic profile between postoperative respiratory failure and matched control patients. These differences can be associated with postoperative respiratory failure risk even before OLV-surgery and may also affect how patients respond to OLV-surgery. Future analyses of this proteomic data can include pre-OLV comparisons in ventilated and operated lung BALF, and plasma proteome to address this limitation.

In addition to *in vitro* validation assays, Dr. Kidane's lab has established an *in vivo* porcine OLV-surgery model.²⁷⁶ This model can be used to validate OLV-responsive markers identified in clinical proteomics and to understand the injury patterns associated with those markers. This model has been subjected to high tidal volume and peak inspiratory pressure – injurious mechanical ventilation – and hyperoxia.²⁷⁶ Biospecimens such as post-OLV: fresh frozen lung tissues, RNA later stored lung tissues, and formalin fixed paraffin embedded lung tissue slides have been harvested from this model.²⁷⁶ These specimens allow detection of tissue-specific and intracellular responses to ventilation-induced lung injury that may not be identified in the current investigation. Additionally, biofluids such as pre- and post-OLV BALF have been collected from both lungs, as well as plasma samples, making the porcine model biofluid inventory directly comparable to clinically obtained fluids.²⁷⁶ This model is ideal for biological validation studies from current proteomic results.

Although not currently established in our lab, a cell culture-based model can provide flexibility for testing various therapeutics before moving to an animal model. The difficulty of establishing such an *in vitro* model is recapitulating the alveolar-capillary barrier in cell culture, including both alveolar epithelial and endothelial cells, while mimicking the impact of ventilation. This difficulty has been addressed by Gabela-Zuniga et al.'s novel ventilator-on-chip model, which simulates application of volutrauma, barotrauma, and atelectrauma to a lung-on-

chip model.²⁷⁷ Replicating this model using primary human alveolar epithelial and endothelial cell lines and culturing them using respiratory failure and matched control BALF and plasma media can allow us to recreate the patient's extracellular environment *in vitro* and monitor cellular responses in real time. By testing the efficacy of different anti-inflammatory therapies in this *in vitro* model, we can assess whether to translate the work in our porcine *in vivo* model, which can mimic both surgical and ventilator trauma.

Another microfluidic device with direct application to our future directions is a neutrophil chemotaxis-based device to detect neutrophil migration from whole blood.²⁷⁸ This method was developed to test neutrophil chemotaxis using COPD patient sputum and whole blood.²⁷⁸ It can easily be repurposed to use BALF and plasma from postoperative respiratory failure patients and matched controls. Using this device can allow efficient testing of anti-inflammatory therapies on neutrophil chemotaxis using patient-derived biofluids and has point-of-care applications to identify patients at risk of postoperative respiratory failure by differences in neutrophil chemotaxis.²⁷⁸

Outside of wet lab experiments for downstream validation, Dr. Kidane's research team has collected real-time ventilator data from OLV study patients. Integration of proteomic data with ventilator data for patients in the current study can shed light on the ventilator dynamics that underlie distinct proteomic profiles. The feasibility of analyzing ventilator data collected in the OR has been shown by So et al.²⁶² Thus, by implementing similar ventilator data analysis pipelines to the current proteomic analysis, we can extract proteomic profiles associated with ventilator patterns in both respiratory failure and matched control patients.²⁶²

Lastly, there remains an unmet need to monitor ventilation and perfusion throughout OLV. In critical care clinical settings and research, electrical impedance tomography (EIT) has been utilized as a novel approach to guide ventilation through PEEP titration and lung recruitment.^{279,280} However, translation of this approach into the operating room requires consideration of the patient's lateral positioning²⁸¹ and interference with the surgical field. Some studies have attempted EIT in lung surgery settings to assess tidal volumes, ensure OLV and PEEP.^{282–284} In our porcine model, we can adjust and modify EIT placement in OLV and lateral positioning to monitor ventilation-perfusion dynamics and correlate them with biomarkers of lung injury. By finding optimal conditions for use of this technology in a preclinical OLV-surgery

animal model, we can inform future randomized controlled trials in clinical OLV settings. Thus, paving the way for approaches that leverage imaging, ventilator waveform, and proteomic data to detect and prevent OLV surgery-induced PPCs.

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Appendix

OLV Clinical Report Form

ONE LUNG VENTILATION 2

CRF 2

PATIENT INITIALS

STUDY NUMBER

ADDRESSOGRAPH

--	--	--

--	--	--

F M L

Date of surgery: _____

Surgery type: _____

Surgeon: _____

Anesthesiologist: _____

O₂ at room air prior to surgery: _____

Steroids Used/Dose: _____

Time surgery began: _____

Time surgery ended: _____

Time TLV started: _____

Time OLV started: _____

Time OLV ended: _____

Time TLV ended: _____

Time of Pre-Op blood: _____

Time of Post-Op blood: _____

Time of Pre - V BAL collection: _____ Volume retrieved: _____ Saline used: _____

Time of Pre - O BAL collection: _____ Volume retrieved: _____ Saline used: _____

Time of Post - V BAL collection: _____ Volume retrieved: _____ Saline used: _____

Time of Post - O BAL collection: _____ Volume retrieved: _____ Saline used: _____

NOTE: please also mark on collection tubes

Ventilated Lung: Right / Left

Operated Lung: Right / Left

Dr. Biniam Kidane

Surgical Peri-Operative Research Team (SPORT)

T: --- ----

Version 1.1 2018-12-18

OXYGEN SATURATION FORM

OLV Oxygen Saturation Form

ONE LUNG VENTILATION 2

CRF 2

PATIENT INITIALS

STUDY NUMBER

ADDRESSOGRAPH

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--	--	--

F M L

TO NURSING STAFF:

1. Please fill out Oxygen Saturation **on Room Air (RA)** for (For RA measurement, remove oxygen for 5 minutes and then measure Oxygen Saturation)

2. Please start recording at 4 hours from end of surgery

3. Please stop recording at 96 hours or 90% on RA **Please give this completed sheet to charge nurse when patient is discharged**

TO WARD CLERK:

1. Please show this to the nurse so it can be added with the nursing vitals sheets

Post-Op Oxygen Saturation on Room Air After End of Surgery						
Nurse's Record	Time After Surgery	Expected Date	Expected Time	%	Recorded Date	Recorded Time
1.	4 hours					
2.	8 hours					
3.	12 hours					
4.	16 hours					
5.	20 hours					
6.	24 hours					
7.	36 hours					
8.	48 hours					
9.	60 hours					
10.	72 hours					
11.	84 hours					
12.	96 hours					
13.	When oxygen saturation $\geq 90\%$:					

Dr. Biniam Kidane

Surgical Peri-Operative Research Team (SPORT)

T: --- --- ---

Version 1.1 2018-12-18

OXYGEN SATURATION FORM

Supplementary Table

Supplementary Table 1. Proteins that failed to converge in dream model

Ventilated Lung BALF	Operated Lung BALF	Plasma
LGALS9.1	IGHV3-74	IGHM.1
RAB27A	MT1A	LPA.1
IGF2R	ITGB2	SAA1.1
HSPA1A.4	BPI	IGHV3-30-3
MAP4	TUBA1A.2	HSPA5
CMPK1	CCT2	IGFBP4
TPI1	NHLRC3	CHI3L1
RHOA.2	DAZAP1	DCBLD1
PITPNA	CALML4	CPB2
TRIM28	AKAP9	GNPTG
PLEC		
TRAF3IP1		
BPIFA1		
LIN7A		
CYRIB		

Supplementary Table 1. List of proteins that failed linear mixed effects model fitting using the Dream function.

Supplementary Table 2. Proteins that become statistically significant (FDR < 0.05) after filtering for proteins present in at least 75% of in ventilated lung BALF samples of at least one condition.

Respiratory Failure Cases Post-Vs-Pre-OLV				Matched Controls Post-Vs-Pre-OLV			
Upregulated	Log ₂ FC	Downregulated	Log ₂ FC	Upregulated	Log ₂ FC	Downregulated	Log ₂ FC
PAK1.3	2.95	SMPDL3B	-1.02	CA3	1.86	SUSD2	-1.01
SART1	2.32	SLC11A2	-1.07	FITM1	1.51	ARRDC1	-1.01
HNRNPR	1.87	SH3BP4	-1.09	RPLP2	1.24	CTSF	-1.02
AGR3	1.85	GGT1	-1.13	KRT7.1	1.24	IGHV3-73	-1.03
FITM1	1.83	LYPD3	-1.14	CFHR5	1.23	CEACAM5.1	-1.11
AKR1C1.3	1.65	PI3	-1.24	AKR1C1.3	1.19	ACP3	-1.14
KRT7.1	1.62	ATP13A3	-1.25	AGR3	1.16		
PTBP1.1	1.59	DTD2	-1.29	SUCLG1	1.13		
SUCLG1	1.56	CRISPLD1	-1.62	AGFG1	1.12		
PLEKHA6	1.47	SPRR3	-1.99	PGLYRP2	1.12		
EPPK1	1.47	KLK1	-2.21	CPSF6	1.11		
ITIH3	1.43	CRIM1	-2.35	NUDT4	1.05		
TUBA1A	1.43	IGHV4-4	-2.51	C1QA	1.02		
CPSF6	1.41			SFN.2	1.02		
CLXN	1.32			BPGM	1.02		
SLC2A14	1.30			VMO1	1.02		
HLA-DQA2	1.28			ECHS1	1.01		

HNRNPA1.2	1.27
RABEP2	1.20
TAGLN	1.18
UCHL1	1.16
CFHR5	1.15
PARP1	1.10
CAPSL	1.04
NPM1	1.00

Supplementary Table 2. List of proteins that become statistically significant when assessing proteins in ventilated lung BALF present in at least 75% of the samples of at least one condition. Post-vs-pre-OLV comparisons were made in postoperative respiratory failure and matched control samples using Dream function.

Supplementary Table 3. Ventilated Lung BALF Proteins that become statistically significant (FDR < 0.05) when lowering fold change threshold to 1.5 ($1.5 \leq FC < 2$).

Respiratory Failure Cases Post-Vs-Pre-OLV							
Upregulated	Log ₂ FC	Downregulated	Log ₂ FC	Downregulated	Log ₂ FC	Downregulated	Log ₂ FC
NPM1	0.999	CD55	-0.587	DUOX1	-0.816	BDH2	-0.979
MECP2	0.992	F11R	-0.635	TMC4	-0.820	SH3BP4	-0.987
CPN1	0.981	MYO1B	-0.653	EPS8L2	-0.823	ABCA13	-0.987
SERPINF2	0.959	MYO1D	-0.662	FSTL1	-0.824		
YWHAE	0.957	CHL1	-0.676	CD82	-0.824		
TTR	0.945	TOM1L1	-0.685	FAM3B	-0.826		
OSTF1	0.932	LYN	-0.689	GNA14	-0.827		
PRDX3	0.929	SNAP23	-0.689	STOML3	-0.829		
SAFB	0.905	JCHAIN	-0.706	GLG1	-0.829		
HS1BP3	0.885	VSIG2	-0.718	SLC15A2	-0.840		
ZYX	0.885	PIGR	-0.721	CTSO	-0.841		
HNRNPC	0.873	SLC34A2	-0.725	SLC44A4	-0.843		
CANX	0.859	LSR	-0.730	SDCBP2	-0.845		
CAPG	0.826	SIAE	-0.730	CIB1	-0.847		
HNRNPM	0.821	PHACTR4	-0.731	TMEM30B	-0.853		
KTN1	0.819	KLK11	-0.733	RRAS	-0.853		
ERH	0.818	ITCH	-0.734	AZGP1	-0.857		
SERPINC1	0.817	GPX4	-0.742	C6orf132	-0.859		
AK2	0.808	GNA15	-0.746	GNAQ	-0.865		
SRSF4.1	0.779	SARG	-0.755	IL6ST	-0.865		
SRSF2	0.778	BBS1	-0.757	ADGRG6	-0.878		
SELENOP	0.744	GIPC1	-0.758	SDCBP	-0.881		
SRSF1	0.742	P0DOX4	-0.761	FRK	-0.882		
THRAP3	0.731	STXBP1	-0.763	IDS	-0.884		
HNRNPK	0.724	PTPRJ	-0.764	DDR1	-0.888		
TTC1	0.686	IGLV4-69	-0.767	AQP5	-0.893		
HNRNPU	0.680	TMC5	-0.769	BAIAP2	-0.897		
SERPINA1	0.677	CLDN3	-0.775	PARD6B	-0.898		
PTBP1	0.591	TSG101	-0.781	SLC31A1	-0.906		
		ALPL	-0.786	DNASE2	-0.906		
		SPINT2	-0.786	ENPP3	-0.913		

SLC6A14	-0.788	CCN2	-0.921
OCC1	-0.789	IGHV3-74	-0.921
TIMP1	-0.789	TMPRSS2	-0.924
NT5E	-0.795	IGFBP2	-0.931
FAM3C	-0.795	STC1	-0.940
GNA11.2	-0.801	SMPDL3B	-0.956
EHD4	-0.801	SMPDL3A	-0.956
EPS8	-0.807	ARRDC1	-0.963
SLPI	-0.809	SLC11A2	-0.963
EPS8L1	-0.814	EVPL	-0.977

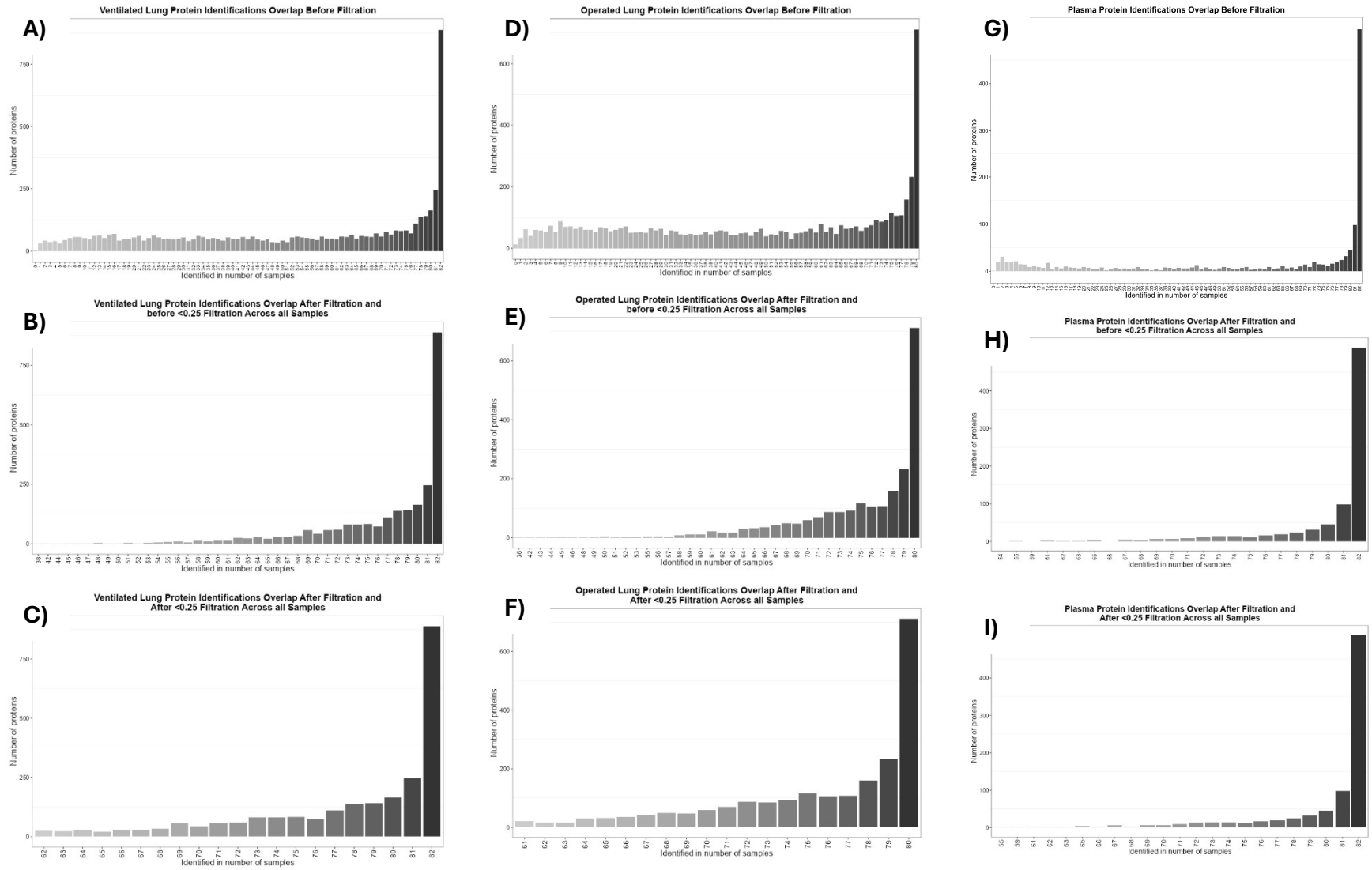
Matched Controls Post-Vs-Pre-OLV

Upregulated	Log ₂ FC	Upregulated	Log ₂ FC	Upregulated	Log ₂ FC	Upregulated	Log ₂ FC
AFM	0.994	SELENOP	0.861	HSPA5	0.762	SDF4	0.669
AGR2.1	0.993	SERPINA7	0.861	OSTF1	0.761	HSPA1A.5	0.668
NUDT4	0.989	PSPC1	0.860	ARHGDI	0.758	ACIN1	0.663
CFAP52	0.989	CES1.1	0.859	SERPINA5	0.757	HP1BP3	0.661
AGFG1	0.988	CFHR3	0.858	ADIPOQ	0.756	CGN	0.661
VTN	0.987	AMBP	0.856	TPD52L2	0.755	CFAP45	0.660
PDIA3	0.983	HSPA1A.6	0.854	NOLC1	0.754	MST1	0.659
VMO1	0.980	CKB	0.851	WDR5	0.754	PTGES3	0.650
PGLYRP2	0.980	SLU7	0.849	CANX	0.751	CFHR1.2	0.641
KTN1	0.969	CD5L	0.849	VAPA	0.749	HNRNPU	0.628
BCLAF1	0.968	RALY	0.849	LZIC	0.744	CFB	0.625
CACNG6	0.966	HP.1	0.845	THRAP3	0.743	PCOLCE	0.623
PI16	0.964	HSPH1	0.845	CYBB	0.743	VASN	0.619
RALYL	0.964	RBM8A	0.843	G3BP1	0.743	SAFB	0.617
APOD	0.963	TARS1	0.840	WDR13	0.742	APOH	0.617
BPGM	0.962	SET.1	0.837	C3	0.741	U2AF2	0.614
C1QA	0.961	C4A.1	0.834	CDA	0.738	LMNB1	0.606
GCA	0.959	EEF1D	0.833	GC	0.736	KIF5A	0.603
VPS29	0.942	ANP32B	0.830	AK2	0.734	HP	0.599
PPP1R7	0.941	ORM2	0.827	IGHG4	0.734	ANXA8.1	0.596
MAN2C1	0.939	NIT1	0.826	VBP1	0.732	LSP1	0.595
IGHG2	0.926	SERPINA1	0.822	RTN4R	0.730	DCTN2	0.594
TNC	0.924	GLUD1.1	0.821	HNRNPC	0.728	DPYSL2	0.593
NPM1	0.923	TMPO.1	0.820	CROCC	0.724	CFHR2	0.592
HSPA4.3	0.921	SKP1	0.805	HSPA1A	0.715	FBLN1	0.586
DNAL1	0.913	C9	0.805	SRSF1	0.712		
SERPINC1	0.910	ACTB.3	0.803	HSPA2.3	0.712		
MB	0.902	SFPQ	0.802	C1QB	0.705		
LAMTOR5	0.901	C1QC	0.799	TPM3	0.702		
SFN.2	0.901	PGAM1	0.799	PFDN2	0.702		
HNRNPR.1	0.900	VAPA.1	0.799	SASH3	0.695		
PDIA4	0.894	GMFB	0.796	DDT.1	0.693		
ERP29	0.889	PRKCSH	0.793	RPS12	0.693		
ENO1	0.888	CNPY2	0.786	DCUN1D1	0.692		
BCHE	0.881	ITGB2	0.778	AKR1C1.2	0.690		
ZYX	0.873	CRMP1.2	0.778	PZP	0.689		
AGR2	0.869	CES1	0.777	TPM1.1	0.685		
MST1.1	0.866	HNRNPA2B1	0.771	ENKUR	0.684		
SRSF4.1	0.864	BUB3	0.768	TMOD3	0.678		

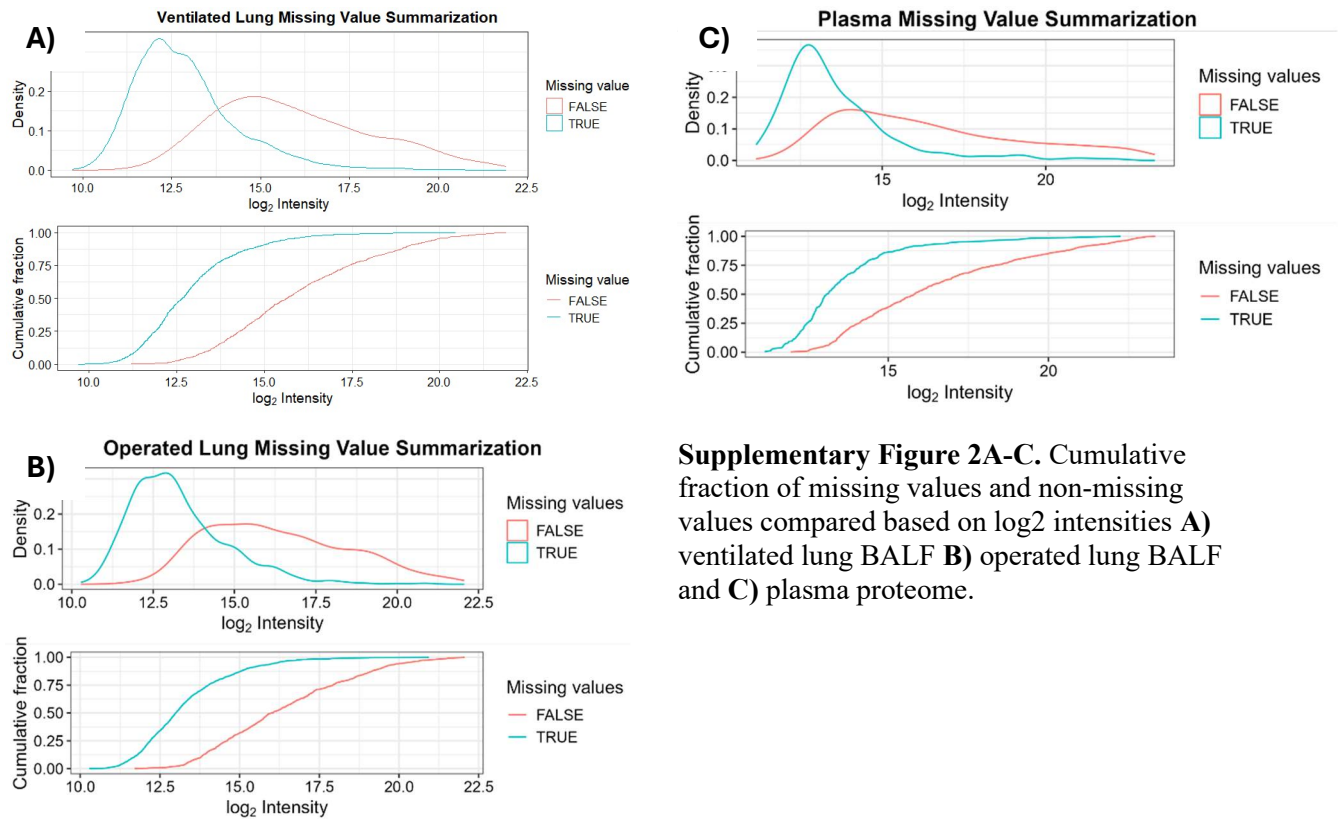
NPEPL1	0.864	ACOT1	0.767	G6PD	0.677		
KRT8	0.862	CNPY3	0.764	SORD	0.670		
Matched Controls Post-Vs-Pre-OLV (continued)							
Downregulated	Log ₂ FC	Downregulated	Log ₂ FC	Downregulated	Log ₂ FC	Downregulated	Log ₂ FC
TPPP3	-0.587	MARCKS	-0.667	CXCL10	-0.753	CDHR4	-0.874
TSG101	-0.587	MANBA	-0.669	DDR1	-0.756	PRSS8	-0.874
CD82	-0.589	CNTN1	-0.675	HSP90AA1.2	-0.761	CYSTM1	-0.880
TFPI	-0.590	FTH1	-0.677	SLC6A14	-0.762	CTSS	-0.880
DPP4	-0.593	GCN1	-0.677	TMC5	-0.764	TMBIM1	-0.894
CRIP2	-0.595	TMEM30B	-0.678	GALNT5	-0.764	CTSL	-0.899
P0DOX3	-0.600	RNASE6	-0.679	CCN2	-0.764	RPL35	-0.901
SIAE	-0.602	VSIG2	-0.683	SLC44A4	-0.765	EPS8L2	-0.907
COL6A1	-0.602	FAM107B	-0.687	EHD1.1	-0.766	PLTP	-0.910
IGHV2-70D.1	-0.603	GNA11.1	-0.690	NPC2	-0.773	IDS	-0.911
SMPDL3A	-0.603	AQP5	-0.691	TCN1	-0.775	MMP7	-0.912
IDUA	-0.605	SLC15A2	-0.692	FSTL1	-0.775	FAM3D	-0.915
GRN	-0.605	GLG1	-0.694	KLK11	-0.778	IGFBP2	-0.917
EFEMP1	-0.608	PYM1	-0.695	EFNB3	-0.780	TNFRSF21	-0.921
IGLV7-43	-0.608	CANT1	-0.696	CDHR3	-0.782	PLAUR	-0.928
GNAQ	-0.609	AZGP1	-0.696	RPL29	-0.783	ABCA13	-0.932
PSMC1	-0.610	CADM4	-0.700	SMAP	-0.785	DNASE2	-0.934
IGFBP5	-0.610	TAX1BP3	-0.703	GNA11.2	-0.789	MUC13	-0.935
CTSB	-0.611	EDF1	-0.707	YBX3	-0.792	IGHV4-28	-0.938
SMPDL3B	-0.617	ACYP2	-0.711	IGLV7-43.1	-0.793	MDK	-0.940
PDCD10	-0.618	MT1E	-0.714	SDCBP	-0.793	PROM1	-0.944
PLSCR1	-0.618	QSOX1	-0.715	TSPAN19	-0.798	KRAS.1	-0.946
GLB1L	-0.619	APLP2.1	-0.717	CD55	-0.801	VSTM2L	-0.946
CTSO	-0.620	FMOD	-0.718	CLDN3	-0.801	GPRC5B	-0.947
TOM1L1	-0.624	RNASE4	-0.719	CADM1	-0.803	BTN2A1	-0.951
AP3B1	-0.630	PIGR	-0.720	CEL	-0.804	MUC15	-0.961
EHD4	-0.632	TMEM59	-0.723	NUCKS1	-0.804	F11R	-0.965
B2M	-0.639	FRZB	-0.729	CRISP2	-0.805	MUC4	-0.967
CTBS	-0.641	ADGRG6	-0.731	PTPRZ1	-0.813	CRISP3	-0.967
STEAP4	-0.645	SLC38A10	-0.735	CIB1	-0.817	SUSD2	-0.967
MYO1E.1	-0.645	SLC34A2	-0.736	SBDS	-0.826	VSIG4	-0.969
TCEAL3	-0.646	MRC1	-0.736	ADGRF5	-0.839	PTMS	-0.970
GOLM1	-0.650	HMGNI	-0.738	CRTAC1	-0.841	ARRDC1	-0.998
SLPI	-0.652	PIP	-0.738	ACE	-0.842		
SLC5A1	-0.652	SPINT1	-0.740	PTPRJ	-0.845		
RIDA	-0.653	SPINT2	-0.746	LTF	-0.848		
NEBL	-0.655	CTSH	-0.748	PRR4	-0.853		
STOML3	-0.658	JCHAIN	-0.748	TMC4	-0.866		
STX2	-0.659	GALNS	-0.748	TNFAIP8L1	-0.866		
EPS8	-0.660	RNASET2	-0.749	TMPRSS2	-0.867		
DCDC2B	-0.660	EHD1.2	-0.750	SPATA33	-0.871		

Supplementary Table 3. Ventilated Lung BALF Proteins that become statistically significant (FDR < 0.05) when lowering fold change threshold to 1.5 ($1.5 \leq FC < 2$). Post-vs-pre-OLV comparisons were made in postoperative respiratory failure and matched control samples using Dream function.

Supplementary Figures

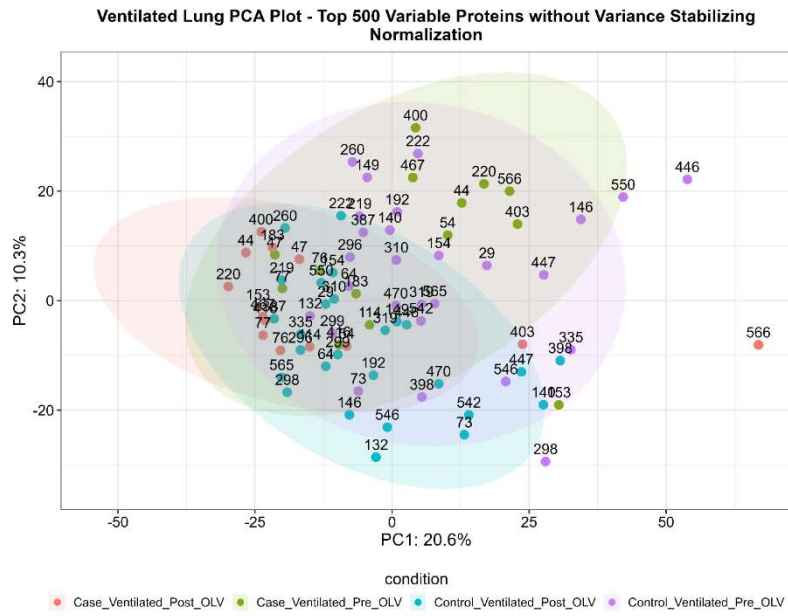


Supplementary Figure 1A. Number of proteins identified before applying any missing value filtration in the ventilated lung BALF proteome. **B)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions in the ventilated lung BALF proteome. **C)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions and less than 25% across all samples in the ventilated lung BALF proteome. **D)** Number of proteins identified before applying any missing value filtration in the operated lung BALF proteome. **E)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions in the operated lung BALF proteome. **F)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions and less than 25% across all samples in the operated lung BALF proteome. **G)** Number of proteins identified before applying any missing value filtration in the plasma proteome. **H)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions in the plasma proteome. **I)** Number of proteins identified after applying missing value filtration less than 25% in at least one out of four conditions and less than 25% across all samples in the plasma proteome.

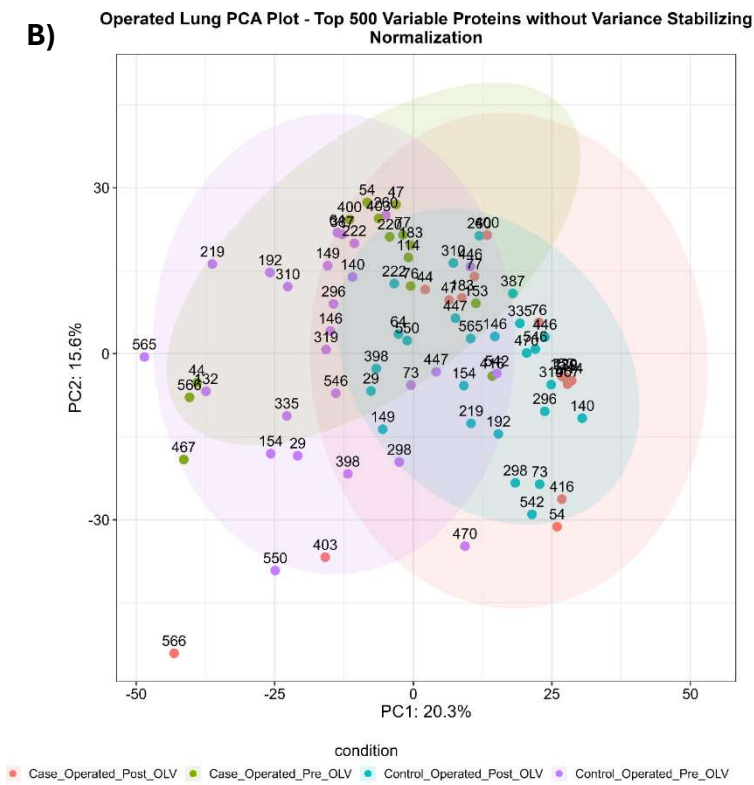


Supplementary Figure 2A-C. Cumulative fraction of missing values and non-missing values compared based on log₂ intensities **A)** ventilated lung BALF **B)** operated lung BALF and **C)** plasma proteome.

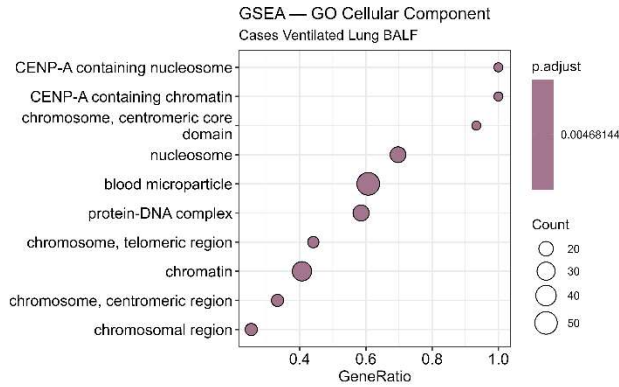
A)



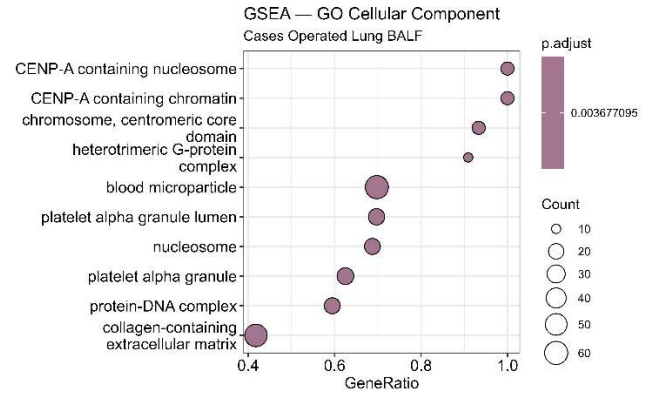
B)



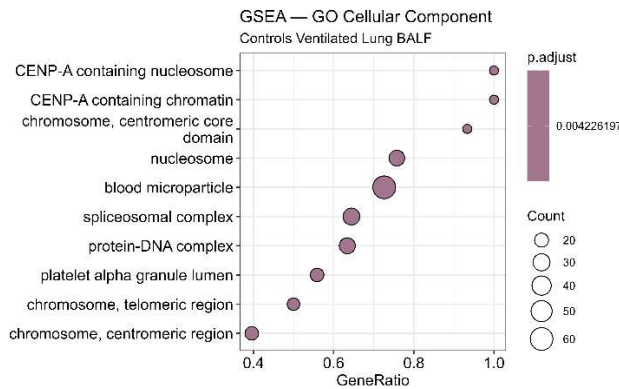
A)



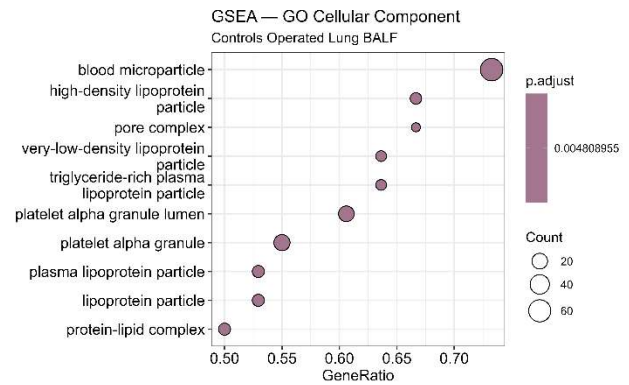
B)



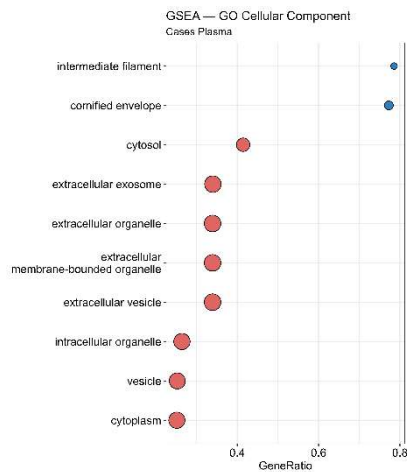
C)



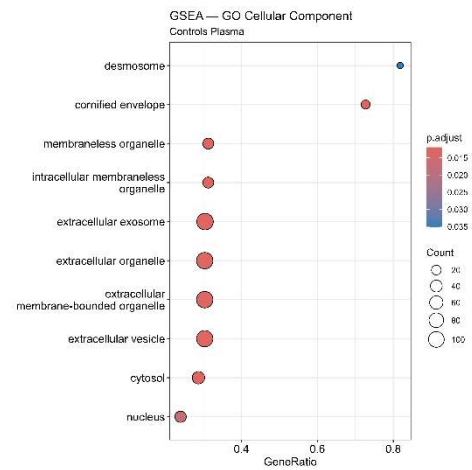
D)



E)



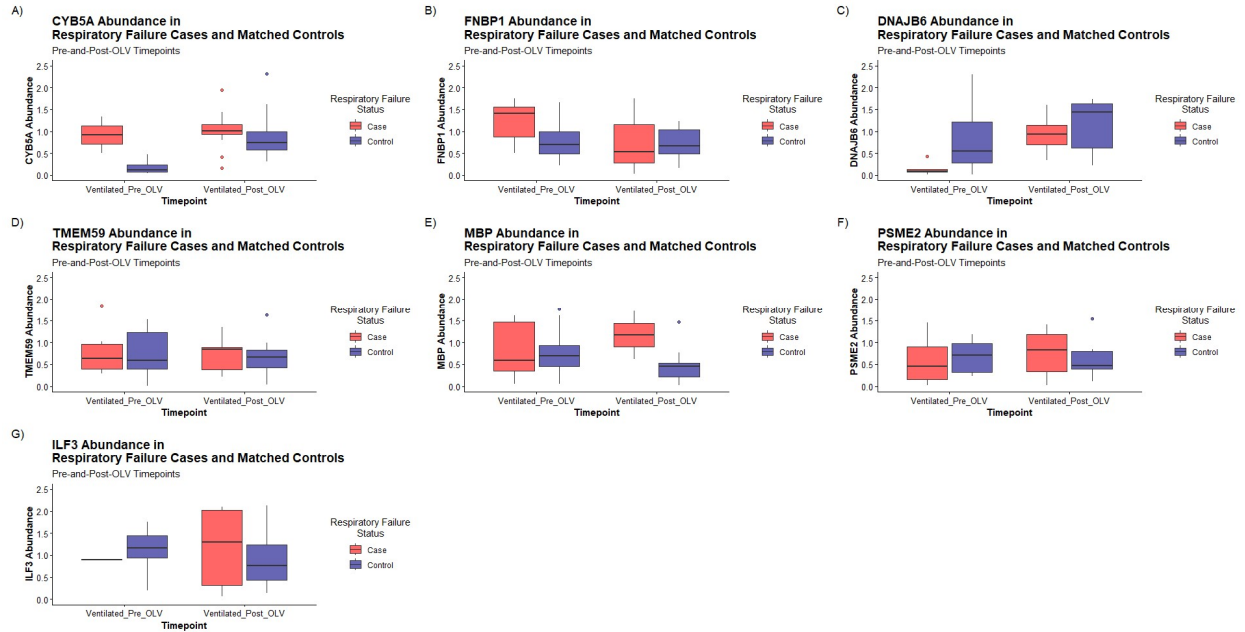
F)



Supplementary Figures 4A-F. Gene Set Enrichment Analysis (GSEA) on Gene Ontologies (GO) Cellular Components for **A)** postoperative respiratory failure cases ventilated lung, **B)** postoperative respiratory failure cases operated lung, **C)** matched controls ventilated lung, and **D)** matched controls operated lung BALF proteome, **E)** postoperative respiratory failure cases plasma, **F)** matched controls plasma, all with FDR < 0.05.

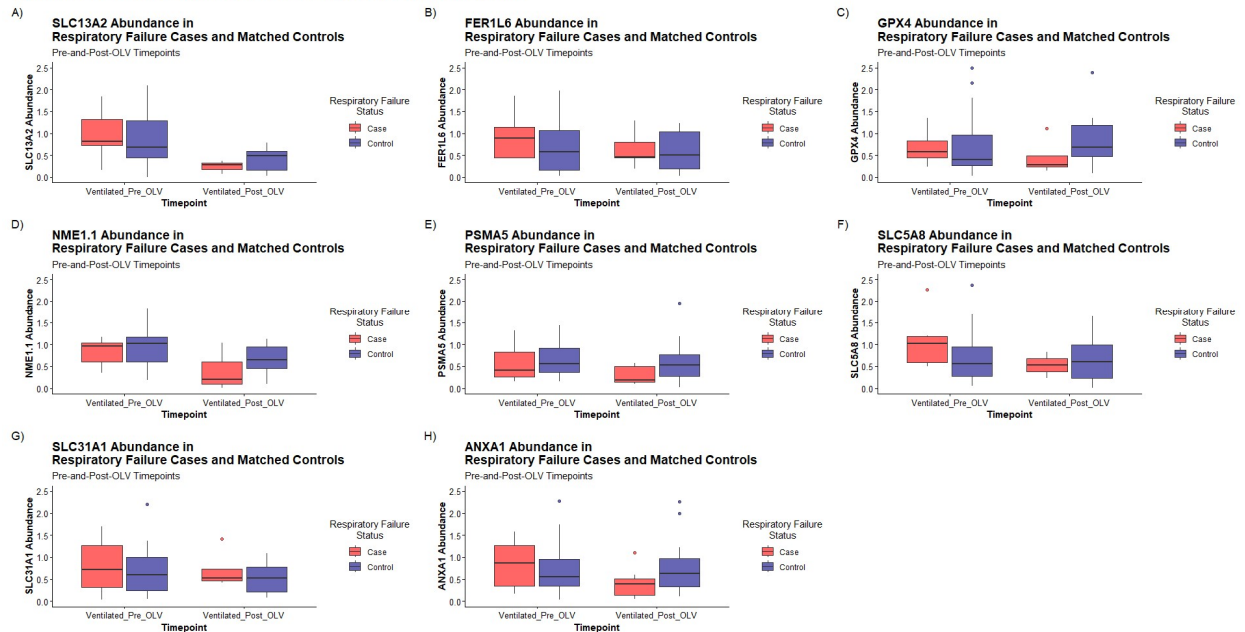
A)

Top 15 Elastic Net Selected Proteins Abundances with Positive Coefficients of Regression



B)

Top 15 Elastic Net Selected Proteins Abundances with Negative Coefficients of Regression



Supplementary Figures 5A-B. Normalized log₂ abundance of top 15 proteins selected through elastic net conditional logistic regression analysis. **A)** Proteins with positive coefficients of regression. **B)** Proteins with negative coefficients of regression.