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Wang, Qi

2026

Document Version:
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Citation for published version (APA):

Wang, Q. (2026). *From Tissue to Insight: Advanced Tissue Processing and Spatial Imaging in Translational Lung Transplantation*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Lund]. Lund University, Faculty of Medicine.

Total number of authors:
1

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From Tissue to Insight
Advanced Tissue Processing and Spatial Imaging
in Translational Lung Transplantation

From Tissue to Insight: Advanced Tissue Processing and Spatial Imaging in Translational Lung Transplantation

Qi Wang



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of
Medicine at Lund University to be publicly defended on September 3rd 2026, at 8:30 in
Belfragesalen, BMC, Sölvegatan 19, 221 84 Lund, Sweden

Faculty opponent

Professor Mihai Oltean, MD, PhD; University of Gothenburg, Sweden

Organization: LUND UNIVERSITY

Document name: Doctoral thesis

Date of issue: 2026-09-03

Author(s): Qi Wang

Sponsoring organization:

Title and subtitle: From Tissue to Insight: Advanced Tissue Processing and Spatial Imaging in Translational Lung Transplantation

Abstract:

Acute lung injury (ALI) and transplantation-related graft dysfunction remain major clinical and scientific challenges. In conditions relevant to donor lung assessment and transplantation, injury can affect multiple tissue compartments, including the alveolar epithelium, vascular endothelium, immune microenvironment, and extracellular matrix (ECM). These alterations can compromise the alveolar-capillary barrier, impair gas exchange and lung mechanics, and reduce donor lung quality or graft function. Understanding these processes requires investigation of how structural, cellular, and molecular changes are spatially distributed within lung tissue.

This thesis investigated tissue-level alterations in translational porcine models of aspiration-induced donor lung injury and complement-mediated graft injury, with a focus on alveolar architecture, barrier integrity, immune activation, and ECM organisation. Tissue-processing and sectioning methods were first evaluated to support histological and fluorescence-based analyses. Isopropanol provided histological quality, sectioning performance, and fluorescence-based measurements comparable to those obtained with xylene. Agarose embedding combined with automated vibratome sectioning enabled the preparation of structurally preserved lung sections for high-resolution multiplex fluorescence imaging.

Quantitative fluorescence imaging showed that aspiration-induced injury increased alveolar wall thickness and reduced alveolar circularity. These features were integrated into a morphological quotient (MQ) that captured injury-related changes in alveolar architecture. Across the aspiration-induced and complement-mediated injury models, spatial analysis revealed alterations involving the alveolar epithelium, epithelial junctions, vascular endothelium, innate immune response, ECM, and contractile structures, with both shared and model-specific patterns. These tissue changes occurred alongside functional deterioration and histological injury in both models.

Overall, the workflows evaluated and applied in this thesis enabled quantitative characterisation of multicompartment lung tissue injury. Spatial tissue analysis provided information complementary to conventional histology and physiological assessment and distinguished tissue responses shared by or specific to the two injury models. This work provides a basis for further validation of spatial imaging approaches in mechanistic studies, donor lung assessment, and evaluation of therapeutic responses in lung transplantation.

Key words: lung transplantation; donor lung evaluation; *ex vivo* lung perfusion; large animal model; tissue processing; vibratome sectioning; multiplex immunofluorescence; quantitative spatial analysis

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language: English

Number of pages: 93

ISSN and key title: 1652-8220

ISBN: 978-91-8021-906-8

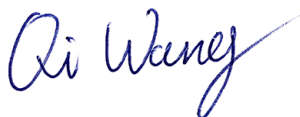
Recipient's notes

Price

Security classification

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Qi Wang



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Paper 5 © 2026 The authors (Manuscript unpublished)

Cover image © Nicholas B. Bèchet and cover composition by Qi Wang. The image was acquired using scanning electron microscopy (SEM) at the Lund University Bioimaging Centre (LBIC).

Published by:

Department of Clinical Sciences Lund

Faculty of Medicine

Lund University

Lund 2026

ISBN 978-91-8021-906-8 (print)

ISBN 978-91-8021-906-8 (electronic)

Lund University, Faculty of Medicine Doctoral Dissertation Series 2026:108

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund, 2026



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致这四年难忘的时光，真挚的感情，和辽阔而宁静的北欧大自然。

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Paper I

Wang Q, Gu R, Olm F, Bèchet NB, Lindstedt S. Xylene versus Isopropanol for Paraffin Wax Processing of Lung Tissue. *Applied Sciences*. 2024;14(5):1726. doi:10.3390/app14051726.

Paper II

Wang Q, Bèchet NB, Lindstedt S. Automated Vibratome Sectioning of Agarose-Embedded Lung Tissue for Multiplex Fluorescence Imaging. *Journal of Visualized Experiments*. 2023;(200). doi:10.3791/65943.

Paper III

Lindstedt S, **Wang Q**, Niroomand A, Stenlo M, Hyllen S, Pierre F, Olm F, Bèchet NB. High resolution fluorescence imaging of the alveolar scaffold as a novel tool to assess lung injury. *Sci Rep*. 2024;14(1):6662. doi:10.1038/s41598-024-57313-6.

Paper IV

Lindstedt S, **Wang Q**, Mittendorfer M, Bodén E, Hirdman G, Niroomand A, Stenlo M, Ghaidan H, Hyllén S, Pierre L, Olm F, Bèchet NB. High Content Pulmonary Spatial Biology in a Translational Organ Donor Aspiration Model. 2026 (manuscript)

Paper V

Wang Q, Gu RC, Bèchet NB, Mittendorfer M, Huzevka T, Edström D, Kjellberg G, Olm F, Lindstedt S. Using Ex Vivo Lung Perfusion as a Platform to Study Hyperacute Rejection in Lung Transplantation. 2026 (manuscript)

List of papers not included in the thesis

Bèchet NB, Celik A, Mittendorfer M, **Wang Q**, Huzevka T, Kjellberg G, Bodén E, Hirdman G, Pierre L, Niroomand A, Olm F, McCully JD, Lindstedt S. Xenotransplantation of mitochondria: A novel strategy to alleviate ischemia-reperfusion injury during ex vivo lung perfusion. *J Heart Lung Transplant*. 2025 Mar;44(3):448-459. doi: 10.1016/j.healun.2024.10.033. Epub 2024 Nov 12. PMID: 39536924.

Hirdman G, Stenlo M, Bèchet NB, Niroomand A, Mittendorfer M, **Wang Q**, Edström D, Ghaidan H, Kjellström S, Pierre L, Olm F, Hyllén S, Lindstedt S. Unraveling Molecular and Functional Responses Across 3 Lung Injury Models to Expand the Donor Lung Pool. *Transplantation*. 2025 Jul 1;109(7):1166-1174. doi: 10.1097/TP.0000000000005353. Epub 2025 Feb 19. PMID: 39969856; PMCID: PMC12180699.

Mittendorfer M, Olm F, Huzevka T, Bèchet NB, **Wang Q**, Gu R, Svereus F, Edström D, Janson EB, Hirdman G, Scheduling S, Hyllén S, Lindstedt S. Early Allogeneic Stromal (Stem) Cell Therapy Mitigates Aspiration-Induced Lung Injury: Closed-System Automated Cell Manufacturing Using the CliniMACS Prodigy. 2026 (manuscript)

Bèchet NB, Huzevka T, Svereus F, Sterner N, Mittendorfer M, **Wang Q**, Gu R, Clarke D, Bodén E, Harry J, Hirdman G, McCully JD, Kjellström S, Olm F, Celik A, Oniscu GC, Lindstedt S. Exogenous Mitochondrial Therapy During Machine Perfusion Restores the Function of Livers donated after circulatory death: A Cross-Circulation Evaluation for Clinical Transplant Suitability. 2026 (manuscript)

Abstract

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Popular science summaries

Popular summary in English

The lungs sustain life by supplying the blood with oxygen and removing carbon dioxide. When end-stage lung disease prevents them from functioning adequately, transplantation may be the only effective treatment. However, suitable donor lungs are scarce, and many are too injured or function too poorly for transplantation. Infection, aspiration of stomach contents, inflammation, and transplantation-related immune reactions can all damage lung tissue.

To improve transplantation outcomes, we need to understand what occurs inside an injured lung. Oxygen crosses a thin barrier between millions of tiny air sacs, called alveoli, and surrounding blood vessels. If damaged, fluid and inflammatory cells may accumulate in the alveoli, making oxygen transfer more difficult. The supporting framework may also be damaged or remodeled, potentially reducing lung elasticity and function.

This thesis developed methods to preserve, visualize, and analyze lung tissue. One study evaluated isopropanol as a less hazardous alternative to xylene in paraffin processing. Another established a method for cutting and staining thick, agarose-embedded tissue sections to examine spatial organization. Together, these approaches preserved tissue structure and fluorescence signals, enabling quantitative analysis of alveoli, blood vessels, immune cells, and supporting tissue.

These methods were applied in two porcine injury models, chosen because pig lungs resemble human lungs anatomically and physiologically. One model examined acute injury caused by aspiration of stomach contents. The other used *ex vivo* lung perfusion to keep lungs ventilated and perfused outside the body, enabling controlled investigation of early complement-mediated, rejection-like injury. Quantitative fluorescence imaging showed that aspiration injury thickened alveolar walls and made alveoli more irregular. Both models showed alterations in cells lining the alveoli and increased inflammatory cell abundance or activity. However, cell junctions, blood vessels, supporting fibers, and contractile structures showed different patterns between the models. These findings demonstrate that different causes of lung injury can produce both shared and distinct tissue changes.

Overall, this thesis provides improved methods for studying lung tissue and advances our understanding of donor lung injury. With further validation, these approaches may support better donor lung assessment, facilitate the development and evaluation of new treatments, and ultimately contribute to improved outcomes after lung transplantation.

Populärvetenskaplig sammanfattning på svenska

Lungorna är livsviktiga för andningen. De förser blodet med syre och hjälper kroppen att göra sig av med koldioxid. För patienter med svår lungsjukdom kan en lungtransplantation vara det enda behandlingsalternativet. Det råder dock brist på lämpliga donatorlungor, och många donatorlungor kan inte användas eftersom de redan är skadade eller inte fungerar tillräckligt bra.

Lungskador kan orsakas av infektioner, inflammation, aspiration av maginnehåll eller immunreaktioner i samband med transplantation. Även om skadorna kan ha olika orsaker påverkar de ofta samma känsliga strukturer i lungan. Normalt passerar syre genom en mycket tunn barriär mellan lungblåsorna och de omgivande blodkärlen. Om denna barriär skadas kan vätska och inflammatoriska celler tränga in i lungblåsorna. Även blodkärlen kan påverkas, vilket försvårar syreupptaget i blodet. Lungans stödjevävnad kan också förändras, vilket kan göra lungan mindre elastisk och försämra dess normala funktion. I avhandlingen undersöktes hur lungskador påverkar lungvävnadens struktur och funktion, med särskilt fokus på skador i donatorlungor och lungtransplantat.

Grismodeller användes eftersom grisens lungor liknar människans i både storlek och struktur. I en modell studerades akut lungskada orsakad av aspiration av maginnehåll. I en annan modell användes ett maskinbaserat system för att hålla lungorna ventilerade och perfunderade utanför kroppen, en metod som kallas ex vivo-lungperfusion. Med hjälp av denna modell studerades tidiga lungskador och strukturella förändringar i lungvävnaden. Genom att kombinera vävnadsberedning, fluorescensmikroskopi och kvantitativ bildanalys undersöktes hur skadorna var fördelade i lungvävnaden och hur de hängde samman med ansamling av immunceller, skador på barriären mellan lungblåsorna och blodkärlen samt förändringar i stödjevävnaden. Detta gjorde det möjligt att studera lungskador inte bara som en generell vävnadsskada, utan också som specifika förändringar i olika delar av lungan.

Sammantaget bidrar denna avhandling med ny kunskap om hur lungskador påverkar olika delar av lungan och hur dessa förändringar kan försämra donatorlungans funktion. På sikt kan resultaten bidra till en bättre bedömning av donatorlungor och till utvecklingen av nya behandlingsstrategier, vilket i förlängningen kan förbättra behandlingsresultaten för patienter som genomgår lungtransplantation.

肺损伤的可视化：为肺移植寻找更清晰的答案

肺是人体维持生命的重要器官。它像一座精密的“气体交换站”，不断把氧气送入血液，同时把二氧化碳排出体外。当严重肺病发展到终末期时，患者的肺功能可能已经无法维持生命，肺移植就成为他们继续生存的重要治疗选择。然而，肺移植面临两个主要困难：一是可用的供肺数量远远不够，二是很多供体肺在移植前已经受到损伤，无法安全用于移植。感染、炎症、胃内容物误吸，以及移植后的免疫反应，都可能造成肺组织损伤。

要改善肺移植效果，首先需要更清楚地了解受损肺组织内部到底发生了什么。肺的结构非常精细，氧气需要从一个个微小的肺泡进入周围的毛细血管。肺泡和血管之间有一层极薄的屏障，就像一扇薄薄的“交换窗”。一旦这扇窗被破坏，液体和炎症细胞就可能进入肺泡腔，使氧气更难进入血液。同时，肺组织中起支撑作用的结构也可能受损或重塑，使肺变得更僵硬，呼吸功能进一步下降。

在本研究中，我主要关注如何更好地保存、观测和分析肺组织。一部分研究探索了更安全的肺组织处理方法，希望在减少有害化学试剂使用的同时，仍然保持肺组织结构和染色质量。另一部分研究建立了新的肺组织切片和染色方法，可以更好的在显微镜下观测到肺组织的空间结构。通过这些方法，我们再结合先进的荧光显微成像技术，观察和评估肺泡结构、血管、免疫细胞以及支撑肺组织的结构成分。

这些方法和技术随后应用于猪肺损伤和肺移植相关模型中。之所以使用猪模型，是因为猪肺在大小和结构上与人肺较为相似，更接近临床肺移植的实际情况。在其中一个模型中，我们研究了胃内容物误吸造成的急性肺损伤；在另一个模型中，我们利用体外肺灌注系统，将供肺在体外进行通气和灌注，用于研究早期免疫损伤和模拟排斥反应的组织改变。

本研究的总体目标是改进肺损伤的观测和评估方法。通过优化肺组织的处理流程，更好地保护肺组织，并采用相对客观，可量化且精确的分析方式研究肺组织内部的结构和分子变化，这项研究有助于加深我们对供肺损伤的理解，改善供肺质量评估，并为未来开发新的治疗策略、提高肺移植效果奠定基础。

Abbreviations

ACR	Acute cellular rejection
AIF-1	Allograft inflammatory factor 1
ALI	Acute lung injury
AMR	Antibody-mediated rejection
AQP5	Aquaporin-5
ARDS	Acute respiratory distress syndrome
ATMPs	Advanced therapy medicinal products
Cdyn	Dynamic compliance
CLAD	Chronic lung allograft dysfunction
COPD	Chronic obstructive pulmonary disease
CO	Cardiac output
DAPI	4',6-diamidino-2-phenylindole
DAMPs	Damage-associated molecular patterns
DCD	Donation after circulatory death
ECM	Extracellular matrix
EVLP	<i>Ex vivo</i> lung perfusion
FEG-SEM	Field-emission-gun scanning electron microscope
FFPE	Formalin-fixed, paraffin-embedded
FiO ₂	Fraction of inspired oxygen
HAR	Hyperacute rejection
HTII-280	Human type II pneumocyte marker
H&E	Haematoxylin and eosin
IPF	Idiopathic pulmonary fibrosis
LEA	Lycopersicon esculentum agglutinin
MAC	Membrane attack complex
MPAP	Mean pulmonary artery pressure
MPO	Myeloperoxidase

MSCs	Mesenchymal stromal cells
OCS	Organ care system
OD	Optical density
PaO ₂	Arterial oxygen partial pressure
PaCO ₂	Arterial partial pressure of carbon dioxide
PBS	Phosphate-buffered saline
PCLS	Precision-cut lung slices
PEEP	Positive end-expiratory pressure
PGD	Primary graft dysfunction
PIP	Peak inspiratory pressure
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
RR	Respiratory rate
α -SMA	Alpha-smooth muscle actin
V _t	Tidal volume
VCV	Volume-controlled ventilation
ZO-1	Zonula occludens-1

Introduction

Lung architecture and pathophysiology

Anatomical and structural characteristics

The lungs are highly specialized organs responsible for gas exchange, enabling oxygen uptake and carbon dioxide elimination^{1–3}. Regulation of carbon dioxide removal also contributes to systemic acid–base homeostasis⁴. These functions depend on the coordinated organization of the conducting airways, distal airspaces, pulmonary vasculature, and supporting extracellular matrix (ECM). The preservation of this structural organisation is therefore essential for normal respiratory function.

At the macroscopic level, the lungs lie within the thoracic cavity. Each lung is covered by the visceral pleura, whereas the parietal pleura lines the inner surface of the thoracic cavity. A thin layer of pleural fluid between these surfaces allows them to glide smoothly over one another, thereby minimizing friction during respiration^{5,6}. The trachea divides into the right and left main bronchi, each entering the corresponding lung. In humans, the right lung has three lobes, whereas the left lung has two⁷. The epithelial lining changes progressively along the branching airway tree. The larger conducting airways are lined by pseudostratified ciliated epithelium and contain goblet cells, submucosal glands, and cartilage, which together support mucociliary clearance and airway stability^{8,9}. As the airways branch into smaller bronchioles, the epithelium becomes simpler and club cells become more prominent, contributing to epithelial protection and repair^{10,11}. The distal airways lead to the alveoli, which are closely associated with the pulmonary capillary network and form the main site of gas exchange (Fig. 1)^{2,12}.

Efficient gas exchange requires an intact alveolar–capillary barrier. This barrier is formed by the alveolar epithelium, a thin interstitial compartment containing basement membranes and other ECM components, and the pulmonary capillary endothelium^{13,14}. The close apposition of the alveolar airspaces and pulmonary capillaries minimises the diffusion distance, allowing efficient exchange of oxygen and carbon dioxide¹⁵. The alveolar epithelium consists predominantly of type I and type II pneumocytes. Type I pneumocytes cover most of the alveolar surface and form the thin interface required for gas diffusion¹⁶. Type II pneumocytes produce

surfactant, which reduces surface tension and maintains alveolar stability, and also act as progenitor cells during epithelial repair after injury^{17,18}. Within the alveolar wall, the ECM provides structural support and contributes to the mechanical properties of the lung. Elastin, in particular, helps maintain alveolar shape and elastic recoil during repeated cycles of inflation and deflation^{19,20}. Disruption of this organisation can impair gas exchange, alter lung compliance, and contribute to pathological remodelling¹³. These structural relationships provide the anatomical basis for interpreting changes in alveolar architecture and lung mechanics, together with the barrier-associated and ECM alterations examined in this thesis.

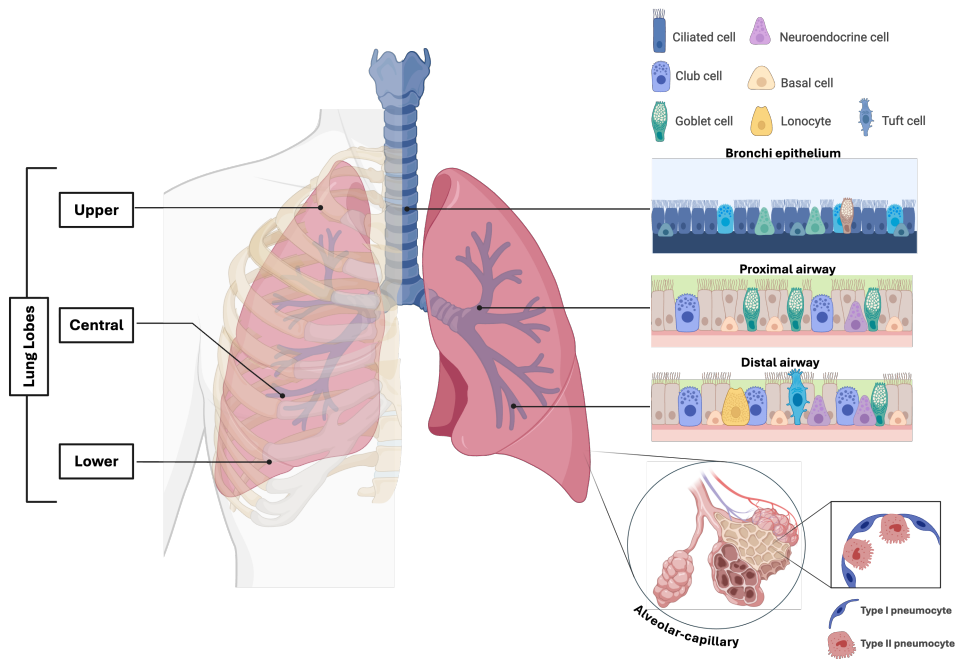


Figure 1. Lung anatomy, airway epithelium, and major epithelial cell types.

Schematic illustration of lung anatomy, showing the right upper, middle, and lower lung lobes, the branching airway system, and the alveolar-capillary unit. The distribution of major epithelial cell populations along the proximal and distal airways is illustrated, together with type I and type II pneumocytes in the alveolar region. Figure created with BioRender.

Pathological alterations in lung injury and transplantation-related graft dysfunction

Disruption of the alveolar–capillary network can impair gas exchange, disturb fluid balance, and alter lung mechanics^{21,22}. Although the initiating insults vary, acute lung injury and transplantation-related graft dysfunction often involve overlapping pathological processes, including barrier dysfunction, inflammation, oedema, and ECM alterations. These responses are closely interconnected: loss of barrier integrity promotes fluid accumulation and inflammatory cell recruitment, while persistent inflammation and dysregulated repair can lead to fibroblast activation, excessive ECM deposition, tissue stiffening, and fibrosis^{23–26}.

These processes are particularly relevant to lung transplantation because pre-existing donor lung injury can reduce organ quality, whereas subsequent graft injury can compromise post-transplant outcomes. Experimental models and platforms, including *ex vivo* lung perfusion (EVLP), allow these processes to be studied under controlled conditions and provide opportunities to evaluate strategies for limiting injury and preserving graft function^{27–29}.

Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is a clinical syndrome of acute hypoxaemic respiratory failure associated with diffuse inflammatory lung injury and increased pulmonary vascular permeability^{30,31} (Fig. 2). It may arise from direct or indirect insults, including pneumonia, aspiration, sepsis, and severe trauma. Primary graft dysfunction (PGD), a form of acute lung injury occurring early after lung transplantation, shares several pathophysiological features with ARDS³². Although these conditions differ in their initiating causes, they can converge on loss of barrier integrity, disruption of alveolar architecture, impaired oxygenation, and altered lung mechanics. These alterations increase the diffusion distance for oxygen, reduce the effective surface area for gas exchange and ultimately lead to worsening respiratory failure. According to the Berlin definition, ARDS develops within one week of a known clinical insult. Chest imaging shows bilateral opacities that are not fully explained by effusions, lobar or lung collapse, or nodules. Respiratory failure is not fully explained by cardiac failure or fluid overload, and impaired oxygenation is assessed using the $\text{PaO}_2/\text{FiO}_2$ ratio³³. Severe cases may require intensive care and mechanical ventilation with positive pressure to support gas exchange, and ARDS remains associated with substantial morbidity and mortality^{34,35}.

Alveolar–capillary barrier dysfunction

Increased permeability of the alveolar–capillary barrier allows protein-rich fluid to accumulate in the pulmonary interstitium and airspaces. This oedema can impair surfactant function, promote alveolar instability and collapse, and contribute to hyaline membrane formation, further compromising gas exchange^{23,24}. Barrier-

associated markers can provide information about the epithelial and endothelial compartments. Aquaporin-5 (AQP5) is a water-channel protein predominantly expressed by alveolar type I epithelial cells and facilitates water transport across the alveolar epithelium³⁶. Zonula occludens-1 (ZO-1) is a tight-junction scaffolding protein involved in paracellular barrier regulation, whereas CD31 is located at endothelial cell junctions and is widely used to visualise pulmonary vascular structures³⁷. Changes in the signal intensity, coverage, or spatial distribution of these markers may indicate alterations in the epithelial, junctional, or endothelial compartments during lung injury³⁸.

Inflammatory cell accumulation and activation

Inflammatory cell recruitment and activation are central features of ARDS. After injury, resident macrophages become activated, while circulating monocytes and neutrophils are recruited to the lung parenchyma, alveolar spaces, and perivascular regions²⁴. These cells release inflammatory mediators, reactive oxygen species (ROS), and proteases, which can amplify epithelial and endothelial injury and sustain local inflammation²³. Allograft inflammatory factor-1 (AIF-1) is associated with activated macrophages and monocytes, whereas myeloperoxidase (MPO) is a neutrophil granule enzyme commonly used as a marker of neutrophil-associated inflammation^{39,40}.

Extracellular matrix remodelling and mechanical dysfunction

As lung injury progresses, persistent injury and dysregulated repair lead to fibroproliferative remodelling, characterised by fibroblast and myofibroblast activation, ECM deposition, alveolar wall thickening, and increased tissue stiffness^{41–43}. These structural changes can reduce lung compliance, impair gas exchange, and complicate mechanical ventilation. Elastin and α -smooth muscle actin (α -SMA) provide information about structural and contractile components of injured lung tissue. Elastin contributes to elastic recoil and alveolar stability, whereas α -SMA is expressed by contractile smooth muscle cells and activated myofibroblasts and can indicate contractile structures or fibroproliferative responses^{19,44}. Together, these pathological processes guided the selection of tissue markers and spatial analyses used to characterise multicompartment alterations in aspiration-induced lung injury and complement-mediated graft injury.

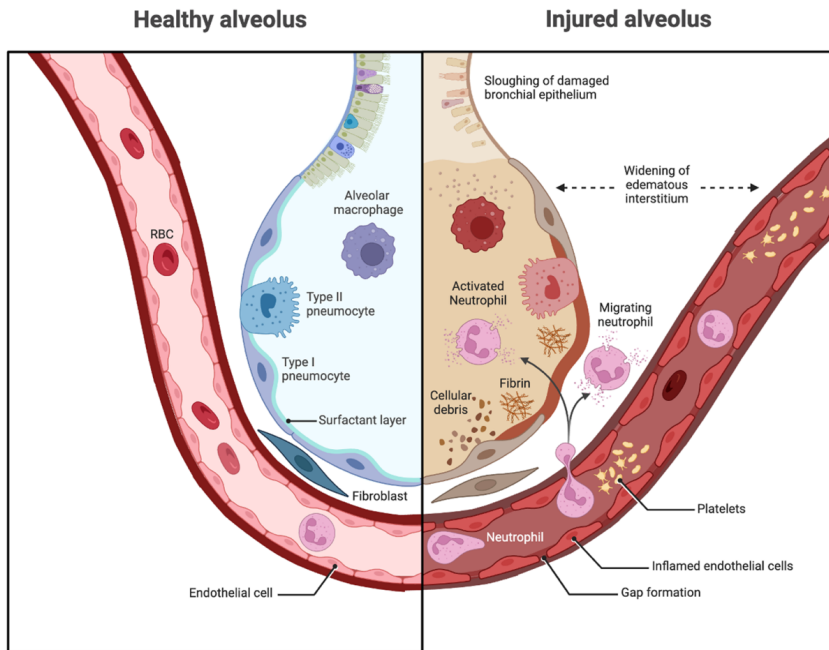


Figure 2. Schematic comparison of healthy and injured alveoli in ARDS.

ARDS is characterized by disruption of the alveolar-capillary barrier, interstitial and alveolar edema, inflammatory cell recruitment, vascular leakage, and accumulation of fibrin and cellular debris. Figure created with BioRender.

Lung transplantation

Historical development

Lung transplantation has developed from an experimental procedure into an established treatment option for selected patients with end-stage lung disease. Early experimental work in vascular anastomosis and animal lung transplantation provided the technical basis for later clinical attempts. The first human lung transplantation was performed by James Hardy in 1963, but early clinical outcomes were poor, mainly due to challenges related to graft preservation, airway healing, infection, and rejection⁴⁵. A major turning point occurred in the early 1980s with improvements in surgical technique, immunosuppression, and perioperative care. In 1983, the Toronto Lung Transplant Group reported the first successful long-term single-lung transplantation, and successful double-lung transplantation followed in the mid-1980s⁴⁶. These advances established lung transplantation as a feasible clinical therapy and laid the foundation for modern single- and bilateral lung

transplantation. In Sweden, lung transplantation was introduced in 1990, and the lung transplantation programme at Lund University Hospital began in January that year with the first single-lung transplantation in the Nordic countries⁴⁷.

Current clinical lung transplantation

Nowadays, lung transplantation is an important treatment for carefully selected patients with advanced lung diseases such as idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), cystic fibrosis, and pulmonary vascular disease⁴⁸. According to the 2025 ISHLT Thoracic Organ Transplant Registry report, the annual number of lung transplant recipients reported to the Registry increased from approximately 600 per year in 1992 to approximately 3,800 per year in 2019, followed by a decline during the COVID-19 pandemic and a gradual recovery thereafter⁴⁹. Among adult lung transplant recipients, IPF and COPD remain leading indications, and most adult recipients receive bilateral lung transplantation. Short-term survival improved over time, with one-year post-transplant survival among adult lung transplant recipients increasing from 75.8% in 1992–2000 to 88.4% in 2018–2023⁴⁹. However, lung transplantation remains limited by donor organ shortage, PGD, rejection, infection, and chronic graft dysfunction^{50,51}. Therefore, improved methods for donor lung evaluation, preservation, and mechanistic assessment of graft injury are still needed. In this context, the tissue-processing, fluorescence imaging, and quantitative spatial analysis approaches developed and applied in this thesis are relevant because they provide tools to investigate lung tissue architecture, immune activation, alveolar–capillary barrier disruption, and structural remodelling in translational models of lung injury and graft dysfunction.

Allograft rejection and graft dysfunction

Immune-mediated rejection and graft dysfunction remain major causes of morbidity and reduced long-term survival after lung transplantation⁵². The transplanted lung is particularly vulnerable to inflammatory injury because it is directly exposed to the external environment through the airways, has an extensive alveolar–capillary surface area, and contains a large population of resident immune cells capable of amplifying inflammatory responses^{53,54}. Ischaemia–reperfusion injury, infection, and other post-transplant complications may further contribute to graft injury and dysfunction^{55,56}.

Allograft rejection may occur as hyperacute rejection (HAR), acute cellular rejection (ACR), or antibody-mediated rejection (AMR), whereas persistent or recurrent injury may contribute to the development of chronic lung allograft dysfunction (CLAD)^{51,57}. Although HAR is now rare in clinical practice, its rapid antibody- and complement-mediated pathophysiology remains relevant to the study

of early graft injury^{58–62}. ACR is primarily T-cell mediated, whereas AMR is associated with donor-specific antibodies; both forms of rejection can impair allograft function and increase the subsequent risk of CLAD^{57,63,64}. CLAD is characterised by a persistent decline in allograft function and remains a major cause of morbidity and mortality beyond the first post-transplant year⁶⁵. Understanding the tissue-level consequences of immune activation is therefore important, particularly in complement-mediated early graft injury.

Complement system in lung transplantation

The complement system is an important component of innate immunity and contributes to host defence, immune complex clearance, and regulation of inflammation⁶⁶. Under physiological conditions, most complement proteins circulate as inactive precursors⁶⁷. Once activated, the complement system triggers a proteolytic cascade that amplifies inflammatory responses and promotes pathogen elimination. However, excessive or uncontrolled complement activation can also cause host tissue injury^{68–70}.

Complement can be activated through three main pathways: the classical, lectin, and alternative pathways⁷¹. All three pathways converge at the cleavage of C3, leading to the formation of C5 convertases that cleave C5 into C5a and C5b. C5b then initiates the assembly of the terminal complement complex C5b-9, also known as the membrane attack complex (MAC)^{72,73}. The soluble terminal complement complex, sC5b-9, can be detected in the fluid phase and is commonly used as a marker of terminal complement activation⁷⁴ (Fig. 3).

In lung transplantation, complement activation contributes to several forms of early graft injury, including ischemia–reperfusion injury, PGD, AMR, and HAR^{58,62,75,76}. HAR is one of the most rapid and severe forms of graft injury and can develop within minutes to hours after reperfusion, when preformed donor-specific antibodies trigger rapid complement activation^{58,76}. This process can damage the endothelium, disrupt the alveolar–capillary barrier, promote increased vascular permeability, edema, haemorrhage, microvascular thrombosis, and increased pulmonary vascular resistance (PVR)^{61,62,77,78}. These alterations impair perfusion and gas exchange and can rapidly cause severe graft dysfunction or graft failure.

Several experimental approaches have been used to investigate the mechanisms underlying HAR, including allotransplantation, xenotransplantation, and *ex vivo* perfusion models^{79,80}. Although *in vivo* models preserve systemic physiological interactions, they are technically demanding and can make it difficult to distinguish lung-specific effects from systemic inflammatory, coagulation, metabolic, and haemodynamic responses⁸¹. EVLP provides a controlled, isolated organ setting while maintaining ventilation, perfusion, and continuous functional monitoring. This makes EVLP a useful translational platform for studying complement-

mediated lung injury⁸². In this thesis, a porcine EVLP model of complement-mediated HAR-like injury was used to investigate changes in epithelial and endothelial barrier integrity, innate immune responses, and ECM and contractile structures.

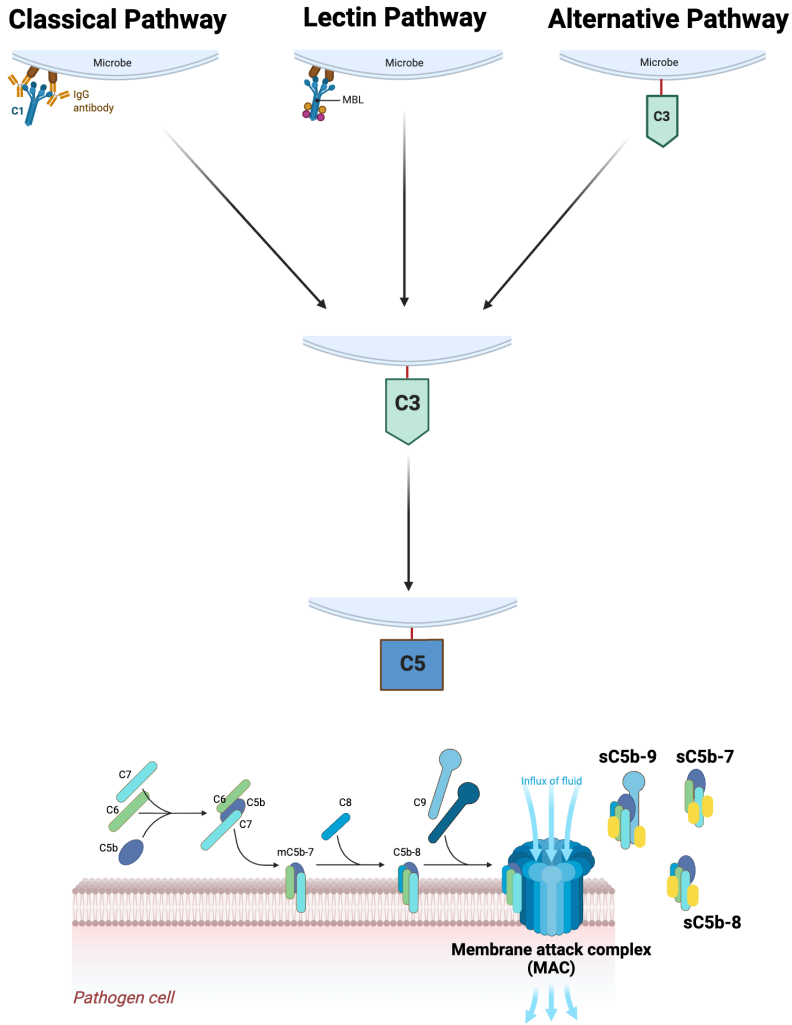


Figure 3. Schematic overview of complement activation pathways.

The complement system can be activated through the classical, lectin, or alternative pathway. All three pathways converge at C3 cleavage, followed by C5 cleavage and assembly of the terminal complement complex C5b–9. When formed on a cell membrane, C5b–9 is known as the membrane attack complex (MAC). Its soluble form, sC5b–9, can be detected in the fluid phase and is commonly used as a marker of terminal complement activation. Figure created with BioRender.

Ex vivo lung perfusion

Ex vivo lung perfusion (EVLP) is a technique that maintains donor lungs outside the body under controlled ventilation and perfusion conditions (Fig. 4). It enables functional assessment, extended preservation, and potential therapeutic intervention before transplantation⁸²⁻⁸⁴. Clinical EVLP was pioneered by Prof. Stig Steen and colleagues in Lund, Sweden, who used it to evaluate lungs retrieved from an uncontrolled donation after circulatory death (DCD)^{85,86}. The technique was subsequently refined by Cypel, Keshavjee, and colleagues at Toronto General Hospital, contributing to the expansion of its clinical and experimental application⁸⁷.

During EVLP, the lung is perfused and ventilated under controlled conditions, allowing dynamic assessment of gas exchange, airway pressure, PVR, and lung compliance^{83,88}. The primary clinical aim is to expand the donor lung pool by assessing and potentially rehabilitating lungs that might otherwise be considered unsuitable for transplantation^{82,89}. The PaO₂/FiO₂ ratio thresholds ranging from 300 to 500 mmHg have traditionally been used as part of the assessment of transplant suitability, depending on the protocol and clinical context⁹⁰. Three main EVLP protocols have been applied in clinical and experimental settings. These differ in perfusate composition, flow strategy, ventilation settings, and evaluation duration, as summarized in Table 1.

Table 1. Comparison between the clinically used EVLP protocols in lung transplantation

	Lund ⁹¹	Toronto ⁸⁷	OCS ⁹²
EVLP time	1-2h	4-6h	Variable; transport-/case-dependent
Perfusate contents	Steen solution +15% hematocrit	Acellular Steen solution	OCS solution +15-20% hematocrit
Pump type	Roller	Centrifugal	Piston (pulsatile)
Target flow (L/min)	100% CO	40% CO	2-2.5L/min
PAP (mmHg)	≤20	≤15	≤20
Left atrium	Open	Closed (3-5mmHg)	Open
Ventilation settings			
Vt (mL/kg)	5-7	7	6
Respiratory rate (per minute)	8	7	10
FiO ₂ (%)	50	21	21
PEEP (cmH ₂ O)	5	5	5
Temperature (°C)			
Start of ventilation	32	32	32
Start of perfusion	15	25	32
Start of evaluation	37	37	37

In addition, EVLP also serves as a translational platform for investigating lung injury, graft dysfunction, and potential therapeutic strategies^{89,93,94}. Continuous monitoring and repeated sampling allow inflammatory responses, ischaemia–reperfusion injury, vascular dysfunction, and tissue repair to be studied over time in an isolated organ^{82,95–97}. This also makes EVLP useful for testing pharmacological and biological therapies before transplantation⁸². Examples include advanced therapy medicinal product (ATMP)-based approaches involving mesenchymal stromal cells (MSCs) or gene therapy vectors, together with emerging mitochondrial and other targeted therapies^{98–101}. In this thesis, porcine EVLP was used to model complement-mediated HAR-like injury and to evaluate physiological dysfunction alongside histological and spatial tissue alterations.

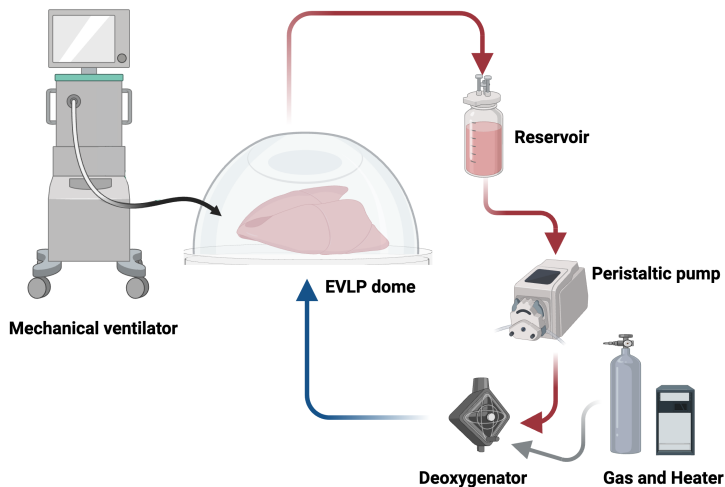


Figure 4. Schematic diagram of *ex vivo* lung perfusion (EVLP) system.

The isolated lung is maintained in an EVLP dome and connected to a closed perfusion circuit. The perfusate circulates from the reservoir through a peristaltic pump, deoxygenator/gas exchanger, and heater before returning to the lung. Mechanical ventilation maintains lung ventilation during perfusion. Adapted from Ghaidan et al. 2022²⁸, licensed under CC BY 4.0. Figure created with BioRender.

Animal models in translational lung research

Mouse

Mouse models are widely used in pulmonary research because they are well established, experimentally tractable, and suitable for controlled mechanistic studies¹⁰². Although mouse lungs differ from human lungs in size, anatomy, and physiology, they provide a useful small-animal system for investigating pulmonary structure and cellular organisation.

Mouse lung tissue was included in Paper II to evaluate whether the agarose-embedding and automated vibratome-sectioning workflow could be applied across species. The workflow was suitable for small and delicate lung samples and preserved overall tissue architecture, enabling fluorescence visualisation of structural components such as the alveolar scaffold, small airways, and blood vessels¹⁰³. The use of available mouse lung tissue also supported the 3Rs principle by increasing the scientific value obtained from existing, otherwise unused tissue and reducing the need for additional animals.

Porcine

Among large animal models, pigs are widely used in translational lung research because their lungs share important anatomical and physiological similarities with human lungs, including comparable organ size, airway branching, vascular organization, and alveolar architecture^{104–106}. These features are particularly valuable in lung transplantation research, as the size and anatomy of porcine lungs allow clinically relevant procedures such as lung procurement, ventilation, vascular cannulation, extracorporeal circulation, and EVLP^{104,107}.

Porcine EVLP provides a controlled platform for assessing donor lung function, studying ischemia–reperfusion injury, modelling graft dysfunction and rejection-related processes, and evaluating potential therapeutic interventions under conditions that closely resemble clinical practice¹⁰⁸. The size of pigs also permits repeated collection of blood, perfusate, bronchoalveolar lavage fluid, and tissue, enabling longitudinal assessment of physiological, inflammatory, and structural changes^{109,110}.

The porcine immune system also shares several important features with the human immune system, supporting its use in studies of inflammation, alloimmune activation, and transplantation-related injury^{104,111}. However, pigs have a tracheal bronchus that supplies the right cranial lobe, which differs from normal human airway anatomy and may affect regional ventilation¹⁰⁵. They also possess a resident population of pulmonary intravascular macrophages that is not normally present in

healthy human lungs and may modify pulmonary inflammatory responses¹¹². These anatomical and immunological differences should be considered when extrapolating experimental findings to humans.

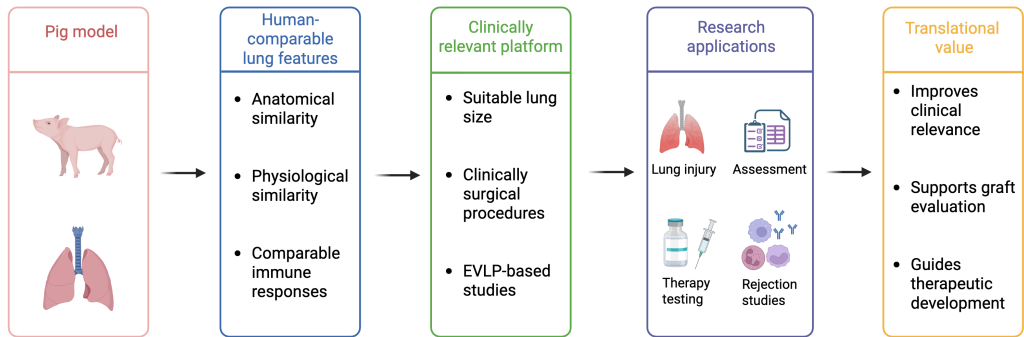


Figure 5. Porcine lung model as a translational research platform.

This schematic summarizes how the human-comparable features of the porcine lung and its suitability for clinically relevant experimental procedures support its use in lung injury, graft assessment, rejection, and therapy-testing studies. Figure created with BioRender.

Histology

An overview

Histology plays a fundamental role in diagnostic pathology and biomedical research by enabling the microscopic examination of tissue structure, cellular organisation, and pathological alterations. In routine histological practice, tissues are prepared using a standardised workflow that typically includes fixation, tissue processing, embedding, sectioning, and staining (Fig. 6). Together, these steps preserve tissue morphology and enable reproducible microscopic evaluation.

Within this workflow, tissue processing prepares fixed specimens for embedding and subsequent sectioning. In paraffin-based workflows, tissue processing involves dehydration to remove water, clearing to replace the dehydrating agent with a medium compatible with paraffin, and infiltration with molten paraffin^{113,114}. After embedding and solidification, the paraffin block provides mechanical support, allowing thin, uniform sections, typically 5–10 µm thick, to be cut with a microtome. The sections are then mounted on glass slides and stained using routine or specialised methods. Haematoxylin and eosin (H&E) staining is one of the most widely used methods for assessing overall tissue architecture and cellular morphology^{115,116}. It provides an important reference for histopathological

evaluation and complements more advanced approaches, including immunofluorescence imaging and quantitative spatial analysis.

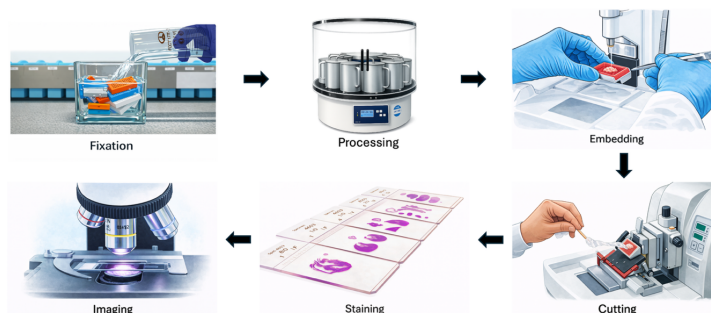


Figure 6. Schematic overview of the histological processing workflow.

The workflow illustrates the main steps of tissue preparation for histological evaluation and image-based analysis, including tissue fixation, automated processing, paraffin embedding, sectioning, staining, and microscopic imaging. Figure created by the author with the assistance of ChatGPT (OpenAI, 2026).

Tissue processing methods

The choice of tissue-processing method can affect tissue morphology, antigen preservation, staining performance, and the reliability of downstream quantitative analysis¹¹⁷. This is particularly relevant to the lung because its large airspaces, delicate alveolar septa, vascular structures, and ECM components are susceptible to distortion during preparation. Processing protocols should therefore be selected according to the tissue type, experimental aim, and intended imaging approach.

Paraffin-based tissue processing and clearing reagents

Formalin-fixed, paraffin-embedded (FFPE) tissue remains one of the most widely used specimen formats in histology and diagnostic pathology. The workflow typically includes formalin fixation, dehydration through graded alcohols, clearing, paraffin infiltration and embedding. Paraffin provides mechanical support for thin sectioning, while FFPE sections are compatible with a wide range of histological stains and downstream analyses¹¹⁸. FFPE blocks can also be stored for many years at room temperature, making them valuable for archival collections, retrospective studies, and re-analysis using new technologies¹¹⁹.

Clearing occurs after dehydration and before paraffin infiltration, replacing alcohol with a reagent compatible with molten paraffin wax. Effective clearing supports uniform paraffin infiltration and subsequent sectioning and staining. Xylene has traditionally been the most widely used clearing reagent because it efficiently replaces alcohol and is compatible with paraffin^{120,121}. However, xylene is a volatile aromatic solvent associated with occupational and environmental hazards. Its use

requires adequate ventilation, careful handling, and regulated waste disposal, which increases the practical burden and may increase the cost of routine processing^{121–124}. These concerns have encouraged the development of xylene-free processing strategies. Several potentially safer substitutes have been investigated, but differences in their chemical properties, tissue penetration, and sample compatibility may affect paraffin infiltration, tissue shrinkage, staining quality, and imaging performance^{125–127}.

Isopropanol has been explored as a practical xylene-free alternative for histological tissue processing¹¹³. Compared with xylene, its generally lower toxicity may reduce some occupational risks associated with routine tissue processing¹²⁸. Its use may also reduce reliance on hazardous aromatic solvents¹²¹. Isopropanol-based protocols have produced sectioning and staining outcomes comparable to those obtained with conventional xylene-based methods^{113,128}. However, isopropanol may be less effective in lipid-rich tissues because it has a lower lipid-extraction capacity than xylene¹²⁹. In such samples, incomplete lipid removal or reagent penetration may impair paraffin infiltration and staining quality. Its performance should therefore be evaluated for the tissue type and downstream analysis, particularly for quantitative immunofluorescence imaging.

Conventional FFPE processing is also time-consuming and labour-intensive, and fixation and organic solvents may mask or alter antigenic epitopes, thereby affecting immunostaining and downstream analyses¹³⁰. These limitations provide a rationale for evaluating alternative workflows for fluorescence-based quantitative tissue analysis.

Agarose embedding and vibratome sectioning

Agarose embedding offers a practical method for preparing fixed lung tissue, particularly when thick sections are required for fluorescence imaging. In this workflow, tissue blocks are embedded in low-melting-point agarose to support the tissue during vibratome sectioning¹⁰³ (Fig. 7).

Precision-cut lung slices (PCLS) are widely used *ex vivo* models, and their preparation typically involves filling the airways with low-melting-point agarose to maintain lung inflation and support the tissue during sectioning^{131,132}. This approach is relatively straightforward in intact small-animal lungs because low-melting-point agarose can be instilled through the trachea to inflate and support the lung before sectioning. However, it is less practical for isolated tissue blocks from large-animal or human lungs when a suitable airway is not available. By contrast, agarose embedding of fixed tissue blocks does not require airway filling. Combined with automated vibratome sectioning, this approach can prepare thick sections of mouse, pig, and human lung tissue for multiplex fluorescence imaging without antigen retrieval¹⁰³. However, diseased lung tissue can be more difficult to section because airway obstruction, pleural thickening, or fibrosis may interfere with blade

movement. These challenges can be reduced by lowering the blade advancement speed, using a new and sharp blade, gently supporting the tissue block during sectioning, trimming excess agarose around the tissue, and removing the pleura before sectioning¹⁰³.

Overall, paraffin-based and agarose-based tissue processing methods provide complementary advantages. FFPE processing is well-suited for routine histology, morphological assessment, and long-term storage, whereas agarose embedding and vibratome sectioning support thick-section fluorescence imaging and spatial analysis. These approaches provided the methodological basis for the histological and fluorescence-based spatial analyses used in this thesis.

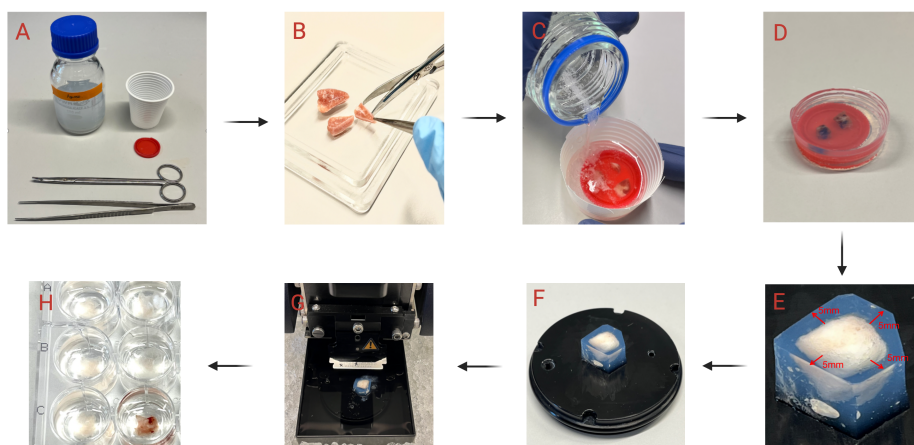


Figure 7. Workflow for preparation, agarose embedding, and vibratome sectioning lung tissue slices.

(A) Materials used for tissue preparation. (B) Lung tissue was dissected into small pieces. (C–D) Tissue pieces were embedded in 3% low-melting-point agarose and removed from the mold after solidification. (E) Excess agarose was trimmed, leaving an approximately 5-mm agarose margin around the tissue. (F–G) The agarose-embedded tissue block was mounted onto the specimen holder and sectioned in ice-cold PBS using a vibratome. (H) Lung tissue slices were collected in a 24-well plate. Adapted from Wang et al¹⁰³, with permission.

Fluorescence imaging for spatial analysis of lung tissue

Lung injury is often heterogeneous, with structural and cellular alterations unevenly distributed across tissue regions^{133,134}. Fluorescence microscopy is therefore valuable for visualising the location and distribution of these changes. Multiplex immunofluorescence allows several markers to be detected within the same tissue section, enabling their distribution and relationships to the surrounding structures to be assessed^{135,136}. This is particularly useful in lung research because lung injury and

transplantation-related graft dysfunction can affect multiple tissue compartments, including the alveolar epithelium, pulmonary endothelium, immune cells, and ECM¹³⁷.

Confocal microscopy is widely used for fluorescence imaging because it reduces out-of-focus fluorescence and enables high-resolution optical sectioning at different tissue depths^{138,139}. It can be used to acquire z-stack images from tissue fields selected using consistent anatomical criteria for the structure being analysed. These images support assessment of alveolar architecture, cellular organisation, and marker distribution¹⁴⁰. Quantitative analysis can then provide measurements of fluorescence intensity, tissue coverage, cell abundance, and morphological features^{141,142}. Standardised staining, image acquisition, and analysis procedures support consistent comparison of these measurements between experimental groups.

In this thesis, multiplex fluorescence imaging was combined with quantitative image analysis to characterise tissue structure and marker distribution in translational models of aspiration-induced lung injury and complement-mediated graft injury. This image-based spatial approach enabled alterations across multiple lung compartments to be examined alongside conventional histological injury scores and physiological measurements.

Aims of the thesis

Paper I

Paper I aimed to evaluate isopropanol as an alternative to xylene in paraffin-based lung tissue processing by assessing its ability to preserve tissue morphology and staining quality.

Paper II

Paper II aimed to establish a reproducible agarose-embedding and automated vibratome-sectioning workflow for lung tissue, with the goal of preserving tissue architecture and enabling fluorescence-based structural visualization across species.

Paper III

Paper III aimed to establish a quantitative and reproducible imaging pipeline for detecting and quantifying injury-related alterations in alveolar architecture during acute lung injury.

Paper IV

Paper IV aimed to apply and extend a multiplex imaging pipeline for the quantitative characterization of tissue-level alterations in a porcine model of acute lung injury. The study investigated injury-associated changes in alveolar architecture, inflammatory cell infiltration, alveolar–capillary barrier integrity, extracellular matrix organization, and tissue ultrastructure.

Paper V

Paper V aimed to establish a controlled porcine EVLP model of complement-mediated HAR-like graft injury and to investigate the associated changes in graft function and spatial tissue alterations, including immune activation, alveolar–capillary barrier disruption, and structural remodelling.

Summary of Results and Discussion

Paper I

Background and study design

Xylene is routinely used as a clearing reagent in paraffin-based tissue processing, but its potential health risks and environmental impact have encouraged the search for safer alternatives. Paper I therefore evaluated whether isopropanol could be used as an alternative to xylene without compromising lung tissue morphology, sectioning performance, histological staining quality, or fluorescence-based analysis.

Lung tissues from donor ($n = 6$) and recipient ($n = 6$) samples were processed using either xylene or isopropanol, allowing comparison between the two clearing methods across different tissue origins. The processed sections were assessed by ease of sectioning, visual histological inspection, blinded H&E histological scoring, and fluorescence-based analyses, including alveolar morphology, fluorescence intensity, and signal-to-noise ratio measurements of α -SMA and elastin staining.

H&E-based assessment of lung tissue morphology and sectioning performance

Brightfield microscopy of H&E-stained lung sections showed that both processing methods preserved the characteristic alveolar architecture, including recognizable alveolar airspaces and alveolar septal structure. No obvious morphological differences were observed between xylene- and isopropanol-processed tissues (Fig. 8). The ease-of-sectioning scores did not differ significantly between tissue blocks processed with xylene and those processed with isopropanol. These results indicate that isopropanol processing preserved lung tissue morphology and provided sectioning performance comparable to that achieved with xylene.

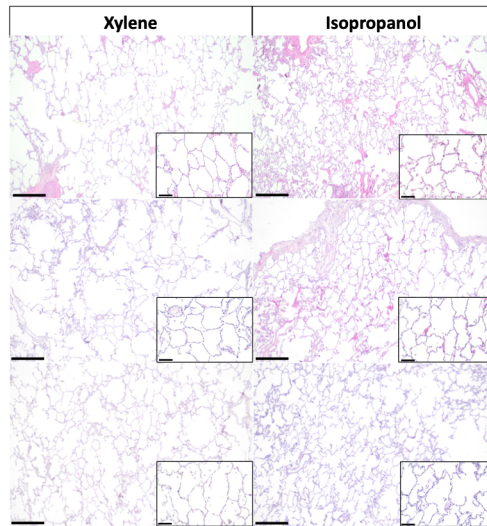


Figure 8. H&E staining of lung tissue sections processed with xylene and isopropanol. Representative haematoxylin and eosin (H&E) stained lung sections processed with xylene (left) and isopropanol (right). Scale bars: 500µm (overview); 100µm (callout). Adapted from Paper I.

To further evaluate histological quality, H&E-stained lung sections processed with xylene or isopropanol were assessed using a standardized scoring system¹²⁸. Sections from donor and recipient lungs were evaluated in a blinded manner by independent experienced observers. The scoring criteria included overall tissue quality, staining quality, tissue architecture, nuclear–cytoplasmic differentiation, and vascular structures. No significant differences were observed between the two processing methods, indicating that isopropanol provided histological quality comparable to xylene (Fig. 9).

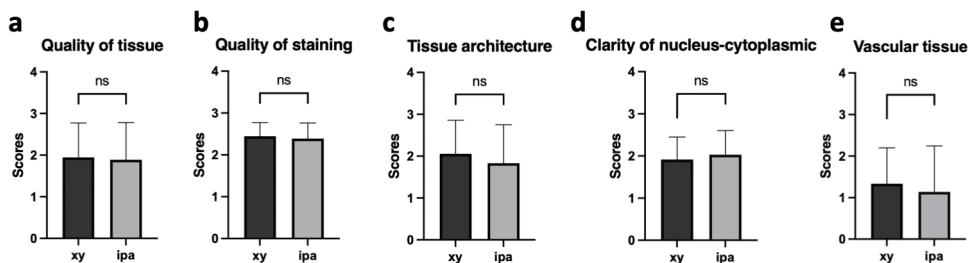


Figure 9. Quantitative comparison of H&E-stained lung tissue sections processed with xylene or isopropanol.

Histological evaluation was performed on H&E-stained donor and recipient lung biopsies. Tissue sections were scored for overall tissue quality (a), staining quality (b), tissue architecture (c), nuclear–cytoplasmic differentiation (d), and vascular structure (e). $n = 12$. ns, not significant ($p > 0.05$). Adapted from Paper I.

Fluorescence staining evaluation

To quantitatively assess whether the clearing reagent affected fluorescence-based tissue evaluation, lung sections stained with LEA and DAPI were analysed (Fig. 10a). Alveolar circularity, wall thickness, and LEA fluorescence intensity were quantified. No significant differences in alveolar circularity or wall thickness were detected between xylene- and isopropanol-processed tissues (Fig. 10b, c).

Similarly, no significant difference in LEA fluorescence intensity was observed between the two processing methods within either the donor or recipient tissue group (Fig. 10f, g). In contrast, recipient lungs showed significantly higher LEA intensity than donor lungs following both processing conditions (Fig. 10d, e; Fig. 10h–j). This indicates that this difference was associated with tissue origin rather than the clearing reagent. Overall, the two processing methods produced comparable fluorescence-based readouts.

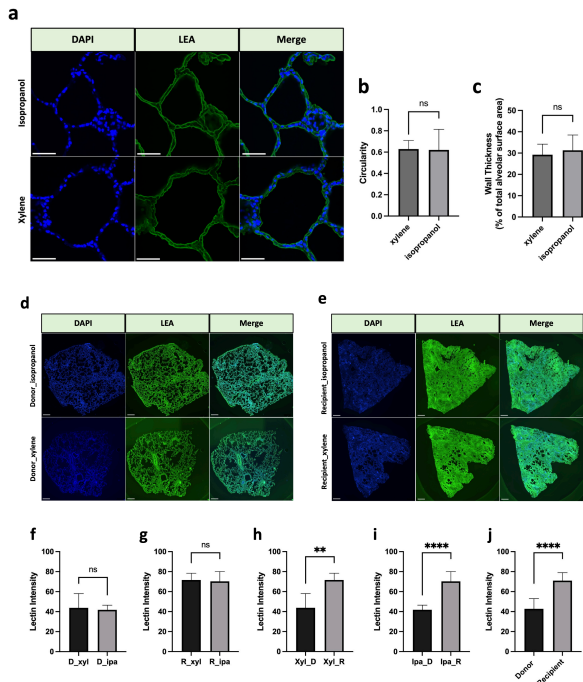


Figure 10. Fluorescence staining analysis of lung sections processed with xylene or isopropanol.

(a) Representative fluorescence images of LEA/DAPI-stained lung sections. Scale bar = 50 μ m. (b, c) Quantification of alveolar circularity and wall thickness in xylene- and isopropanol-processed tissues. (d, e) Representative fluorescence images of donor and recipient lung sections stained with LEA and DAPI. Scale bars: 1000 μ m. (f, g) LEA fluorescence intensity in donor and recipient lungs processed with xylene or isopropanol. (h, i) LEA fluorescence intensity compared between donor and recipient tissues within each clearing reagent. (j) LEA fluorescence intensity compared between donor and recipient tissues across both processing methods. ns, not significant; ** $p < 0.01$; **** $p < 0.0001$. D, donor; R, recipient; Xyl, xylene; lpa, isopropanol; Adapted from Paper I.

Comparable immunofluorescence signal quality in xylene- and isopropanol-processed tissues

To assess whether the clearing reagent affected immunofluorescence signal quality, lung tissue sections were stained for α -SMA and elastin (Fig. 11a, b). Signal quality was quantified using the signal-to-noise ratio (SNR). For both markers, no significant differences in SNR were detected between the two processing methods (Fig. 11c, d).

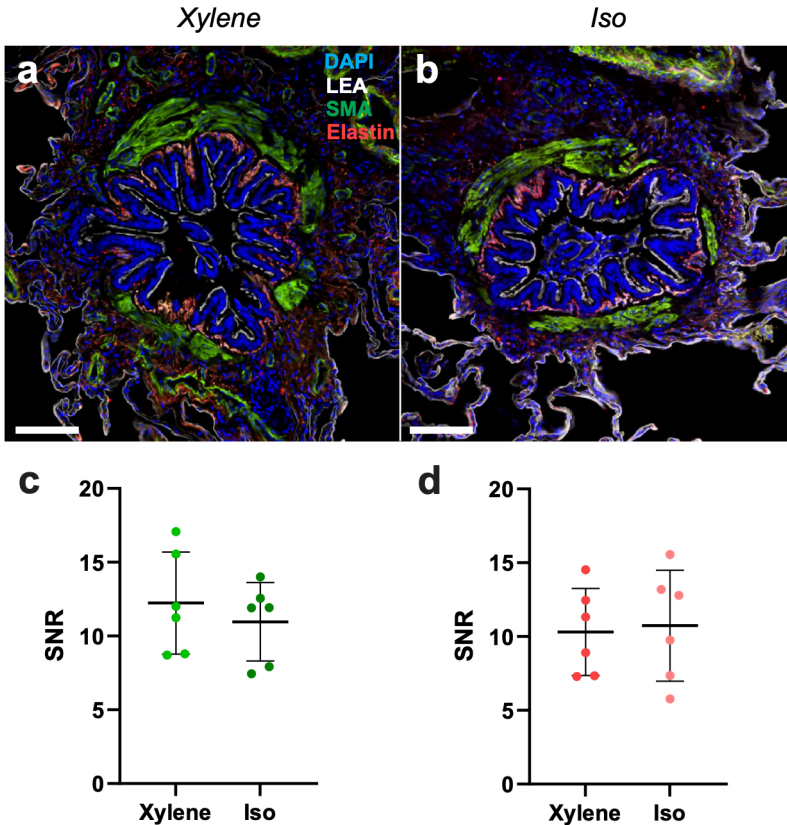


Figure 11. Immunofluorescence staining performance in xylene- and isopropanol-processed lung tissue.

(a, b) Representative fluorescence images of bronchiolar regions stained for α -SMA, elastin, lectin, and DAPI after processing with xylene (a) or isopropanol (b). Scale bars: 100 μ m. (c, d) Quantification of the signal-to-noise ratio (SNR) for α -SMA (c) and elastin (d) staining. $n = 6$. DAPI, 4',6-diamidino-2-phenylindole; LEA, Lycopersicon esculentum agglutinin; α -SMA, alpha-smooth muscle actin; SNR, signal-to-noise ratio. Adapted from Paper I.

Paper II

Background and study design

Lung tissue is fragile and contains large air-filled spaces, making it technically challenging to prepare sections while preserving its native architecture. Conventional paraffin-based histology and precision-cut lung slices (PCLS) are useful for lung tissue assessment, but paraffin processing can be time-consuming, whereas PCLS preparation can be technically demanding, particularly for porcine and human biopsy samples. Therefore, a practical and standardised workflow that supports fragile lung tissue during sectioning and enables multiplex fluorescence staining and volumetric imaging was needed.

In Paper II, a vibratome-based workflow was established and applied to generate lung tissue sections from fixed mouse, porcine, and human lung samples. Tissue blocks were embedded in 3% low-melting-point agarose to provide support and were then sectioned into 200 μm slices using optimized vibratome settings. The sections were stained with α -SMA, elastin, LEA, and DAPI to visualise pulmonary structures, while HTII-280 was used to identify type II pneumocytes in human lung tissue. Fluorescence imaging and image-based analyses were used to visualise structural organisation and quantify type II pneumocyte abundance in adult and infant human lung samples.

Agarose-embedding and vibratome sectioning workflow

Lung tissue samples were embedded in 3% low-melting-point agarose, which provided mechanical support for the fragile tissue and helped preserve lung architecture during sectioning. After the excess agarose was trimmed, the embedded blocks were mounted and sectioned into 200 μm slices in cooled PBS using optimised vibratome settings. The resulting slices remained structurally intact and were prepared for subsequent staining and imaging (Fig. 12a–d).



Figure 12. Workflow for agarose embedding and vibratome sectioning of lung tissue.

(a) Materials were prepared, and lung tissue was dissected into smaller blocks. (b) Tissue blocks were embedded in agarose. (c) The agarose-embedded block was trimmed, mounted onto the tissue holder, and sectioned using a vibratome. (d) Lung tissue slices were prepared for subsequent immunofluorescence staining. Vibratome settings: section thickness, 200 μm ; frequency, 100 Hz; amplitude, 1.3 mm; forward speed, 0.02–0.04 mm/s. Adapted from Paper II.

Structural characterization of lung tissue sections using immunofluorescence imaging

Immunofluorescence imaging of 200 μm lung sections from mouse, porcine, and human samples visualised the distinct distribution of α -SMA and elastin across bronchioles, blood vessels, alveolar ducts, and alveoli in relation to the LEA-labelled bronchoalveolar scaffold (Fig. 13a–i). These immunofluorescence images showed that this method provides consistent and reliable staining performance in lung tissue from different species.

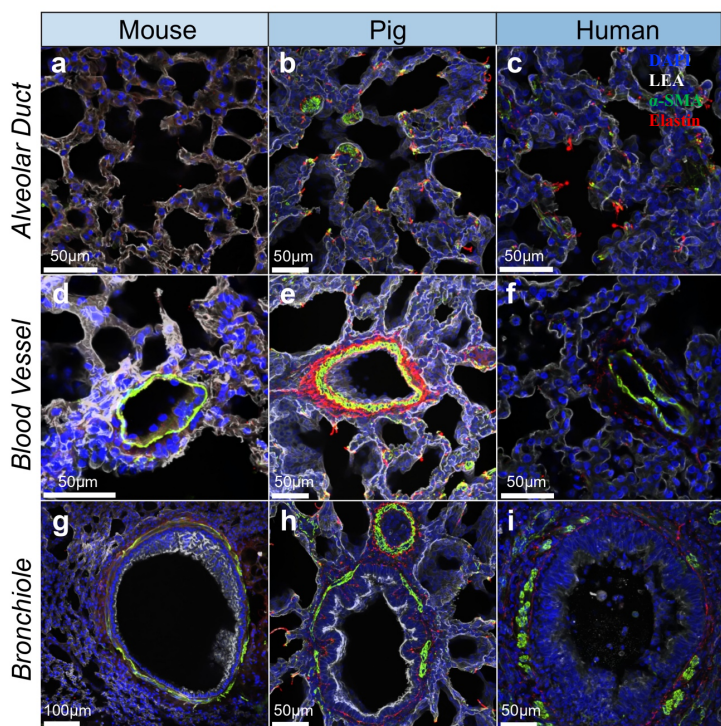


Figure 13. Immunofluorescence visualization of lung structure across species.

(a–i) Representative immunofluorescence images of alveolar ducts, blood vessels, and bronchioles in mouse, pig, and human lung tissue slices stained for DAPI, LEA, α -SMA, and elastin. Scale bars = 50 μ m (a–f, h–i) and 100 μ m (g). DAPI, 4',6-diamidino-2-phenylindole; LEA, Lycopersicon esculentum agglutinin; α -SMA, alpha-smooth muscle actin. Adapted from Paper II.

Quantitative analysis of type II pneumocyte distribution in human lung tissue

To demonstrate the quantitative application of this imaging approach, sections from adult and infant human lung tissue were stained with the human-specific HTII-280 antibody to identify type II pneumocytes (Fig. 14a–d). Infant lung tissue contained significantly more HTII-280-positive cells and a greater scaffold-covered area than adult lung tissue. After normalisation to scaffold-covered tissue area, HTII-280-positive cell density remained significantly higher in the infant sample (Fig. 14e–g). This higher density in the infant sample was consistent with previous reports of an age-related decline in type II pneumocyte density¹⁴³.

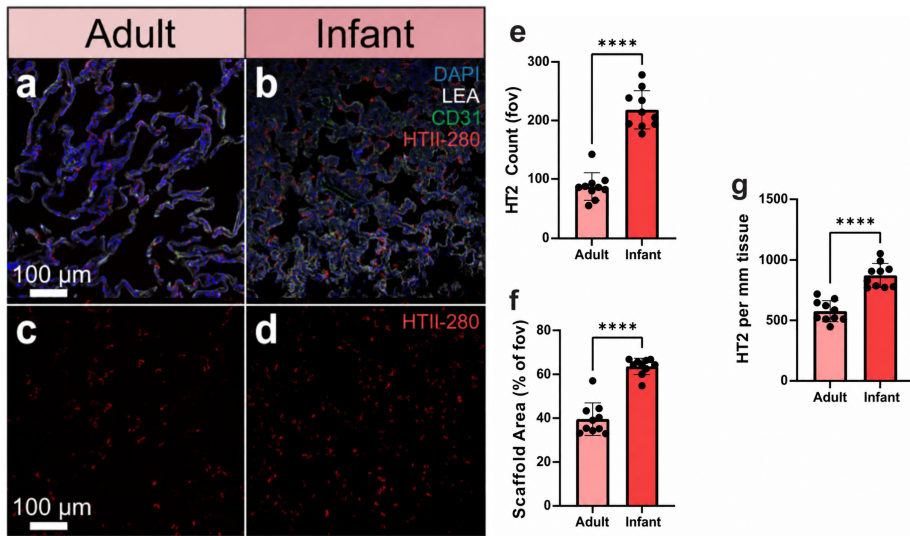


Figure 14. Quantitative analysis of HTII-280⁺ type II pneumocytes in adult and infant human lung tissue.

(a–d) Representative immunofluorescence images of adult and infant human lung tissue stained for HTII-280, CD31, LEA and DAPI. (e–g) Quantitative comparison of HTII-280-positive cell counts, scaffold coverage, and area-normalized HTII-280-positive cell density between adult and infant lung tissue (n=10 FOVs per sample). Scale bars: 100 μ m (a–d). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. FOV, field of view; CD31, cluster of differentiation 31; DAPI, 4',6-diamidino-2-phenylindole; HTII-280, human type II pneumocyte marker; LEA, *Lycopersicon esculentum* agglutinin. Adapted from Paper II.

Paper III

Background and study design

Acute lung injury is associated with structural disruption of the alveolar compartment, including alveolar wall thickening, collapse, and distortion of normal airspace architecture. Although conventional histological scoring can assess the overall tissue injury, it provides semiquantitative scores and is partly observer dependent. Quantitative image analysis may therefore complement conventional scoring by providing continuous measurements of injury-related changes in alveolar morphology.

In Paper III, high-resolution fluorescence imaging was used to quantify alveolar morphology in porcine lung tissue. Fluorescence staining with LEA enabled visualization and tracing of individual alveoli, allowing alveolar wall thickness and circularity to be measured. The morphological quotient (MQ) was introduced to integrate wall thickness and circularity into a single composite readout of alveolar morphology. Reference values for wall thickness and circularity were established

by analysing 212 alveoli from 10 healthy pigs. The same analysis was then applied to compare five baseline pigs from this cohort with five pigs in the gastric aspiration injury group, with 10 alveoli analysed per animal, to assess injury-induced alveolar structural changes. To assess the reproducibility of the analysis, a researcher with no previous imaging or image-analysis experience traced four randomly selected alveoli per animal in both groups.

LEA delineates the alveolar scaffold in lung tissue

To visualize the alveolar scaffold, 200 μm lung sections prepared using the vibratome-based workflow were incubated with DAPI and LEA (Fig. 15a). Confocal imaging showed distinct LEA labelling of the bronchoalveolar scaffold, including bronchioles, alveolar ducts, alveolar septa, and alveoli (Fig. 15b, c).

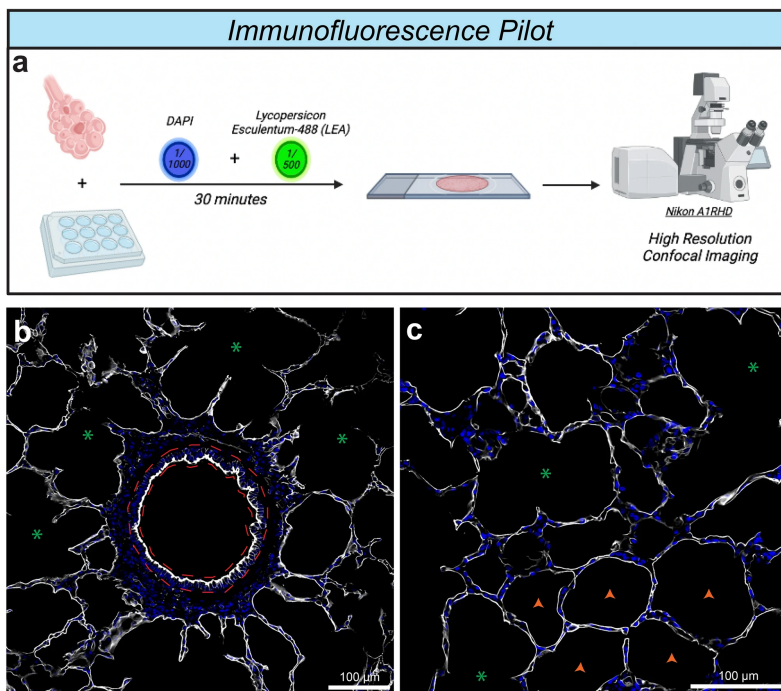


Figure 15. LEA staining delineates bronchoalveolar structures.

(a) Overview of the staining and imaging workflow for assessing alveolar architecture. (b, c) Representative LEA-stained lung tissue images showing airway-associated structures, terminal bronchiolar regions (green asterisks) and alveolar epithelium (orange arrowheads). Scale bars: 100 μm . DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin. Adapted from Paper III.

High-resolution imaging enables quantitative analysis of alveolar morphology

High-resolution imaging showed individual alveoli (Fig. 16a, b) and allowed their inner and outer borders to be traced using image-analysis software to quantify alveolar morphology (Fig. 16c, d). Alveolar wall thickness was calculated as a relative wall area by subtracting the inner alveolar area from the outer alveolar area and normalized to the total outer alveolar area (Fig. 16e). Circularity was quantified from the inner alveolar border, with a value of 1.00 representing a perfect circle (Fig. 16f).

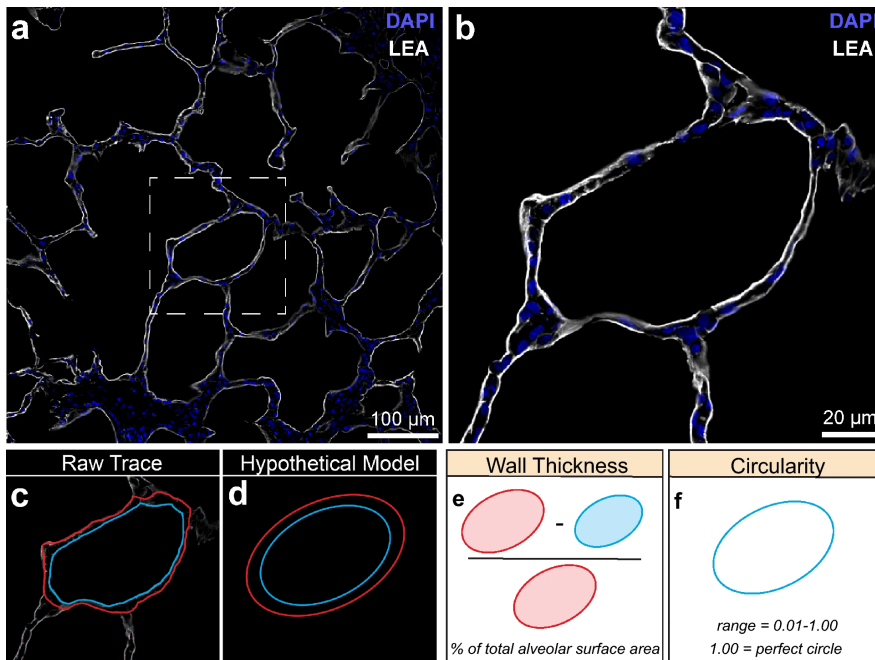


Figure 16. Quantitative image analysis of alveolar morphology in lung tissue.

(a) Representative fluorescence image showing individual alveoli. (b) Magnified view of a single alveolus from the boxed region in (a). (c) Raw traces of the inner and outer alveolar borders generated from the LEA signal. (d) Simplified schematic representation of the traces shown in (c). (e) Schematic illustrating the calculation of alveolar wall thickness. (f) Schematic illustrating alveolar circularity. Scale bars: 100 μ m (a) and 20 μ m (b). DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin. Adapted from Paper III.

Aspiration injury increases alveolar wall thickness and reduces alveolar circularity

The imaging pipeline was subsequently applied to compare alveolar morphology between the baseline and gastric aspiration injury groups. High-resolution confocal images revealed morphological differences between the groups (Fig. 17a–d).

Quantitative analysis showed that aspiration injury significantly increased relative alveolar wall thickness and reduced alveolar circularity (Fig. 17e, f).

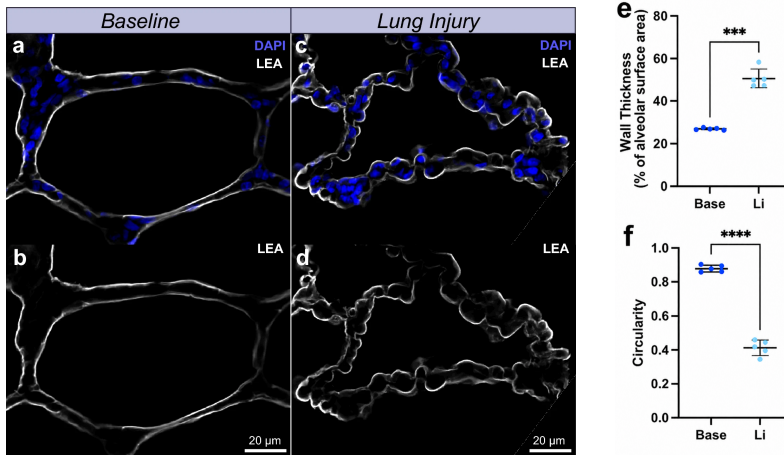


Figure 17. High-resolution assessment of alveolar morphology following aspiration injury.

(a–d) Representative high-resolution confocal images of individual alveoli from baseline and aspiration injury groups. Scale bars: 20 μm. (e, f) Quantification of alveolar wall thickness and circularity (n = 5 animals per group). Base, baseline; Li, lung injury; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin. ***p < 0.001, ****p < 0.0001. Adapted from Paper III.

Reproducibility of the alveolar morphology image-analysis pipeline

To assess whether the results could be reproduced by an inexperienced user, a researcher with no previous imaging or image-analysis experience independently analysed four randomly selected alveoli per animal. The independent analysis also showed increased alveolar wall thickness and reduced circularity in the aspiration injury group, consistent with the primary analysis (Fig. 18a, b). When the baseline and aspiration injury groups were analysed separately, alveolar wall thickness and circularity measurements from the randomly selected subset were comparable to those from the full dataset (Fig. 18c–f).

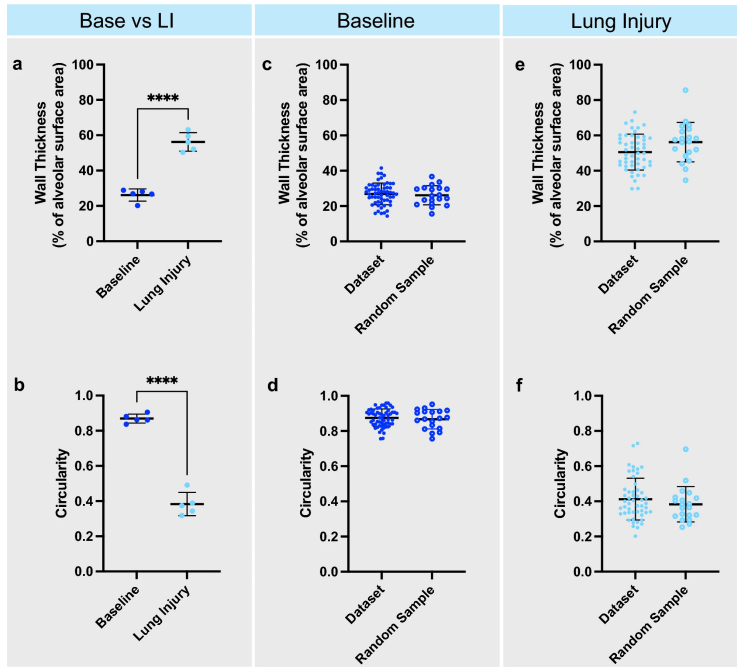


Figure 18. Reproducibility of the alveolar morphology analysis pipeline.

(a, b) Alveolar wall thickness and circularity in baseline and lung injury groups, independently measured by a researcher with no prior experience with the pipeline using a randomly selected subset of alveoli ($n = 5$ animals per group, 4 alveoli per animal). (c–f) Comparison of the primary full-dataset analysis and the independent subset analysis for alveolar wall thickness and circularity in baseline and lung injury groups. Base, baseline; LI, lung injury. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Adapted from Paper III.

Morphological quotient provides an integrated measure of alveolar structural injury

MQ was calculated as alveolar circularity divided by alveolar wall thickness (Fig. 19a). Higher MQ values represent more circular alveoli with thinner walls, consistent with better preserved alveolar structure. MQ was significantly lower in the injury group (Fig. 19b). Across all analysed alveoli, MQ values were generally lower in the injury group than in the baseline group (Fig. 19c).

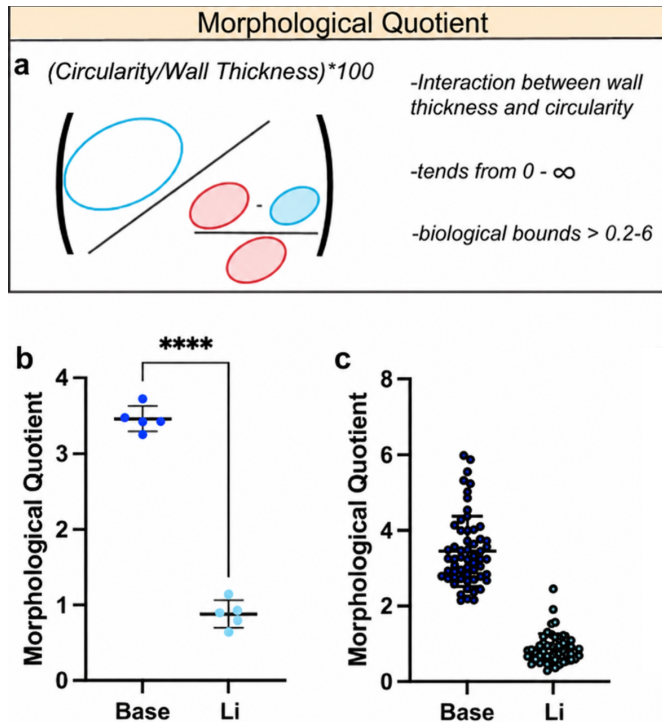


Figure 19. Morphological quotient calculation and comparison between baseline and aspiration injury groups.

(a) Schematic illustration of MQ calculation based on alveolar wall thickness and alveolar circularity. (b) Quantification of MQ in baseline and lung injury groups ($n = 5$ animals per group). (c) Distribution of MQ across all analysed alveoli ($n = 50$ alveoli per group). Base, baseline; Li, lung injury. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Adapted from Paper III.

Paper IV

Background and study design

Aspiration-induced acute lung injury is clinically relevant to donor lung assessment and transplantation because gastric aspiration can cause epithelial damage, inflammation, impaired gas exchange, and disruption of lung structure. These changes may compromise donor lung quality and contribute to early graft dysfunction. A translational large-animal model is therefore useful for investigating how aspiration injury affects lung structure and the tissue microenvironment.

Paper IV used a porcine gastric aspiration-induced acute lung injury model comprising healthy control animals ($n = 8$) and aspiration-injured animals ($n = 5$).

The study combined physiological measurements and histological injury scoring with fluorescence-based scaffold imaging, multiplex immunofluorescence, and scanning electron microscopy. This design allowed for validation of acute lung injury and spatial assessment of injury-related changes in the alveolar epithelium, vascular endothelium, immune microenvironment, ECM, and contractile-associated structures.

Gastric aspiration induces pulmonary dysfunction and histological lung injury

Adult pigs were allocated to control and gastric aspiration injury groups (Fig. 20a). Compared with controls, the injury group showed a significantly lower $\text{PaO}_2/\text{FiO}_2$ ratio and higher PVR (Fig. 20b, c), indicating impaired gas exchange and altered pulmonary haemodynamics. Histopathological analysis further revealed microscopic changes consistent with acute lung injury and significantly higher lung injury scores in the aspiration group (Fig. 20d, e).

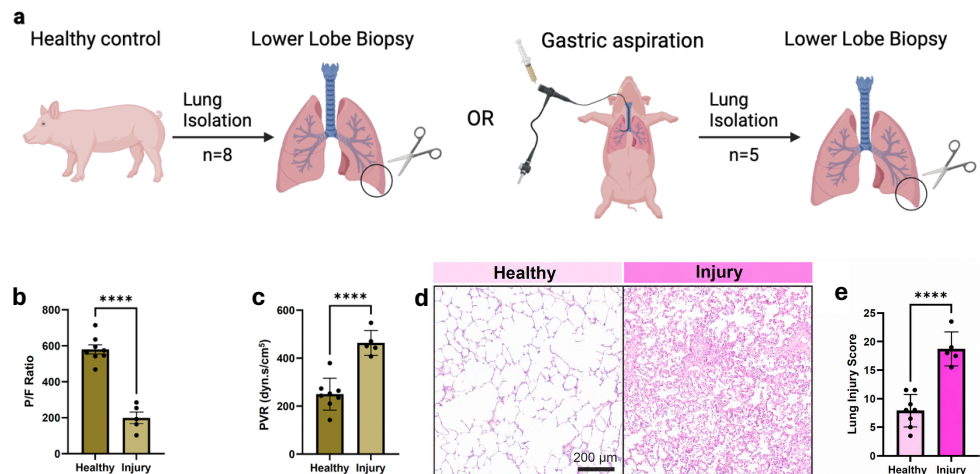


Figure 20. Functional and histological validation of a porcine gastric aspiration-induced acute lung injury model.

(a) Schematic overview of healthy control animals ($n = 8$) and gastric aspiration injury animals ($n = 5$). (b, c) Quantification of the $\text{PaO}_2/\text{FiO}_2$ ratio and pulmonary vascular resistance (PVR). (d) Representative H&E-stained lung sections from healthy and injured animals. Scale bars: 200 μm . (e) Quantification of lung injury scores. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Adapted from Paper IV.

Fluorescence imaging reveals alveolar scaffold alterations following aspiration injury

LEA staining clearly delineated the bronchiolar and alveolar scaffold (Fig. 21a). Compared with control tissue, aspiration-injured samples exhibited alveolar septal thickening and distortion (Fig. 21b, c). Alveolar scaffold coverage, quantified as the percentage of LEA-positive area within each field of view (FOV), was significantly higher in injured samples (Fig. 21d).

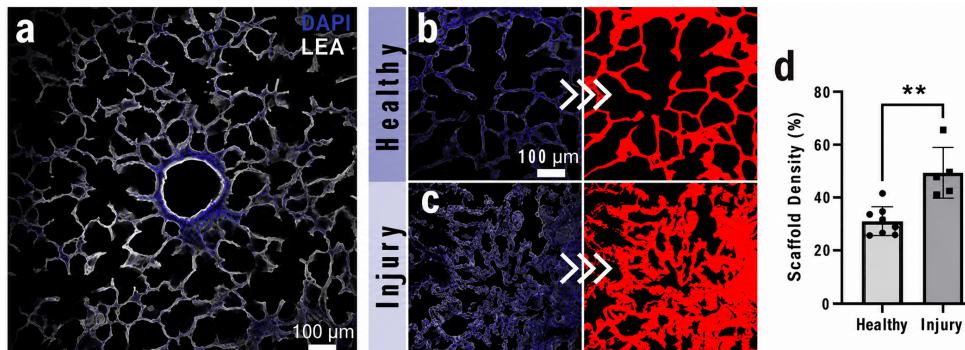


Figure 21. Fluorescence imaging and quantification of alveolar scaffold coverage after aspiration-induced lung injury.

(a) Representative LEA/DAPI-stained image showing the broncho-alveolar scaffold. (b, c) Representative LEA-stained images from healthy and injured lung samples, with corresponding threshold-based scaffold masks in healthy and injured lung samples. (d) Quantification of scaffold density in healthy and injured samples. Scale bars: 100 μm. Healthy, n = 8; injury, n = 5. ** $p < 0.01$. DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; FOV, field of view. Adapted from Paper IV.

Scaffold-normalised analysis reveals differential changes in alveolar barrier-associated markers

AQP5, a marker associated with the alveolar type I epithelial surface, was analysed relative to LEA-labelled scaffold coverage (Fig. 22a). Before normalisation, AQP5 fluorescence intensity was higher in injured tissue; however, this difference largely reflected the greater scaffold coverage in injured lungs. After scaffold normalisation, AQP5 intensity and coverage were significantly lower in injured tissue, and AQP5-positive particle size was also reduced, consistent with alteration of the alveolar type I epithelial compartment (Fig. 22b–g). In contrast, scaffold-normalised ZO-1 intensity and coverage were increased in injured tissue, suggesting altered tight-junction organisation (Fig. 22h–l). Scaffold normalisation was used to express marker signals relative to LEA-positive scaffold coverage, thereby accounting for differences in tissue coverage between FOVs.

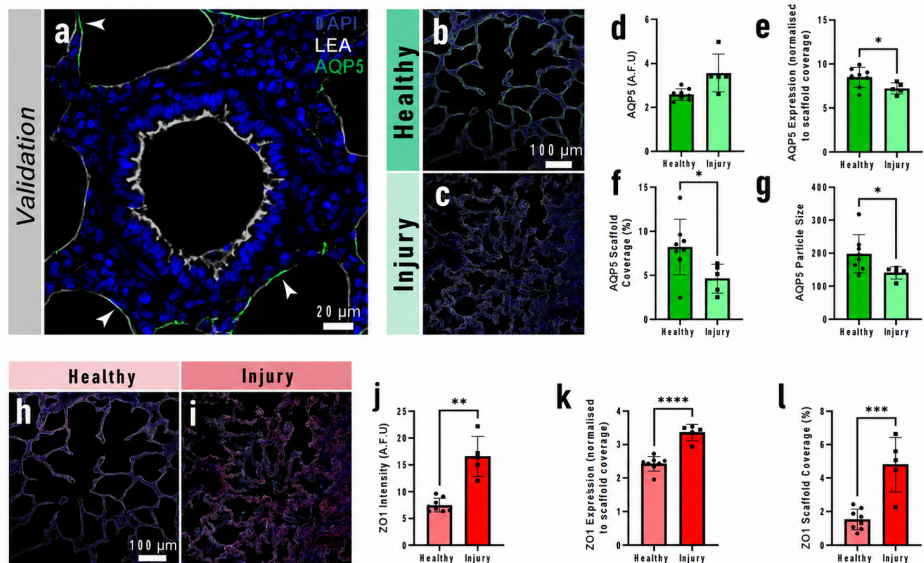


Figure 22. Scaffold-normalized fluorescence analysis of AQP5 and ZO-1 expression after aspiration-induced lung injury.

(a) Representative AQP5-stained image showing AQP5 signal mainly along the alveolar surface adjacent to the bronchiolar region. Scale bars: 20 μ m. (b, c) Representative AQP5-stained images from healthy and injured samples. Scale bars: 100 μ m. (d–g) Quantification of AQP5 fluorescence intensity, scaffold-normalized expression, scaffold coverage, and particle size. (h, i) Representative ZO-1-stained images from healthy and injured samples. Scale bars: 100 μ m. (j–l) Quantification of ZO-1 fluorescence intensity, scaffold-normalized expression, and scaffold coverage. Healthy, $n = 8$; injury, $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. AFU, arbitrary fluorescence units; AQP5, aquaporin-5; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; ZO-1, zonula occludens-1. Adapted from Paper IV.

CD31 signal in the alveolar capillary network following aspiration injury

CD31 was used as a vascular endothelial marker to assess the alveolar capillary network after lung injury. Quantitative analysis showed scaffold-normalised CD31 coverage was higher in the injury group than in the healthy controls, but the difference was not statistically significant (Fig. 23a–c).

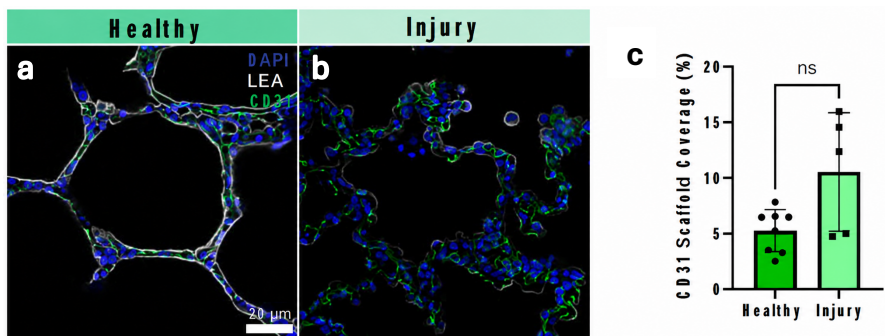


Figure 23. CD31-based assessment of vascular coverage after aspiration-induced lung injury. (a, b) Representative CD31-stained images from healthy and injured samples. Scale bars: 20 μm. (c) Quantification of CD31-positive coverage. Healthy, n = 8; injury, n = 5. ns, not significant. CD31, cluster of differentiation 31; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin. Adapted from Paper IV.

Alterations in elastin and α-SMA signals after aspiration-induced lung injury

To assess ECM and contractile structures, immunofluorescence staining was performed for elastin and α-SMA. Large-field-of-view imaging illustrated the distribution of both markers in healthy and injured lung tissue (Fig. 24a, b). Scaffold-normalized quantification showed no significant difference in overall elastin signal between the groups, whereas α-SMA signal was significantly lower in injured than in healthy tissue (Fig. 24c, d).

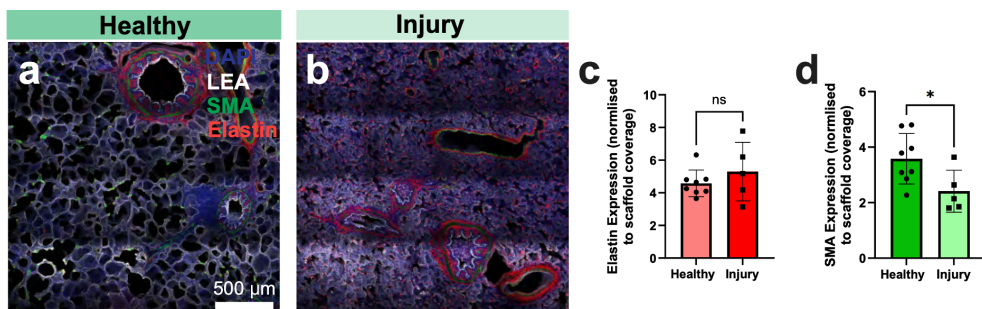


Figure 24. Spatial distribution and scaffold-normalized quantification of α-SMA and elastin. (a, b) Representative large field-of-view images showing α-SMA and elastin staining in healthy and injured lung samples. Scale bars: 500 μm. (c, d) Quantification of scaffold-normalized elastin and α-SMA fluorescence intensity in healthy and injured samples. Healthy, n = 8; injury, n = 5. ns, not significant; *p < 0.05. DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; α-SMA, α-smooth muscle actin. Adapted from Paper IV.

To further assess compartment-specific changes in ECM and contractile structures, elastin and α-SMA were analysed within defined lung compartments (Fig. 25a, b; Fig. 25f, g). In alveolar regions, there was no significant difference in scaffold-

normalized elastin particle count after injury (Fig. 25c), whereas particle intensity was significantly lower and particle size significantly greater in injured tissue (Fig. 25d, e). These changes may result from gastric acid- and protease-mediated damage to elastin fibres, as well as increased exposure of elastin due to surface tissue damage¹⁴⁴. In alveolar ducts, α -SMA-positive nodes showed reduced size and intensity after injury, suggesting early alterations in α -SMA-associated contractile structures following gastric aspiration (Fig. 25h, i).

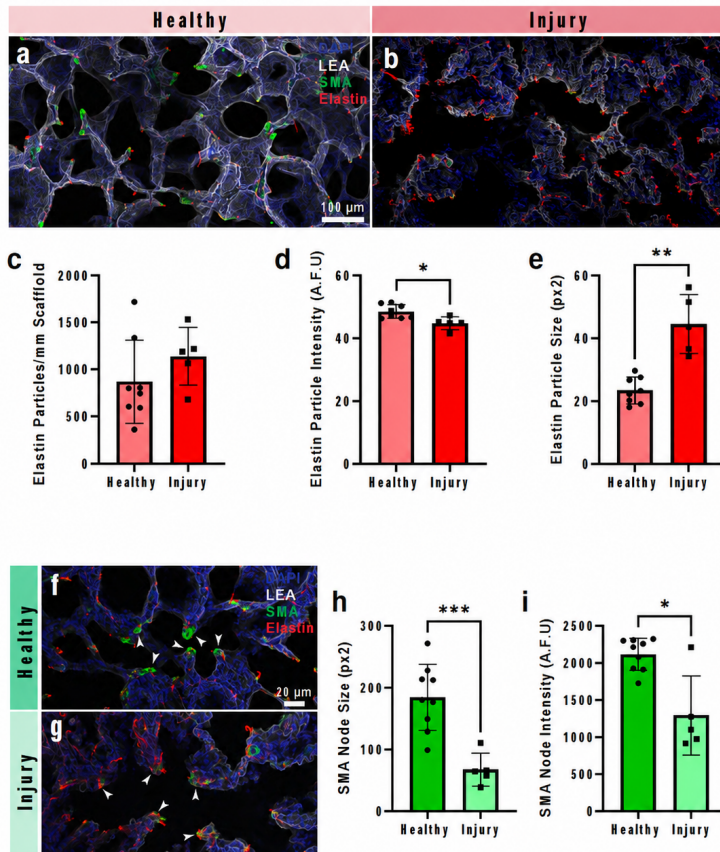


Figure 25. Alterations in elastin and α -SMA-associated structures in distinct lung regions after aspiration-induced lung injury.

(a, b) Representative alveolar regions from healthy and injured lung samples showing DAPI, LEA, α -SMA, and elastin staining. Scale bars: 100 μ m. (c–e) Quantification of scaffold-normalized elastin particle number, elastin particle intensity, and elastin particle size. (f, g) Representative high-resolution images of alveolar ducts from healthy and injured samples. Scale bars: 20 μ m. (h, i) Quantification of α -SMA-positive node size and intensity in alveolar ducts. Healthy, n = 8; injury, n = 5. * p < 0.05, ** p < 0.01, *** p < 0.001. DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; α -SMA, α -smooth muscle actin. Adapted from Paper IV.

Aspiration-induced lung injury is associated with immune cell accumulation and NET-like structures

AIF-1 and MPO were used to assess macrophage/monocyte-associated cell accumulation and neutrophil-associated responses, respectively. Fluorescence imaging showed increased accumulation of AIF-1-positive cells and enhanced MPO signal in injured alveolar regions. Quantitative analysis showed significant increases in both AIF-1-positive cell numbers and MPO fluorescence intensity in the injured group (Fig. 26a–e). SEM imaging further revealed filamentous extracellular structures within the alveolar spaces that were morphologically consistent with NET-like structures, providing ultrastructural evidence of injury-associated immune activation (Fig. 26f–i).

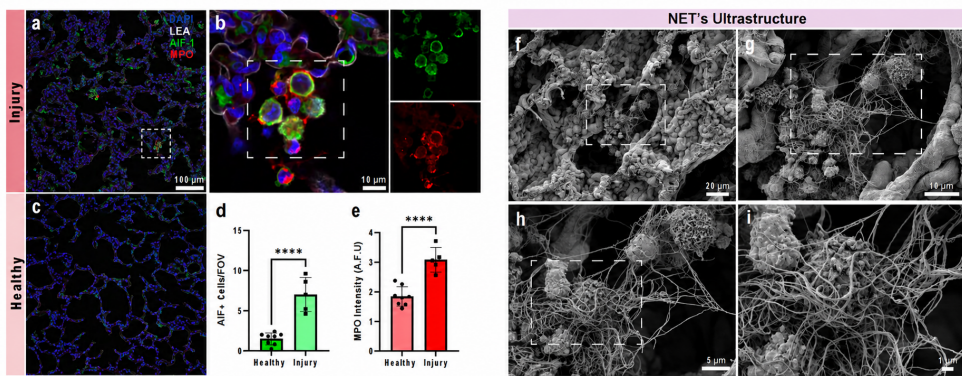


Figure 26. Immune cell infiltration and NET-like structures after aspiration-induced lung injury. (a–c) Representative AIF-1 and MPO fluorescence images from healthy and injured lung samples, including a magnified callout of AIF-1- and MPO-positive cells. Scale bars: 100 μ m (overview) and 10 μ m (callout). (d, e) Quantification of AIF-1-positive cells per field of view and MPO fluorescence intensity. (f–i) SEM images showing NET-associated filamentous extracellular structures within alveolar regions. Scale bars: 20 μ m, 10 μ m, 5 μ m, and 1 μ m, respectively. Healthy, n = 8; injury, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. AIF-1, allograft inflammatory factor-1; DAPI, 4',6-diamidino-2-phenylindole; FOV, field of view; LEA, *Lycopersicon esculentum* agglutinin; MPO, myeloperoxidase; NET, neutrophil extracellular trap; SEM, scanning electron microscopy. Adapted from Paper IV.

Paper V

Background and study design

HAR is a severe form of early graft injury in which complement activation plays a central role. However, the early tissue-level effects of complement-mediated lung injury are difficult to examine directly in patients or conventional *in vivo* models. In Paper V, EVLP was used as a controlled platform to model complement-mediated HAR-like injury while maintaining ventilation and perfusion. This setup allowed

lung function to be monitored throughout EVLP and associated structural and spatial alterations to be examined at the tissue level.

Twelve porcine donor lungs were included and divided into a rejection group and a non-rejection control group, with six pigs in each group. After donor lung retrieval, all lungs underwent normothermic EVLP for 4 hours. The rejection group received allogeneic porcine DNA to induce complement activation, whereas the control group received placebo treatment. Lung function and blood gas parameters were monitored throughout EVLP, while perfusate samples were collected to assess complement activation. Endpoint lung biopsies were then analysed by histology, SEM, and multiplex immunofluorescence to assess structural injury, immune cell activation, and alterations in the alveolar–capillary barrier, ECM, and contractile-associated structures.

Establishment of an EVLP-Based HAR-like Model

An EVLP-based porcine lung model was established to investigate complement-mediated HAR-like injury under controlled *ex vivo* conditions. Twelve porcine donor lungs were assigned to a rejection group and a non-rejection control group ($n = 6$ per group) and perfused for 4 hours on the EVLP platform. Complement activation was induced in the rejection group by adding allogeneic porcine DNA to the perfusate, while control lungs received vehicle treatment. Lung function was monitored, arterial blood gases were analysed, and perfusate samples were collected throughout EVLP. At the end of EVLP, lung biopsies were collected for histological and imaging analyses (Fig. 27).

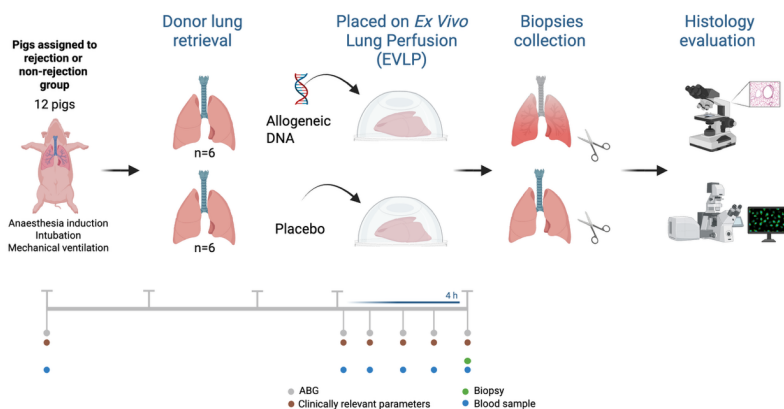


Figure 27. Experimental workflow of the porcine EVLP HAR-like model.

Twelve donor lungs were assigned to rejection or non-rejection groups. After donor lung retrieval, the lungs were connected to the EVLP platform and perfused for 4 h. Allogeneic DNA was added to the perfusate in the rejection group, whereas placebo was administered in the non-rejection control group. Arterial blood gas measurements and physiological parameters were recorded, and perfusate samples were collected at predefined time points during EVLP. Endpoint biopsies were collected for histology and

fluorescence imaging analyses. EVLP, ex vivo lung perfusion; ABG, arterial blood gas. Adapted from Paper V.

Histological and functional validation of the EVLP model

Compared with non-rejection lungs, rejection lungs showed marked histological changes and significantly higher lung injury scores (Fig. 28a, b). During EVLP, rejection lungs showed progressive functional deterioration: dynamic compliance (C_{dyn}) was significantly lower from 2 hours onward, PVR showed an upward trend, and gas exchange was impaired, as indicated by reduced PaO₂/FiO₂ ratios at 3 and 4 hours and a downward trend in CO₂ elimination (Fig. 28c). SEM further revealed thickened, irregular alveolar walls and cellular debris within the airspaces in rejection lungs, whereas alveolar architecture was relatively preserved in non-rejection controls (Fig. 28d).

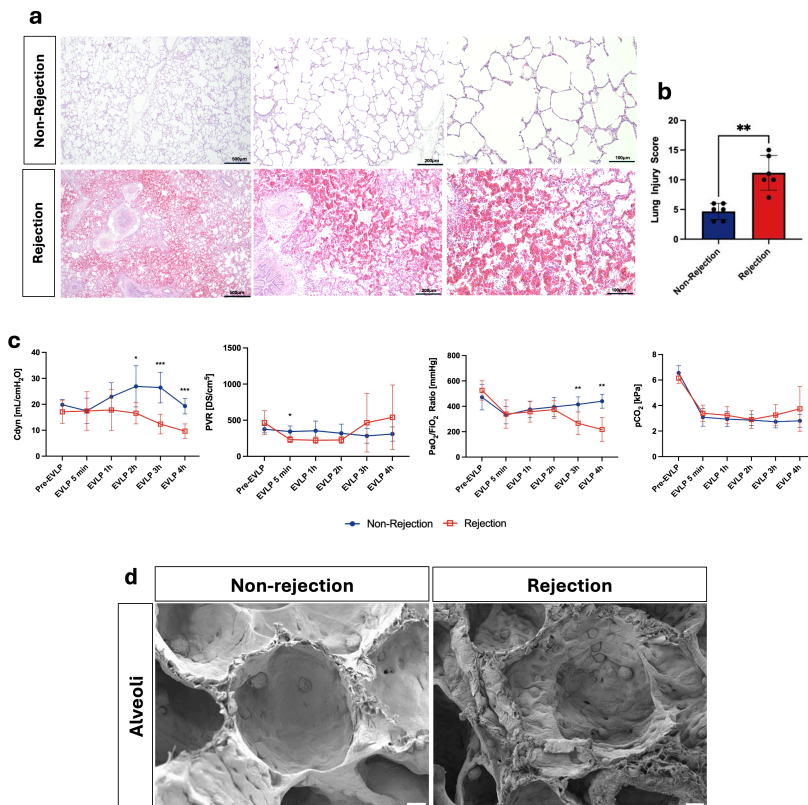


Figure 28. Functional and structural assessment of lung injury in the EVLP-based HAR-like model.

(a) Representative H&E-stained lung sections from non-rejection and rejection groups. Scale bars: 500 μ m, 200 μ m, and 100 μ m. (b) Quantification of histological lung injury scores. (c) EVLP functional parameters over time, including C_{dyn}, PVR, PaO₂/FiO₂ ratio, and PaCO₂. (d) Representative SEM images of alveolar ultrastructure in non-rejection and rejection lungs. Scale bars: 10 μ m. * p < 0.05, ** p <

0.01, *** $p < 0.001$. Cdyn, dynamic compliance; EVLP, *ex vivo* lung perfusion; H&E, haematoxylin and eosin; PVR, pulmonary vascular resistance; SEM, scanning electron microscopy. Adapted from Paper V.

Validation of complement activation in the EVLP model

Perfusate samples were analysed for C3 and soluble C5b-9 to assess complement activation during EVLP. Western blot showed significantly lower C3 levels in the rejection group, consistent with C3 consumption. In parallel, soluble C5b-9 levels were significantly increased, indicating activation of the terminal complement pathway. Together, these findings confirmed activation of the complement cascade following administration of allogeneic porcine DNA during EVLP (Fig. 29a–c).

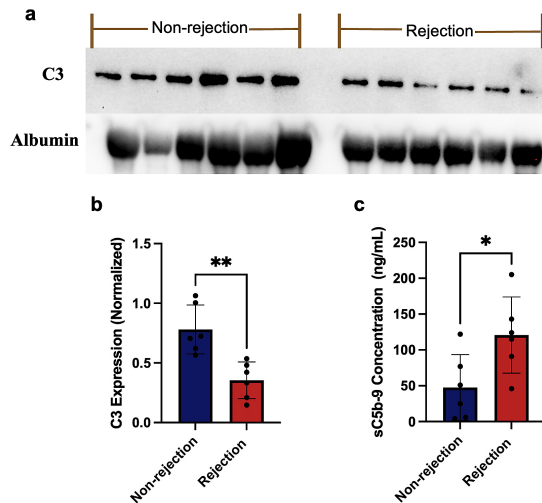


Figure 29. Validation of complement activation in EVLP perfusate.

(a) Representative Western blot analysis of C3 in EVLP perfusate. (b) Densitometric quantification of albumin-normalized C3 expression. (c) ELISA quantification of soluble C5b-9 (sC5b-9) levels in EVLP perfusate. * $p < 0.05$, ** $p < 0.01$. EVLP, *ex vivo* lung perfusion; sC5b-9, soluble C5b-9. Adapted from Paper V.

Immune activation in the complement-activated EVLP model

Following the observation of inflammatory cell infiltration by histological analysis, immunofluorescence staining was performed to further characterize innate immune cell-associated changes. AIF-1-positive cells were quantified as an indicator of monocyte/macrophage-associated cell abundance, whereas MPO fluorescence intensity was measured as an indicator of neutrophil-associated inflammatory activity (Fig. 30a). Compared with the non-rejection group, the rejection group showed significantly more AIF-1-positive cells (Fig. 30b) and markedly higher

MPO fluorescence intensity (Fig. 30c). Together, these findings supported enhanced innate immune activation in the rejection group.

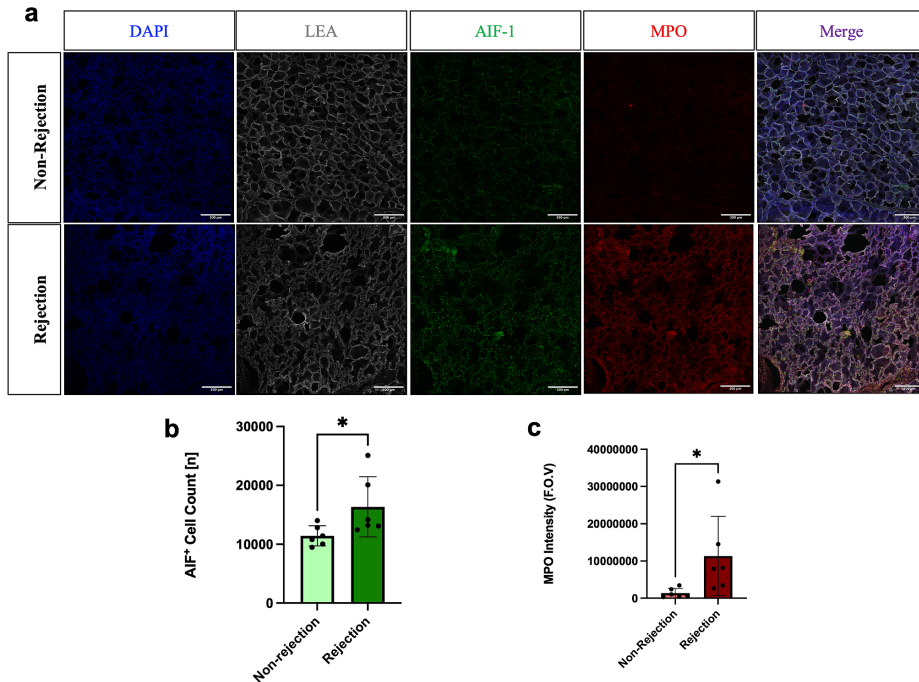


Figure 30. Immune activation during complement-mediated HAR-like lung rejection.

(a) Representative immunofluorescence images of AIF-1, MPO, DAPI, and LEA staining in non-rejection and rejection lungs. Scale bars: 300 μ m. (b, c) Quantification of AIF-1⁺ cell count and MPO fluorescence intensity. Non-rejection, n = 6; rejection, n = 6. *p < 0.05. AIF-1, allograft inflammatory factor-1; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; MPO, myeloperoxidase. Adapted from Paper V.

Alterations in the alveolar vascular endothelium in the complement-activated EVLP model

To investigate how complement-mediated rejection affected endothelial and microvascular organization of alveolar capillaries, we stained for CD31, an endothelial junctional marker involved in regulating immune cell transmigration and maintaining vascular integrity. Compared with the non-rejection group, the rejection group showed significantly lower scaffold-normalized CD31 fluorescence intensity, consistent with alteration of the endothelial compartment (Fig. 31a, b).

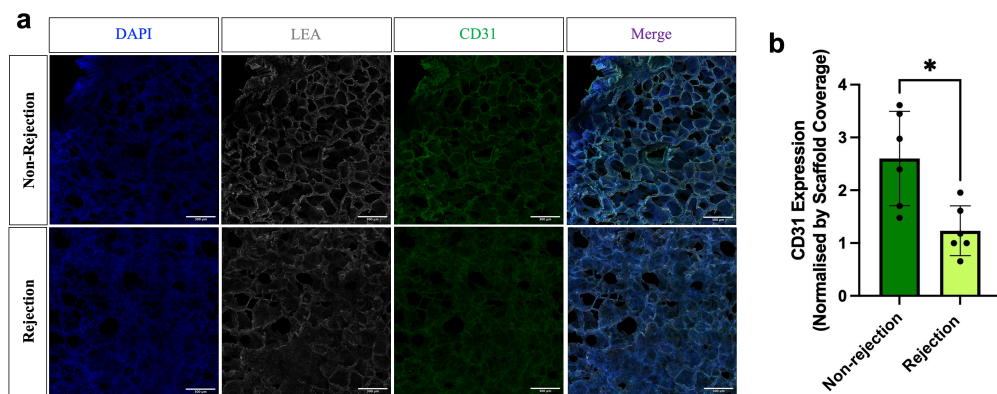


Figure 31. Alterations in the vascular endothelium during complement-mediated HAR-like lung rejection.

(a) Representative CD31 immunofluorescence images from non-rejection and rejection lungs. Scale bars: 300 μm . (b) Quantification of scaffold-normalized CD31 expression. Non-rejection, $n = 6$; rejection, $n = 6$. * $p < 0.05$. CD31, cluster of differentiation 31; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin. Adapted from Paper V.

Alterations of alveolar-capillary barrier-associated markers

Alveolar–capillary barrier integrity was assessed by immunofluorescence staining of AQP5, a type I alveolar epithelial marker, and ZO-1, a tight junction-associated marker expressed at epithelial and endothelial barriers (Fig. 32a). Scaffold-normalized AQP5 fluorescence intensity and AQP5-positive scaffold coverage were significantly reduced, consistent with alteration of the alveolar type I epithelial compartment (Fig. 32b, c). Similarly, the rejection group exhibited lower scaffold-normalized ZO-1 fluorescence intensity and reduced ZO-1-positive scaffold coverage (Fig. 32d, e). These findings demonstrated epithelial and junctional alterations within the alveolar–capillary barrier during complement-mediated HAR-like injury.

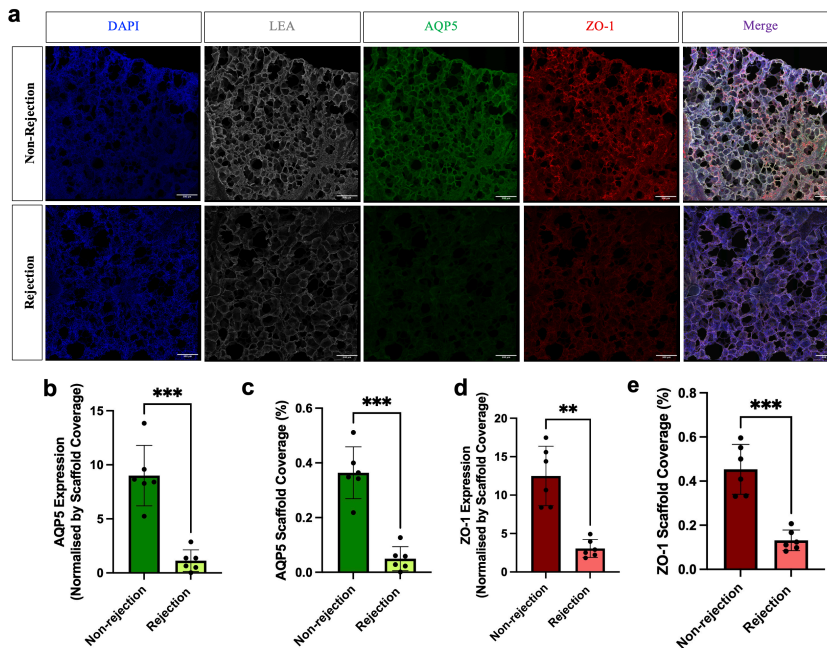


Figure 32. Alterations in alveolar–capillary barrier during complement-mediated HAR-like lung rejection.

(a) Representative AQP5 and ZO-1 immunofluorescence images from non-rejection and rejection lungs. Scale bars: 300 μ m. (b, c) Quantification of scaffold-normalized AQP5 expression and scaffold coverage. (d, e) Quantification of scaffold-normalized ZO-1 expression and scaffold coverage. Non-rejection, $n = 6$; rejection, $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. AQP5, aquaporin-5; DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; ZO-1, zonula occludens-1. Adapted from Paper V.

ECM and contractile structures in the complement-activated EVLP model

Elastin and α -SMA were analysed to assess ECM and contractile-associated structures during complement-mediated rejection injury. Elastin was used as an essential component of ECM, while α -SMA was used as a contractile-associated marker related to smooth muscle cells and myofibroblast-like cells. Large field-of-view imaging showed broad distribution of both markers across lung compartments (Fig. 33a).

In alveolar regions, scaffold-normalized elastin signal was significantly decreased in the rejection group, while elastin particle size was markedly increased, and elastin particle count showed an increased trend in the rejection group, but the difference was not statistically significant (Fig. 33b-e). α -SMA-positive node-like structures were mainly located at the tips of alveolar duct and appeared closely associated with elastin-rich ECM clusters. In alveolar duct regions, α -SMA fluorescence intensity showed a non-significant decreasing trend in the rejection group, whereas the size of α -SMA-positive structures remained comparable between groups (Fig. 33f-h).

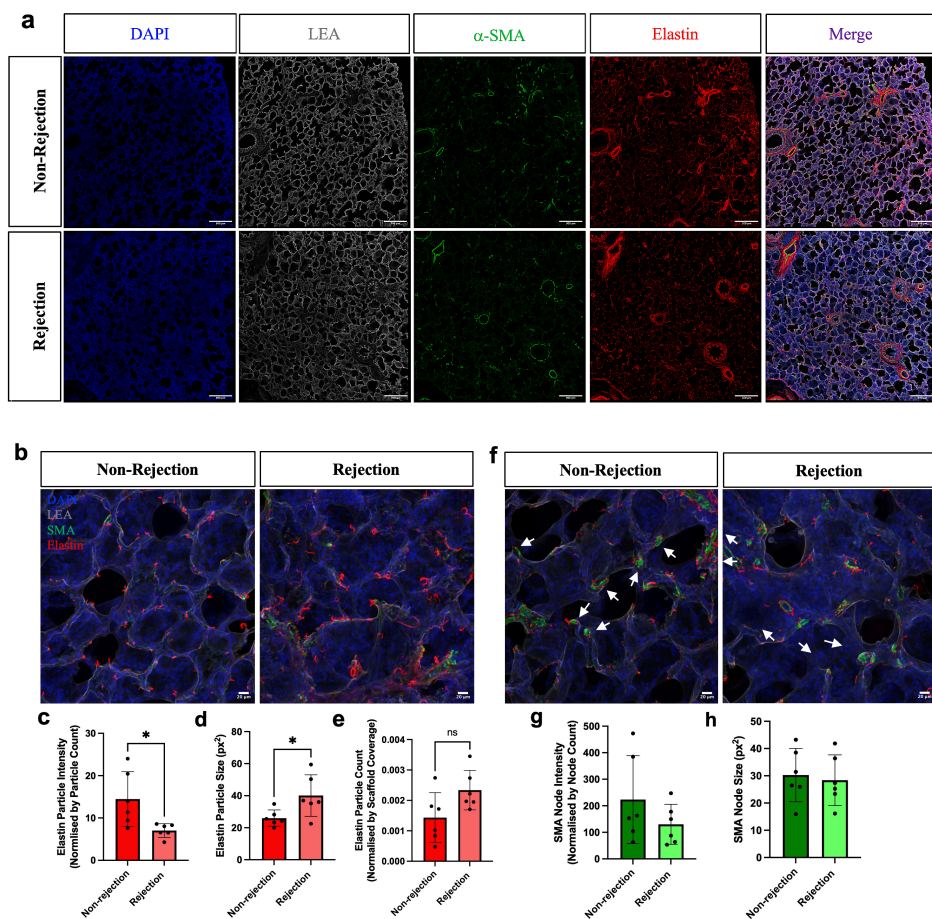


Figure 33. Alterations in elastin and α -SMA-associated structures in distinct lung regions during complement-mediated HAR-like lung rejection.

(a) Representative confocal images showing DAPI, LEA, α -SMA, elastin, and merged staining in non-rejection and rejection lung samples. Scale bars: 300 μ m. (b) Higher-magnification images showing elastin distribution in alveolar regions. Scale bars: 20 μ m. (c–e) Quantification of scaffold-normalized elastin expression, elastin particle size, and elastin particle count. (f) Higher-magnification images showing α -SMA-positive node-like structures in alveolar duct regions. Scale bars: 20 μ m. (g, h) Quantification of scaffold-normalized α -SMA fluorescence intensity and α -SMA-positive node-like structure size. ns, not significant; * $p < 0.05$. DAPI, 4',6-diamidino-2-phenylindole; LEA, *Lycopersicon esculentum* agglutinin; α -SMA, α -smooth muscle actin. Adapted from Paper V.

Discussion

Lung injury remains a major challenge in lung transplantation because it can limit donor lung utilization and compromise graft function. Although initiated by different insults, injury can affect multiple interconnected tissue compartments, including the alveolar epithelium, vascular endothelium, innate immune compartment, extracellular matrix (ECM), and contractile structures. Conventional histology and individual molecular markers provide important information, but an integrated tissue-level pipeline is needed to characterize how these alterations occur together and are spatially distributed within the lung.

Within this context, this thesis investigated structural and spatial tissue alterations in translational porcine models of aspiration-induced acute lung injury and complement-mediated graft injury. To enable this analysis, tissue-processing and sectioning workflows were first evaluated and established to preserve lung morphology and fluorescence signals for quantitative imaging. These workflows were then applied to quantify architectural changes and characterize epithelial, endothelial, inflammatory, ECM, and contractile responses. Together, the five studies establish an integrated framework for tissue-level analysis. In this thesis, this framework revealed both shared and model-specific changes across multiple tissue compartments, including alveolar deformation, barrier-associated alterations, innate immune activation, and changes in ECM and contractile structures. Therefore, these tissue-level findings provide a more integrated understanding of lung injury relevant to donor lung assessment and graft function.

Lung tissue can be difficult to process because of its delicate alveolar architecture. Its thin alveolar walls and large airspaces make the tissue prone to collapse and distortion, while processing may also reduce antigen detectability and fluorescence signal quality. Papers I and II addressed these challenges by evaluating an alternative clearing reagent for paraffin processing and developing an agarose-based sectioning workflow, respectively. These approaches preserved lung morphology and supported histological and fluorescence-based analyses. In paraffin-based processing, xylene is widely used as a clearing reagent to enable paraffin wax infiltration and promote sectioning but concerns about occupational exposure and environmental impact have motivated the evaluation of alternatives^{120,121,124}. Among the available alternatives, isopropanol is commonly used in laboratory practice, because it facilitates paraffin infiltration, has lower toxicity, environmental impact, and cost than xylene^{113,128}. Paper I therefore examined whether isopropanol could

replace xylene in lung tissue processing without compromising tissue processing or downstream histological and fluorescence-based assessment. No significant differences between the two methods were detected in histological quality, sectioning performance, fluorescence-based morphological measurements, or the signal-to-noise ratios for α -SMA and elastin. Importantly, differences between donor and recipient samples remained detectable with both clearing agents, indicating that isopropanol did not obscure biologically relevant variation. These findings support isopropanol as a practical alternative that may reduce reliance on xylene without compromising histological or fluorescence-based assessment.

Paper II developed a workflow combining low-melting-point agarose embedding with automated vibratome sectioning to preserve three-dimensional lung architecture in thick sections for fluorescence imaging. This workflow enables direct embedding of fixed tissue in low-melting-point agarose, thereby shortening sample preparation and reducing chemical use compared with paraffin processing, while avoiding the airway cannulation and inflation required for conventional PCLS preparation^{145,146}. This approach may be particularly advantageous for large-animal and human specimens, in which identifying and cannulating an accessible airway can be difficult^{131,147}. The method preserved tissue architecture without the antigen retrieval often required for paraffin-based immunostaining and generated thick sections suitable for volumetric fluorescence imaging in mouse, porcine, and human lung tissue. Importantly, quantification of HTII-280-positive type II pneumocytes in adult and infant human lungs further demonstrated that the workflow supported high-resolution imaging and quantitative analysis of cell distribution. Although pathological changes in diseased lung tissue, such as fibrosis, pleural thickening, and increased tissue stiffness, may complicate vibratome sectioning, these difficulties can be managed by optimizing the cutting procedure, such as using a sharper blade, adjusting cutting parameters, improving tissue support, or removing the pleura before sectioning. Overall, Paper II provides a practical and reproducible workflow for preserving lung tissue structures and generating lung sections. It served as a foundation for subsequent high-resolution imaging and quantitative spatial analysis, which was further developed in Paper III for the quantitative assessment of alveolar scaffold morphology in acute lung injury.

Conventional H&E staining remains essential for histological evaluation¹⁴⁸. However, it relies largely on categorical scoring and visual assessment, which may be less objective and limit the detection of subtle structural variation. Paper III employed a high-resolution fluorescence imaging pipeline to obtain continuous measurements of alveolar wall thickness and circularity in a porcine model of aspiration-induced acute lung injury. Acute lung injury increased alveolar wall thickness and reduced alveolar circularity, consistent with both septal expansion and deformation of the alveolar airspace¹⁴⁰. The MQ integrated these two features into a single value and was markedly reduced after injury, reflecting the combination of thicker walls and less circular alveolar airspaces. Because the MQ served as a

structural indicator rather than a direct measure of lung function, its relationship with physiological changes requires further investigation. An independent second observer repeated the measurements using the same criteria and identified similar differences between the baseline and injured groups, supporting the reproducibility of the analysis. However, alveolar selection and tracing of the inner and outer boundaries remained partly manual, making the measurements sensitive to image quality, section orientation, and alveolar integrity. Automated analysis may reduce user-dependent variability, but it would require careful validation because injured alveoli often have irregular or incomplete borders. Overall, quantitative measurements of alveolar wall thickness and circularity provided additional structural detail alongside conventional injury scoring. This analysis was subsequently expanded in Paper IV to include multiplex assessment of epithelial, endothelial, immune, ECM, and contractile alterations.

Aspiration is clinically relevant to donor lung evaluation because it can impair lung function and reduce donor lung utilization^{82,149}. To examine the tissue-level consequences of this injury, Paper IV combined physiological and histological assessment with multiplex spatial imaging in a pre-clinical porcine model of aspiration-induced acute lung injury. The porcine model allowed the tissue responses to aspiration injury to be investigated in lungs that resemble human lungs in anatomy and physiology¹⁰⁴.

At the organ level, aspiration injury was associated with impaired oxygenation, increased PVR, and higher histological injury scores¹⁰¹. Multiplex spatial analysis showed that these changes occurred alongside alterations in epithelial and endothelial markers associated with the alveolar–capillary barrier, activation of innate immune responses, and changes in ECM and contractile structures.

Reduced scaffold normalised AQP5 signal was consistent with alteration of the type I alveolar epithelial compartment, in which AQP5 is predominantly expressed and contributes to alveolar fluid transport¹⁵⁰. Its reduction may reflect alveolar epithelial injury and impaired fluid homeostasis, contributing to oedema formation, impaired gas exchange, and the observed decrease in the PaO₂/FiO₂ ratio¹⁵¹. Increased scaffold-normalised ZO-1 coverage may reflect redistribution or disorganisation of epithelial junctions during injury, as ZO-1 undergoes dynamic reorganisation during epithelial injury and repair¹⁵². Additionally, scaffold-normalised CD31 coverage showed a non-significant increasing trend. This may reflect the redistribution of existing vascular structures or increased tissue density following alveolar collapse and septal thickening rather than microvascular remodelling³⁸.

Aspiration injury also affected ECM and contractile-associated structures^{144,153}. The lower alveolar elastin intensity and larger particles were more consistent with disruption or reorganisation of the elastin network than with increased elastin expression. These alterations may result from acidic insult and protease-mediated degradation of elastin fibres, together with greater exposure of elastin following

disruption of the alveolar epithelial surface^{144,154,155}. The reduced size and intensity of α -SMA-positive structures further suggested alteration in the spatial organisation of contractile-associated structures. These findings indicate that aspiration injury extended beyond the epithelial barrier to structural components involved in maintaining alveolar architecture and mechanical stability¹⁹.

The inflammatory response was characterised by increased numbers of AIF-1-positive cells and higher MPO signal. Together with NET-like structures identified by SEM, these findings supported macrophage- and neutrophil-associated inflammation after aspiration injury¹⁵⁶. The simultaneous presence of inflammatory changes with epithelial and ECM alteration suggests that aspiration injury progressed through interacting tissue processes. The acidic insult may have produced direct epithelial and structural damage, followed by recruitment and activation of inflammatory cells and formation of NET-like structures. These responses may subsequently have amplified local tissue injury and matrix disturbance, thereby contributing to impaired lung function¹⁵⁶. However, because the tissue was examined only at the end of the experiment, the study could not determine which changes occurred first or how much of the damage was caused by the gastric contents and how much by the inflammatory response. Collectively, the combined tissue-level and functional findings provide a more comprehensive understanding of aspiration-induced lung injury as a multicompartment process and may inform future approaches to donor lung assessment.

Paper V then investigated another form of lung injury arising from a different initiating mechanism: complement-mediated HAR-like injury during EVLP. Clinical HAR is typically initiated by pre-existing recipient antibodies that bind to graft antigens and activate the complement cascade, leading to rapid endothelial injury, thrombosis, oedema, and graft dysfunction⁵⁸. In this paper, allogeneic porcine DNA was introduced into the perfusion circuit to activate complement, creating a HAR-like model focused on complement-mediated injury rather than the full clinical process of HAR. Despite their different initiating mechanisms, gastric aspiration and complement-mediated injury can both rapidly affect the alveolar–capillary barrier and impair gas exchange^{76,157}.

EVLP provided a controlled *ex vivo* platform for serial physiological assessment and the collection of perfusate and tissue samples for biochemical, histological, and spatial analyses^{83,158}. Compared with controls, the rejection group showed a progressive decline in dynamic compliance, impaired oxygenation, a trend towards increased PVR, and higher histological injury scores. Reduced C3 signal in the perfusate, consistent with complement consumption, together with increased sC5b-9, provided biochemical evidence of complement activation^{71,159}. Together, these findings indicated that the model reproduced key biochemical, functional, and histological features of complement-mediated HAR-like injury.

Multiplex imaging demonstrated innate immune activation in the HAR-like injury group. Increased numbers of AIF-1-positive cells and higher MPO signal indicated macrophage-associated inflammation and neutrophil activation^{160–162}. NET-like structures identified by fluorescence imaging and SEM provided further evidence of neutrophil involvement. Complement activation can directly stimulate immune cells and promote the release of inflammatory mediators, while damaged endothelial and epithelial structures may further enhance immune recruitment.

At the alveolar–capillary barrier, reductions in scaffold-normalised AQP5, ZO-1, and CD31 signals were consistent with injury to epithelial, junctional, and endothelial structures^{37,163,164}. Reduced AQP5 suggested alteration of the type I alveolar epithelial compartment, reduced ZO-1 was compatible with junctional disruption, and reduced CD31 was consistent with endothelial injury or loss of microvascular organisation. Taken together, these changes supported early disruption of barrier-associated structures. These tissue alterations accompanied pulmonary oedema and impaired oxygenation, but the study did not determine whether changes in individual markers were directly related to functional deterioration.

Complement-mediated rejection also affected ECM organization and contractile-associated structures. Reduced elastin intensity together with increased particle size suggested loss and reorganisation of the elastin network, which could compromise tissue elasticity and structural integrity. This pattern may be related to neutrophil activation and the associated release of proteases, which contribute to elastin degradation and ECM damage^{165,166}. In contrast, α -SMA⁺ contractile structures were relatively preserved, showing a non-significant decreasing trend in fluorescence intensity and comparable structural size between groups. These findings suggest that the elastin-rich ECM may be more vulnerable to complement-mediated injury, whereas α -SMA⁺ structures remain comparatively stable at this stage.

Comparison of Papers IV and V revealed both shared and model-specific tissue responses. In the aspiration model, gastric contents directly contacted the airway and alveolar surfaces, whereas complement-mediated injury was initiated in the perfusion circulation and therefore more closely associated with the vascular compartment. Additionally, the models also differed in experimental setting: aspiration injury was studied *in vivo*, while complement-mediated injury was examined *ex vivo* during EVLP. Therefore, the different types of injury and experimental settings may have contributed to the distinct tissue responses observed in the two models.

Despite these differences, both models showed reduced scaffold-normalised AQP5 signal, increased numbers of AIF-1-positive cells and higher MPO signal, indicating shared patterns of alveolar epithelial injury and innate immune activation^{157,167}. ZO-1 and CD31, however, differed between the two models. Scaffold-normalised ZO-1 coverage increased after aspiration, whereas ZO-1 signal and coverage decreased

in the rejection group. CD31 showed a non-significant increasing trend after aspiration but a significant reduction during complement-mediated injury, suggesting model-specific effects on epithelial junctions and endothelial integrity^{38,152}. The structural markers also showed model-specific responses. Elastin showed similar alterations in both models, although the underlying mechanisms may differ, whereas α -SMA-positive contractile structures were reduced after aspiration but relatively preserved during complement-mediated injury^{154,155,166}. These findings suggest that different initiating insults may converge on common epithelial and inflammatory responses while producing distinct patterns of barrier and structural injury. Collectively, the two models provide distinct translational platforms for investigating shared and mechanism-specific tissue responses in transplant-associated lung injury.

Limitations

Although the studies in this thesis provide new insights into tissue-level alterations in aspiration-induced and complement-mediated donor lung injury, several limitations should be considered.

The tissue-processing and sectioning workflows evaluated in Papers I and II were examined under specific experimental conditions. Their performance may vary with tissue characteristics, fixation, staining protocols, and laboratory procedures. In addition, thick-section imaging may be affected by uneven antibody penetration and reduced fluorescence signal in deeper tissue layers. The reproducibility of these workflows across other disease conditions, staining panels, and laboratories therefore requires further validation.

The imaging analyses in Papers III-IV were based on selected alveoli and tissue regions and may therefore not fully represent the spatial heterogeneity of injury across the whole lung. In Paper III, manual selection of alveoli and boundary tracing may have introduced observer-dependent variability, while the measurements may also have been influenced by section orientation. Wall thickness, circularity, and the MQ should therefore be interpreted as quantitative measures of injury-associated alveolar morphology rather than as direct measures of lung function or specific injury mechanisms.

The injury studies in Papers III-V involved relatively small porcine cohorts, and species-related differences may limit direct translation to human donor lungs. In addition, the early experimental endpoints limited the assessment of longer-term injury progression and recovery. The multiplex panels in Papers IV and V included selected markers and therefore did not capture the full cellular and molecular complexity of injury. Furthermore, the two models differed in both the type of injury and the experimental setting: aspiration was studied *in vivo*, whereas complement-

mediated injury was examined *ex vivo* during EVLP. It was therefore not possible to determine whether the different marker patterns resulted from the injury mechanism, the experimental setting, or a combination of both.

Paper V modelled complement-mediated HAR-like injury rather than the complete clinical process of HAR. The use of allogeneic DNA to activate complement bypassed the initial antibody–antigen interaction, while the short-term EVLP setting did not reproduce the full systemic immune, coagulation, and haemodynamic environment present *in vivo*. Because no selective complement inhibitor was used, the study could not determine to what extent complement activation contributed to the observed graft injury.

Conclusions and Future Perspectives

This thesis established and applied an integrated framework for analysing structural and spatial tissue alterations in lung injury relevant to donor lung assessment and transplantation. Papers I and II established tissue-processing and sectioning workflows that preserved lung morphology and supported fluorescence imaging. Paper III showed that injury-associated alveolar wall thickening and deformation could be quantified using wall thickness, circularity, and the MQ. Papers IV and V extended this framework to multiplex spatial analysis of aspiration-induced and complement-mediated HAR-like lung injury.

Across the two injury models, alveolar epithelial alterations and innate immune activation were shared features, whereas epithelial junctions, vascular endothelium, the ECM, and contractile structures were affected differently by aspiration-induced and complement-mediated injury. Overall, these findings show that lung injury affects several tissue compartments, with tissue-level alterations occurring alongside impaired lung function. By integrating conventional histology with quantitative morphometry and multiplex spatial imaging, this thesis provides a more detailed and comprehensive assessment of lung injury relevant to donor lung evaluation and transplantation.

Future studies should assess the reproducibility of these workflows using standardised protocols across laboratories. The injury-related tissue findings should also be evaluated in larger cohorts, increased tissue sampling, broader marker panels, advanced spatial analysis, and human donor lungs. Samples should be collected and analysed from multiple lung regions to determine whether changes observed in individual samples represent the condition of the whole lung. Automated image analysis and validated machine-learning approaches may reduce observer-dependent variability, improve throughput, and identify tissue patterns associated with injury or recovery.

Spatial tissue findings should be analysed together with EVLP physiology, perfusate biomarkers, and molecular readouts and related to functional recovery and post-transplant outcomes. Mechanistic studies, including selective complement inhibition and *in vivo* validation, are needed to clarify causal pathways. The framework developed in this thesis provides a basis for future studies evaluating whether tissue-level analysis can support donor lung assessment and help assess recovery and responses to targeted reconditioning before transplantation.

Material and methods

Ethical considerations

Mice

No mice were specifically bred or sacrificed for the purpose of collecting lung tissue for this study. Instead, unused mouse lungs were obtained from sacrificed mice of other researchers who were using different organ systems from the same animals in ethically approved studies under approved animal ethical permits. This approach was used to support the principles of the 3Rs: Replacement, Reduction, and Refinement, by maximizing the scientific value obtained from each animal and reducing the need for additional animals.

Pigs

The studies were approved by the local Ethics Committee for Animal Research (Dnr 5.2.18-4903/16 and Dnr 5.2.18-8927/16). All experimental procedures were performed in accordance with the approved ethical permits, applicable Swedish animal-welfare legislation, and Directive 2010/63/EU, and were reported in accordance with the ARRIVE guidelines. An on-site veterinarian monitored the experiments and ensured compliance with the approved ethical permit. The principal investigator was present throughout all procedures, and all personnel involved were appropriately trained and licensed to work with porcine models.

All animals included in Papers II–V were locally sourced, adult, farm-raised Yorkshire pigs (*Sus scrofa domestica*). During the procedures, all pigs were maintained under general anaesthesia, with anaesthesia and vital physiological parameters continuously monitored and managed by specialist anaesthesiologists and thoracic clinicians experienced in thoracic intensive care. The experimental design adhered to the principles of the 3Rs (*Replacement, Reduction, and Refinement*). *Reduction*: The animal disease models were carefully designed to maximize the scientific outcomes from each study. Within the same experimental framework, multiple readouts, including physiological, histological, molecular, imaging, and ultrastructural data, were collected from the same experimental material, thereby reducing the need for additional animals. *Replacement*: It was

supported by *ex vivo* and tissue-based methods, including agarose-embedded lung samples and vibratome-based tissue sectioning in Paper II, which enabled methodological optimization and tissue analysis outside living animals. *Refinement*: It was addressed through appropriate animal housing, continuous monitoring, and specialized veterinary and clinical care. All procedures were performed by trained and specialized personnel, with appropriate measures applied to minimize pain, distress, and unnecessary suffering.

Patients

In Papers I and II, human lung tissue was collected from donor lungs deemed unsuitable for transplantation, as well as from diseased explanted lungs of transplant recipients. Ethical approval was granted by the Swedish National Ethics Committee (Dnr 2021-00016; Dnr 2017/396; Dnr 2018/845; and Dnr 2022-05028-02).

Animal preparation

Mice

In Paper II, the mouse lung samples were obtained from another laboratory where the lungs were not required for the original experiment. These unused lungs were therefore included in our study, maximizing tissue use and supporting the 3Rs principle.

Pigs

In Papers III-V, adult pigs (*Sus Scrofa Domesticus*) of both sexes, weighing 40–45 kg were used for the experiments. All the pigs underwent premedication with xylazine (Rompun® vet. 20mg/mL; Bayer AG, Leverkusen, Germany; 2 mg/kg) and ketamine (Ketaminol® vet.100mg/mL; Farmaceutici Gellini S.p.A., Aprilia, Italy; 20mg/kg). Peripheral intravenous (IV) lines were inserted into the earlobe, and a urinary catheter was inserted into the bladder. General anaesthesia was accomplished with ketamine (Ketaminol® vet), midazolam (Midazolam Panpharma®, Oslo, Norway), and fentanyl (Leptanal®, Lilly, France) infusions. Mechanical ventilation was established using a Siemens-Elema ventilator (Servo 900 C, Siemens, Solna, Sweden) after intubation with a 7.5mm endotracheal tube. The pigs were then ventilated using volume-controlled ventilation (VCV), a tidal volume (Vt) of 6-8ml/kg, a positive end-expiratory pressure (PEEP) of 5 cmH₂O, an inspiratory-expiratory ratio (I:E) of 1:2, a respiratory rate (RR) of 22-25 breaths/min, and a fraction of inspired oxygen (FiO₂) of 0.5. Ventilation was

adjusted to maintain carbon dioxide levels (PaCO_2) between 33–41 mmHg. Haemodynamic monitoring was established by placing an arterial catheter in the right common carotid artery and a pulmonary artery catheter (Swan-Ganz CCombo V with Introfex by Edwards Lifesciences Services GmbH, Germany) via the right internal jugular vein. Arterial blood gas measurements were taken throughout the experiments by drawing blood from the arterial catheter and measuring with the blood gas machine (ABL90FLEX PLUS blood gas analyser, Radiometer Medical ApS, Denmark).

Induction of lung injury

In Papers III and IV, lung injury was induced in pigs under general anaesthesia by bronchoscopic administration of gastric contents into both lungs. Gastric contents were previously collected from other pigs and stored at -80°C until use. Before administration, the samples were thawed, filtered through a paper filter to remove particulates, and titrated with hydrochloric acid (HCl) to a controlled pH of 2. The prepared gastric contents were warmed to approximately body temperature and administered into both lungs at a dose of 3 mL/kg/lung to mimic clinical aspiration. After injury induction, the animals remained under anaesthesia and mechanical ventilation for 6 hours, with ventilator settings adjusted according to clinical needs by an anaesthesiologist. In Paper IV, chest X-rays were obtained before aspiration and after 6 hours of acute lung injury.

Lung procurement

In Paper V, lung procurement was performed according to standard clinical procedures through median sternotomy. The pulmonary artery was cannulated through the right ventricle using a 28Fr cannula secured with a purse-string suture at the pulmonary artery outflow tract. The superior and inferior vena cava, and ascending aorta were clamped, while the left atrium was opened to allow drainage. The lungs were antegradely flushed with 4 L of pre-cooled Perfadex® PLUS solution (XVIVO Perfusion, Sweden) at a low perfusion pressure (<20 mmHg). After flushing, the lungs were procured *en bloc* and submerged in cold Perfadex® PLUS solution maintained under static cold storage at $4-8^\circ\text{C}$ for 1 hour before connection to the EVLP circuit.

***Ex vivo* lung perfusion**

The EVLP system used in Paper V has been described previously²⁸. The system was primed with Steen™ Solution (XVIVO Perfusion) supplemented with donor-derived red blood cells to achieve a haematocrit of 15–20%. The perfusion flow rate was set to 40% of cardiac output (CO). Ventilation parameters were set to a tidal

volume of 7 mL/kg donor body weight, a respiratory rate of 7 breaths/min, a positive end-expiratory pressure (PEEP) of 5 cmH₂O, and a FiO₂ of 21%. The lungs were maintained on EVLP for 4 hours with continuous perfusion and ventilation. Physiological parameters, haemodynamics, and arterial blood gases were recorded at regular intervals. Tissue biopsies and perfusate samples were collected for downstream analyses. Additional Steen™ Solution was added when the reservoir volume decreased below 300 mL to maintain the circuit volume.

Monitoring and Downstream Analyses

Physiological and Haemodynamic Monitoring

During EVLP, perfusate samples were obtained for blood gas measurements at pre-EVLP, 5 min, 1 h, 2 h, 3 h, and 4 h after the initiation of EVLP. Respiratory and haemodynamic parameters were monitored and documented at each time point, including PaO₂, PaCO₂, dynamic compliance (C_{dyn}), pulmonary vascular resistance (PVR), and PaO₂/FiO₂. The calculation of C_{dyn} was based on tidal volume (V_t), peak inspiratory pressure (PIP), and positive end-expiratory pressure (PEEP) using the formula: $C_{dyn} = \frac{TV}{PIP-PEEP}$. PVR was calculated based on mean pulmonary artery pressure (MPAP), wedge pressure (WP) and cardiac output (CO) using the formula: $PVR = \frac{(MPAP-WP) \times 79.92}{CO}$. The PaO₂/FiO₂ ratio was calculated by dividing PaO₂ by FiO₂.

Lung tissue processing and sectioning

Lung tissue samples were obtained from human donor and explanted recipient lungs (Paper I, II), unused mouse tissue from other approved studies (Paper II), and porcine lungs used for methodological validation, aspiration-injury experiments, and EVLP (Paper II-V). Depending on the study, samples were obtained from existing tissue material or collected by biopsy or wedge resection. Following fixation, samples intended for histological and fluorescence analyses were processed by either paraffin embedding followed by microtome sectioning or agarose embedding followed by vibratome sectioning.

For paraffin processing, samples were fixed in 4% paraformaldehyde for 48 hours, dehydrated through a graded ethanol series, cleared, embedded in paraffin, and sectioned at a thickness of 5 µm using a rotary microtome (Leica, Germany). For vibratome sectioning, samples were fixed in 4% paraformaldehyde for 48 hours and stored in PBS containing 0.01% sodium azide. The tissue was embedded in 3% low-melting-point agarose (Sigma-Aldrich, Darmstadt, Germany) to provide structural

support and sectioned at a thickness of 200 μm using a vibrating microtome (Leica VT1200S).

Western blot

Complement protein C3 was assessed in perfusate samples collected at the end of EVLP. Briefly, perfusate samples were diluted in sample loading buffer at 1:100 for target protein C3 detection and 1:1000 for albumin detection, with albumin used as a reference protein for normalization. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. Primary antibody incubation was conducted at 4°C overnight targeting C3 protein (1:10,000, Proteintech, USA) and albumin (1:1,000, Invitrogen, USA). Secondary antibody incubation (Goat anti-rabbit, 1:10000; Goat anti-mouse, 1:10000) was conducted for 1 h at room temperature, followed by signal detection and imaging using ChemiDocTM MP Imaging System (Bio-Rad, USA). C3 levels were normalized to albumin levels to ensure comparability across samples.

Enzyme-linked immunosorbent assay

Soluble C5b-9 (sC5b-9), a marker of terminal complement activation, was measured in EVLP perfusate samples using a commercially available sandwich ELISA kit (Nordic Biosite, Sweden, Art. No. CSB-E15837p-96) following the manufacturer's instructions. Samples were measured in duplicate, and concentrations were calculated from a standard curve based on the mean optical density (OD) values. Absorbance was measured at 450 nm with reference correction at 540 nm using a microplate reader.

Scanning electron microscopy

Samples were first washed twice in 0.1M Sorensen's buffer pH 7.4 to remove media, then fixed in approximately 10 times the sample volume of "SEM fix" (0.1M Sorensen's phosphate buffer pH 7.4, 2% formaldehyde and 2% glutaraldehyde) at room temperature for at least 30 min. After fixation, the samples were washed twice in 0.1M Sorensen's buffer pH 7.4 to remove excess fixative. Samples were then dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90% and twice in 100%) and subsequently critical point dried before being mounted on aluminium stubs, sputtered with 10nm Pt/Pd (80/20) in a Quorum Q150T ES turbo pumped sputter coater. Imaging was done on a Jeol JSM-7800F Field-emission-gun scanning electron microscope (FEG-SEM).

Immunofluorescence staining

According to standard protocols, the paraffin sections were deparaffinized, rehydrated, and washed before fluorescence staining. Fluorescent staining was conducted via DAPI (4',6-diamidino-2-phenylindole, 1:1000, ThermoFisher Scientific, Waltham, MA, USA) and *Lycopersicon esculentum* agglutinin (LEA) (1:500, ThermoFisher Scientific, Waltham, MA, USA) diluted in PBS for 30 min and then washed in PBS. Sections were mounted with fluoromount-G. The vibratome slices were permeabilized and blocked for 45 min at 4 °C under gentle shaking in a solution of 1% bovine serum albumin +0.5% Triton + 5% normal goat serum in PBS. Primary antibodies (MPO, 1:300; AIF-1, 1:750; AQP5, 1:250; ZO-1, 1:200; CD31, 1:100; elastin, 1:250; α -SMA, 1:500; HTII-280, 1:250) were added in PBS and incubated overnight at 4 °C on a rocking table. Secondary antibodies (Goat Anti-mouse 647, 1:1000; Goat Anti-Rabbit 568, 1:1000) were added for 90 min at 4 °C on a rocking table. Finally, sections were incubated with DAPI (1:1000) and LEA-488 (1:500) for 30 min at room temperature before final washing in PBS and mounting with Fluoromount-G.

Imaging acquisition and analysis

Bright-field images were acquired using an Olympus CKX53 microscope (Olympus Life Sciences, Tokyo, Japan) with 4 $\times$, 10 $\times$, and 20 $\times$ objectives. Fluorescence images of stained microtome and vibratome sections were acquired using a Nikon Eclipse Ti2 microscope or a Nikon A1R HD confocal microscope (Nikon, Japan).

Sections were stained with H&E and imaged by bright-field microscopy. The images were anonymised and randomised before being independently scored by observers who were blinded to the experimental groups and experienced in porcine lung histology. Histological lung injury was assessed based on inflammatory cell infiltration, hyaline membrane formation, proteinaceous debris, alveolar wall thickening, and haemorrhage. Ease of sectioning was assessed by an experienced researcher using a five-point scale: 1 = poor, 2 = average, 3 = good, 4 = very good, and 5 = excellent¹⁶⁸.

Fluorescence images were analysed in Fiji using custom scripts and predefined marker-specific thresholds. Quantified outcomes included fluorescence intensity, signal coverage, particle size, and cell number. For marker-based analyses, the LEA-labelled alveolar scaffold was first measured in each field of view (FOV). Scaffold coverage was calculated as the LEA-positive area divided by the total FOV area. The fluorescence intensity of each marker was then normalised to LEA-positive scaffold coverage to account for variation in tissue amount between images. Marker coverage was calculated as the marker-positive area relative to the LEA-positive scaffold area. For particle and cell counting analysis, the signals were binarized, and the same marker-specific inclusion criteria were applied to all

experimental groups. Additionally, alveolar morphology was quantified by tracing the outer and inner alveolar borders using the polygon selection tool. Alveolar wall thickness was estimated as a wall area fraction by subtracting the inner alveolar area from the outer alveolar area and expressing the difference as a percentage of the total alveolar area. Circularity was calculated from the inner border using the shape descriptor function. The MQ was calculated as the ratio of circularity to the wall thickness estimate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10. Data normality was assessed using the Shapiro–Wilk test. Normally distributed data were analysed using an unpaired Student's t-test and non-normally distributed data were analysed using the Mann–Whitney U test. Repeated-measures data were analysed using two-way ANOVA with Holm–Šidák multiple-comparisons testing. Data are presented as mean $\pm$ standard deviation (SD) or median with interquartile range, as appropriate. A p -value < 0.05 was considered statistically significant.

Artificial Intelligence (AI) tools

During the preparation of this thesis, OpenAI's ChatGPT (GPT-5) was used to support language refinement and editing, including grammar, sentence structure, clarity, overall flow and figure formatting. All AI-generated suggestions were critically reviewed, revised, and incorporated at the author's discretion.

Acknowledgements

Sandra, there is so much I would like to say, but the simplest and most sincere words I can offer are **thank you**. Thank you for your understanding, patience, kindness, encouragement, and guidance throughout these four years.

As a clinician myself, I understand how demanding your clinical responsibilities are. What I admire most is that, despite these many demands, you approach both clinical work and research with remarkable passion, energy, and determination. I have always been inspired by your vitality. As a surgeon, you demonstrate exceptional professionalism and dedication; as a researcher, you continue to explore, question, and innovate; and as a PI, you have built a warm, inclusive, and close-knit research group.

I feel deeply fortunate to be your PhD student. As my four years of PhD study in Sweden draw to a close, I will carry the values and inspiration I have gained from you with me when I return to clinical work. I will continue to learn and grow, and I will always look to you as a role model throughout my career.

Franzi, as my co-supervisor, I would also like to express my sincere gratitude for your guidance, care, and support. You have always shown such thoughtfulness towards everyone in the group. When I first arrived, you were worried that I might experience culture shock, and you kindly introduced me to Hongzhe. Every Chinese New Year, you sent warm wishes to the group, and you always remembered each of our birthdays.

When I first started, you patiently guided me through many unfamiliar tasks while I was still inexperienced. During my first summer in Sweden, we spent an unforgettable and intensive week collecting lung transplant samples together. That experience taught me a great deal and helped me grow. Your rigour, meticulousness, and dedication to research have set an example that I will always remember and continue to learn from.

Nic, there is also so much I would like to thank you for. During my first summer in Sweden, you joined the group as well. Thank you for teaching me practical experimental techniques and for guiding me in analysing those fascinating images from our staining experiments. With your guidance and support, we published my first paper and took part in filming the video for the journal. This was a completely new and exciting experience for me and has become a precious memory.

Thank you for introducing me to your Chinese friends from your previous group. Getting to know them enriched my life abroad and made me feel less lonely. Whenever I felt stressed or anxious, your encouragement and support always helped me calm down and regain a sense of direction. Thank you for helping me understand how to find a better balance between work and life: to accept that “it is what it is”, to let things unfold naturally, and to believe that there is always a way forward.

Maggie, you were the very first person I met when I joined the group, and I can still clearly remember how you met me at the hospital entrance and brought me to the lab. You also taught me that, in Sweden, a cup of coffee in the morning is very important. Thank you for your help, encouragement, and care over the past four years. You have always been so patient when helping me solve problems. I am also deeply grateful that you have shared your valuable experience in paraffin sectioning with me. You always managed to cut perfect sections. Working with you has always been both efficient and joyful. We truly made a great team.

Gabriel, you were also one of the first people I met when I joined the group. Sometimes you seem quiet, but whenever I talk with you, I feel that you are such a bright, cheerful, and positive Swedish guy. I will always remember the night shifts we spent together during each pig week, standing side by side and taking care of everything together.

Anna, it feels like your defence was only yesterday, and now, somehow, it is my turn. It is amazing how quickly time has passed. I still miss the days when we cut sections and prepared PCLS together. Wherever you were, there was always a joyful atmosphere, and even the most repetitive work became interesting. I think that is your unique charm. I sincerely wish you all the best in your clinical work, and I hope you will continue moving towards your dreams and fulfilling your aspirations.

Embla, you truly are a special presence in our group. You always bring energy, freshness, and vitality to everyone around you. I always noticed the little creative touches you added to our sample boxes during pig weeks. Somehow, even ordinary sample boxes seemed to come alive because of you.

Runchuan, thank you for all your help and support in my research. You are truly meticulous, rigorous, and deeply dedicated to work. Working with you has always been highly efficient, with everything well organized and carried out in an orderly manner. I have learned many valuable qualities from you.

Fanny, we usually met during pig weeks. When I first started taking part in our pig week, whenever I encountered something uncertain, you always provided timely help and guidance. You patiently answered my questions and helped ease my nervousness at the beginning.

Sujeeth and Derek, thank you both for patiently helping me with all kinds of questions, whether they concerned ordering experimental reagents or overcoming technical difficulties in the lab. You are both reliable and trustworthy colleagues

and friends. You are both also full of passion for life, which reminds me of the spirit of the anime you love: “俺は海賊王になる男だ！”. I will always remember the joyful moments we shared while playing Nintendo games and celebrating Halloween together.

Candela and Daniel, you have truly shown me the warmth, energy, and passion of Spanish culture. At the same time, I have seen how careful and dedicated you are in the lab and how thoughtful you are towards the people around you. I feel very happy and lucky to have become your colleague and friend. Although I do not understand or speak Spanish, I really enjoy listening to the two of you chatting in Spanish. For some reason, I simply like the rhythm and sound of the language. I hope that one day I will have the chance to visit Spain and experience its warmth and passion in person.

Tugba and Farima, welcome to the group. Although the time I have spent with you has been limited, as an old Chinese saying suggests, every meeting carries a sense of serendipity. I am very happy to have become your colleague and friend.

Leif, you are such a kind, approachable, and warm person. It was a great honour to share night shifts with you while watching over EVLP systems, and I am also very grateful that you taught me some of the basic principles of EVLP at that time. You have not truly left us. The valuable experience and techniques you passed on will continue to guide us and help us move further forward.

Gunilla, although we usually only met during pig weeks, talking with you always made me feel relaxed and at ease, especially during those busy and slightly stressful moments.

Tibor, you have always been so kind to us. Thank you for your generous encouragement and help. You shared with me, without reservation, so much knowledge about perioperative monitoring, and you patiently answered my questions about porcine lung transplantation during pig weeks. It has been a great honour to work with you.

Dag, I am very glad that I had the chance to be on night shifts with you during pig weeks. Thank you for teaching me how to handle abnormal alarms from the monitoring system. I believe that this knowledge and experience will also be helpful for my future clinical work.

Haider, as someone who also works in thoracic surgery, I always felt a sense of warmth and familiarity when talking with you. Thank you for sharing with me your experience of visiting Shanghai, as well as your insights into clinical work in Swedish thoracic surgery.

Mohammed, we mainly met during pig weeks, and mostly during night shifts. I will always remember the night shift when we tried our best to save that pig.

Although the pig did not survive until the morning, that experience somehow brought me back to my previous days of being on duty in the clinical department.

Annika, although we have not known each other for very long, the time we spent together during pig week was both memorable and enjoyable. Thank you for patiently explaining what might have caused the pig that required emergency treatment that morning to become critically ill. I also learnt several important aspects of postoperative monitoring, and I believe that this knowledge will be valuable in my future clinical work.

Evamarie, every time I saw you, I could feel your positive and optimistic attitude towards life. Thank you for sharing that strength with me. Your Raku studio is another reason why I admire you so much. Thank you for inviting us to your home and for teaching me how to make Raku pottery. I truly enjoyed the whole process. It was a new, fascinating, and truly memorable experience.

Professor Gabi, thank you for serving as the chairperson at my defence. I am truly grateful for your time, support, and presence on this important day.

Mathias, I will remember and miss the week we spent together in Gothenburg for the microsurgery training course. Thank you for sharing your experience with me and for your kind help.

弘喆姐，沁雨姐和马芷老师，你们是我刚来瑞典时就在 BMC 认识的中国同胞。感谢你们在我刚来的那段时间里给予的关心和照顾，帮助我渐渐适应了异国他乡的生活，和你们一起过了我在瑞典的第一个中秋节和圣诞节，偶尔约个午饭，小聚畅聊也是格外令人怀念，还有特别感谢你们在实验上对我的指导和帮助，感谢你们的耐心倾听和给出建议。

同样感谢一路陪伴，帮助和支持我的中国小伙伴们，能认识大家真是太棒了！致我的诸位好友，娜导，薛扬，邓老师，越哥，田儿，束老师，李敏，邵可，大诚子，响子，李梓楠，赵老师，欣瑞，金田和小吴，解玉文，大霖子和圆圆，陆姐，刘洋师兄，天实，猛哥，平哥，昊哥，白哥，小舞，涛哥，华哥和晓妮姐，浩洋，感谢大家！与你们相识在这异国他乡真是一种缘分。虽然大家来自不同的省份，也有着不同的过往经历，但彼此相处得十分融洽，相聚的时间里充满快乐和能量。我会记得那些我们走过的路，翻过的山，经历的风雨甚至冰雹，看过的风景，组过的饭局，聊过的梗儿和冷笑话，以及那些抽象又搞笑的事，所有这些生活片段筑成了我们这几年的美好回忆，或许我们只是彼此人生旅途中的短暂过客，或许随着时间的流逝，有些记忆会渐渐淡去，但我相信，对于这段经历，每个人都会保存属于自己的那份感动和美好，多年以后的某个时刻再次回想起这一段段的过往，也会津津乐道吧。真心的祝愿大家在今后的日子里繁花似锦，再相逢依旧如故。

我还要感谢我的爸爸妈妈，感谢你们对我的付出，关心和理解。这些年我们聚少离多，大学毕业之后就一直在外面学习，生活和工作，不知不觉我也成了那个漂泊在外的东北孩子。但你们给了我足够的自由去闯，去体验，不论遇到什么困难，你们永远是最坚实的后盾，感谢爸妈的支持和鼓励，我也会努力成为你们的依靠和骄傲。

最后还要感谢当初没有放弃和坚持到现在的自己。在安逸和稳定之间却选了挑战，人这一生，有些事，总要自己去亲身经历和感受一下，也只有自己才能深刻的体会这一路走来是什么感觉。四年的时间，可以是备战一场比赛，筹备一部电影，也可以是在新的环境中磨练和成长，这些经历都将成为难忘，美好且有力量的回忆，在今后遇到困难时给自己继续前进的动力和勇气，在享受幸福时也懂得珍惜和感恩。

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