Investigating Biological Sex Differences in a Preclinical Model of Acute Lung Injury

Eva Kuhar

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Preface

In this thesis, I present the culmination of several research projects that I have led under the guidance of my supervisors. My involvement encompassed all phases of these projects, including the formulation of research questions, assembling a multidisciplinary team, and organizing and planning meetings. Each manuscript included in this thesis contains a detailed description of my contributions and those of my co-authors, which can be found in the preface and contribution section of each work.

Abstract

Despite decades of research, Acute Respiratory Distress Syndrome (ARDS) remains a leading cause of mortality in critically patients, with treatments limited to supportive care. Biological sex is increasingly recognized as a key variable influencing disease progression and therapeutic outcomes, but its role in ARDS is underexplored. Clinical findings have been inconsistent, highlighting the need for robust preclinical research. This thesis addresses these gaps through two studies: 1) a systematic review revealing that few studies of LPS-induced acute lung injury (ALI) models report sex stratified data, but the available data suggests males tend to fare worse; and 2) experimental studies refining the LPS administration routes in this model. These refinements reduced unwanted variability and enhanced the detection of meaningful biological differences, finding that male mice exhibited more severe lung injury. Together, these findings lay the groundwork future research (e.g., a multilaboratory studies) that can further address these gaps.

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List of Abbreviations

ALI	Acute Lung Injury
AMECO	Animals, Models, Exposure, Comparison, And Outcome
ANOVA	Analysis Of Variance
ARDS	Acute Respiratory Distress Syndrome
ATS	American Thoracic Society
BALF	Bronchoalveolar Lavage Fluid
BCA	Bicinchoninic Acid Assay
CCL2	Chemokine Ligand 2
CI	Confidence Intervals
CIHR	Canadian Institutes Of Health Research
COVID-19	Coronavirus Disease Of 2019
DAMPs	Damage-Associated Molecular Patterns
ELISA	Enzyme-Linked Immunosorbent Assay
H&E	Hematoxylin And Eosin
ICU	Intensive Care Unit
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IRAK1	Interleukin-1 Receptor-Associated Kinase
LPS	Lipopolysaccharide
LUNG SAFE	Large Observational Study To Understand The Global Impact Of Severe Acute Respiratory Failure
MD2	Myeloid Differentiation Protein 2
MyD88	Myeloid Differentiation Protein 88
NF-kB	Nuclear Factor-Kappa-B
NIH	National Institutes Of Health
OCT	Optimal Cutting Temperature
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PEEP	Positive End-Expiratory Pressure
PI3K	Phosphatidylinositol-3-Kinase
PKB	Protein Kinase B
PRISMA	Preferred Reporting Items For Systematic Reviews And Meta-Analysis
PRESS	Peer Review Of Electronic Search Strategy
ROS	Reactive Oxygen Species
SABV	Sex As A Biological Variable
SMD	Standardized Mean Difference
SYRCLE	Systematic Review Centre For Laboratory Animal Experimentation
TGF-β	Transforming Growth Factor Beta
TLRs	Toll-Like Receptor

TNF- α	Tumor Necrosis Factor Alpha
VILI	Ventilator-Induced Lung Injury

Chapter 1 - Introduction

1.1 Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) is a life-threatening condition characterized by acute lung inflammation and respiratory failure. First described by Ashbaugh and colleagues in 1967, ARDS can arise from various causes, including sepsis, pneumonia, trauma, and viral infections. (1) Despite decades of research and advances in critical care, ARDS continues to have high mortality and long-term morbidity. Current treatment approaches remains primarily supportive, highlighting the urgent need for novel therapeutic interventions. One significant gap in current research is the under-exploration of biological sex differences in ARDS pathogenesis and outcomes. This thesis seeks to address this gap by using a widely accepted preclinical model of acute lung injury (ALI) to investigate sex-specific differences, with the goal of refining these preclinical models to improve the relevance of experimental findings and contribute to the development of targeted therapeutic strategies. In this introductory chapter, I will first provide an overview of ARDS, covering its definition, epidemiology, biological sex differences, management, and pathophysiology. I will then discuss the potential to enhance the rigor of preclinical ARDS research through multilaboratory studies, systematic reviews, and refining existing models of experimental ARDS.

1.1.1 Definition and Diagnosis

Building on the early observations by Dr. Ashbaugh and colleagues, ARDS was first defined through their documentation of twelve ICU patients with severe respiratory distress due to conditions like viral pneumonia, trauma, and acute pancreatitis. Despite varying causes, these patients shared common features: severe dyspnea, tachypnea, hypoxemia, and the need for mechanical ventilation with positive end-expiratory pressure (PEEP). (1) Chest radiographs typically showed diffuse alveolar infiltrates and lung consolidation, indicating pulmonary edema, while histology confirmed the presence of hyaline membranes and reduced lung compliance — both now hallmark characteristics of ARDS. (1) These early observations helped define ARDS as a distinct clinical syndrome.

As the understanding of ARDS has evolved, so too have the diagnostic criteria. In 1994, the American-European Consensus Conference formalized guidelines, which were updated in 2012 by the ARDS Definition Task Force wich introduced the '**Berlin Definition**.' (2, 3) This framework categorizes ARDS into mild, moderate, and severe, based on the PaO2/FiO2 (P/F) ratio: 201-300 mmHg defines mild ARDS, 101-200 mmHg defines moderate ARDS, and ≤100 mmHg defines severe ARDS. The Berlin Definition also requires that bilateral opacities on chest imaging, acute onset within one week of a known clinical insult, and exclusion of heart failure or fluid overload as primary causes. While these updates have improved diagnostic consistency, ARDS remains clinically heterogeneous, complicating recognition and management.

1.1.2 Epidemiology, Causes, and Risk Factors

The incidence of ARDS varies significantly across populations and regions. A population-based study in Olmsted County, Minnesota, reported an incidence of 38.9 per 100,000 person-years, emphasizing the substantial community burden. (4) Similarly, a multicenter study across three Australian states found the incidence to be higher than previously estimated, with differences in definitions and study designs contributing to the variability. (5) The global burden of ARDS was further highlighted by the LUNG SAFE study, a international observational study, which found that ARDS accounted for 10.4% of all ICU admissions and 23.4% of patients requiring mechanical ventilation. (6) Mortality rates for ARDS remain concerningly high ranging from 35-46%, with a median time to death of 7 days from diagnosis, primarily due to multiple organ dysfunction and sepsis. (1, 2)

ARDS is predominantly caused by bacterial and viral pneumonia, as well as non-pulmonary sepsis. (7, 8) It is also frequently linked with clinical conditions such as gastric aspiration, severe trauma (e.g., blunt force injuries and severe burns), and, less commonly, blood product transfusion, drug overdose, inhalation of toxic agents, and acute pancreatitis. (7, 9) Comorbidities such as chronic alcoholism, tobacco use, and environmental pollutants further complicate the clinical picture, as they are associated with increased mortality in ARDS patients. (9–11) Despite advances in identifying these risk factors, their prognostic utility in predicting ARDS progression remains limited.

1.1.3 Management

The management of ARDS requires a multifaceted approach, combining supportive care, mechanical ventilation strategies, pharmacological interventions, and adjunctive therapies. Supportive focuses on fluid management, preventing complications, and ensuring adequate nutrition. (12) Conservative fluid management is recommended to prevent exacerbation of pulmonary edema and worsening gas exchange, with fluid-restrictive strategies shown to shorten mechanical ventilation duration and improve oxygenation. (13, 14) Mechanical ventilation remains central to ARDS management, with low tidal volume ventilation (4-6 mL/kg) now standard practice to reduce mortality and prevent ventilator-induced lung injury (VILI). (15–17) PEEP is critical for maintaining alveolar recruitment and improving oxygenation, though optimal PEEP levels may need to be individualized. (3) Prone positioning has also proven effective in severe ARDS, enhancing ventilation-perfusion matching and reducing mortality by redistributing lung densities and mitigating VILI. (19, 20) Pharmacological treatments are largely supportive, as no single drug has been universally effective. Early, short-term use of neuromuscular blockade can improve oxygenation and reduce mortality by minimizing patient-ventilator asynchrony. (21) The use of corticosteroids remains controversial, with some studies showing reduced inflammation early in the disease, while others find no mortality benefit. (22) Vasodilators like inhaled nitric oxide and prostacyclin may improve oxygenation in refractory hypoxemia, though they have limited effects on mortality. (23, 24) Emerging therapies, such as mesenchymal stem cell treatments, show potential for modulating immune responses and promoting lung repair. Additionally, precision medicine approaches targeting specific molecular pathways are being explored, with personalized strategies based on genetic and biomarker profiles offering hope for improving treatment efficacy and outcomes. (25–27)

1.1.4 Biological Sex Differences in ARDS

Biological sex likely plays a significant role in ARDS outcomes, although research has yielded mixed results. Some studies suggests that male patients have higher mortality rates compared to female patients, while others indicate that females may face a higher risk of mortality in severe cases. (28, 29) For instance, the LUNG SAFE prospective observational cohort study found that

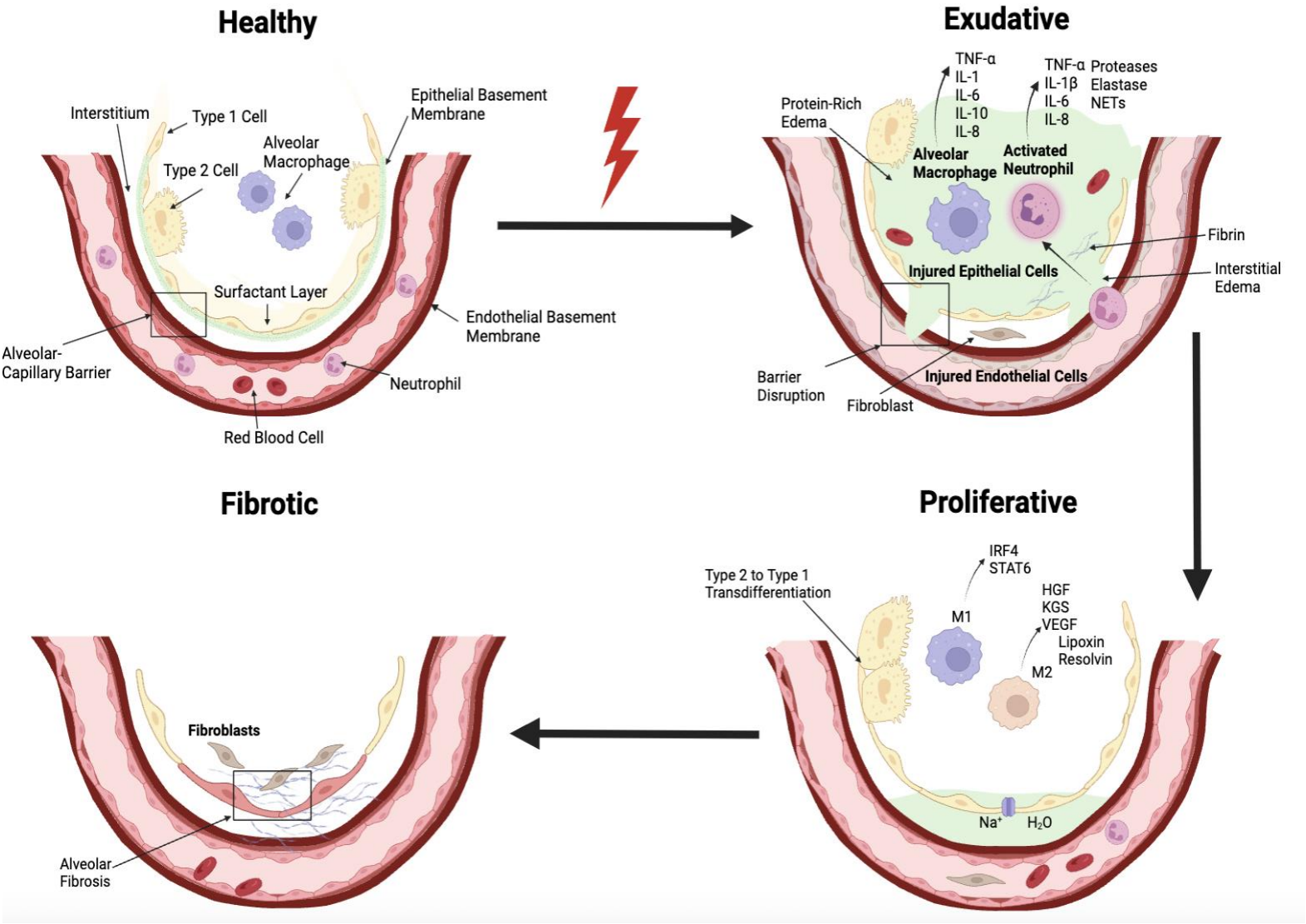
shorter patients, predominantly female, were often subjected to higher tidal volumes than taller patients, who were predominantly male. (6) While overall mortality rates between sexes were similar, females with severe ARDS exhibited higher mortality, potentially due to disparities in ventilatory parameters. The COVID-19 pandemic further highlighted sex-based disparities, with male patients exhibiting more severe disease progression and higher mortality rates. (30, 31) Biological sex differences in ARDS outcomes are likely influenced by hormonal, genetic, and immune factors. Hormones like estrogen and testosterone may modulate immune responses, affecting cytokine production and immune cell recruitment between sexes. (4, 5) However, the mechanisms behind these variations remain unclear, and clinical studies offer conflicting evidence. To explore these biological sex differences, it is crucial to first understand the pathophysiology of ARDS, which is briefly reviewed in the next section.

1.1.5 Pathophysiology

ARDS manifests as acute, diffuse, bilateral inflammatory lung injury with impaired gas exchange and respiratory failure. Inflammatory mediators play a central role, driving leukocyte accumulation and increasing vascular permeability. (7, 34, 35) The pathologic progression of ARDS occurs in three distinct phases, as outlined in **Figure 1**. The *exudative phase* occurs within 24 hours of lung injury, characterized by immune cell-mediated damage to the alveolar endothelium and edema fluid accumulation in the alveoli. (35, 36) Resident alveolar macrophages detect pathogen-or damage-associated molecular patterns (PAMPs/DAMPs) and release proinflammatory cytokines (e.g., IL-6, TNF- α) and chemokines (e.g., IL-8, CCL2), which recruit additional immune cells. This inflammatory cascade compromises the alveolar-capillary membrane, leading to hypoxemia and impaired gas exchange. The *proliferative phase* follows within 7-14 days after the intial insult. During this phase, tissue repair processes are activated, aimed at restoring alveolar integrity. (35, 37) Type II pneumocytes proliferate and differentiate into type I pneumocytes to restore alveolar epithelium and re-establish gas exchange. Type II pneumocytes also produce surfactant, which helps reduce surface tension in the alveoli and maintains alveolar stability. Increased ion channels and aquaporin expression clears intra-alveolar fluid, reducing edema. (35, 37) Endothelial progenitor cells promote repair of the alveolar-capillary barrier, improving gas exchange. Resolution of this phase is often

marked by clinical improvement and reduced respiratory support. In some patients, ARDS progresses to the *fibrotic phase*, marked by unresolved lung injury and tissue repair failure. (35) Fibroblast activation leads to excessive collagen deposition, resulting in lung fibrosis and stiffening of lung tissue. This fibrosis further disrupts pulmonary architecture, severely impairing gas exchange and increasing the risk of prolonged respiratory failure and death. (38) Prolonged mechanical ventilation and ventilator-induced lung injury (VILI) can exacerbate these fibrotic processes, compounding the damage in patients who do not recover during the proliferative phase. (38)

Figure 1. Phases of ARDS Progression : From Injury to Fibrosis



1.2 Bridging the Translational Gap: Enhancing Preclinical Rigor for Effective ARDS Therapies

Much of the pathophysiology described in the previous section was uncovered through the use of preclinical animal models of ARDS. These preclinical acute lung injury (ALI) models reproduce key clinical features of ARDS, such as lung inflammation, alveolar permeability, and hypoxemia. (6, 7) Of note, however, is that many interventions found to be promising in these ALI models have failed to demonstrate efficacy in human trials. (8) There are multiple reasons that have contributed to this challenge of translating preclinical ARDS discoveries. (8, 9) For instance, species differences between animal models and humans—such as variations in immune responses and lung structure—are recognized limitations in preclinical research. (8) Small animal models like mice are frequently used due to their accessibility and cost-effectiveness, despite anatomical differences that limit their ability to fully mimic human ARDS. (10–12) Larger animal models offer better physiological comparisons but are far more resource-intensive. (13, 14) However, the primary challenge lies not in species differences alone, but are compounded by a lack of rigor and reproducibility, as well as poor experimental design and reporting standards across studies.

This is not an issue isolated to preclinical ARDS research. Preclinical research across various domains has demonstrated a lack of standardized protocols, robust statistical designs, and transparency required to produce reliable, reproducible data. (15) This inconsistency weakens the credibility of preclinical findings and complicates their translation into clinical practice. (16–18) Additionally, the frequent exclusion of both male and female animals limits the generalizability of results, ignoring the significant role of biological sex in disease progression and treatment response. (19–21) To overcome these obstacles, preclinical studies have been encouraged to adopt the same level of rigor as clinical trials. (9, 22–24) Standardizing methodologies, improving transparency, and accounting for key biological variables like sex will enhance reproducibility and strengthen the link between preclinical findings and clinical outcomes,

ultimately advancing the development of effective therapies. Multilaboratory studies, described in the next section, offer one potential solution to address these challenges.

1.2.1 Multilaboratory Preclinical Studies: A Solution to Translational Challenges

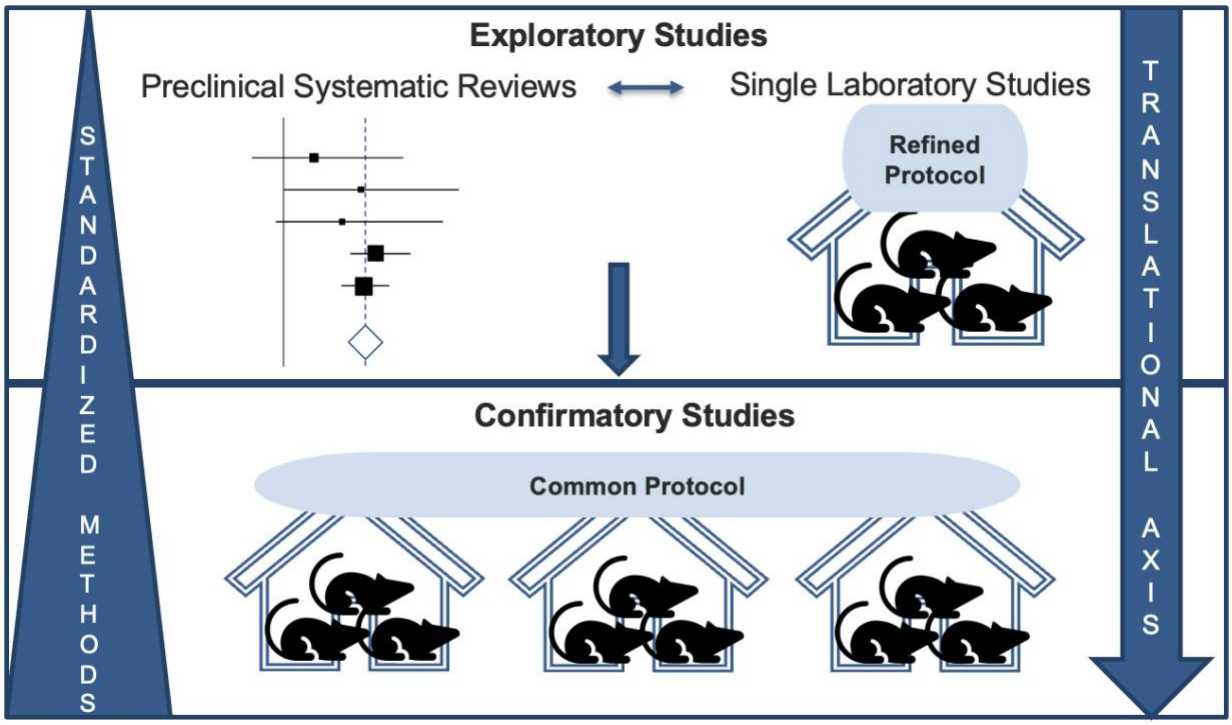
Single-laboratory preclinical studies face significant limitations that hinder the reproducibility and generalizability of their findings due to a lack of study design rigour, small sample sizes, and inadequate statistical power, which make it challenging to detect meaningful effects. (25, 26) Additionally, the controlled experimental conditions unique to individual laboratories often limit the broader applicability of results, as they fail to account for biological variability across different settings. It is widely believed that these issues have contributed to the ongoing replication crisis in preclinical research, with less than 5% of high-impact preclinical reports successfully translating from bench to bedside. (25–27) In clinical research, single-center clinical studies have been frequently criticized along similar lines, such as a higher risk of bias and smaller sample sizes that can distort outcomes, and inflate effect sizes. (17, 28, 29) Multicenter clinical studies mitigate these limitations by increasing sample sizes and facilitating cross-site comparisons to assess the generalizability of findings across diverse clinical environments. This design also accounts for biological variability and enhances statistical power, ultimately improving reproducibility and reducing bias, while strengthening the confidence in translating results into clinical practice.

While multicenter clinical studies have made significant strides in addressing issues of bias, generalizability, and reproducibility, similar strategies are now being adopted in preclinical research. Multilaboratory preclinical studies represent a promising solution to these rigor and reproducibility challenges that have hindered the translation of preclinical ARDS research into clinical practice. Approximately 50% of preclinical studies fail to produce consistent results, contributing to translational failures in clinical trials. (53, 54) By coordinating experiments across multiple independent laboratories using standardized protocols, multilaboratory studies reduce bias and improve the reliability of results. This collaborative approach may enhance the

likelihood of identifying the most promising therapies for early-phase clinical trials. (16, 30) Moreover, the added statistical power from larger sample sizes in multilaboratory studies increases the ability to detect subtle yet meaningful effects, such as sex-based differences in disease response. As funding agencies like the NIH and CIHR increasingly emphasize standardized methodologies and the consideration of biological sex in research, multicenter studies are uniquely positioned to drive progress in translating preclinical findings to clinical applications. (31, 32)

To address these challenges and set the stage for a future multilaboratory study, my thesis follows a framework similar to that of clinical trial development, where I started with a systematic review and then conducted a single laboratory study to refine methods and generate pilot data. **Figure 2** illustrates the key steps in my thesis framework, highlighting the progression from the systematic review to the refinement of methods and generation of pilot data in the single laboratory study, ultimately laying the groundwork for our future multilaboratory study. Below I describe: 1) experimental models of ALI, 2) important outcome measures in experimental models of ALI, and 3) the advantages of preclinical systematic reviews.

Figure 2. Framework for Developing a Multilaboratory Study: From Systematic Review to Pilot Data Generation



1.3 Experimental Models of Acute Lung Injury

1.3.1 Overview

While no single model can perfectly replicate all characteristics of human ARDS, various models emphasize different aspects of the disease. (33, 34) The most effective ALI models closely mirror the acute onset of pulmonary inflammation, vascular hyperpermeability, and diffuse alveolar damage seen in ARDS. However, selecting a model that captures these complexities while ensuring reproducibility and clinical relevance remains a challenge. Common ALI models used in such research include systemic or pulmonary administration of lipopolysaccharide (LPS), airway instillation of live bacteria, and high tidal volume mechanical ventilation that causes VILI. In the following section, I describe the LPS-induced ALI model that I used in my experiments, explaining its relevance in replicating key ARDS features. I also provide an

overview of two other commonly used models other ALI models, highlighting their respective advantages and limitations, to place our findings within the broader context of preclinical ARDS research.

1.3.2 Endotoxin Induced ALI

Lipopolysaccharide (LPS), a key component of the outer membrane of Gram-negative bacteria, induces lung injury through several interconnected mechanisms involving Toll-like receptors (TLRs), particularly TLR4. (35, 36) TLR4 plays a critical role in pathogen recognition by the innate immune system and is found on the surface of monocytes, macrophages, and dendritic cells. When TLR4 binds with LPS in association with myeloid differentiation protein 2 (MD2), it activates the adaptor protein MyD88, triggering pro-inflammatory signaling cascades. (35–37) This leads to the production of cytokines and chemokines like TNF-α, IL-1β, and IL-6, which recruit neutrophils and other immune cells to the lungs. The influx of immune cells amplifies lung injury by increasing reactive oxygen species (ROS) and protease production, which damages the alveolar-capillary barrier. Additionally, activation of pathways such as phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB) enhances inflammation via NF-κB signaling. Disruption of endothelial and epithelial junctions further increases vascular permeability, resulting in edema, impaired gas exchange, and alveolar flooding. These processes contribute to hypoxemia and diffuse alveolar damage, hallmark features of ARDS. Of note although this is the most widely used model of ALI, methods and routes of delivering LPS remain understudied although it has been suggested this may significantly influence the severity and distribution of lung injury. (38) As such, as described in a later section, addressing this knowledge gap became a focus of one of my thesis studies.

1.3.3 ALI Induction by Airway Instillation of Live Bacteria

Airway instillation of live bacteria, such as *Escherichia coli, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*, is a widely used model for inducing ALI by mimicking bacterial pneumonia. This method introduces live bacteria directly into the airways, allowing researchers

to study the host response to active bacterial infections and how it contributes to lung injury. (33) The bacterial load plays a critical role in determining the severity of infection, with higher bacterial concentrations potentially leading to systemic effects beyond localized lung injury. The route of administration significantly influences the outcome of the infection. Intratracheal instillation of bacteria results in localized pneumonia and diffuse alveolitis, characterized by neutrophil infiltration, increased inflammatory markers, heightened microvascular permeability, and pulmonary edema. (6, 39) This approach allows for focused infection and inflammation within the lung's alveolar spaces, closely modeling bacterial pneumonia that can progress to ARDS. In contrast, housing animals in an aerosol chamber for bacterial exposure facilitates a more diffuse pulmonary infection, often resulting in widespread inflammation and systemic involvement. This variation in infection route provides insight into different disease dynamics, offering a versatile platform for investigating bacterial lung infections and their role in the development of ARDS.

Model selection depends on the research question. While the airway instillation of live bacteria model provides valuable insights into pathogen-host interactions and bacterial causes of ARDS, we selected the LPS-induced model to focus on sterile inflammatory processes. This choice allows for precise control over lung injury and inflammation, making it ideal for studying key ARDS mechanisms without the added complexity of infection or mechanical stress.

1.3.4 ALI Induced by High Tidal Volume Mechanical Ventillation

High tidal volume mechanical ventilation is an important model for studying ventilator-induced lung injury (VILI), which contributes to the progression of ARDS. This model induces lung damage by applying elevated tidal volumes, leading to alveolar overdistension, barotrauma, and disruption of the alveolar-capillary barrier. (6, 33) The mechanical stress inflicted by overventilation activates inflammatory pathways, resulting in cytokine release and immune cell recruitment, which further exacerbates lung injury. (40, 41) The severity of lung injury can be controlled by adjusting the tidal volumes, with higher volumes causing more pronounced inflammation, pulmonary hemorrhage, hyaline membrane formation, and reduced lung compliance. However, it also presents several technical challenges. Setting up mechanical

ventilation systems in rodents is complex, and continuous anesthesia can lead to complications such as hypothermia and respiratory depression. Furthermore, the relatively short duration of mechanical ventilation in rodent models does not fully replicate the prolonged ventilation typically seen in clinical settings. (33)

While mechanical ventilation models accurately simulate VILI, we chose the LPS-induced model to specifically target the inflammatory processes that precede VILI. Unlike ventilator models, which introduce confounding factors such as mechanical stress and barotrauma, the LPS model allows us to isolate the inflammatory cascade central to ARDS development. Additionally, LPS models are more reproducible and controllable, facilitating a focused investigation of early immune responses that complements the mechanistic insights from ventilator studies.

1.3.5 Summary of Experimental ALI Models

Each experimental ALI model offers distinct advantages depending on the research focus. These models collectively highlight the multifactorial nature of ARDS, where infection-driven processes and mechanical injury both contribute to disease development. The choice of model depends on the specific mechanisms being investigated. For my studies, we selected the LPS model due to its precision in replicating the key inflammatory features of ARDS. In the next section I summarize relevant outcomes in models of ALI, highlighting features that became the focus of my studies.

1.4 Assessing Preclincial Outcomes of ALI

Replicating ARDS symptoms in animal models presents challenges due to the limitations of diagnostic tools and complexities in measuring lung compliance and hypoxemia. Furthermore, preclinical ARDS research often suffers from inconsistent outcome measurements, which can obscure the reliability of findings. Recognizing these challenges, the American Thoracic Society (ATS) has established guidelines focusing on the most relevant and feasible preclinical ALI

outcomes. (6) These guidelines emphasize standardizing key measures of histological lung injury, vascular permeability, and inflammation, ensuring that preclinical models accurately capture the essential features of ARDS, despite the limitations posed by small animal studies.

1.4.1 Histological Evidence of Tissue Injury

Histological evaluation remains a cornerstone for diagnosing ALI in animal models. (6) Standardized scoring systems are often used to assess lung sections (e.g., prepared from paraffin-embedded tissues and stained with hematoxylin and eosin). Key histological features such as intra-alveolar neutrophil accumulation, interstitial neutrophil infiltration, alveolar septal thickening, hyaline membrane formation, and the presence of proteinaceous debris are commonly scored. (6) My thesis studies relied on these semi-quantitative methods, which can provide reliable histopathological data across experiments. Beyond my studies, other researchers also examine gross pathological features, such as pulmonary hemorrhage, cellular infiltration, and pneumocyte hyperplasia, to provide a more complete picture of ALI pathology. These features, in combination with histological lung injury scores, help to characterize the extent of lung injury in preclinical models.

1.4.2 Measuring Alterations in Permeability

Vascular permeability is a hallmark of ARDS, driven by the breakdown of the alveolar-capillary barrier, which leads to the leakage of protein-rich fluid into the alveolar spaces. In our experimental studies, we measured total protein concentration in bronchoalveolar lavage fluid (BALF), a well-established indicator of protein leakage and vascular permeability. (6, 33) This measurement allowed us to quantify the extent of damage to the alveolar-capillary barrier in our model. While we did not employ other common measurements in our experiments, we assessed all permeability measurements in our systematic review. These methods include measuring the wet-to-dry weight ratio of lung tissue, which reflects the degree of pulmonary edema; assessing albumin concentration and other high molecular weight proteins such as IgM, which serve as indicators of vascular leakage due to their size and inability to cross an intact alveolar-capillary

barrier; and conducting Evans Blue dye assays, where dye accumulation in the lung indicates increased vascular permeability. Together, these complementary methods provide valuable insights into permeability changes during ALI.

1.4.3 Measuring Inflammation

Pulmonary inflammation is a central feature of ALI, characterized by neutrophil infiltration and the release of inflammatory mediators. (6, 33) In our experimental studies, we assessed total cell counts in BALF to measure overall cellular responses. Additionally, we quantified IL-6 levels in BALF, a key proinflammatory cytokine that signals neutrophil recruitment and amplifies the inflammatory response. To gain a comprehensive understanding of the inflammatory processes in LPS-induced ALI models, our systematic review evaluated all measurements related to pulmonary inflammation, as detailed below. Other common techniques include flow cytometry for precise quantification of neutrophils and the measurement of myeloperoxidase levels in BALF as a surrogate for neutrophil activation. (6, 33) Proinflammatory cytokines such as IL-1β, IL-8, TNF-α, and TGF-β are also frequently measured via ELISA or Western blot to provide a more detailed view of the inflammatory milieu in ALI models.

1.4.4 Measurements of Physiological Dysfunction

Hypoxemia and alveolar–arterial oxygen gradients are critical markers of physiological dysfunction in ARDS. (6, 33) However, due to challenges with in vivo assessment—including variability in technician skill, anesthesia, and equipment—these measures can be difficult to standardize across small animal models. While these parameters were not the focus of either our experimental studies nor our systematic review, they are frequently used to assess the degree of lung dysfunction in other models, particularly when focusing on gas exchange and oxygenation. Although these measures are clinically relevant, robust evidence of ALI is often better confirmed by pronounced histological damage, significant vascular hyperpermeability, and marked pulmonary inflammation, as was the case in my thesis studies.

1.5 The Role of Systematic Reviews and Meta-analysis in Enhancing Preclincial Rigour

As illustrated in Figure 1, the systematic review I conducted not only synthesized the existing literature but also laid the groundwork for our single-laboratory pilot studies, thereby reinforcing the critical role of systematic reviews in improving study design and addressing gaps in preclinical ARDS research. Systematic reviews synthesize all available studies on a given topic using predefined criteria to ensure the process is thorough, unbiased, and replicable. (42–44) This methodical approach ensures that new experiments are grounded in current evidence, improving the design and relevance of future studies by identifying gaps, inconsistencies, and trends in the literature. (42–44) In preclinical ARDS research, where experimental variability and inconsistent outcome measures are prevalent, systematic reviews refine research methods and resolving conflicting findings. (45, 46) Meta-analyses build on systematic reviews by statistically combining data from multiple studies, enhancing the power of the conclusions drawn and helping to detect patterns or treatment effects that may be obscured in individual studies. (45) For my thesis, I conducted a systematic review to evaluate the existing literature on biological sex differences in preclinical ARDS research. Just as clinical trials often begin with a systematic review to inform study design and identify gaps in the literature, our approach mirrors this foundational step. By systematically evaluating the existing preclinical data, we laid the groundwork for our single center pilot studies that delved deeper into these differences. This strategic approach not only strengthens the rationale for our research but also sets the stage for our future multilaboratory study.

1.6 Summary

This introduction has outlined the key considerations for modeling ALI in preclinical research. From the selection of appropriate ALI models—such as LPS instillation, live bacteria exposure, or mechanical ventilation—to the identification of key outcomes like lung injury, inflammation, and vascular permeability, each decision shapes the relevance of experimental findings to clinical ARDS. In this context, LPS-induced ALI provides a well-controlled model for studying

sterile inflammation, making it a strong choice for investigating the immune processes central to ARDS. By adopting the standardized approaches described throughout this chapter, and incorporating histological scoring, inflammatory analysis, and permeability assessments, my thesis studies focused on refining this model for enhanced reproducibility and relevance to clinical ARDS, while also assessing potential biological sex differences.

1.7 Thesis Objectives

1.7.1 Description of Rationale for Thesis

The underlying rationale for this thesis lies in the need to improve preclinical to clinical translation in ARDS research. Current models, particularly the LPS-induced ALI model, are plagued by variability and lack of standardization, which likely hampers their clinical translation. By refining this model, my thesis aims to establish a robust foundation for a future laboratory study that will assess the model and outcomes on a larger scale. Furthermore, the impact of biological sex on ALI outcomes has often been overlooked in preclinical research, despite clinical evidence suggesting that males and females may respond differently to ARDS. This thesis investigated sex-specific responses in the LPS model, enhancing our understanding of how biological sex influences disease progression and informing future multilaboratory studies that incorporate sex as a critical variable.

1.7.2 Specific Objectives for Each Thesis Project

Objective 1-Systematic Review on Biological Sex Differences in Preclinical ARDS Models: I conducted a systematic review to assess how biological sex modifies outcomes in the LPS-ALI model. This review revealed significant gaps in how sex differences were reported and addressed across studies. By evaluating the extent of biological sex inclusion in existing research, I underscored the need for greater attention to sex-based analysis in ARDS studies. The findings advocate for the consistent integration of sex as a critical variable in future preclinical studies to improve the relevance and accuracy of research outcomes**.**

Objective 2-Refining the LPS-Induced ALI Model:

Building on insights from the systematic review, I refined and standardized the LPS-induced ALI model to address key gaps. I optimized experimental protocols, including the LPS administration route and lung injury evaluation methods, while incorporating both male and female mice to explore sex-specific differences. These refinements improved the model's reproducibility and provided important pilot data to understand how biological sex influences ALI outcomes.

Chapter 2 - A preclinical systematic review and meta-analysis assessing the effect of biological sex in lipopolysaccharide-induced acute lung injury

2.1 Preface

The systematic review outlined in this chapter was conducted to better understand the influence of biological sex on the progression and severity of ALI in preclinical models. In this review, I systematically examined the existing body of literature on LPS-induced ALI models, with a specific focus on identifying gaps related to how biological sex has been incorporated into experimental designs and reported outcomes.

This work not only provides a critical analysis of the current state of preclinical biological-sex ALI research but also serves as a foundational step for the future refinement of preclinical models. By identifying inconsistencies and highlighting the underreporting of sex-specific data, the findings of this review emphasize the need for more rigorous consideration of biological sex in experimental designs.

Thus, this systematic review sets the stage for the experimental investigations detailed in subsequent chapters, where I will focus on optimizing the LPS-induced ALI model to enhance its reliability, rigor, and relevance for both male and female subjects.

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Eva Kuhar[1,2], Nikesh Chander[1], Duncan J Stewart [2,3,11], Forough Jahandideh[1], Haibo Zhang[4,5], Arnold S. Kristof[6], Julie A. Bastarache[7], Eric P. Schmidt[8], Monica Taljaard[9], Bernard Thebaud[3,10], Doreen Engelberts[1], Dean A. Fergusson[1,9,11,12], Manoj M. Lalu[1,2,3,9,13]

Author Affiliations

[1]Clinical Epidemiology Program, Blueprint Translational Research Group, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
[2]Department of Cellular and Molecular Medicine, University of Ottawa, Ontario, Canada
[3]Regenerative Medicine Program, The Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
[4]Keenan Research Centre for Biomedical Science, St. Michael's Hospital, Unity Health Toronto, Ontario, Canada
[5]Departments of Anesthesiology and Pain Medicine and Physiology, Interdepartmental Division of Critical Care Medicine, University of Toronto, Toronto, Ontario, Canada
[6] Meakins-Christie Laboratories and Translational Research in Respiratory Diseases Program, Research Institute of the McGill University Health Centre, Faculty of Medicine, McGill University, Montreal, Quebec, Canada.
[7]Vanderbilt University Medical Center, Tennessee, United States
[8]Harvard University School of Medicine, Cambridge, United States
[9]School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario, Canada
[10]Department of Pediatrics, The Ottawa Hospital and the Children's Hospital of Eastern Ontario
[11]Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada
[12]Department of Surgery, University of Ottawa, Ottawa, Ontario, Canada
[13]Department of Anesthesiology and Pain Medicine, The Ottawa Hospital, University of Ottawa, Ottawa, Ontario, Canada

Contributions (using CRediT Taxonomy)

Guarantor: MML; Conceptualization: EK, MML, DAF, and DS; Methodology: EK, MML, and DAF; Investigation: EK and NC; Writing – original draft: EK, MML, FJ; Writing – review and editing: all authors; Supervision: MML, DAF, and DS; Resources: DAF and MML.

2.2 Abstract

It is unclear what effect biological sex has on the outcomes of acute lung injury (ALI). Clinical studies are confounded by their observational design. We addressed this knowledge gap with a preclinical systematic review of ALI animal studies. We searched MEDLINE and Embase for studies of intratracheal/intranasal/aerosolized lipopolysaccharide (LPS) administration, the most common ALI model, and reported sex-stratified data. Screening and data extraction were conducted in duplicate. Our primary outcome was histological tissue injury and secondary outcomes included alveolar-capillary barrier alterations and inflammatory markers. We used a random effects inverse variance meta-analysis, expressing data as standardized mean difference (SMD) with 95% confidence intervals (CI). Risk of bias was assessed using the SYRCLE tool. We identified six studies involving 132 animals across 11 independent experiments. A total of 41 outcomes were extracted, with the direction of effect suggesting greater severity in males than females in 26/41 outcomes (63%). One study reported on lung histology and found that male mice exhibited greater injury than females (SMD 1.61, 95% CI 0.53 to 2.69). Meta-analysis demonstrated significantly elevated albumin levels (SMD 2.17, 95% CI 0.63 to 3.70) and total cell counts (SMD 0.80, 95% CI 0.27 to 1.33) in bronchoalveolar lavage fluid from male mice compared to females. Most studies had an 'unclear risk of bias'. Our findings suggest sex-related differences in ALI severity. However, these conclusions are drawn from a small number of animals and studies. Further research is required to address the fundamental issue of biological sex differences in LPS-induced ALI. **Registration:** PROSPERO CRD42022329067

New and Noteworthy

We performed a preclinical systematic review to understand the effect of biological sex on outcomes in acute lung injury (ALI). Following best practices for systematic reviews and meta-analysis, we identified six ALI studies that reported sex-stratified data. Overall, our results suggested male mice may develop more severe ALI phenotype than female mice. However, the paucity of published data emphasizes the need for further rigorously designed studies to assess biological sex differences in ALI.

Keywords: acute lung injury; ALI; acute respiratory distress syndrome; ARDS; biological sex

2.3 Introduction

Clinical investigations have produced inconsistent findings regarding the impact of biological sex on the pathogenesis of acute respiratory distress syndrome (ARDS). Some studies suggest that male patients exhibit a higher mortality rate from ARDS compared to female patients. (47, 48) Conversely, other studies have shown that females are at a higher risk of mortality in severe ARDS. (49, 50) For instance, within the LUNG SAFE prospective observational cohort study, investigators found that shorter patients, most often females, were subjected to inappropriately high tidal volumes more frequently than taller patients, who were most often males. While mortality rates were comparable between both sexes, females experiencing severe ARDS had higher mortality rates, possibly due to disparities in ventilatory parameters. (51)

These reported discrepancies may reflect the inherent limitations in observational designs and susceptibility to confounding factors in these studies. To address this knowledge gap, preclinical models of acute lung injury (ALI) offer a controlled setting that minimizes confounding factors present in the clinical setting. Features of ARDS can be studied in the laboratory by inducing ALI through the administration of proinflammatory agents such as lipopolysaccharide (LPS), hydrochloric acid, and bleomycin. Notably, the airway instillation of bacterial LPS is one of the most commonly used ALI models and can be reliably replicated in the laboratory. (33, 52) LPS is a well-established activator of the neutrophilic inflammatory response, which damages alveolar epithelial and lung endothelial cells, resulting in the accumulation of protein-rich inflammatory fluid within the alveolar space. This process mirrors aspects of clinical ARDS that ultimately lead to respiratory failure. (6)

In this study, we investigated potential biological sex differences in the LPS model of ALI. We performed a preclinical systematic review to synthesize the existing body of literature, identify knowledge gaps, and provide guidance for future laboratory studies. This approach to preclinical knowledge synthesis has been widely endorsed to strengthen and improve the translational potential of experimental findings. (24, 53, 54) We also performed this study to ‘evidence inform’ a forthcoming multilaboratory study that will investigate the interaction between biological sex and ALI outcomes. Although systematic reviews are typically advocated as a

prerequisite before initiating a clinical study, (55) its application here is innovative as an initial step to shape and inform a laboratory study.

Our focused question was, "*In animal models of LPS-induced ALI, what is the effect of biological sex on histological evidence of lung injury (primary outcome), alterations of the alveolar-capillary barrier, and inflammatory response (secondary outcomes*)?"

2.4 Material and Methods

2.4.1 Systematic Review Protocol and Registration

We registered our protocol in the International Prospective Registry of Systematic Reviews (PROSPERO CRD42022329067). We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (see **Supplemental Figure 1**).

2.4.2 Eligibility Criteria

We used the Animals, Models, Exposure, Comparison, and Outcome (AMECO) framework to define our eligibility criteria.

2.4.3 Animals and Models

We included *in vivo* animal models of experimentally induced ALI via intratracheal/intranasal/aerosolized LPS administration. We excluded indirect methods of ALI (e.g., intravenous and intraperitoneal LPS administration), as these usually result in a milder form of ALI. We included all mammalian species of all ages. Studies needed to include and report on both male and female animals.

2.4.4 Exposure and Comparators

In this systematic review, we compared the responses of male animals to female animals following LPS exposure. Other exposures/interventions were not considered.

2.4.5 Outcomes

We included studies that reported data stratified by biological sex for any of the outcomes listed below. These are based on three domains that reflect key pathological features of ALI, as defined

by the American Thoracic Society (ATS) guidelines on features and measurements of experimental ALI. (7)

1. Evidence of histological tissue injury (lung injury score) was our primary outcome, as it is the most relevant domain of ALI according to the ATS consensus statement. (7) We extracted the overall lung injury and lung inflammation scores.
2. Alterations of the alveolar-capillary barrier was measured via bronchioalveolar lavage fluid (BALF) as a secondary outcome. Examples of data extracted included total protein, albumin, and IgG.
3. The presence of an inflammatory response was another secondary outcome and was measured by BALF and plasma cytokine levels (e.g., IL-6, IL-1β, and TNF-α). All data for secondary outcomes were continuous.

Data for secondary outcomes were stratified by a window of outcome ascertainment (<24 h, 24-72 h, >72 h).

2.4.6 Publication Type

We excluded studies reporting only *in vitro* or *ex vivo* data. We considered only full publications of primary studies; we did not include abstracts, letters, review articles, and editorials. Any articles not written in English or French were excluded from this review.

2.4.7 Search Strategy

We developed the search strategy in collaboration with an information specialist experienced in preclinical systematic searches (Risa Shorr, MLS, Ottawa Hospital Library Services). To provide an accurate literature search, we used keywords related to the focus of the systematic review (see **Supplemental Figure 2**). As an example, we used precise vocabulary related to *in vivo* preclinical research on ARDS (e.g., acute lung injury) or acronyms (e.g., ALI). We applied additional validated search filters for preclinical animal studies on a per-database basis. We searched two databases from 1946 to March 2023: MEDLINE (OVID interface, including In-

Process and Epub Ahead of Print) and Embase (OVID interface). We submitted the final search strategy to another librarian for validation by the Peer Review of Electronic Search Strategy (PRESS) checklist. (56)

2.4.8 Study Records

Data Management:

We uploaded the results of our search to DistillerSR (Evidence Partners, Ottawa, Canada), a cloud-based platform. DistillerSR is auditable and designed specifically for the conduct of systematic reviews.

Study Selection:

Two reviewers independently screened titles and abstracts, referencing the previously stated inclusion and exclusion criteria. Reviewers then retrieved and screened full texts of abstracts that met the inclusion criteria. Any conflicts in article inclusion/exclusion were resolved by the senior author. We documented and justified all full-text article exclusions.

Data Collection Process and Data Items:

We created standard data extraction forms in DistillerSR to record data on animal and intervention characteristics, outcomes, and risk of bias. Two reviewers independently extracted relevant data items in duplicate and resolved discrepancies. Data categories included: study characteristics (e.g., number of animals per group), publication characteristics (e.g., the country where the study was conducted), study population (e.g., animal strain), route of LPS administration, outcomes, and risk of bias. We used Engauge Digitizer (Mitchell et al., 2023) software to extract any data that was only presented in graphical format (e.g., lung histology scores). A second reviewer then audited the extracted numerical data. In cases of doubt, study authors were contacted for clarification.

Statistics and Data Analysis:

When feasible, we meta-analyzed outcomes using standardized mean differences (SMD) with inverse variance random effects modelling (Comprehensive Meta-Analysis, version 3; BioStat

Inc., USA). Of note, in clinical systematic reviews SMDs of 0.2, 0.5, and 0.8 are considered small, moderate, and large effects respectively. (57) To assess the statistical heterogeneity of effect sizes, we used the Cochrane I^2 statistic.

2.4.9 Risk of Bias Assessment

We assessed risk of bias and study quality using the tool developed by the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE). (58) We evaluated the following: baseline characteristics, selective outcome reporting, blinding, random housing, and incomplete outcome data. We did not evaluate allocation concealment and randomization since we exclusively focused on the effects of biological sex (i.e., randomization to biological sex is not possible). Each included study was assigned an overall risk of bias value of low, high, or unclear using the highest risk obtained across all domains. Two reviewers independently evaluated each domain, and the senior author resolved any disagreements.

2.5 Results

2.5.1 Study Characteristics

Our systematic literature search yielded 1,679 unique citations, and six studies met our eligibility criteria (Figure 1). These studies were published between 2001-2023, with five from the United States and one from Canada. Three studies were funded by government and the remaining three studies receiving funding from a combination of sources (Table 1).

2.5.2 Experimental Model and Study Design

The age of mice included in studies ranged between 6 and 36 weeks (Table 1) with a median across all studies of 11 weeks. One study investigated biological sex and age differences and conducted separate experiments using young (8–12 weeks old) and aged (30–36 weeks old) mice. The most common route of LPS administration was intratracheal instillation (n=4/6), while intranasal instillation and oropharyngeal aspiration of LPS were used in one study each. The median LPS dose across studies using intratracheal instillation was 2.25 mg/kg (range 0.7 mg/kg to 3 mg/kg).

2.5.3 Outcomes

We extracted 41 outcomes from the six included studies. A heat map of outcomes is depicted in Figure 2. Overall, we found that the direction of the measure of effect for 26/41 (63%) outcomes demonstrated males developed a more severe phenotype of ALI.

Primary Outcome:

Histological evidence of lung injury:

Three studies assessed lung histology. One study used a scoring system to quantify the extent of lung injury. The scoring system assessed perivascular neutrophils, perivascular hemorrhage, and neutrophilic margination. (Card et al., 2006) This study found that male mice exhibited higher lung

injury compared to female mice (SMD 1.61, 95% CI 0.53 to 2.69). The two other studies provided representative images but did not comment on potential differences between males and females.

Secondary Outcomes:

Measurements of Alterations of the Alveolar-Capillary Barrier:

Albumin leakage and BALF total protein:

Three experiments from two studies reported BALF albumin levels, a measure of alveolar permeability. Overall, male mice had elevated albumin levels in BALF compared to female mice (pooled analysis, SMD 2.17, 95% CI 0.63 to 3.70, I^2 = 87%, Figure 3A). Stratified by the timing of outcome assessment, this effect was preserved in early (<24 h) but not later timepoints (24-72 h).

Four experiments from three studies reported BALF total protein concentrations at different time points. Overall, there was no significant difference in BALF total protein between the two sexes (SMD -0.20, 95% CI -0.68 to 0.29, I^2 = 36%, Figure 3B). Stratified by time, the single study at the earlier time point (<24 h) demonstrated higher protein concentrations in female mice. The pooled analysis of three studies at later time points (24-72h) did not demonstrate a significant difference between male and female mice.

Inflammatory Markers:

BALF Total Cell Counts:

Seven independent experiments from four studies reported BALF cell counts as a measure of inflammation. Overall, we found that male mice had higher BALF cell counts than female mice (SMD 0.80, 95% CI 0.27 to 1.33, I^2 = 73%, Figure 4). Stratified by time, this effect was seen in early (<24 h, SMD 0.79, 95% CI 0.24 to 1.34) but not later timepoints (24-72 h, SMD 0.93, 95% CI -1.07 to 2.91). At the later time points Moitra et al. demonstrated a potential interaction between age and sex, as young male mice demonstrated higher cell counts than young female mice, but this effect was not seen in older mice.

BALF Proinflammatory Cytokines: IL-6, TNF-α, and IL-1β

Proinflammatory IL-6 levels in BALF demonstrated no differences between male and female mice (4 studies, 6 independent experiments, SMD 0.23, 95% CI -0.49 to 0.95, I^2 = 57%, Figure 5A). Similarly, we found no differences when stratifying data by time of outcome assessment. A single study at six hours post-LPS administration did demonstrate male mice had significantly higher levels of IL-6 in BALF relative to females. TNF-α levels in BALF <24 h after LPS administration presented no significant differences between male and female mice (3 studies, 5 independent experiments, SMD 0.48, 95% CI -0.46 to 1.42, I^2 = 76%, Figure 5B). IL-1β levels in BALF between males and females also presented no significant differences (2 studies, SMD 1.86, 95% CI -1.37 to 5.08, I^2 = 88%, Figure 5C).

Immune Cell Markers: BALF Total Neutrophils and Total Macrophages, and Tissue Myeloperoxidase:

Overall, there was no significant difference between male and female mice regarding total neutrophils in BALF (4 studies, 8 independent experiments, SMD 0.61, 95% CI -0.08 to 1.30, I2 = 72%, Figure 6A), total macrophages in BALF (1 study, 3 independent experiments, SMD 0.30, 95% CI -0.52 to 1.13, I^2=76%, Figure 6B), and lung myeloperoxidase (1 study, 2 independent experiments, SMD -0.14, 95% CI -1.02 to 0.73, I^2=0%, Figure 6C).

2.5.4 Risk of Bias Assessment

A majority of studies were deemed to have an 'unclear' risk of bias across most domains (Figure 7). Three studies presented a low risk for baseline characteristics. Three studies reported blinded assessments (i.e., detection bias) only for selected outcomes (e.g., histological injury), while no studies reported blinding for all outcome assessments. Similarly, three studies had consistent n-values (sample sizes) between the methods and the results and were assessed as having a low risk of bias from incomplete outcome data.

2.6 Discussion

We investigated biological sex differences in LPS-induced ALI, a well-established preclinical model that effectively activates the immune response. (6) We included studies assessing outcomes that mirror key pathophysiologic features of ARDS. (7) The majority of extracted outcome point estimates suggested male mice administered LPS developed a more severe ALI phenotype compared to female mice. The sole study reporting lung injury scores also demonstrated more severe ALI histopathology in male mice. This trend persisted in our metaanalysis of BALF total cell counts and albumin leakage. However, we found no significant differences between male and female mice for all other outcomes that were meta-analyzed. Our study, however, was limited by an overall scarcity of data, small sample sizes within these preclinical studies, and differences in disease induction. This likely contributed to imprecise pooled estimates and high heterogeneity in the majority of our analyses.

It is concerning that one of the most commonly used ALI models lack studies that have examined the influence of biological sex. In 2022, 544 studies employing the LPS ALI model were published, yet a lack of consideration for biological sex persists with not a single study in 2022 reporting sex-stratified data. This gap is particularly troubling given the increasing recognition that biological sex must be considered in preclinical research. Incorporating sex differences enhances research excellence, rigor, and relevance, aligning with guidelines from major funding agencies such as the National Institutes of Health, the Canadian Institutes of Health Research, and the European Commission. (55, 59) While there have been efforts to incorporate male and female animals into preclinical studies, our study aligns with a larger cross-sectional analysis conducted by Woitowich et al. in 2020, which identified that a minority of studies use both sexes and report data in a sex-stratified manner. Failing to report sex-stratified data hinders external validity by failing to identify potential sex-dependent differences in outcomes. Ultimately, gaining a deeper understanding of whether fundamental biological differences exist between males and females may help address the limitations of observational clinical studies on this issue. Identifying whether sex-dependent differences are present may also avoid sex-dependent disparities in the efficacy of downstream therapies, a problem that has notably impacted research in cardiovascular health, cancer, and mental health. (60–62)

We identified several knowledge gaps across all included studies. None of the studies demonstrated alterations in at least three domains that reflect key pathophysiological features of ALI (Figure 2). The American Thoracic Society recommends that at least three domains must be assessed in a model in order to qualify as true "experimental ALI". (7) Furthermore, they emphasize the significance of histological evidence of tissue injury as the foremost defining feature of ALI. Only one study in our review used a scoring system for this purpose. Although there is currently no standardized/validated scoring system for quantifying lung injury, it is recommended that such a system be employed to assess the extent of histological lung injury. (7) In addition, none of the studies were methodologically rigorous in terms of implementing methods to reduce the risk of bias. Methods such as blinding have been clearly associated with less exaggerated effect sizes. (63, 64)

Despite these shortcomings, our results provide evidence, although sub-optimal, suggesting that male mice develop a more severe phenotype than female mice. Biological sex affects both innate and adaptive immune responses, shaping disparities in autoimmunity and infection reactions. (65, 66) The X chromosome harbours crucial immune genes like toll-like receptors (TLR7, TLR8) and interleukin-1 receptor-associated kinase (IRAK1). With two X chromosomes, females boast diverse and redundant immune traits, while males, bearing only one X chromosome, possess 5% fewer gene variants, possibly heightening infection susceptibility and mortality risk. (67) Sex hormones, such as estrogen, progesterone, and testosterone, wield direct influence over immune cell function via their respective receptors in immune cells like monocytes, B cells, and T cells, activating hormone-responsive genes. (68–70) Indeed, in one of the included studies, separate experiments in gonadectomized male mice demonstrated decreased ALI-induced inflammation (i.e., TNF-α and BALF total cell count) compared to intact male mice. (71) Furthermore, another study featured in our review indicated a correlation between resistance to injury and the presence of either endogenous or exogenous estrogen, suggesting a protective role of estrogens against acute inflammation. (72) Interestingly, they also uncovered a suppressive effect of exogenous estrogen on the production of IL-6 and IL-1β both *in vivo* and *in vitro*. This finding highlights a novel pathway through which estrogen appears to mediate LPS-induced lung injury. These results align with other studies demonstrating that exogenous

estradiol administration had a protective effect in male mice after trauma-hemorrhage, resulting in significantly reduced pulmonary edema and neutrophil infiltration. (73–75) Additionally, the ability of estradiol to protect the lung in three different models of lung injury has been identified. (76)

Although the deliberately narrow scope of our review (a direct LPS-induced ALI) could be perceived as a limitation of our study, we will be using this model in a confirmatory multi-laboratory study to examine the effects of biological sex. Thus, we have heeded recommendations to synthesize existing laboratory data prior to considering larger 'confirmatory' studies. (53) Our systematic review has clearly identified knowledge gaps that our confirmatory study will address. First, there is a pressing need to determine whether sex-dependent differences exist in this model of ALI. The paucity of data available suggests that further exploration of this issue in laboratory animals is ethically justified. Similarly, our review has identified the need for studies conducted in a low risk of bias manner. Confirmatory studies will also need to measure an adequate number of domains recommended by ATS to ensure true experimental ALI has been induced. Additionally, effect sizes calculated in our systematic review will be used to inform power analyses and sample sizes for future confirmatory studies. In conclusion, our findings highlight potential sex-dependent differences that warrant further exploration. Our results can serve as a resource for guiding future studies aimed at elucidating sex-related differences and facilitating the development of more effective interventions.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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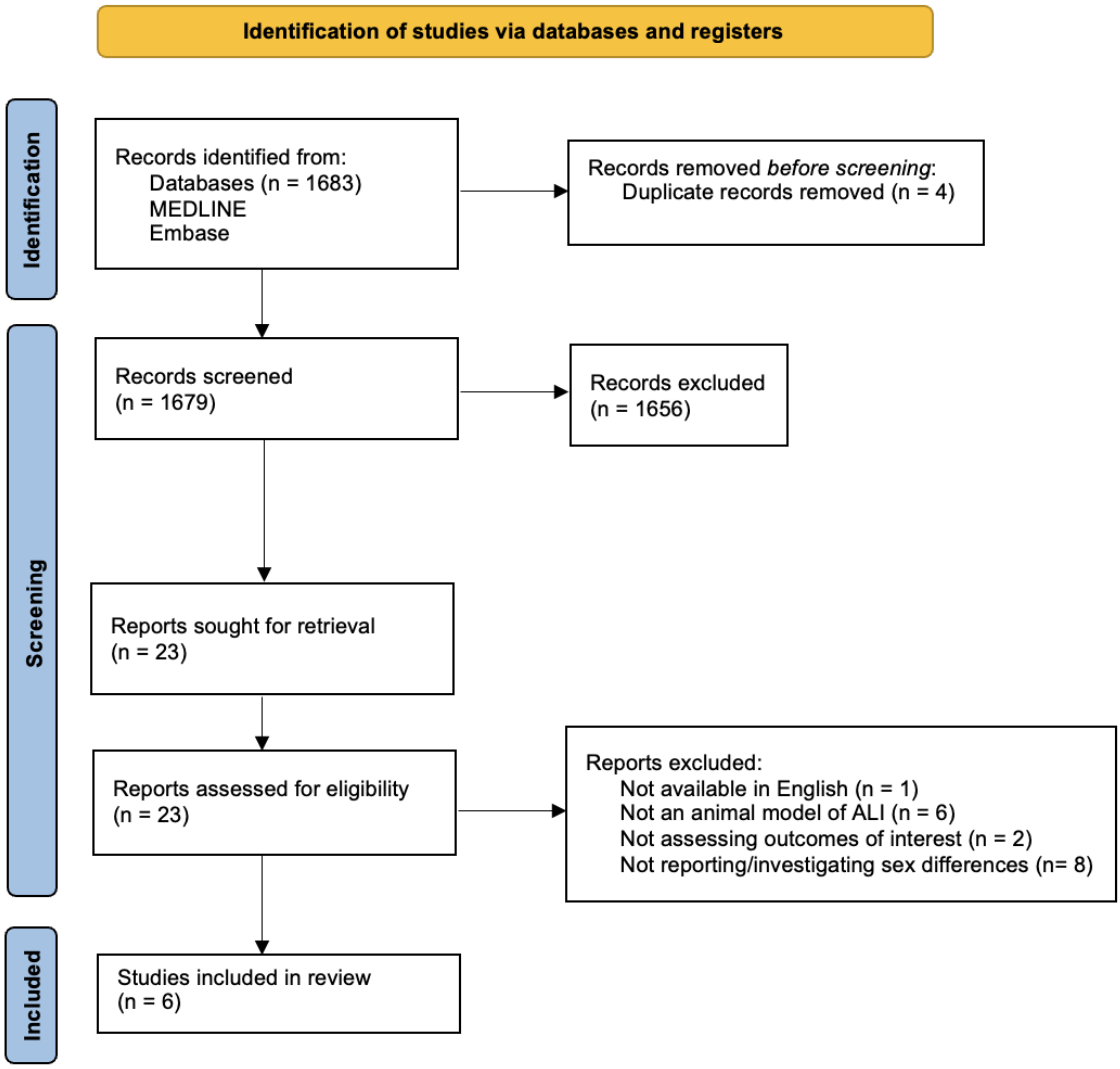

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram detailing study screening and selection.

Table 1: Summary of Study Characteristics of All Included Articles

First Author	Year	Country	Animal Model	Strain	Age (Weeks)	Model of Injury	Reported Dose of LPS	Dose of LPS/kg	Doses (#)	Endpoint (Hours)	Source of Funding
Tesfaigzi, Y	2001	United States	Mouse								Government
Experiment A				C57BL/6	6-8	Intranasal	60µg	2.81mg/kg*	1	24	Foundation
Experiment B				C57BL/6	6-8	Intranasal	60µg	2.81mg/kg*	1	48	
Experiment C				C57BL/6	6-8	Intranasal	60µg	2.81mg/kg*	1	72	
Speyer, C	2004	United States	Mouse	C57BL/6	6-8	Intratracheal	1 mg/kg	1 mg/kg	1	6	Government
Card, J.	2006	United States	Mouse								Government
Experiment A				C57BL/6	8-10	Oropharyngeal aspiration	50 µg	2.25 mg/kg*	1	6	
Experiment B				C57BL/6	8-10	Oropharyngeal aspiration	50 µg	2.25 mg/kg*	1	6	
Experiment C				BALB/c	8-10	Oropharyngeal aspiration	50 µg	2.25 mg/kg*	1	6	
Moitra, J.	2008	United States	Mouse								Government
Experiment A				C57BL/6J	8-12	Intratracheal	2.5 mg/kg	2.5 mg/kg	1	24	
Experiment B				C57BL/6J	30-36	Intratracheal	2.5 mg/kg	2.5 mg/kg	1	24	
Puntorieri, V	2016	Canada	Mouse	C57/BL6	16	Intratracheal	0.5 mg/mL	0.71 mg/kg*	1	18	Academic, Government Foundation
Mock, J. R	2023	United States	Mouse	C57BL/6J	10-12	Intratracheal	3 mg/kg	3 mg/kg	1	72	Academic, Government

* The estimated LPS dose was calculated based on the reported LPS amount and the average body weight of mice within the same strain and age

Outcomes Measured:		Included Studies:										
		Tesfaigzi, Y: A	Tesfaigzi, Y: B	Tesfaigzi, Y: C	Speyer, C	Card, J: A	Card, J: B	Card, J: C	Moitra, J: A	Moitra, J: B	Puntorieri, V	Mock, J
Histological Evidence of Tissue Injury	Histological lung injury score					1.61						
Alterations of the Alveolar Capillary Barrier	Albumin leakage				3.3				1.4	-1.2		
	Total protein								0.61	-0.37	-1.15	-0.08
Inflammatory Response	Cell counts					1.32	1.12	0.67	4.32	-0.66	-0.17	0.01
	IL-1β				3.64	0.34						
	IL-6	0.47	-0.58	0.87	1.16	0.67					-1.4	
	TNF-α				0.06	1.88	0.67	0.9			-1.5	
	Total macrophages	-0.2	0.03	1.2								
	Total neutrophils	0.63	-0.14	0.59	14.63	1.35	1.38	0.7			-0.39	
	Tissue Myeloperoxidase								-0.23	-0.01		

Male Worse Outcome:	Female Worse Outcome:	Effect Size Range:
		> 0.8
		0.5-0.8
		0.2-0.5
		< 0.2
		Outcome not Measured

Figure 2: Heat map illustrating direction of effect in acute lung injury outcomes.The direction of the effect is visually represented by color: blue signifies that males had more severe outcomes, while red indicates that females had more severe outcomes. The magnitude of the effect size is depicted through the intensity of shading.

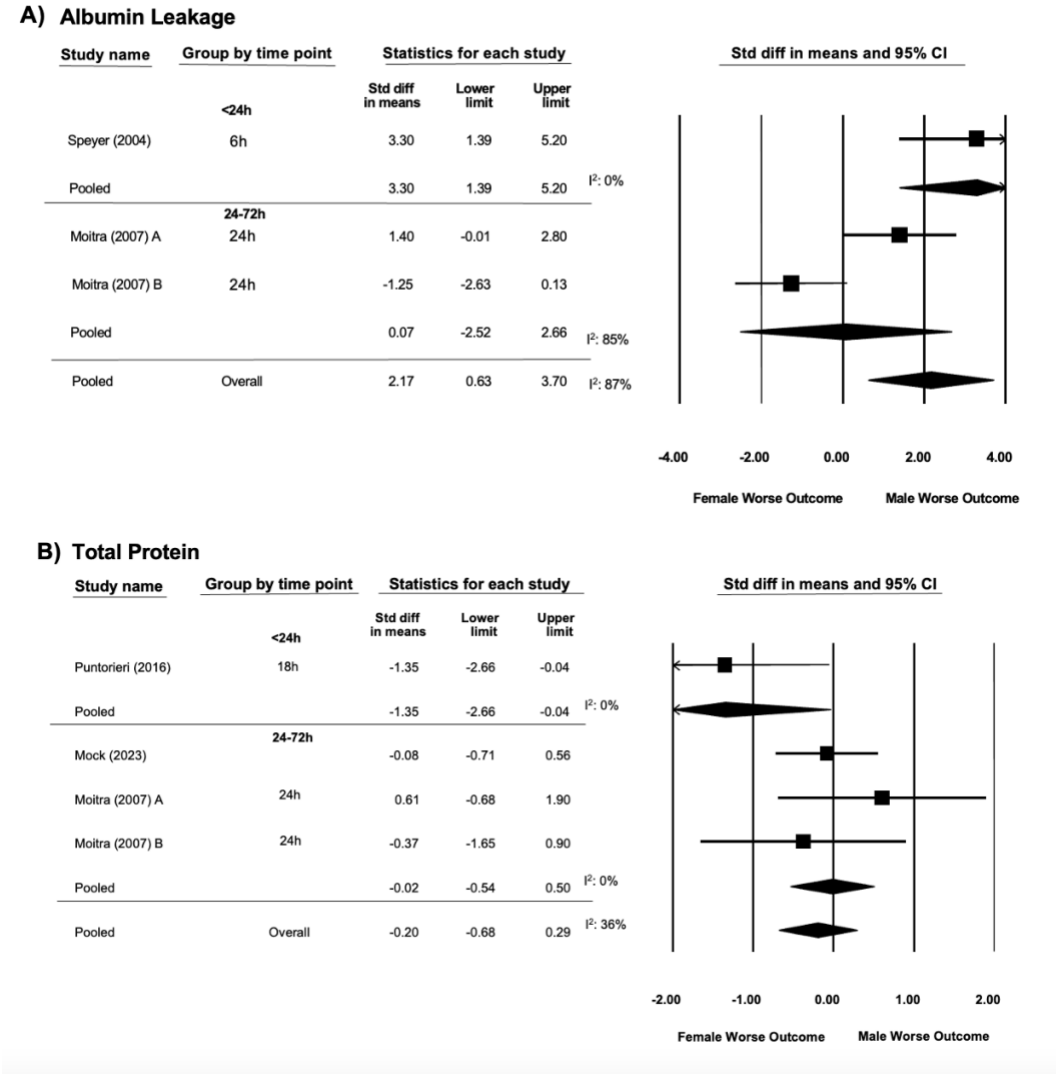

Figure 3: Metaanalysis of all included studies of LPS-induced lung injury that reported the outcome of albumin leakage (A) and bronchoalveolar lavage fluid (BALF) total protein (B). Data are presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favour female animals having worse outcomes and >0 favour males having worse outcomes. The I^2 value represents the statistical heterogeneity.

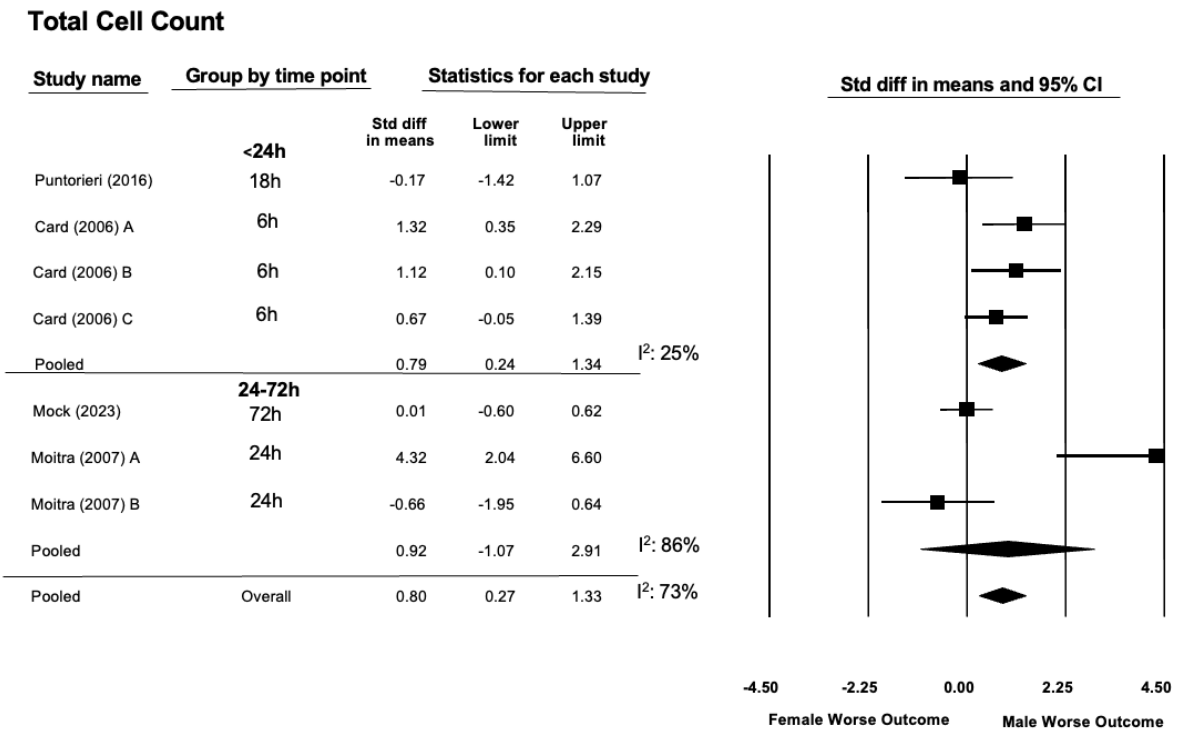

Figure 4: Metaanalysis of all included studies of LPS-induced lung injury that reported the outcome of bronchoalveolar lavage fluid (BALF) total cell counts. Data are presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favour female animals having worse outcomes and >0 favour males having worse outcomes. The I^2 value represents the statistical heterogeneity.

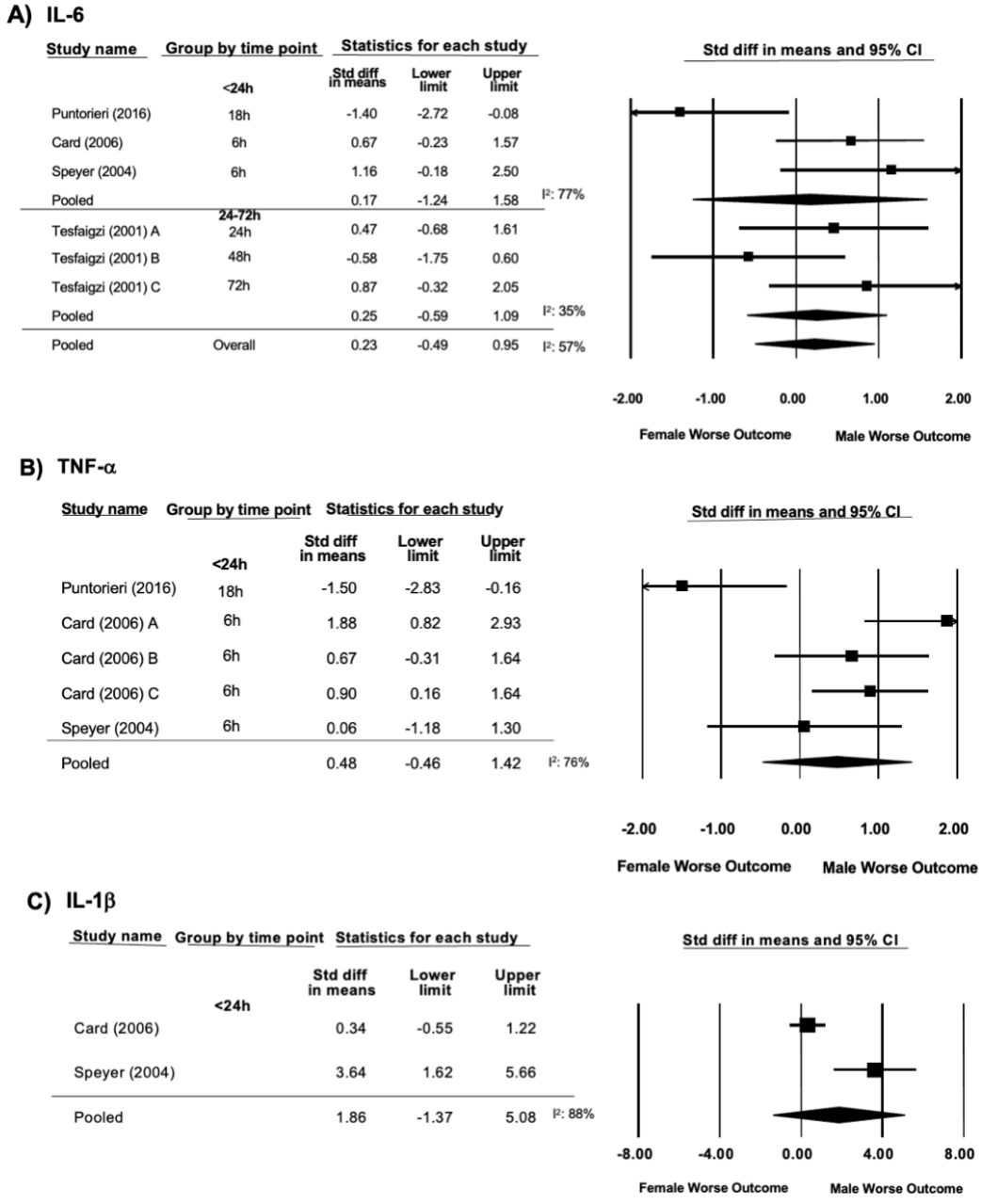

Figure 5: Metaanalysis of all included studies of LPS-induced lung injury reporting bronchoalveolar lavage fluid (BALF) IL-6 (A), TNF-α (B), and IL-1β (C) concentration. Data are presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favour female animals having worse outcomes and >0 favour males having worse outcomes. The I^2 value represents the statistical heterogeneity.

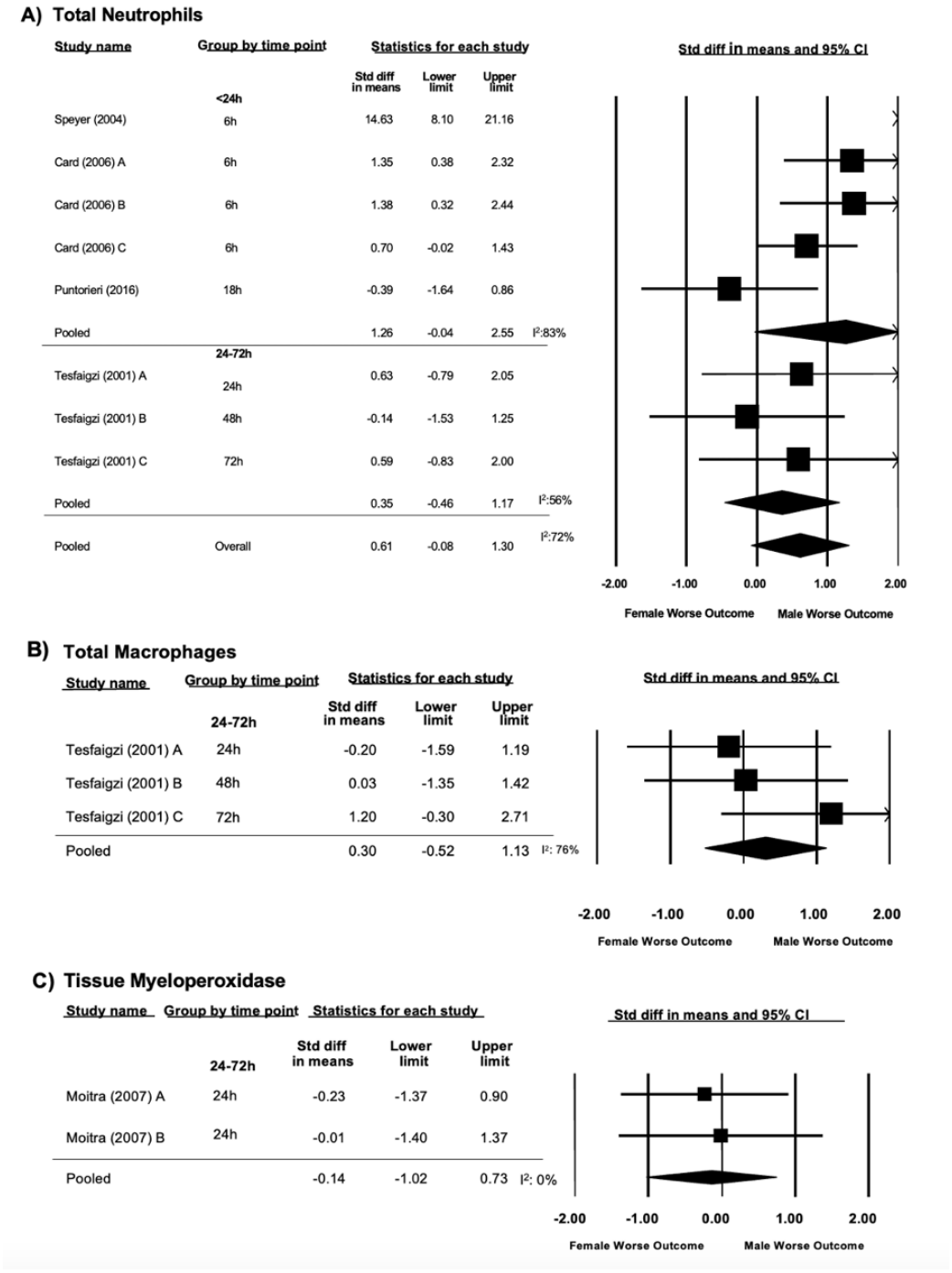

Figure 6: Metaanalysis of all included studies of LPS-induced lung injury that reported the outcome of total neutrophils (A), total macrophages (B), and tissue myeloperoxidase content (C). Data are presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favour female animals having worse outcomes and >0 favour males having worse outcomes. The I^2 value represents the statistical heterogeneity.

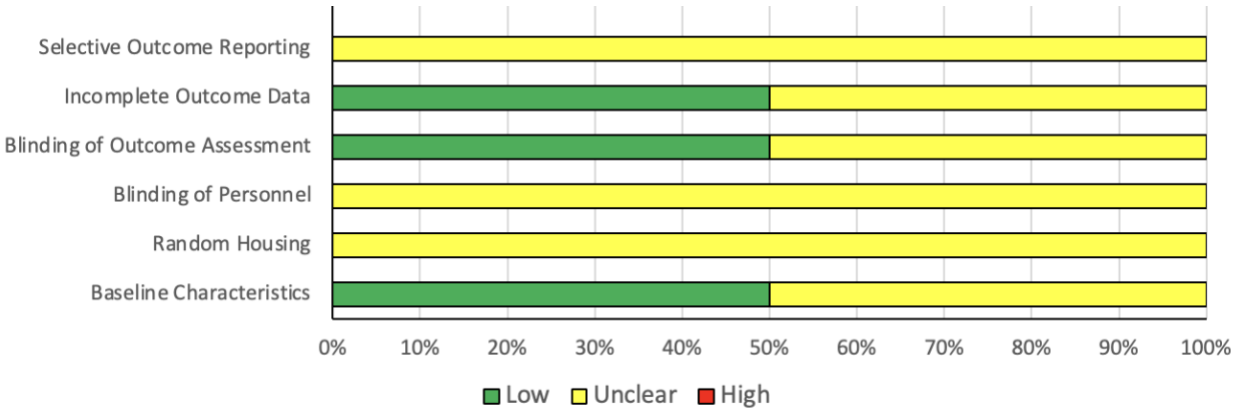

Figure 7: Risk of Bias Assessment

Supplemental Figure 1. Search Strategy

MEDLINE/Ovid <1946 to March 20, 2023>

1 exp Respiratory Distress Syndrome, Adult/ or exp Acute Lung Injury/ 47035

2 ((lung or pulmonary) adj3 (injur* or damage or inflammation)).tw,kf. 62738

3 ((acute or adult) adj respiratory distress syndrome).tw,kf. 26338

4 ards.tw,kf. 18931

5 ali.tw,kf. 10027

6 exp endotoxins/ 116872

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Supplemental Figure 2. PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Pages 3-4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Pages 5-6
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 6
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 6, Supp. File 2
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Pages 7-8
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Pages 7-8
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Pages 5-6
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Pages 5-6
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 8
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Pages 7-8
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Pages 5-8
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Pages 5-8

Section and Topic	Item #	Checklist item	Location where item is reported
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Pages 5-8
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Pages 5-8
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	N/A
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 8
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 9, Figure 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Figure 1
Study characteristics	17	Cite each included study and present its characteristics.	Pages 9-12 Table 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Pages 20-21 Figure 7
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Pages 14-21 Figures 3-6
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Pages 12-20
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Pages 12-20
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Pages 18-21
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	N/A
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Pages 22-23
	23b	Discuss any limitations of the evidence included in the review.	Pages 22-23

Section and Topic	Item #	Checklist item	Location where item is reported
	23c	Discuss any limitations of the review processes used.	Pages 22-23
	23d	Discuss implications of the results for practice, policy, and future research.	Pages 23-25
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Page 2
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Page 2
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Page 25
Competing interests	26	Declare any competing interests of review authors.	Page 25
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Page 25

Chapter 3 – Comparative evaluation of LPS administration routes for inducting acute lung injury in murine models: efficacy, consistency, and technical considerations

3.1 Preface

This manuscript presents a detailed comparative analysis of different routes of LPS administration in inducing ALI in murine models. A critical focus of this work is to address the inconsistencies observed in preclinical studies by systematically evaluating the impact of varying LPS delivery methods on the severity and reproducibility of lung injury. Specifically, we compare intratracheal intubation, intranasal instillation, and surgical trans-tracheal routes via either needle or catheter methods, to understand how these different approaches influence the extent of lung injury and model variability.

The results of this study are essential for refining experimental techniques in the LPS-induced ALI model. By identifying the most consistent method of LPS delivery, we aim to reduce unwanted experimental variability. Moreover, by including both male and female animals, I aimed to better understand the effect of biological sex results observed when varying administration routes. This begins to address the significant knowledge gap identified in my previous chapter.

This manuscript contributes to the growing body of literature aimed at improving preclinical rigor and also lays a necessary foundation for our future multilaboratory studies. By establishing standardized protocols for LPS administration, this manuscript provides a framework that will guide collaborative research across multiple laboratories. This standardization, and reduction of unwanted variability, is key to address reproducibility. The comparative evaluation presented here supports the broader objectives of my thesis: to refine preclinical models of ARDS and ensure their reliability for both male and female subjects.

This manuscript will be submitted for peer-reviewed publication.

Eva Kuhar[1,2], Duncan J. Stewart[2,3,4], Doreen Engelberts[1], Forough Jahandideh[1], Matthew S. Jeffers[1,5], Haibo Zhang[6,7], Julie Khang[6], Arnold S. Kristof[8], Bernard Thebaud[3,9], Arul Vadivel[3], Dean A. Fergusson[1,4,5,10], Manoj M. Lalu[1,2,3,5,11]

Author Affiliations

[1]Clinical Epidemiology Program, Blueprint Translational Research Group, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
[2]Department of Cellular and Molecular Medicine, University of Ottawa, Ontario, Canada
[3]Regenerative Medicine Program, The Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
[4]Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada
[5]School of Epidemiology and Public Health, University of Ottawa, Ottawa, Ontario, Canada
[6]Keenan Research Centre for Biomedical Science, St. Michael's Hospital, Unity Health Toronto, Ontario, Canada
[7]Departments of Anesthesiology and Pain Medicine and Physiology, Interdepartmental Division
[8]Meakins-Christie Laboratories and Translational Research in Respiratory Diseases Program, Research Institute of the McGill University Health Centre, Faculty of Medicine, McGill University, Montreal, Quebec, Canada.
[9]Department of Pediatrics, The Ottawa Hospital and the Children's Hospital of Eastern Ontario
[10]Department of Surgery, University of Ottawa, Ottawa, Ontario, Canada
[11]Department of Anesthesiology and Pain Medicine, The Ottawa Hospital, University of Ottawa, Ottawa, Ontario, Canada

Contributions (using CRediT Taxonomy)

Guarantor: MML; Conceptualization: EK, MML, DAF, and DS; Methodology: EK, MML, and DAF; Investigation: EK and DE; Writing – original draft: EK, MML, FJ; Writing – review and editing: all authors; Supervision: MML, DAF, and DS; Resources: DAF and MML.

3.2 Abstract

Background:

Preclinical lipopolysaccharide (LPS) acute lung injury (ALI) models are commonly used to study acute lung injury and acute respiratory distress syndrome. However, even subtle differences between preclinical models can significantly affect severity and reproducibility of lung injury. We characterized the effect of varying the method of lung delivery of LPS to facilitate comparison of data from different preclinical ALI models.

Objective:

To compare the severity and variability of ALI across different routes of LPS administration in mice.

Methods:

We administered LPS (2.25 mg/kg) to male and female C57BL/6 mice via four routes: 1) intratracheal installation by intubation; 2) intranasal instillation, 3) surgical trans-tracheal injection by needle puncture or 4) catheter placement. We assessed the severity and variability of ALI at 72 hours post-treatment by histological scoring, bronchoalveolar lavage fluid (BALF) measurements, which included total protein concentration and inflammatory markers such as total cell counts and IL-6 levels. The relative distribution of Evans Blue to the lungs and stomach was also assessed for each model.

Results:

The four routes resulted in different lung injury patterns, with the trans-tracheal catheter route causing significantly greater lung injury scores compared to the intratracheal intubation and intranasal routes (0.76 ± 0.03, vs. 0.67 ± 0.03, and 0.59 ± 0.05, mean ± SEM). Both trans-tracheal routes were characterized by greater alveolar neutrophil counts, increased proteinaceous debris, fewer hyaline membranes, and lower inter-subject variability than non-surgical routes. The trans-tracheal with catheter route showed higher BALF total cell counts ($7.15 \times 10^6 \pm 3.5 \times 10^5$ vs. $5.5 \times 10^6 \pm 3.0 \times 10^5$) and IL-6 levels ($185 \pm 21.3$ vs. 95.7 ± 21.3) compared to the intratracheal intubation route, with more localized Evans Blue dye distribution in the lungs and

minimal leakage into the stomach compared to other routes. Male mice exhibited more severe lung injury scores and higher protein concentrations than females.

Conclusion:

Surgical trans-tracheal administration of LPS produced the most robust and least variable ALI phenotype in our study. While both surgical trans-tracheal routes offer precision and consistency, practical considerations such as procedural complexity, resource requirements, and the learning curve should also guide model selection. Our results will allow researchers to better tailor their choice of model to align with their specific study objectives and downstream translational goals. In addition, our findings should prompt further exploration of sex-specific responses in ALI.

3.3 Introduction

Laboratory animal models of Acute Lung Injury (ALI) serve as crucial preclinical tools to study Acute Respiratory Distress Syndrome (ARDS), providing essential insights into disease pathology and therapeutic potential. (2, 33, 39, 77) Among these models, the lipopolysaccharide (LPS) model is one of the most widely used. LPS, derived from the outer membrane of Gram-negative bacteria, triggers a robust inflammatory response that closely parallels the systemic inflammation seen in ARDS. (6, 33, 78, 79) Various methods of administration have been employed to deliver LPS to the lungs, including intratracheal intubation, intranasal instillation, and trans-tracheal instillation. Understanding the impact of different LPS administration methods on the severity and overall model phenotype is crucial for refining experimental techniques and minimizing unwanted variability.

Researchers have investigated various LPS delivery methods, each with distinct advantages and challenges. Delivery via an endotracheal catheter provides precision in localization and dosage control, which can enhance efficacy and reproducibility compared to oropharyngeal aspiration. (80–82) However, intubation requires technical expertise and carries risks of trauma and interoperator variability. Intranasal instillation is commonly used for its less invasive nature, but may result in inconsistent delivery and potential gastrointestinal contamination, which can alter lung injury severity, as observed in several studies. (80, 82, 83) Trans-tracheal instillation, offers direct and controlled administration (i.e., ensuring consistent lung delivery) but demands advanced surgical skills and the use of constantly monitored deep anesthesia, introducing procedural variability that may affect reproducibility.

Despite extensive research on LPS delivery methods, a comprehensive comparison of the various routes of administration has not been conducted. (80, 83–86) We hypothesized that different routes of LPS administration not only influence the severity and reproducibility of acute lung injury (ALI) but also affect specific histological features of lung injury (e.g., alveolar hemorrhage, neutrophil infiltration, and hyaline membrane formation), as well as a comparison of variability between routes. (6) As well, we investigated how these routes of delivery influenced the distribution of solutions between the lungs and extra-pulmonary tissue. By testing

this hypothesis, we aim to refine our understanding of how delivery routes shape lung pathology. Finally, we investigated whether biological sex acts as an effect modifier in these outcomes, given emerging evidence of sex differences in inflammatory responses.

3.4 Materials and Methods

This report follows the ARRIVE 2.0 Guidelines (completed checklist in Supplemental 1). (87)

3.4.1 Animals, Housing, and Husbandry

We conducted all animal experiments in accordance with the Canadian Council of Animal Care Guide to Care and Use of Experimental Animals and received approval from the Institutional Animal Care and Veterinary Services at the University of Ottawa (Protocol #OHRI 3501). We used 9- to 11-week-old male and female C57BL/6 mice weighing 17-24g (Charles River Laboratories, Laval, QC, Canada). Mice were individually housed in Tecniplast ventilated cages (Sealsafe Plus GM500) in a temperature and humidity-controlled facility with a 12-hour light-dark cycle. Mice were provided with ad libitum access to food, water, and enrichment materials (cardboard hut, 2 nestlets, and loose crinkle paper).

3.4.2 Experimental Groups and Methods Of Administration

Mice were assigned to four separate experimental groups, each corresponding to a different route of LPS administration, with each route having its own dedicated control group. The total number of animals allocated, including both experimental and control groups, are detailed in Supplemental Table 1. To induce ALI, mice received 2.25 mg/kg lipopolysaccharide (LPS; Escherichia coli, O55:B5, Millipore Sigma, Cat# L4524) in a vehicle of 40 μL phosphate buffered saline (PBS). Control animals received 40 μL PBS vehicle alone. Following all administration procedures described below, we placed all mice in a cage on a warming blanket set to 37°C, monitored them until fully mobile, and we then returned the mice to standard housing.

We initially characterized the LPS-ALI model using the intratracheal intubation with catheter method. We sacrificed mice at 6-, 24-, 48-, and 72-hours post-administration. In subsequent experiments comparing methods of administration, we sacrificed animals at the 72 h timepoint where we saw the most significant histological evidence of lung injury. The four methods to administer LPS that we compared are described in detail below and depicted in Figure 1. Note

that anesthesia and analgesia needed to be varied between methods to comply with institutional requirements, match invasiveness, procedural length, and post-procedural pain/discomfort. All methods of administration were completed by a single research animal technician (DE) with over 20 years of experience with small animal models of ALI.

***Intratracheal instillation- intubation with catheter*:**

We anesthetized each mouse with 75 mg/kg ketamine and 5 mg/kg xylazine (intraperitoneally) and then placed it in an isoflurane induction chamber (1 to 2% concentration) until surgical plane of anesthesia was achieved, confirmed by the absence of response to a toe pinch. We then positioned the mouse on an angled intubation board tilted to approximately 60 degrees, illuminating the neck with a focused bright light (Figure 1A). Using gentle finger retraction of the tongue, we placed a small depressor at the back of the tongue to aid in visualizing the vocal cords. We then inserted a 24-gauge angiocatheter into the trachea using an optical light to guide the intubation needle. We removed the stylet and then attached a 1cc syringe containing a 60 µL meniscus of water in the barrel. Correct intratracheal placement of the angiocatheter resulted in movement of meniscus in sync with the respiratory cycle. We then removed the 1 cc syringe and deposited the LPS or PBS into the catheter hub, followed by a gentle air puff using a resuscitator (bulb of a plastic disposable transfer pipette) to ensure complete delivery of the fluid to the lung.

Intranasal instillation:

We placed each mouse in an isoflurane induction chamber (3-5% concentration) until fully anesthetized, confirmed by the absence of response to a toe pinch. Once anesthetized, we held the mouse upright in the technician's left hand (Figure 1B). Using a fine 20 µL pipette tip, we applied a small drop of the LPS or PBS solution to the edge of the right nostril, allowing the mouse to inhale the droplet with normal respiration. This process was repeated until the full volume of 40 µL was inhaled. If the mouse began to emerge from anesthesia during the procedure, we returned it to the isoflurane chamber until fully re-anesthetized.

Trans-tracheal instillation-needle or catheter:

We administered 0.1 mg/kg buprenorphine subcutaneously to each mouse one hour before surgery. At the time of surgery, we placed the mouse in an isoflurane induction chamber (3-5%) until unresponsive to a toe pinch. We positioned the mice on a surgical table with an isoflurane nose cone (2-3%). The neck area was shaved, disinfected with 70% ethanol, and a 5-8 mm midline incision was made above the trachea. The trachea was exposed by blunt dissection of the thin muscle above the trachea. For "trans-tracheal with needle" route we inserted a 25-gauge needle into the trachea under direct visualization (Figure 1C). For "trans-tracheal with catheter" route we inserted a 24-gauge angiocatheter with rigid stylet under direct visualization; once the catheter was in place, the stylet was removed (Figure 1D). We then administered either LPS or PBS, followed by 150 μL of air to ensure dispersion of the solution. We closed the incision using 4-0 Vicryl suture and applied topical bupivacaine (7.5%) immediately after suturing and again 6 hours post-surgery.

Plasma Collection:

At sacrifice, we anesthetized the mice (150 mg/kg ketamine and 10 mg/kg xylazine), removed midline skin, and opened the thorax via a lateral rib incision to expose the heart. We then performed cardiac puncture collected in a heparin coated syringe and immediately placed the collected blood on ice for processing. We centrifuged the whole blood at 1500g for 10 minutes at 4°C to collect plasma, which was then stored at −80°C until subsequent analysis.

BALF Supernatant Collection:

After collecting the blood, we inserted and secured an angiocatheter into the trachea and placed an aneurysm clip (Micro Serrefines, 8x 2 mm Cat# 18055-02, Fine Surgical Instruments) on the left main stem bronchus. We then lavaged the right lung by instilling and withdrawing 0.5 mL normal saline (0.9%) three times. We collected the bronchioalveolar lavage fluid (BALF) and immediately put it on ice for processing. After determining total cell count of the recovered BALF, we centrifuged it at 500g for 10 minutes at 4°C. We collected the BALF supernatant and stored it at −80°C for subsequent analyses.

Lung Tissue Sample Collection:

After lavaging the right lung, we clamped the right main stem bronchus using a mosquito forcep and removed the aneurysm clip from the left main stem bronchus. We prepared syringes containing 0.5mL of optimal cutting temperature (OCT) medium (1:1 ratio with PBS) and inserted them into the angiocatheter. We injected approximately 350-500 µL OCT until the lung appeared fully inflated. We dissected the left lobe and carefully removed the bronchi. We then sectioned the left lobe transversely into five equal parts. We submerged middle sections 2 and 4 in 4% paraformaldehyde for approximately 24-48 hours.

3.4.3 Histologic Analysis - Primary Outcome

The fixed lungs were rinsed in PBS, dehydrated in 70% ethanol, and embedded in paraffin. Transverse sections of 5 µm thickness were cut from each mouse lung and stained with hematoxylin and eosin (H&E) before digital scanning using the Panoramic DESK scanner (3DHISTECH). We evaluated five randomly selected, non-overlapping fields per mouse using a semiquantitative, weighted scoring system adapted for mouse models of acute lung injury (ALI). (6) This system assessed five parameters: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris in the airspaces, and alveolar septal thickening. Each parameter was scored for each field, where a score of 0 indicated absence, 1 indicated mild presence, and 2 indicated severe presence of the feature. (6) The overall lung injury score was calculated by summing the weighted scores of these features and normalizing the total to the number of fields evaluated, resulting in a continuous score between 0 and 1, with a score of 1.0 representing the most severe lung injury. The evaluation was conducted by a blinded lab member who was unaware of the treatment group assignments. To further investigate whether the different routes of administration demonstrated distinct phenotypes in the individual parameters, each of the five parameters was analyzed separately. For each mouse, the five fields could collectively yield a maximum score of 15 per parameter.

3.4.4 Assessment Of LPS-Induced Airway Inflammation

We analyzed the recovered BALF total cell count using an automatic cell counter (Invitrogen Thermo). We analyzed both BALF supernatant and plasma for IL-6 levels using a commercially available ELISA assay (ThermoFisher, Cat #216-16-10UG).

3.4.5 Assessment of Alveolar Permeability

We measured the total protein content in BALF samples from all experiments using a Bradford protein assay (BIO-RAD, Cat # 5000001).

3.4.6 Evans Blue Administration

We administered Evans Blue dye to evaluate the efficiency of the four administration methods in delivering instilled solutions to the lungs. We randomly assigned 16 male and female mice, weighing between 20-23g, to groups receiving intratracheal intubation, intranasal, trans-tracheal with needle, and trans-tracheal with catheter administration. Each mouse received 40 μL of 0.3% Evans Blue dye. Fifteen minutes post-administration, we anesthetized the mice with 150 mg/kg ketamine and 10 mg/kg xylazine and euthanized them by exsanguination through the carotid artery. We opened the abdominal and thoracic cavities to expose the organs and flushed the lungs by injecting 1 mL saline into the right ventricle and out through a small incision in the left atrium. We then removed the lungs and stomach (including 9 mm segment of intestine), weighed the tissues, and homogenized them to extract Evans Blue (1 mL formamide at 55 °C for 18-24 hours). We centrifuged the homogenates at 5,000 *g* and assessed absorbance of the supernatant using a spectrophotometer. (620 nm) The concentration was determined by comparing to a standard curve generated with known concentrations of Evans Blue.

3.4.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.1. For the time course and outcomes across the four models, data were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test, with $P < 0.05$ considered statistically significant. Variance between routes of administration was assessed using the Fligner-Killeen test to evaluate the homogeneity of variances across the groups. For the Evans Blue assay, statistical comparisons were conducted using two-way ANOVA followed by Tukey's post hoc test to assess differences among groups. All tests applied a significance level of $P < 0.05$.

3.5 Results

3.5.1 Characterization of Inflammatory and Lung Injury Outcomes in the Intratracheal Instillation-Intubation Model

We sacrificed mice 6, 24, 48, and 72 h following LPS administration via the intratracheal - intubation model to assess the time course of lung injury.

Histological Evidence of Tissue Injury:

Histological analysis revealed progressive lung injury over time. Lung injury scores increased from 6 to 72 hours post-LPS, with mild edema and cellular infiltration observed at 6 hours. By 24 hours, inflammatory cell accumulation became more pronounced, culminating in the most severe injury at 72 hours, characterized by widespread tissue damage and extensive inflammatory cell infiltration (Figure 2A-F).

Measurement of Alterations of the Alveolar Capillary Barrier:

BALF protein concentrations increased significantly over time, with levels rising from control values to significantly higher concentrations between 24 and 72 hours post-LPS, indicating a disruption of the alveolar-capillary barrier (Figure 2G).

Inflammatory Biomarkers:

Total cell counts in BALF increased significantly at 6 hours after LPS exposure and remained elevated through 72 hours, reflecting an influx of inflammatory cells (Figure 2H). Both BALF and plasma IL-6 levels peaked at 6 hours post-LPS and decreased over the subsequent time points, though they remained significantly elevated compared to control animals (Figure 2I, 2J).

3.5.2 Comparison of Four Routes of LPS Administration On Outcomes of ALI

Histological Evidence of Tissue Injury:

We compared lung injury severity at 72 hours, the time point selected based on our preliminary time course experiments and existing literature (6, 88), across different routes of LPS administration. While all four routes resulted in significant lung injury, we observed notable differences in lung injury scores between the administration routes. The trans-tracheal method with catheter produced the highest injury score (0.76 ± 0.03, mean ± SEM), significantly higher that with delivery by intubation (0.67 ± 0.03) or intranasal instillation (0.59 ± 0.05) (Figure 3A). The trans-tracheal with needle route showed similar scores to the trans-tracheal catheter route (0.72 ± 0.07).

Further examination of individual histological parameters (6, 33) revealed that the trans-tracheal with catheter route resulted in significantly higher neutrophil counts in both alveolar and interstitial spaces compared to the intubation and intranasal routes (Figure 3B, 3C). This route also caused substantial increases in proteinaceous debris and thickening of the alveolar septa (Figure 3E, 3F). Interestingly, despite the increased severity of these markers, the trans-tracheal routes were associated with significantly fewer hyaline membranes (Figure 3D), suggesting a distinct injury phenotype characterized by pronounced inflammation and less detectable hyaline membrane formation.

The intratracheal intubation and trans-tracheal with catheter routes showed sex-dependent differences in histological lung injury scores and BALF protein concentrations, with male mice exhibiting more severe lung injury (Supplemental Figure 1A, 1D).

Variability analysis using the Fligner-Killeen test indicated that differences in variance were observed between the trans-tracheal routes and non-trans-tracheal routes of LPS administration (Table 1). This suggested that magnitude of between animal variability was greater in the intranasal and intubation groups versus either the trans-tracheal with needle or trans-tracheal with catheter routes.

Taken together, results of histological analysis suggested that trans-tracheal routes, particularly the catheter method, induced more severe lung injury with less variability.

Secondary Outcomes: Inflammatory Markers and Alveolar Permeability

At 72 hours, all administration routes resulted in a significant increase in total BALF cells, with the trans-tracheal catheter route leading to a significantly higher cell count compared to the intubation and trans-tracheal with needle routes ($7.15 \times 10^6 \pm 3.5 \times 10^5$ cells vs. $5.5 \times 10^6 \pm 3.0 \times 10^5$ cells and $4.31 \times 10^6 \pm 5.9 \times 10^5$ cells, respectively, Figure 4A). No significant sex differences were observed in BALF cell counts.

IL-6 concentrations in BALF increased significantly across all administration routes at 72 hours, with the trans-tracheal catheter route exhibiting significantly higher levels compared to the intubation route (185 ± 21.3 vs. 95.7 ± 21.3 pg/mL, Figure 4B). No significant sex differences were observed in IL-6.

All routes of LPS administration increased BALF protein concentration, indicating disruption of the alveolar-capillary barrier. The intubation, intranasal, trans-tracheal with needle, and trans-tracheal catheter routes showed similarly elevated protein levels (1.88 ± 0.11, 1.55 ± 0.31 µg/µL, 1.66 ± 0.78 µg/µL, and 2.24 ± 0.16 µg/µL, respectively, Figure 4C). The intranasal route displayed sex-dependent differences in BALF protein concentration (Supplemental Figure 1B), while the trans-tracheal catheter route demonstrated no sex-dependent differences (Supplemental Figure 1C).

3.5.2 Evans Blue Distribution in Different Routes of LPS Administration

Given the observed differences in lung injury severity, we compared the efficiency of pulmonary delivery by administering Evans Blue dye via the via four different routes: intratracheal instillation-intubation (Figure 5A), intranasal instillation (Figure 5B), trans-tracheal instillation-needle (Figure 5C), and trans-tracheal instillation-catheter (Figure 5D) In the images, "L" indicates lungs and "S" indicates the stomach (including a 9 mm segment of intestine). The intratracheal intubation group effectively targeted the lungs, with some spillover into the stomach (0.37 ± 0.41 vs. 0.315 ± 0.217, Figure 5A, 5E). The intranasal route led to minimal uptake of dye in the lungs and stomach, suggesting poor inhalation of LPS, or its absorption in

the upper respiratory tract (0.508 ± 0.218 vs. 0.798 ± 0.362, Figure 5B, 5E). The trans-tracheal with needle route demonstrated significantly greater lung distribution with minimal spillover into the stomach (3.15 ± 0.940 vs. 0.218 ± 0.193, Figure 5C, 5E). The trans-tracheal with catheter route yielded the most intense lung distribution and negligible spillover to the stomach (5.73 ± 0.61 vs. 0.170 ± 0.145, Figure 5D, 5E).

3.6 Discussion

In this study, we assessed the efficacy of four LPS administration routes—intratracheal intubation, intranasal instillation, and trans-tracheal instillation via needle or catheter—in a preclinical model of LPS-induced ALI. Our results demonstrate that each administration route induces distinct lung injury phenotypes, characterized by varying severity, variability, and distribution of administered solution. **Figure 6** provides a comprehensive summary of the advantages and disadvantages of each administration route, highlighting technical considerations alongside their effects on lung injury severity and variability. Notably, the trans-tracheal with catheter and needle routes induced significantly higher lung injury scores compared to non-surgical routes. Trans-tracheal routes led to significantly less inter-subject variability compared to non-surgical routes. Both trans-tracheal routes were associated with greater delivery of solution to the lungs, and a distinct lung injury phenotype, characterized by significantly higher neutrophil counts in the alveolar (vs. intratracheal intubation and intranasal) and interstitial (vs. intratracheal intubation) spaces, and fewer hyaline membranes, compared to non-surgical routes. Additionally, our findings reveal that biological sex may significantly influence injury severity, with male mice exhibiting a more severe phenotype compared to females. These findings underscore the importance of carefully selecting the LPS administration route and considering biological sex in ALI models, as each factor uniquely influences injury severity, phenotype, and variability.

Despite the widespread use of the LPS ALI model, we were only able to identify two studies that compared routes of administration, none of which compared multiple routes as we did. Liu et al. compared intratracheal intubation and trans-tracheal instillation via catheter, using 5 mg/kg LPS and a 24-hour endpoint. Similar to our results, the trans-tracheal route with catheter caused

significantly more pulmonary inflammation. In addition, they found the trans-tracheal route resulted in increased neutrophil infiltration, exudative pulmonary edema, and a higher lung wet/dry weight ratio. BALF analysis further confirmed more severe cell injury, with elevated IL-8, lactate dehydrogenase, alkaline phosphatase, and total protein levels. (89) Conversely, Khadangi et al. compared intratracheal and intranasal LPS administration, finding that while both routes induced neutrophilic inflammation and lung damage, intratracheal intubation showed greater variability, which they attributed to technical challenges like visualizing the trachea and catheter placement. (80) This suggests that technical factors, such as catheter placement and inconsistent delivery, likely contribute to variability.

Beyond LPS studies, a broader body of literature has investigated other instilled substances such as silica, microparticles, and live bacteria. Evidence suggests that intratracheal instillation, compared to intranasal delivery, results in more efficient and uniform lung deposition, producing stronger inflammatory responses with silica administration, deeper pulmonary penetration of microparticles, and more consistent lung colonization in infection models such as Acinetobacter baumannii. (83, 86, 90) Conversely, Lakatos et al. (2006) found that oropharyngeal aspiration led to more uniform silica distribution and lower inter-animal variability than intratracheal instillation, which exhibited greater variability and less distal lung distribution. (91) This discrepancy may be due to the particulate nature of silica, which may disperse more uniformly in the lungs during oropharyngeal aspiration compared to intratracheal instillation.

Although our study found that trans-tracheal routes are superior in terms of increased severity and reduced variability, we would stress that selecting the most appropriate model for LPS-induced ALI should take into account practical considerations such as ease of use, procedural time, cost, and the learning curve. Based on our experience, trans-tracheal routes require approximately 10-15 additional minutes per animal, incur procedural costs about three times higher due to analgesia, work hours, and materials. These invasive routes also demand advanced training, to avoid procedural pitfalls. Indeed, despite experience, our technician needs frequent practice with these methods to achieve reliable results, underscoring their technical complexity. Ultimately, the choice of LPS route of administration should also align with specific research

objectives and resource availability, ensuring that the selected model best meets a particular study's needs and constraints.

One limitation of our study is the selection of the 72-hour time point for assessing lung injury. This time point was chosen based on preliminary experiments and existing literature, which indicate significant injury severity at this stage. However, different time points represent distinct stages of ALI, each with its own injury patterns and severities. For example, earlier time points might capture initial inflammatory responses, while later ones might reveal chronic injury or recovery phases. (6, 33, 71, 92) Additionally, our use of C57BL/6 mice of a specific age and a particular LPS concentration was guided by their extensive use in preclinical research, which facilitates comparability with other studies. (89, 91, 93) Despite this, LPS responses can vary between strains, and our results may not fully generalize to other species or dosing regimens. (94) While we focused on histological scores, total protein, total cell counts, and IL-6 content, incorporating additional metrics such as extravascular lung water content, protein accumulation in airspaces, and inflammatory markers like total neutrophil counts and myeloperoxidase could provide a more comprehensive evaluation of lung injury and inflammation. (6, 33, 88) Although our study was designed to model severe and consistent lung injury with a focus on histological validation, we recognize that different models, time points, or outcome measures might yield varying results. Therefore, the choice of methodology should be tailored to align with the specific objectives of each study to ensure the most accurate and relevant outcomes.

Future research should address the gaps identified in our study by investigating key factors influencing ALI models. These include exploring different time points, LPS concentrations, mouse strains, and species, as well as evaluating alternative outcomes relevant to ALI. Additionally, the practicality, reproducibility, and standardization of the trans-tracheal with catheter model needs further exploration. Our research group plans to conduct a rigorously designed multilaboratory study to address these aspects by evaluating several key factors. We will investigate the ease of across different research settings, assess the severity of injury outcomes using standardized LPS sources, and examine the variability in model performance among different research groups.

Figure 1. Diagram of four routes of LPS administration

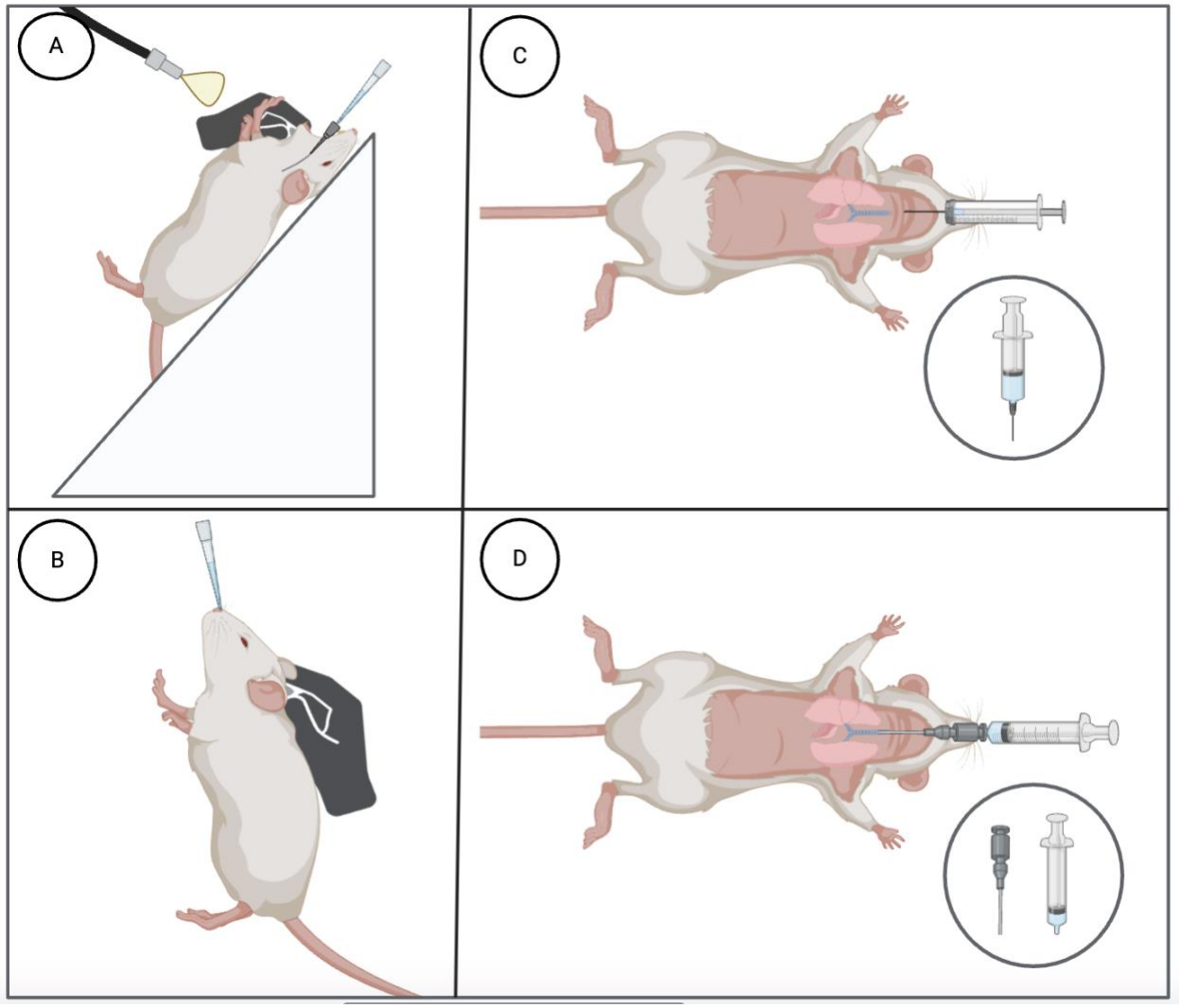


Figure 1. Diagram of four routes of LPS administration

The routes of administration are as follows: A) intratracheal instillation-intubation; B) intranasal instillation; C) trans-tracheal instillation with needle; D) trans-tracheal instillation with catheter. The diagram was created using BioRender.

Figure 2. Time-course assessment of inflammatory and lung injury measurements progression following LPS administration via intratracheal-intubation route

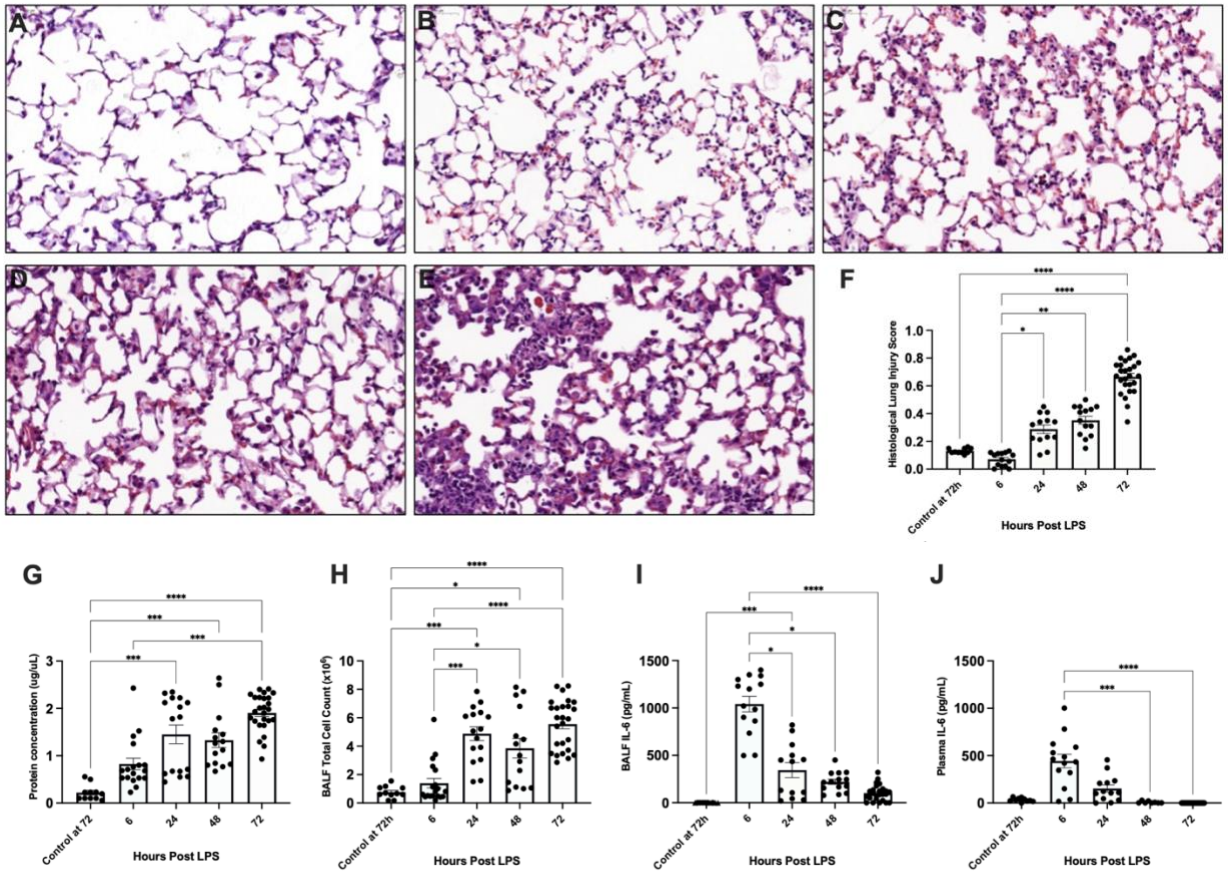


Figure 2. Time-Course Assessment of Inflammatory and Lung Injury Measurements Progression Following LPS Administration Via Intratracheal-Intubation Route

Representative lung sections from the time-course: A) Control, B) 6 hours, C) 24 hours, D) 48 hours, and E) 72 hours post lipopolysaccharide (LPS) administration. Histological lung injury score (F), protein concentration in bronchioalveolar lavage fluid (BALF) (G), BALF total cell count (H), and proinflammatory cytokine IL-6 levels in BALF (I) and plasma were measured at different time points following LPS administration via the intratracheal intubation route to assess lung injury progression. Control groups were included for baseline comparisons. Lung tissue sections were stained with hematoxylin and eosin (H&E) and scored, with results expressed as mean ± SEM (n = 10–15 per group). Total protein concentration in BALF was determined using the bicinchoninic acid assay (BCA) and presented as mean ± SEM (n = 10–15 per group). BALF total cell count, indicating cell influx, was measured using a Cell Countess and reported as mean ± SEM (n = 10–15 per group). Proinflammatory cytokine IL-6 levels in BALF were quantified using an enzyme-linked immunosorbent assay (ELISA), with results shown as mean ± SEM (n = 10–15 per group). Statistical analyses were conducted using the Kruskal-Wallis test followed by Dunn's post hoc test, with $P < 0.05$ considered statistically significant. Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Table 1: Fligner-Killeen test results comparing variability in lung injury markers across four routes of LPS administration

Routes P value of Fligner-Killeen test	Intratracheal-Intubation	Intranasal	Trans-Tracheal with Needle	Trans-Tracheal with Catheter
Intratracheal-Intubation	-	0.643	0.017	0.041
Intranasal	0.643	-	0.005	0.008
Trans-Tracheal with Needle	0.017	0.005	-	0.707
Trans-Tracheal with Catheter	0.041	0.008	0.707	-

P Value	**Color**	**Significance**
$p > 0.1$		Variances across the groups are likely similar.
$0.01 < p \leq 0.10$		It is likely that the variances across the groups are different.
$p \leq 0.01$		The variances across the groups are almost certainly different.

Figure 3. Comparison of four routes of LPS administration on outcomes of ALI: Histological evidence of tissue injury

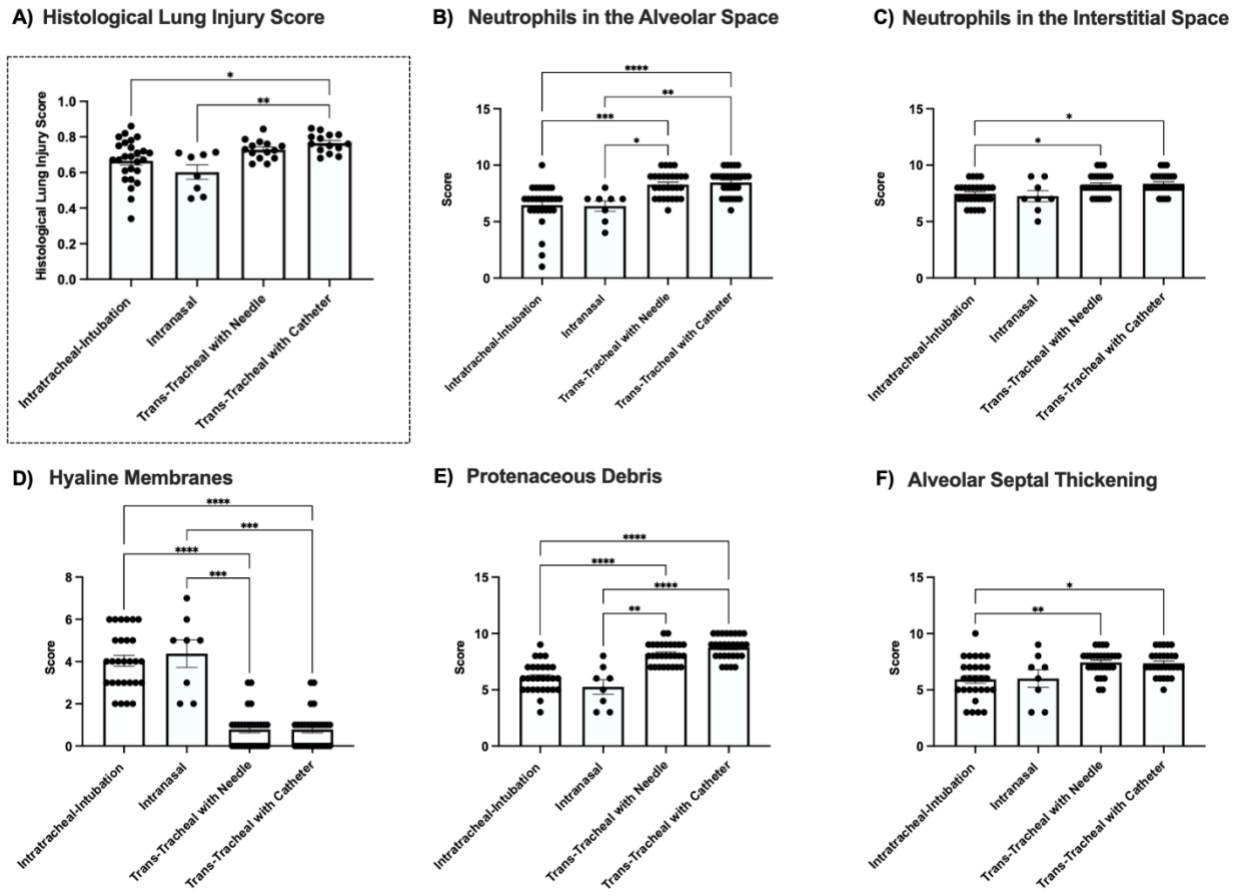


Figure 3. Comparison of four routes of LPS administration on outcomes of ALI: Histological evidence of tissue injury

Histological lung injury score (A)**,** parameter 1) neutrophils in the alveolar space (B), parameter 2) neutrophils in the interstitial space (C), parameter 3) hyaline membranes (D), parameter 4) proteinaceous debris (E), parameter 5) alveolar septal thickening (F) were assessed in mice 72 hours after LPS administration via four different routes: intratracheal intubation, intranasal, trans-tracheal with needle, and trans-tracheal with catheter. Lung tissue sections were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope for histological scoring, with scores presented as mean ± SEM (n = 8–15 per group). Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's post hoc test to compare differences among groups, with $P < 0.05$ considered statistically significant. Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure 4. Comparison of four routes of LPS administration on outcomes of ALI: Alveolar permeability and inflammatory markers

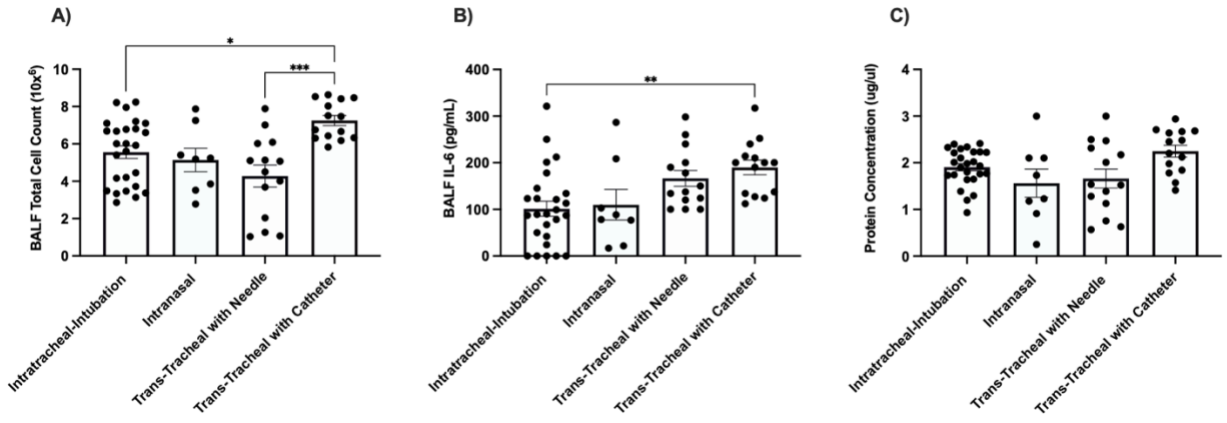


Figure 4. Comparison of Different Outcomes Across LPS Administration Routes: Alveolar permeability and inflammatory markers

Bronchoalveolar Lavage Fluid (BALF) total cell count (A)**,** IL-6 activity in BALF (B), and Protein concentration in BALF (C)**,** were measured in mice 72 hours after Lipopolysaccharide (LPS) administration via four different routes: intratracheal intubation, intranasal, trans-tracheal with needle, and trans-tracheal with catheter. BALF total cell count, an indicator of cell influx, was assessed using a Cell Countess and presented as mean ± SEM (n = 8–15 per group). Levels of the proinflammatory cytokine IL-6 in BALF were quantified using enzyme-linked immunosorbent assay (ELISA), with results shown as mean ± SEM (n = 8–15 per group). Total protein concentration in BALF was measured using the bicinchoninic acid assay (BCA) and expressed as mean ± SEM (n = 8–15 per group). Statistical analyses were performed using the Kruskal-Wallis test followed by Dunn's post hoc test to compare differences among groups, with $P < 0.05$ considered statistically significant. Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure 5. Evans blue dye distribution and quantification in different routes of LPS administration

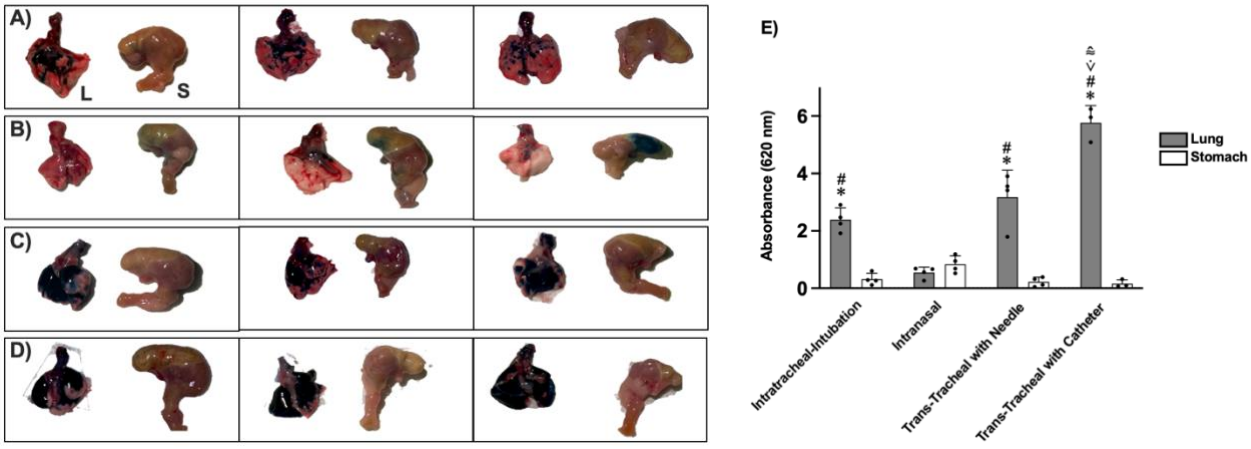


Figure 5. Evans blue dye distribution and quantification in different routes of LPS administration

Representative images (n = 3 mice per route) show the distribution of Evans Blue dye in the lungs and stomach 15 minutes after administration via four different routes: intratracheal intubation (A), intranasal (B), trans-tracheal with needle (C), and trans-tracheal with catheter (D). The images illustrate the extent of dye penetration, highlighting distribution patterns specific to each administration route. (E) Quantification of Evans Blue dye in lung and stomach tissues for each administration route. Dye concentration was assessed by measuring absorbance at 620 nm, providing an evaluation of tissue distribution. Results are presented as mean ± SEM (n = 3-4 per group). Statistical analyses were conducted using two-way ANOVA followed by Tukey's post hoc test to compare differences among groups. Statistical significance is indicated as follows: * Indicates a significant difference compared to the stomach tissue within the same route. # Indicates a significant difference compared to the lung tissue of the intranasal instillation group. v̇ Indicates a significant difference compared to the lung tissue of the intratracheal intubation group. ≅ Indicates a significant difference compared to the lung tissue of the surgical intratracheal needle group.

Figure 6. Comparison of Advantages and Disadvantages Across Four LPS Administration Routes in a Preclinical ALI Model

Route	Advantages	Disadvantages
Intratracheal Intubation	• Short procedure time = higher throughput • Significant lung injury	• Significant inter-subject variability • Less consistent injury = poor precision • Patchy Evans Blue distribution in lungs • Risk of tracheal damage if intubation is done incorrectly • Anesthesia required (Ketamine/Xylazine) = longer wake-up time
Intranasal	• Short procedure time = higher throughput • Non-invasive • Ease of administration • Cost-effective	• Significant inter-subject variability • Less consistent injury = poor precision • Minimal Evans Blue distribution in lungs – LPS lost to other areas • Variability in delivering dose
Trans-Tracheal with Needle	• Significant lung injury • Minimal inter-subject variability • Significant Evans Blue lung targeting • High precision	• Invasive procedure • Technical skills required • Anesthesia requirement (constant sedation with Isoflurane) • Time-consuming (approx. 20 min/mouse)
Trans-Tracheal with Catheter	• Significant lung injury • Minimal inter-subject variability • Significant Evans Blue lung targeting • High precision	• Invasive procedure • Technical skills required • Anesthesia requirement (constant sedation with Isoflurane) • Time-consuming (approx. 20 min/mouse)

Supplemental 1. ARRIVE 2.0 Guidelines

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including:	Page 8
		a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	Page 8
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Page 8
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	Page 8
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	S. Table 1
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	N/A
		b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	N/A
		c. For each analysis, report the exact value of *n* in each experimental group.	Figures
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	N/A
		b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Page 8
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Page 9,12
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	Page 10-13
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	Page 1
		b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	Page 8
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	N/A
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	
		a. What was done, how it was done and what was used.	Page 8-13
		b. When and how often.	
		c. Where (including detail of any acclimatisation periods).	
		d. Why (provide rationale for procedures).	
Results	10	For each experiment conducted, including independent replications, report:	Page 8-13
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	Figures
		b. If applicable, the effect size with a confidence interval.	

Text

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

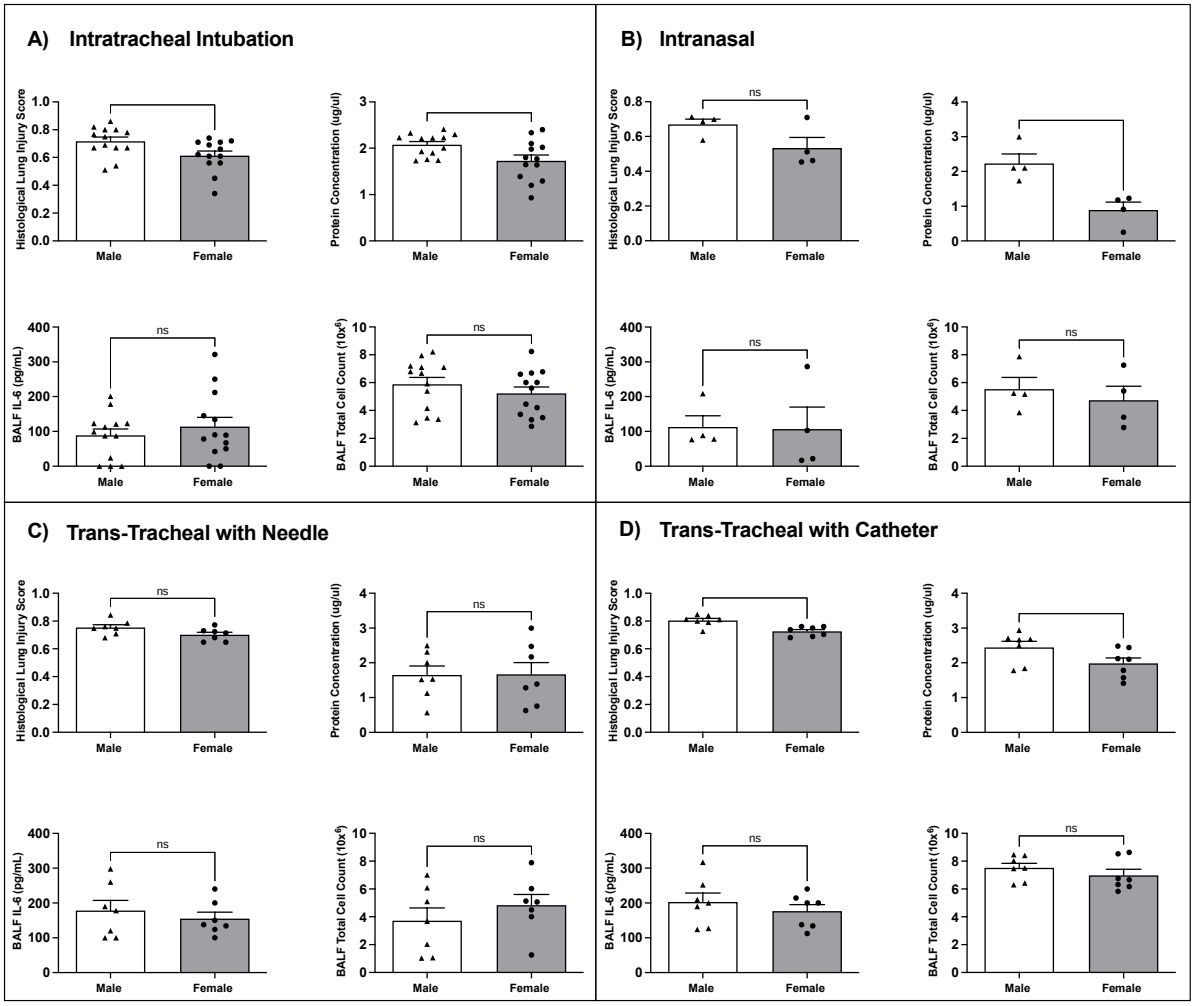
Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Page 4
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	Introduction Introduction
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Introduction
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	Methods
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	Methods
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	Methods Meth/Discuss Methods
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	Discussion Discussion
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	Discussion
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	N/A
Data access	20	Provide a statement describing if and where study data are available.	N/A
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	Page 2 Page 1-2

Supplemental Table 1. Sample Size

Group	Sex	Intratracheal Intubation (6h)	Intratracheal Intubation (24h)	Intratracheal Intubation (48h)	Intratracheal Intubation (72h)	Intranasal	Trans-Tracheal with Needle	Trans-tracheal with Catheter
LPS	Male	9	8	8	13	4	7	7
	Female	10	8	7	13	4	7	7
PBS	Male	-	-	-	6	1	2	2
	Female	-	-	-	5	1	2	2

- Indicates that no control group was matched for that timepoint. All control mice were sacrificed at 72h.

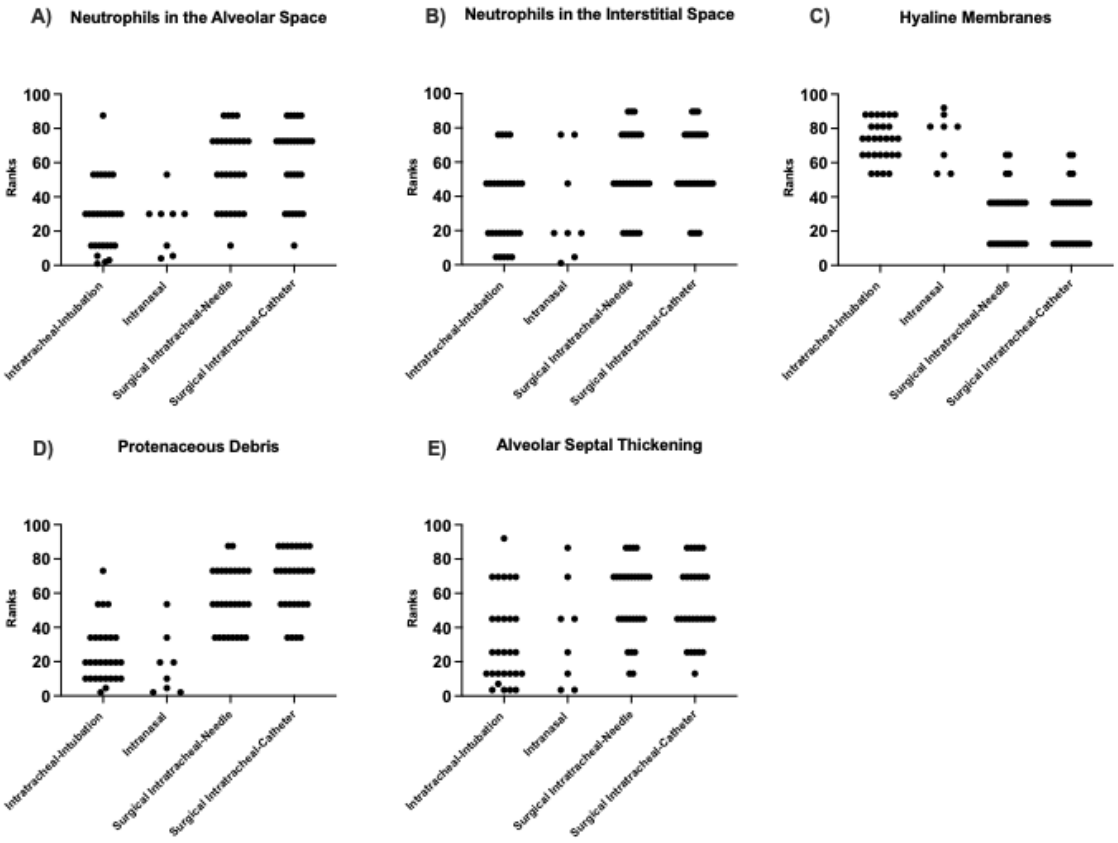
Supplemental Figure 1. Comparison of biological sex in ALI outcomes across four routes of LPS administration



Supplemental Figure 1. Comparison of biological sex in ALI outcomes across four routes of LPS administration

Intratracheal intubation (A), intranasal (B), trans-tracheal with needle (C), and trans-tracheal with catheter (D) routes were used to assess acute lung injury (ALI) 72 hours after lipopolysaccharide (LPS) administration. Lung tissue sections were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope for histological scoring, with scores presented as mean ± SEM (n = 4–12 per group). Total protein concentration in bronchioalveolar lavage fluid (BALF) was measured using the bicinchoninic acid assay (BCA) and expressed as mean ± SEM (n = 4–12 per group). Levels of the proinflammatory cytokine IL-6 in BALF were quantified using enzyme-linked immunosorbent assay (ELISA), with results shown as mean ± SEM (n = 4–12 per group). BALF total cell count, an indicator of cell influx, was assessed using a Cell Countess and presented as mean ± SEM (n = 4–12 per group). Statistical analyses were conducted using the Kruskal-Wallis test followed by Dunn's post hoc test, with $P < 0.05$ considered statistically significant. Significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Supplementary Figure 2. Rank Plots of Histological Parameters Across Four Routes of LPS



Chapter 4 - Integrated Discussion

4.1 Introductory Section

This chapter integrates findings from both my systematic review and experimental studies addressing two central themes: (1) the influence of biological sex on acute lung injury (ALI) outcomes, and (2) the refinement of the lipopolysaccharide (LPS)-induced ALI model to better detect model and sex-based differences. These studies underscore the need for more precise preclinical models to accurately evaluate sex-dependent variations, particularly in ALI progression and response to interventions. By refining the LPS model and minimizing unwanted variability, we provide a robust platform for studying sex-specific differences in ALI. These findings serve as a critical first step towards our planned multi-laboratory study, where we will further validate these sex-dependent differences. Additionally, the generalizability of our results will be tested across different sites and operators, ensuring that our findings are applicable across varied research settings. This approach will also help evaluate the reproducibility of the results and guide future preclinical research efforts.

4.2 Individual Summary of Each Article

Key Insights from Systematic Review: Gaps in Reporting and Sex-Based Differences in ALI

Our preclinical systematic review aimed to evaluate the representation and impact of biological sex in the preclinical LPS-induced ALI models. The review revealed significant underreporting of sex differences in these models. Among the 1,679 studies screened, only six accounted for biological sex as a critical variable. Despite this limited dataset, our meta-analysis suggested that male mice exhibited more severe lung injury, characterized by elevated histological injury scores, higher bronchoalveolar lavage fluid (BALF) albumin levels, and increased total cell counts compared to females. These findings indicate that sex-specific inflammatory responses may influence ALI outcomes, but the underrepresentation of sex-based analysis significantly hampers the generalizability of this evidence. This gap underscores an urgent need for systematic integration of sex-based analyses in future ALI research.

Refining the LPS-Induced ALI Model: Improved Precision in Studying Sex Differences

The experimental component of this thesis refined the LPS-induced ALI model, evaluating four administration routes: intratracheal intubation, intranasal instillation, and trans-tracheal instillation via needle or catheter methods. Our findings showed that trans-tracheal routes produced a more severe and less variable lung injury phenotype, characterized by higher histological injury scores, increased neutrophil infiltration, and more pronounced proteinaceous debris. The trans-tracheal catheter method, in particular, provided the most precise pulmonary delivery as confirmed by Evans blue dye distribution.

This refinement addresses a critical challenge in ALI modeling: variability in LPS administration can obscure true sex-based differences, leading to confounded outcomes. By optimizing the LPS delivery route and improving model precision, we provide a stronger framework for investigating sex-dependent inflammatory responses. The refinements we made ensure that observed differences in lung injury outcomes are not attributable to variability in LPS delivery but are instead reflective of biological phenomena related to sex.

4.3 Main Points of Integrated Discussion

The Influence of Biological Sex on ARDS Outcomes: Potential Implications for ALI Research

Clinical evidence has shown that **biological sex may** influence outcomes in **ARDS outcomes**. Some studies suggest that **male patients** often experience worse outcomes, including higher mortality rates and more severe ARDS presentation, while other studies, such as the **LUNG SAFE study**, suggest that **females** may fare worse under certain circumstances, potentially due to differences in **ventilatory management strategies**. (50, 51, 95) This variability in clinical findings reflects the complexity of sex-based differences in ARDS, which may be influenced by multiple confounding factors in clinical settings.

In contrast, the laboratory setting provides a controlled environment where these biological differences can be more clearly examined. By tightly controlling variables such as environmental influences, disease induction methods, and animal selection, the lab allows for a more accurate

investigation of sex-specific differences that may be harder to detect in clinical research. This precision in preclinical models is essential for isolating how biological sex influences ALI outcomes.

Our research builds on these clinical observations and leverages the control offered by the laboratory setting to demonstrate that male mice exhibit more severe lung injury than females in ALI models. Both our systematic review and experimental studies confirmed that male mice consistently displayed elevated histological injury scores, higher BALF protein levels, and greater inflammatory cell infiltration. Several sex-dependent biological pathways are likely at play in driving these differences in lung injury severity. One key pathway involves the heightened pro-inflammatory cytokine response in males, with increased levels of TNF-α, IL-6, and IL-1β driving tissue damage through NF-κB activation, which leads to more severe inflammation. (96–99) In contrast, estrogen in females attenuates this inflammatory response by downregulating NF-κB and boosting antioxidant defenses, resulting in less tissue injury. (96–99) Furthermore, testosterone in males, while initially immunosuppressive, eventually contributes to a delayed but amplified immune response that exacerbates lung injury. (100, 101) This hormonal modulation is compounded by sex-related differences in immune cell populations, where females exhibit a more efficient T-cell and macrophage response, enabling faster resolution of inflammation. Additionally, X-chromosome mosaicism in females allows for a more diverse immune response, offering them greater flexibility in managing inflammatory challenges during ARDS. (97) These interconnected biological pathways highlight the importance of considering sex as a critical variable in preclinical ALI research. Future investigations should focus on further elucidating these mechanisms to better understand their role in modulating lung injury severity.

This variability in sex-specific outcomes further underscores the need for standardized reporting and robust sex-based analyses in ARDS research. As highlighted in our systematic review, many preclinical studies either fail to report the sex of the subjects or do not stratify their findings by sex, which limits the scope and applicability of their conclusions. Transparent reporting of the sex of experimental animals is essential for ensuring reproducibility, while stratifying data by sex provides a more nuanced understanding of how biological sex may influence disease

progression, treatment responses, and outcomes. The absence of consistent sex-based reporting in ALI studies reflects a broader issue observed in other fields, such as sepsis research, where sex-based differences in treatment outcomes are increasingly recognized. For example, some studies have demonstrated that sex-specific responses to fluid management and treatment in sepsis models significantly altered both inflammatory markers and survival rates, reinforcing the importance of considering sex as a critical variable in preclinical research. (102–104)

Optimization of LPS Administration Routes for ALI Models: Balancing Precision and Biological Relevance

The LPS-induced ALI model remains the most widely used preclinical model for studying inflammation and lung injury, but our study is the first to compare all direct lung injury routes comprehensively. We found that trans-tracheal administration, particularly using a catheter, resulted in more severe and consistent injury outcomes than intranasal or intratracheal intubation methods. This refinement reduces model-related variability, allowing for more precise investigations of sex-based differences.

While the trans-tracheal catheter route provided the most controlled LPS delivery, it also produced distinct lung injury phenotypes, characterized by increased neutrophil infiltration and proteinaceous debris, and fewer hyaline membranes compared to other methods. In addition, this route was inherently more invasive than non-surgical methods, which may limit its applicability in certain contexts. These phenotypic and technical differences highlight the importance of matching the appropriate LPS administration route to experimental goals and available resources. Understanding these differences is crucial for ensuring precise LPS delivery and improving the reproducibility and clinical relevance of ALI models. Recognizing the nuances of these models is critical for enhancing their translational relevance, ensuring that research outcomes are both reliable and may be more likely to be reproducible.

Achieving accurate LPS delivery is crucial for minimizing technical variability, but models must also account for biological factors to reflect real-world clinical conditions. ARDS is a complex, multifactorial disease influenced by sex, age, and genetics. (49) Replicating this variability in

preclinical models ensures translational relevance and prevents oversimplification, which could limit the applicability of findings to clinical scenarios. Age and genetic background play a significant role in inflammatory responses, with younger animals typically showing more robust immune reactions, while older animals may have age-related immune changes. (33, 105, 106) Likewise, different strains, such as C57BL/6, exhibit controlled inflammatory responses compared to the heightened reactions seen in BALB/c mice. (33, 94, 107) In our studies, we used younger male and female C57BL/6 mice, which are commonly used in preclinical research, to ensure our findings are relevant to a broad range of studies. Our systematic review revealed that few studies consider biological sex, making this a crucial starting point for examining sex-based differences in ALI outcomes. By controlling for age and genetic variability, we ensured that the observed differences were driven by sex, providing a strong foundation for future research to explore more diverse genetic backgrounds and age groups. Selecting the appropriate strain and animal characteristics is key to aligning the model with the study's objectives and ensuring the relevance of findings in clinical ARDS research.

In addition to biological variability, flexibility in model design is essential for capturing the complex physiological responses of ARDS. Our studies showed that the route of direct LPS administration significantly affects the severity and consistency of lung injury. Specifically, trans-tracheal routes resulted in the least variable and most severe lung injury, providing a more controlled environment for studying sex-based differences. In contrast, systemic models—such as sepsis-induced ALI—better mimic the multisystemic inflammatory responses seen in clinical ARDS, but often result in milder lung injury which may obscure these differences. (33) These findings underscore the importance of selecting the appropriate LPS administration route based on the study's specific goals. By refining these routes in our experiments and considering both biological and technical factors, we demonstrated how researchers can improve the reproducibility and clinical relevance of ALI models, ultimately strengthening the bridge between experimental findings and patient care.

4.4 Strengths and Limitations

This thesis presents several key strengths, particularly in its comprehensive approach to addressing the role of biological sex in ALI. The systematic review provided valuable insights into how sex differences influence ALI outcomes, highlighting gaps in existing research and the need for more consistent reporting of sex-specific data. When integrated with the experimental study, which refined the LPS-induced ALI model to reduce variability and improve reproducibility, the biological sex dependent differences noted make a strong case for incorporating biological sex as a critical factor in preclinical ALI research. Together, these two components strengthen the overall understanding of sex differences and underscore the importance of reliable modeling for studying ARDS.

However, there are some limitations to acknowledge. The systematic review was constrained by the lack of studies that included both sexes, reported the sex of the animals, or conducted sex-stratified analyses, making it challenging to draw broad conclusions. Additionally, the review had a narrow focus, as it primarily examined studies using LPS-induced ALI, which aligns with the experimental component of this thesis. The experimental study, while providing a refined model, requires significant technical expertise, which may introduce variability based on operator skill and could affect the reproducibility of the results in different settings. Moreover, both the systematic review and the experimental study focused primarily on short-term outcomes, leaving the long-term implications of sex differences and model refinements on recovery unexplored.

4.5 Implications

Implications for ARDS Research and Practice: Influencing Research Practice, Policy, and Future Research

Despite policies such as the NIH's Sex as a Biological Variable (SABV) policy and CIHR's guidelines on sex and gender-based analysis, our systematic review reveals a significant gap between these mandates and their implementation in preclinical ALI research. (108, 109) Although LPS-induced ALI is one of the most commonly used models to study lung injury, a PubMed search from 2016 (when the NIH SABV policy came into effect) to 2024 yielded over

2,570 studies, yet our systematic review found that only six studies included and stratified outcome measurements for both male and female animals. This glaring underrepresentation of sex-based analyses reveals a critical disconnect between policy and practice, which not only limits the discovery of important sex-based differences but also hampers efforts to conduct meta-analyses. The lack of consistent sex-specific data reduces the ability to pool data across studies and draw meaningful conclusions about sex-based differences in ALI outcomes, potentially diminishing the impact of the research.

This gap is particularly concerning given the well-documented influence of sex hormones on immune responses. Research shows that men and women exhibit different reactions to inflammatory stimuli, with females often displaying heightened immune activity, which can significantly alter the trajectory of diseases like ARDS. (21, 110, 111) Ignoring these differences risks producing incomplete or biased findings, limiting the translatability of preclinical research into effective treatments for both sexes.

To address this, a cultural shift is needed in the research community—beyond policy enforcement—to incentivize the inclusion of sex-based analyses. For instance, research has shown that when policies like SABV are backed by clear expectations and funding rewards, researchers are more likely to integrate sex-based stratification into their designs. (59, 112, 113) CIHR data also demonstrates that incentivizing sex and gender integration has led to increased compliance and output in sex-specific research. (114) Moreover, fostering researcher education and training on the importance of biological sex in disease outcomes will be crucial for changing the research culture. Journals, peer reviewers, and funding bodies must also play an active role by requiring transparency and justification when sex is not included in study designs.

The findings of our systematic review provide a clear call to action: policies alone are insufficient without greater incentivization, cultural shifts, and consistent application of sex-based analyses in research practices. Only by bridging this gap between policy and practice can we build a body of evidence that is reliable. This shift is critical not only for improving preclinical research but also for advancing the translational impact of our findings, ultimately leading to better outcomes for all patients, regardless of sex.

4.5 Future Directions

Preparing for Future Studies: Addressing Research Gaps and Building Collaborative Foundations

Looking ahead, this thesis lays the groundwork for a future multilaboratory studies aimed at investigating sex differences in ALI outcomes through rigorous, low-risk-of-bias methodologies. Our approach began with a comprehensive systematic review, which highlighted the limited exploration of sex-specific differences in preclinical ALI research. Despite this paucity of data, the review suggested that sex-based influences likely affect ALI outcomes. These findings underscored the need for further studies and prompted the refinement of our LPS model in my second study. This refinement was designed to optimize the model for reproducibility in future multi-laboratory settings.

Additionally, we aligned our outcome measurements with the American Thoracic Society (ATS) guidelines for ALI models. (6) This ensured that the selected outcomes were both clinically relevant and meaningful, laying a strong foundation for future research. Furthermore, the data obtained from this single-laboratory study provides critical effect sizes, which will allow for sample size calculations in future multi-laboratory studies. This step is essential for ensuring that the study is sufficiently powered to detect meaningful differences in ALI outcomes based on biological sex.

The outcomes of this single-laboratory study will also serve as a feasibility test, identifying potential logistical and methodological challenges that may arise in a multi-laboratory setting. By refining these protocols early, we aim to standardize procedures across sites, reducing variability and ensuring consistency in future studies. This preparatory work will also help mitigate risks related to variability in LPS administration and outcome measurement. By identifying and addressing these challenges in advance, we reduce the likelihood of inconsistencies across laboratories, further enhancing the reliability and interpretability of the study outcomes.

Reflecting on the challenges encountered in other multi-laboratory studies emphasizes the importance of robust pilot testing and careful planning. For instance, the large-scale preclinical stroke trial led by Lyden et al. demonstrated that insufficient model validation and poorly chosen outcome measurements can obscure important differences, ultimately leading to inconclusive results. (115) In that case, the trial failed to show significant differences between control and stroke groups, partly due to inadequate validation of the stroke model and the use of outcome measures that were not suited to assess stroke severity.

By learning from these past experiences, our study takes crucial steps to ensure success in future multi-laboratory settings. The refinement of the LPS model and validation of outcome measures ensure that both the severity of the ALI model and the selected measurements are appropriate and reliable. Additionally, this work sets the stage for the harmonization of protocols across collaborating laboratories. Ensuring consistent application of methodologies, from LPS delivery to data collection, is vital for reducing unwanted variability and increasing the reproducibility of results across study sites. This approach significantly enhances the likelihood of generating meaningful, translatable results when the study expands to multiple laboratories.

4.5 Conclusions

This thesis demonstrates the critical role of biological sex and methodological refinement in influencing ALI outcomes. The systematic review revealed major gaps in sex-specific reporting in preclinical studies, underscoring the need for consistent integration of sex-based analyses to improve the translational relevance of findings. Our experimental work, focused on refining the LPS-induced ALI model, minimized variability and improved the detection of sex-based differences. These findings lay a strong foundation for future multi-laboratory studies, ensuring that both biological variability and rigorously validated models drive more effective, personalized therapeutic strategies for ARDS patients.

Chapter 5 - References

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