Development of techniques to determine extracellular matrix alterations in acute and chronic lung diseases and bioengineered tissues

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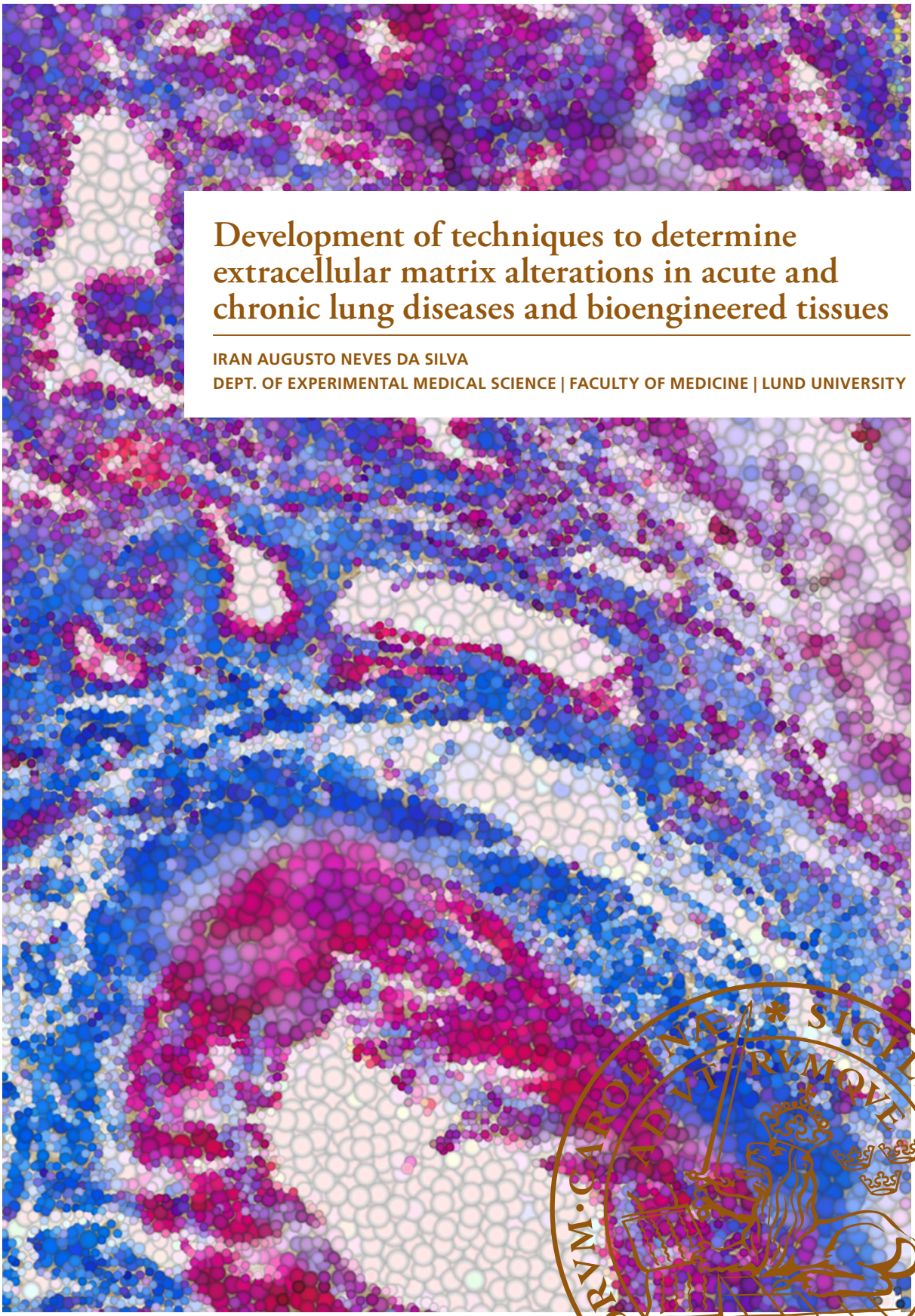

Development of techniques to determine extracellular matrix alterations in acute and chronic lung diseases and bioengineered tissues

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Abstract:

Acute and chronic lung diseases are a major cause of global mortality. While pharmacological approaches exist, no therapies are curative. The only option at end-stage disease is lung transplantation which is hampered by a chronic shortage of donor organs. Therefore, there is a high interest to develop alternative approaches to use regenerative medicine approaches to generate new lung tissue in the lab or to deliver cells which can participate in structural repair. In parallel to this clinical need, these new technologies, and the animal models which are used to assess their efficacy, require the development of new evaluation methods.

One of the most important methods for evaluating these therapies is histological assessment, as it can provide direct information at the tissue and cellular level information across all stages of the bioengineered tissue: from manufacture through evaluation in pre-clinical animal models. However, many of these potential therapies are comprised of a mix of cells, extracellular matrix and biomaterials (i.e. polymers in the case of soft tissues). Standard histological approaches have been developed for use with native animal and human tissues and organs, based on chemical moieties which are ubiquitous in animal tissues (e.g. amine or carboxylic acid groups).

Biomaterials (e.g. synthetic or natural polymers), on the other hand, have diverse chemical moieties that may not always be compatible with standard fixatives and tissue processing. Furthermore, the chemical composition of the solutions used in fixation or tissue processing, even at trace amounts, may alter biomaterials which have been used for bioengineering tissue or in vitro models.

Therefore, this thesis aimed to develop new methods to histologically assess native and bioengineered lung tissue, with a particular focus on developing methods which preserve cell-extracellular matrix or cell-biomaterial interactions for light and electron-based microscopy.

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Dedicated to Her and all the ones that support and believed in me during this journey. To all the ones who already born without chances, but still fights and perseverates.

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Preface

This thesis composite has the objective to summarize the work that I have been involved within the past years. The initial part aims to summarize the main questions that arose during this journey. Many of the questions still need more research, but I hope that my contributions may provide tools for the ones that also have similar questions. Further, I navigate through the aims and the approach that was used to achieve it, as well as my contribution for the field gaps. Finally, I conclude with the overview of the main articles that support my findings and efforts to contribute to achieve the Agenda 2030 SDGs.

This investigation gave me the opportunity to connect various fields, such as sustainability, histology, lung acute and chronic diseases, animal modelling, and bioengineering with hydrogels, where I made the effort to find solutions or develop tools such as score system, formalin-free and xylene-free histological techniques to allow the development, optimization and exploration of the subjects that were investigated.

I sincerely hope that you appreciate this journey, of course is not possible to summarize all the things that I learned and contribute, but for sure I'm more than happy to discuss and reflect on these topics as well.

Popular summary - EN

Acute respiratory distress syndrome (ARDS) is a highly heterogeneous and life-threatening disease with mortality rates of around 30-50% worldwide. Characterized by diffuse damage in the lung structures which causes a rapid decline in lung function. Caused by a variety of infectious and non-infectious injuries (e.g., trauma, surgery, burn wounds, gastric aspiration, or inhalation of highly toxic substances), infectious causes of ARDS may be direct lung injury through different pathogens, including viral (e.g., SARS-CoV-2), bacterial (e.g., *Streptococcus pneumoniae*), and fungal colonization (e.g., aspergillosis in mechanically ventilated patients).

While *in vitro* models can recapitulate some features of ARDS, the disease is multifactorial and therefore small and large animal models remain the main preclinical research tools; each, has its own benefits and limitations. In order to deal with these limitations, investigations on biomaterials polymers such as, alginate-based hydrogels, may offer the possibility as well to investigate another interaction of cells in a 3D environment.

Observation of the tissue (histological) assessment of features at different time points can help distinguish ARDS from alternative diagnoses by revealing evidence of lung tissue injury, such as accumulation of inflammatory cells. However, histology is time consuming and a multi-step process prone to variability, besides the fact that the traditional methodologies to use histological examinations uses hazard chemicals that might be toxic both for the environment and the user, besides the possible interaction with components of interest presents in the tissue.

Semi-quantitative scoring systems performed by independent observers are the most common form of histologic analyses used in pre-clinical animal models of ARDS, but high levels of expertise are not always available in all laboratories and human bias is known to be problematic.

The main aim of the project is to develop methods to perform histopathological analyses of samples from animal studies to develop and validate a histological scoring system applicable to machine learning approaches to speed up the diagnosis of samples with different lung structure changes. In addition to apply more sustainable histological techniques to bioengineered and native tissues. Future work will aim to further develop techniques to understand the composition and role of the deranged lung structures components in the development of acute and chronic lung diseases in clinical human disease.

Populärvetenskaplig sammanfattning - SV

Akut respiratoriskt stressyndrom (ARDS) även kallat akut andnödssyndrom, är ett samlingsnamn för livshotande lungsjukdom där lungorna inte kan tillgodose syresättningen av kroppens olika organ. Dödligheten i ARDS är 30–50%. Sjukdomen karakteriseras av diffusa skador i lungan vilka orsakar en snabb försämring av lungfunktionen. Dessa skador kan ha många olika bakomliggande orsaker som tex fysiska skador (yttrevåld, operation, brännskador, att maginnehållet når lungan eller inhalering av en giftig substans, mm). ARDS kan också bero på skador som uppkommit vid infektioner som orsakats av virus (tex SARS-CoV-2), bakterier (tex bakteriell lunginflammation), eller svamp (tex hos personer som ligger i respirator).

In vitro modeller kan återspegla vissa aspekter av ARDS men då sjukdomen beror på många samspelande faktorer är fortfarande djurmodeller det huvudsakliga verktyget då ARDS studeras. För att hantera dessa begränsningar kan undersökningar av biomaterialpolymerer såsom alginatbaserade hydrogeler erbjuda möjligheten att undersöka andra interaktioner mellan celler i en 3D-miljö.

Att titta på sjuk eller skadad lungvävnad med hjälp av histologi (infärgning av specifika komponenter i vävnad) kan avgöra om det är ARDS eller någon annan sjukdom som gett upphov till besvären. Rör det sig om ARDS syns det tecken på skador i lungvävnaden som tex ansamlingar av inflammatoriska celler.

Histologi är dock tidskrävande och en process i flera steg med benägenhet att variera, förutom det faktum att de traditionella metoderna för att använda histologiska undersökningar använder farliga kemikalier som kan vara giftiga både för miljön och användaren, förutom den möjliga interaktionen med komponenter i intresse finns i vävnaden. Vanligen avläses resultaten med semi-kvantitativa metoder och av olika personer, detta gör att det är svårt att få konsekventa resultat, speciellt som det inte alltid finns personer med hög expertis att tillgå.

Det huvudsakliga målet med detta avhandlingsarbete var att utveckla metoder för histopatologisk-analys av vävnadsprover samt ta fram och validera ett histologiskt poängsystem som skulle gå att använda i maskininlärning.

Att använda maskinbaserade metoder kvalitetssäkrar processen, genom att ta bort den mänskliga faktorn, och kortar tiden för diagnos av förändringar i lungan. I framtida arbete kommer vi att vidareutveckla tekniker för att förstå hur olika skador uppstår, vad som kännetecknar dem och vilken roll de spelar i utvecklingen av akut eller kronisk lungsjukdom hos människa.

Resumo popular – PT-BR

A síndrome do desconforto respiratório agudo (SDRA) é uma doença altamente heterogênea e potencialmente fatal, com taxas de mortalidade em torno de 30-50% em todo o mundo. Caracterizado por danos difusos nas estruturas pulmonares que causam um rápido declínio da função pulmonar. Causada por uma variedade de lesões infecciosas e não infecciosas por exemplo, (trauma, cirurgia, queimaduras, aspiração gástrica ou inalação de substâncias altamente tóxicas). As causas infecciosas da SDRA podem ser lesões pulmonares diretas por diferentes patógenos, incluindo viral por exemplo, (SARS-CoV-2), bacteriano, por exemplo (*Streptococcus pneumoniae*) e colonização fúngica por exemplo, (aspergilose em pacientes ventilados mecanicamente).

Embora os modelos *in vitro* possam recapitular algumas características da SDRA, a doença é multifatorial e, portanto, modelos animais pequenos e grandes continuam sendo as principais ferramentas de pesquisa pré-clínica; cada um, tem seus próprios benefícios e limitações. Para lidar com essas limitações, investigações em polímeros de biomateriais, como hidrogéis à base de alginato, podem oferecer a possibilidade também de investigar outras interações de células em um ambiente 3D.

A observação da avaliação tecidual (histológica) das características em diferentes momentos pode ajudar a distinguir SDRA de diagnósticos alternativos, revelando evidências de lesão do tecido pulmonar, como o acúmulo de células inflamatórias. No entanto, a histologia é demorada e um processo de várias etapas sujeito a variabilidade, além do fato de que as metodologias tradicionais para usar exames histológicos utilizam produtos químicos perigosos que podem ser tóxicos tanto para o meio ambiente quanto para o usuário, além da possível interação com componentes de interesse presentes no tecido.

Sistemas de pontuação semiquantitativos realizados por observadores independentes são a forma mais comum de análises histológicas usadas em modelos animais pré-clínicos de ARDS, mas altos níveis de especialização nem sempre estão disponíveis em todos os laboratórios e o viés humano é conhecido por ser problemático.

O principal objetivo do projeto é desenvolver métodos para realizar análises histopatológicas de amostras de estudos com animais para desenvolver e validar um sistema de pontuação histológica aplicável a abordagens de aprendizado de máquina para acelerar o diagnóstico de amostras com diferentes alterações na estrutura pulmonar. O trabalho futuro terá como objetivo desenvolver técnicas para entender a composição e o papel dos componentes das estruturas pulmonares perturbadas no desenvolvimento de doenças pulmonares agudas e crônicas em doenças humanas clínicas.

Resumen popular - ES

El síndrome de dificultad respiratoria aguda (SDRA) es una enfermedad altamente heterogénea y con tasas de mortalidad de alrededor del 30-50% en todo el mundo. Está caracterizado por un daño difuso en las estructuras pulmonares que provoca una rápida disminución de la función pulmonar. Causado por una variedad de lesiones infecciosas y no infecciosas (p. ej., trauma, cirugía, heridas por quemaduras, aspiración gástrica o inhalación de sustancias altamente tóxicas), las causas infecciosas del SDRA pueden ser lesiones pulmonares directas a través de diferentes patógenos, incluyendo viruses (p. ej., SARS-CoV-2), colonización bacteriana (p. ej., Streptococcus pneumoniae) y fúngica (p. ej., aspergilosis en pacientes con ventilación mecánica).

Si bien los modelos in vitro pueden recapitular algunas características del SDRA, la enfermedad es multifactorial y, por lo tanto, los modelos animales pequeños y grandes siguen siendo las principales herramientas de investigación preclínica; cada uno tiene sus propios beneficios y limitaciones. Para hacer frente a estas limitaciones, las investigaciones sobre polímeros de biomateriales, como hidrogeles a base de alginato, también pueden ofrecer la posibilidad de investigar interaccioes de interésn de las células en un entorno 3D.

La evaluación histológica de las características del tejido en diferentes momentos puede ayudar a distinguir el SDRA de diagnósticos alternativos al revelar lesión del tejido pulmonar, como esla acumulación de células inflamatorias. Sin embargo, la histología requiere mucho tiempo y puede dar lugar a variabilidad en la interpretacion histologica. Esto es debido a los muchos pasos que esta técnica requiere, además del hecho de que las metodologías tradicionales para usar exámenes histológicos utilizan químicos peligrosos que pueden ser tóxicos tanto para el medio ambiente como para el usuario. Otro punto a tener en cuenta es la posible interacción con componentes que presenta interés en el tejido.

Los sistemas de puntuación semicuantitativos realizados por observadores independientes son la forma más común de análisis histológicos utilizados en los modelos preclínicos de SDRA, pero no siempre se dispone de altos niveles de experiencia en todos los laboratórios para ello dando lugar a un problemático sesgo humano.

El objetivo principal del proyecto es desarrollar métodos para realizar análisis histopatológicos de muestras de estudios en animales y validar un sistema de puntuación histológica aplicable al aprendizaje automático que acelere y realice un diagnóstico más consistente. El trabajo futuro tendrá como objetivo seguir desarrollando técnicas más sostenibles para comprender la composición y el papel de de las disitintas estructuras pulmonares afectadas en el desarrollo de enfermedades humanas pulmonares agudas y crónicas.

Gbajumo Lakotan- YO

Arun aibanujẹ atẹgun nla (ARDS) jẹ orisirisi pupọ pupọ ati arun eewu-aye pẹlu awọn oṣuwọn iku ti o wa ni ayika 30-50% ni agbaye. Ti ṣe afihan nipasẹ ibajẹ kaakiri ninu awọn ẹya ẹdọfóró eyiti o fa idinku iyara ni iṣẹ ẹdọfóró. Ti o fa nipasẹ ọpọlọpọ awọn ipalara ati awọn ipalara ti ko ni arun (fun apẹẹrẹ, ibalokanjẹ, iṣẹ abẹ, awọn ọgbẹ sisun, ifunra inu, tabi ifasimu ti awọn nkan majele ti o ga julọ), Awọn okunfa aarun ti ARDS le jẹ ipalara ẹdọfóró taara nipasẹ awọn orisirisi pathogens, pẹlu gbogun ti (fun apẹẹrẹ, SARS-CoV-2), kokoro arun (fun apẹẹrẹ, Streptococcus pneumoniae), ati imunisin olu (fun apẹẹrẹ, aspergillosis ni awọn alaisan ti o ni ẹrọ atẹgun).

Lakoko ti awọn awoṣe in vitro le ṣe atunṣe diẹ ninu awọn ẹya ti ARDS, arun na jẹ multifactorial ati nitorinaa awọn awoṣe ẹranko kekere ati nla wa awọn irinṣẹ iwadii iṣaaju akọkọ; kọọkan, ni o ni awọn oniwe-ara anfani ati idiwọn. Lati le koju awọn idiwọn wọnyi, awọn iwadii lori awọn polima biomaterials gẹgẹbi, hydrogels-based alginate, le funni ni iṣeeṣe daradara lati ṣe iwadii ibaraenisepo miiran ti awọn sẹẹli ni agbegbe 3D kan.

Ṣiṣayẹwo ti iṣan-ara (histological) ti awọn ẹya ara ẹrọ ni awọn aaye akoko orisirisi le ṣe iranlọwọ lati ṣe iyatọ ARDS lati awọn ayẹwo miiran nipa fifihan ẹri ti ipalara ti iṣan ẹdọfóró, gẹgẹbi ikojọpọ awọn sẹẹli iredodo. Bibẹẹkọ, itan-akọọlẹ n gba akoko ati ilana igbesẹ pupọ ti o ni itara si iyipada, ni afikun si otitọ pe awọn ilana ibile lati lo awọn idanwo itan-akọọlẹ nlo awọn kemikali eewu ti o le jẹ majele mejeeji fun agbegbe ati olumulo, ni afikun ibaraenisepo ti o ṣeeṣe pẹlu awọn paati ti anfani iloju ni àsopọ.

Awọn eto igbelewọn ologbele-pipo ti a ṣe nipasẹ awọn alafojusi ominira jẹ ọna ti o wọpọ julọ ti awọn itupalẹ itan-akọọlẹ ti a lo ninu awọn awoṣe ẹranko iṣaaju-iwosan ti ARDS, ṣugbọn awọn ipele giga ti oye ko wa nigbagbogbo ni gbogbo awọn ile-iṣere ati aibikita eniyan ni a mọ lati jẹ isoro. Ero akọkọ ti iṣẹ akanṣe naa ni lati ṣe agbekalẹ awọn ọna lati ṣe awọn itupalẹ itan-akọọlẹ ti awọn ayẹwo lati awọn ikẹkọ ẹranko lati ṣe agbekalẹ ati fọwọsi eto igbelewọn itan-akọọlẹ kan ti o wulo si awọn isunmọ ikẹkọ ẹrọ lati mu iyara ayẹwo ti awọn ayẹwo pẹlu awọn ayipada eto ẹdọfóró orisirisi. Iṣẹ iwaju yoo ṣe ifọkansi lati ṣe agbekalẹ awọn imuposi siwaju lati ni oye akopọ ati ipa ti awọn ẹya ara ẹdọfóró ti o baje ninu idagbasoke awọn arun ẹdọfóró nla ati onibaje ni arun eniyan ile-iwosan.

List of Papers

Paper I

Iran A. N. Silva, Nika Gvazava, Deniz Bölükbas, Martin Stenlo, Jiao Dong, Leif Pierre, Sandra Lindstedt, Darcy Wagner*. A semi-quantitative scoring system for green histopathological evaluation of large animal models of acute lung injury. Bio-protocol. 2022 Aug 20;12(16): e4493.

Paper II

Iran A. N. Silva, Salma Kazemi Rashed, Ludwig Hedlund, August Lidfeldt, Nika Gvazava, John Stegmayr, Valeriia Skoryk, Sonja Aits*, Darcy E Wagner*. Development of a machine learning system for rapid detection of features of lung injury in animal models of acute lung injury. (manuscript)

Paper III

Martina M. De Santis, Hani N. Alsafadi, Sinem Tas, Deniz A. Bölükbas, Sujeethkumar Prithiviraj, **Iran A. N. Da Silva,** Margareta Mittendorfer, Chiharu Ota, John Stegmayr, Fatima Daoud, Melanie Königshoff, Karl Swärd, Jeffery A. Wood, Manlio Tassieri, Paul E. Bourgine, Sandra Lindstedt, Sofie Mohlin, Darcy E. Wagner*. (2021). Extracellular-matrix-reinforced bioinks for 3D bioprinting human tissue. Advanced materials, 33(3), 2005476.

Paper IV

Iran Augusto Neves Da Silva, Nika Gvazava, Indra Putra Wendi, Rodrigo Guinea, Francisco García Giménez, John Stegmayr, Oxana Klementieva, Darcy E. Wagner*. Formalin free fixation and xylene free tissue processing preserves cell-hydrogel interactions for histological evaluation of 3D calcium alginate tissue engineered constructs. Frontiers in Biomaterials Science 2023 April 17; 2: 4.

Other Publications

1. Deniz Bölükbas, **Iran Augusto Neves Da Silva**, Kristina Rydell-Törmänen, Darcy E. Wagner (2019). Preclinical Evidence for the Role of Stem/Stromal Cells in COPD. In: Burgess, J., Heijink, I. (eds) Stem Cell-Based Therapy for Lung Disease. Springer, Cham.
2. Martin Stenlo, Snejana Hyllén, **Iran A. N. Silva**, Deniz A. Bölükbas, Leif Pierre, Oskar Hallgren, Darcy E. Wagner, and Sandra Lindstedt*. (2020). Increased particle flow rate from airways precedes clinical signs of ARDS in a porcine model of LPS-induced acute lung injury. American Journal of Physiology-Lung Cellular and Molecular Physiology, 318(3), L510-L517.
3. Martin Stenlo, **Iran A. N. Silva**, Snejana Hyllén, Deniz A. Bölükbas, Anna Niroomand, Edgars Grins, Per Ederoth, Oskar Hallgren, Leif Pierre, Darcy E. Wagner, Sandra Lindstedt*. (2021). Monitoring lung injury with particle flow rate in LPS-and COVID-19-induced ARDS. Physiological Reports, 9(13), e14802.
4. Sandra Lindstedt*, Edgar Grins, Hillevi Larsson, Johan Nilsson, Hamid Akbarshahi, **Iran Silva**, Snejana Hyllen, Darcy Wagner, Johan Sjögren, Lennart Hansson, Per Ederoth, Ronny Gustafsson. (2021). Lung transplant after 6 months on ECMO support for SARS-CoV-2-induced ARDS complicated by severe antibody-mediated rejection. BMJ Open Respiratory Research, 8(1), e001036.
5. Haider Ghaidan, Martin Stenlo, Anna Niroomand, Margareta Mittendorfer, Gabriel Hirdman, Nika Gvazava, Dag Edström, **Iran AN Silva**, Ellen Broberg, Oskar Hallgren, Franziska Olm, Darcy E Wagner, Leif Pierre, Snejana Hyllén, Sandra Lindstedt*. (2022). Reduction of primary graft dysfunction using cytokine adsorption during organ preservation and after lung transplantation. Nature Communications, 13(1), 4173.
6. Hani N Alsafadi, John Stegmayr, Victoria Ptasinski, **Iran Silva**, Margareta Mittendorfer, Lynne A Murray, Darcy E Wagner*. (2022). Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations. Stem Cell Reports, 17(12), 2718-2731.
7. Nika Gvazava, Sabine Konings, Efrain Cepeda-Prado, Valeriia Skoryk, Chimezie H Umeano, Jiao Dong, **Iran AN Silva**, Daniella Rylander Ottosson, Nicholas D Leigh, Darcy E Wagner, Oxana Klementieva*. (2023). Label-free high-resolution infrared spectroscopy for spatiotemporal analysis of complex living systems. bioRxiv, 2023-01.

* indicates corresponding author

Abbreviations

COPD	Chronic obstructive pulmonary diseases
ECM	Extracellular matrix
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ECMO	Extracorporeal Membrane Oxygenation
LPS	lipopolysaccharide
PASC	post-acute sequelae of SARS-CoV-2 infection
H&E	Hematoxylin-Eosin
OCT	Optimal Cutting Temperature
FFPE	Formalin fixed and paraffin embedded (FFPE)
SED	Socioeconomic deprivation
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced vital capacity
TLC	Lung capacity or total lung capacity
VC	Vital Capacity
WHO	World Health Organization
SDGs	Sustainable Development Goals (SDGs)
rECM	Reinforced Extracellular Matrix Hydrogel
SEM	Scanning Electron Microscopy

Introduction

Lung as a bridge with the external world

The lung, as a major part of the respiratory system, acts as a constant bridge between the body and with the external world. Due to the role that the lung plays as a barrier between the body and the external world but also as a main site of exchange between the internal and external world (i.e. primarily gases but also the internalization and/or release of diverse particles), it naturally may be in some cases the primary organ affected by external causes. However, it is also known to be susceptible to systemic disease processes such as sepsis. The lung has an extremely well-organized architecture, composed by an interwoven branching network of airways and vessels (**Figure 1**). The airways begin with a larger diameter in the trachea and then dividing into the main bronchi (right and left lungs in humans) and becoming smaller in diameter until they open up to the alveoli in the distal region, where the air comes in contact with the capillaries to execute the passive exchange of oxygen and carbon dioxide.

However, in addition to the important role the airways and alveoli play in gas exchange, they also serve an important function as a filter between the internal and the external world. They protect the internal organs from environmental insults such as virus, bacteria and particles (e.g. pollution) that might damage the lung tissue and impair the body's ability to perform gas exchange (Johnson 2011). The lung mainly performs filtering through mucociliary clearance in the upper airways to constantly filter the air and prevent pathogens, allergens and particles from reaching the distal lung where they may cross the thin alveolar-capillary barrier and enter the rest of the body. Therefore, changes in the modern world related to climate change, urbanization, indoor pollution associated with social inequalities, industrialization, increased mobility, etc. have a major impact on the lung. Many aspects to our lung health are related to the Agenda 2030 sustainability development goals (SDGs) set forth in 2015 by the United Nations. Increased knowledge about how the lung responds to these challenges and better ways to study this are urgently needed. This thesis has a main focus to try develop techniques, using a sustainable approach, to help us better understand how to evaluate the changes in the architecture of the lung when the external world damages our bridge (Weibel 2013).

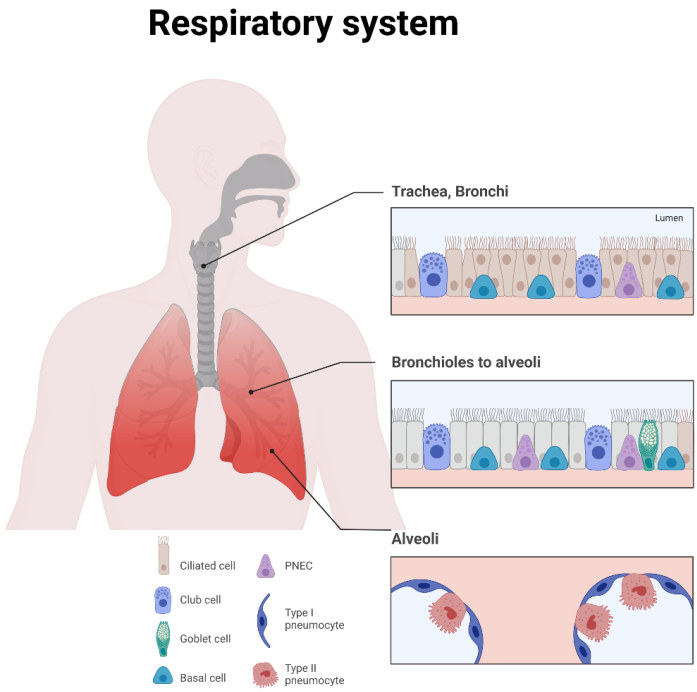


Figure 1 Overview of the respiratory system
Created with BioRender.com

Acute and chronic lung diseases

Lung diseases are known to be complex and to have both genetic and environmental components. They can be separated in two different types of diseases, acute and chronic. These diseases can begin from different injuries that can be created from inhalation of cigarette smoke, bacteria and virus, as well as direct injuries which can cause acute and life-threatening changes (e.g., trauma, gastric content aspiration or drowning).

Both can have an acute phase of inflammation that can either worsen acutely, slowly resolve or can continue and lead to chronic disease. Chronic lung diseases such as, asthma, pulmonary fibrosis, and chronic obstructive pulmonary disease (COPD) are a result of cumulative injuries that each person responds to differently due to individual aspects such as age, sex, gender and type/duration of exposure. All of these personal differences affect the way that the lung reacts to injury and may result in an increased or decreased ability to regenerate and heal. While little is known about the exact phases of chronic lung disease development, the timeline of changes

in ARDS are a little better characterized, especially when diffuse alveolar damage (DAD) is present(Thille, Esteban et al. 2013) . The progression of ARDS injury and features can be divided in three interrelated and overlapping phases of the diseases: *exudative* (day 1-7) featured by the presence of edema and hemorrhage, leucoagglutination, necrosis, hyaline membranes and platelet-fibrin thrombi (Katzenstein, Bloor et al. 1976). *Proliferative* (day 7-21) featured by the interstitial myofibroblast reaction, luminal organizing fibrosis, chronic inflammation, parenchymal necrosis, type II pneumocyte hyperplasia, and macrothrombi (Nash, Blennerhassett et al. 1967). *Fibrotic* phase end-stage of the diseases featured by the collagenous fibrosis, microcystic honeycombing, traction bronchiectasis, arterial tortuosity, and medial hypertrophy (Pratt, Vollmer et al. 1979, Tomashefski 2000).

In addition, it is important to highlight that individual differences are known to be very important in determining how a patient responds to an injury. This is very much exemplified by the ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic and how differently each person responded to the virus. Increasing research shows how individual differences related to both genetics (e.g. polymorphisms, race and sex) and environmental/lifestyle factors such as gender, ancestry and socioeconomic status (Linnea, Sophia et al. 2022). In particular, it is interesting to consider the impact of environmental factors, indoor and outdoor pollution particles and gases, which exposes large populations of persons to injurious agents (Svedahl, Svendsen et al. 2013). Pollution can be categorized broadly in different geographic regions but there is also knowledge about how pollution can differ locally within a city (e.g. city playgrounds) (Sheridan, Roscoe et al. 2019). These studies have contributed to our knowledge on the role that pollution plays at both a population, subpopulation, and individual level. One prominent example is the correlation between air pollution and premature deaths in countries with socioeconomic inequalities (Collaborators 2020). While we have increasing knowledge about the importance of these multiple factors on the onset of lung disease from an epidemiological perspective, most molecular studies of human samples or animal models are insufficient/underpowered to distinguish the relative impact of these on a biological level. It is important to keep these different aspects in mind as both limitations of our current knowledge and as opportunities for future study. It is important to understand that these topics are connected and related to the onset and progression of both acute and chronic lung diseases.

Chronic Lung Diseases

Obstructive lung diseases are characterized by the obstruction of the airways, with reduced exhalation. This occurs when inflammatory process and thickening of the wall and thus narrowing of the lumen diameter due to airway remodelling, causing difficulties to expel the air out of the lungs (Barnes, Burney et al. 2015). This can

result in a high volume of residual air remaining in the lungs, leading to hyperinflation, and extracellular matrix (ECM) remodelling that contributes to the worsening of the respiratory symptoms (i.e. COPD, chronic bronchitis, Asthma, bronchiectasis, bronchiolitis, cystic fibrosis). In contrast, restrictive lung diseases are characterised by a reduction of the lung capacity to fill the lungs due to remodelling which results in stiffening of the lung tissue (**Table 1)** (Paraskeva, Borg et al. 2011, Barnes, Burney et al. 2015).

Table 1 Obstructive versus restrictive lung diseases

Obstructive Airway Disease	Restrictive Lung Diseases
Definition:	
Increased resistence to airflow secondary to obstruction airways	Decreased lung volume and capacity
Pulmonary function tests (spirometry)	
FEV1/FVC ratio is decreased	Decreased TLC and VC
Examples:	
Chronic obstructive airway disease	Chest wall disordes
• **Asthma**	• Obesity
• **Chronic bronchitis**	
• **Emphysema**	Interstitial/ infiltrative diseases
• **Bronchiectasis**	• ARDS, pneumoconiosis
	• Pulmonary fibrosis

Table modified from Paraskeva MA (Paraskeva, Borg et al. 2011)

Chronic obstructive pulmonary diseases (COPD) are known as one of the major causes of death worldwide. The development of COPD is correlated with several risk factors, such as cigarette smoking, exposure of environmental or occupational pollutants (passive and thirdhand smoke) and gases from volatile chemicals such as formaldehyde (Buesa 2008), indoor air pollutants and repetitive infections, example presented in **Figure 2**.

The characteristic symptoms of COPD are reduction in airflow due to chronic bronchitis characterized by, inflammation (swelling) and irritation of the bronchial airways, causing accumulation of mucus, as well as airspace enlargement in the distal lung, which results in reduction of the surface area available for gas exchange. In addition, some of the most relevant genetic factors linked to COPD are the deficiency of serine protease α1 antitrypsin (A1AT), as well as upregulation of transforming growth factor-β1 (TGFB1) and tumour necrosis factor α (TNF α) at the plasma level in patients. The presence of TNF-α can induce inflammatory responses and has been shown to increase neutrophils present in the airways. On the other hand, TGβ1 can be related with submucosal collagen expression which is increased leading to deposition of extra cellular matrix in the airways (Mannino and Buist 2007, Mak, Chan-Yeung et al. 2009).

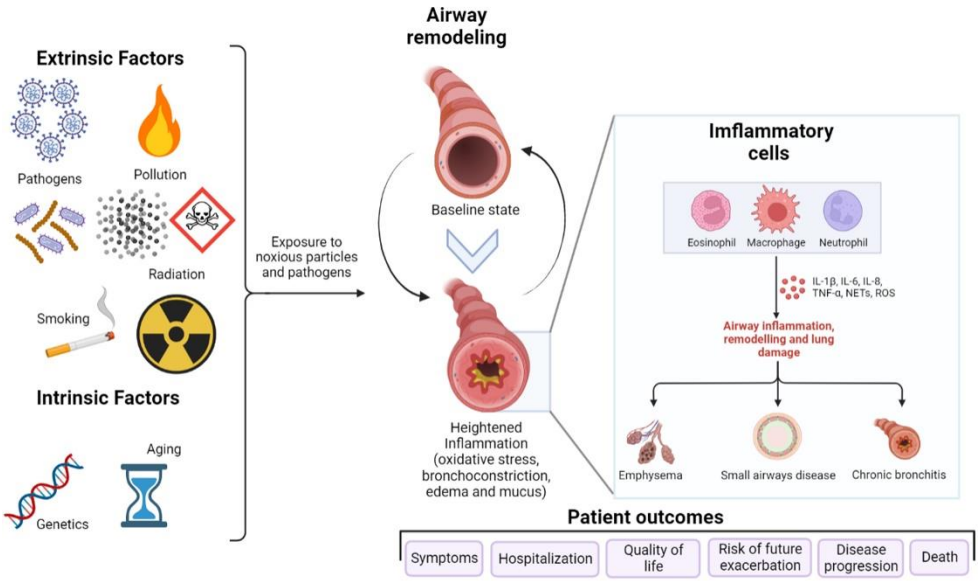


Figure 2 Factors associated with COPD progression and patient outcome.
Association of extrinsic and intrinsic factors that lead to the progression of COPD. Adapted from (Wagner DE. Encyclopedia of Respiratory Medicine 2022) Created with BioRender.com

In addition to upregulation of cytokines due to newly acquired infections, some recent studies have shown that nanoparticle exposure can reactivate latent viruses, which may result in a pulmonary inflammatory response leading to ongoing and further tissue damage in the absence of a newly acquired infection (Ni, Chuang et al. 2015).

While ECM remodeling is known to be prevalent in COPD and asthma (airway remodeling as well as destruction of the distal alveoli)(Larsson-Callerfelt, Weitoft et al. 2015), there is a need of more investigations to elucidate the specific correlation between alterations in ECM and the cells present in the lung that might to participate on the disease's pathology. Thus far, multiple cell types have been identified to play a role in disease, especially inflammatory and resident cell types for both airway and parenchymal remodeling. Structural cells such as epithelial cells, endothelial cells and fibroblasts are also known to be affected in COPD and have been shown to chronically release inflammatory mediators which may enhance inflammation, highlighting the close connection between inflammation and remodeling (Mercado, Ito et al. 2015, Sattler, Moritz et al. 2017, Berggren-Nylund, Ryde et al. 2023).

Patients can live many years with COPD, but the disease is currently irreversible. Many patients ultimately die following an acute exacerbation which greatly

accelerates the decline of their symptoms. These patients have an increased risk for hospitalization and subsequent risk of future exacerbations which accelerate the disease progression until the death. Exacerbations have a heterogenic character such as those due to hypoxia and hyperoxia, cardiac instability, and degree of inflammation; this is both due to and related to the substantial risk of increased prevalence of underlying health conditions and immune responses to respiratory infections (Bhat, Panzica et al. 2015).

COPD is a co-morbidity for increased risk of ARDS

As has been previously reported, there is a high risk of poor outcome of patients with COPD developing ARDS and in particular relevance to the ongoing pandemic, severe COVID-19. COPD patients have increased hospitalizations, ICU admission and higher mortality rates (Gerayeli, Milne et al. 2021). Severe cases of cardiac instability, hypoxia or hyperoxia associated with COPD can also lead to both acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) that is related to high morbidity and mortality (Burge and Wedzicha 2003, Clark, Jit et al. 2020, Polverino and Kheradmand 2020).

ARDS

Acute respiratory distress syndrome (ARDS) is a widely heterogenous and life-threatening disease with mortality rates of around 30-50% worldwide (Gonzales, Lucas et al. 2015, Maca, Jor et al. 2017, Potere, Valeriani et al. 2020). ARDS can be trigged by a wide source of infectious as well as non-infectious injuries and is histologically characterized by diffuse alveolar damage (DAD) characterized by the presence of e.g., edema, inflammation, hyaline membranes, and hemorrhage, which causes a rapid decline in lung function (Kao, Hu et al. 2015, Cardinal-Fernandez, Lorente et al. 2017, Matthay, Zemans et al. 2019, Konopka, Nguyen et al. 2020). Infection-related ARDS can result from direct injury to the lung by different pathogens, including viruses (e.g., SARS-CoV-2), bacterial (e.g., Streptococcus pneumoniae), and fungal colonization (e.g., Aspergillosis in mechanically ventilated patients).

In addition, ARDS is also commonly caused by indirect lung injury from systemic infection (e.g., urinary tract, skin infections and soft tissue), fragments of pathogens (e.g., lipopolysaccharide (LPS)) and inflammatory cytokines in patients in sepsis (Lee 2017). ARDS can be induced when the lung is damaged by other sources, e.g., by gastric aspiration, drowning, traumatic injury, burn wounds, surgery, or inhalation of highly toxic substances, such as xylene or other solvents (Buesa and Peshkov 2009).

ARDS initially presents itself with local or systemic pathophysiological features which depend on the source of the injury. The acute phase consists of deregulated inflammatory activity, surfactant deficiency, activation of leukocytes, coagulation and alterations of the permeability alveolar endothelium and epithelial barriers disruption **Figure 3**. (Ware and Matthay 2000, Matthay, Ware et al. 2012).

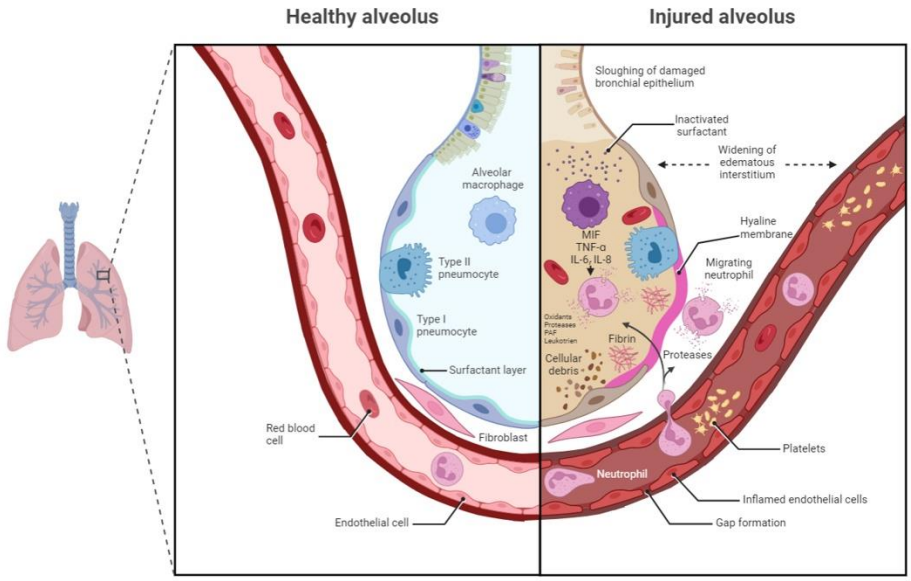


Figure 3 Pathophysiology of ARDS
Observation of changes on the alveolar level during the exudative phase. Adapted from (Ware et al, NEJM 2000) Created with BioRender.com

The treatment to patients in this condition requires meticulous observation and control, depending on the condition of the patient, in case of patients with early phase of severe ARDS the appropriate use of sedation and neuromuscular blockage to avoid patient movements, management of the hemodynamic, prone positioning, support nutritional and observation of blood gases and glucose levels (Artigas, Bernard et al. 1998).

Human studies observing the progression of the inflammatory and physiological changes in the lung have provided an important amount of descriptive information that led to the formation of hypotheses concerning injury mechanisms, such as the examination of the presence of DAD histological hallmarks of ARDS.

Interestingly, some reports have found that the features of DAD may not be present in all patients (56.4%) with ARDS which which suggests that the current criteria may be mischaracterizing about half of the patients (Lorente, Ballen-Barragan et al. 2015, Lorente, Cardinal-Fernández et al. 2015). However, most of the hypotheses formed from clinical studies, face a main challenge of how to evaluate these

hypotheses in human studies due to the many clinical variables that accompany the patients that already are in a critical state. Further, there is a main challenge of obtaining biopsies during disease development to study how the disease develops. Due to these difficulties, animal modelling has emerged as a link between the clinic and the laboratory bench and will be discussed later in the thesis(Matute-Bello, Frevert et al. 2008).

Sex and Gender Differences in Lung Disease

The impact of sex and gender are an understudied aspect of acute and chronic lung diseases. They represents an important aspect to be observed closely in lung diseases. One of the main challenges in studying the effect of sex and/or gender in lung disease is that it is currently not possible to study gender in animal models. While biological sex is assigned at birth due to the presence of specific physical features, gender relates to a social, cultural and self-identification aspect (Caracta 2003, Gharaee-Kermani, Hatano et al. 2005, Card, Carey et al. 2006). As it is not possible to ask animals how they identify and no biomarkers have been identified to date which distinguish genders, the study of gender is restricted to study in human populations or cellular models derived from humans where this can be asked. Until quite recently, sex and gender have been used interchangeably and that is reflected in older literature relating to animals where sex is incorrectly referred to as gender in animal models. Terminologies need to be constantly revisited according with the where society finds itself; the awareness of topics such as, integrated nature of the Sustainable Development Goals (SDGs), diversity and equality, are highly important to guide new generations of researchers using an appropriate terminology and reflecting on these important aspects.

The importance to observe sex as a determinant to the prevalence of some lung diseases is based on average differences in anatomical sizes but also features which affect physiology to differentiate female and male organisms. On average, male lungs are larger than female lungs and therefore have greater lung volume capacity (Somayaji and Chalmers 2022). COPD and asthma are the main contributors to death due to lung disease and 6.2% and 5.2% of females versus males of all deaths.,(Heron 2018, Chowdhury, Guntur et al. 2021). In the past, the direct correlation among elevated prevalence of COPD in males was associated with their higher cigarette smoking habits; however, this has been slightly changed. More recent data shows that smoking habits in females living in some high-income countries has grown such as in Austria (Schirnhofer, Lamprecht et al. 2007), and Spain (Haeberer, Leon-Gomez et al. 2020), but not Norway where it has been decreasing for both males and females (Lund, Lund et al. 2009). Interestingly, while the prevalence of smoking is decreasing for both male and females in Spain, the prevalence of males smoking is decreasing faster, but the rates of smoking prevalence are increasing in females over 45 years old (Haeberer, Leon-Gomez et

al. 2020). Due to these changes in both lifestyle and environment, new epidemiological estimates now show that in some regions COPD is equally common in males and females (Haeberer, Leon-Gomez et al. 2020). Moreover, females may show in some cases a worse outcome than males leading to death (Schirnhofer, Lamprecht et al. 2007, Somayaji and Chalmers 2022).

Histologically sex-specific differences in disease features have also been identified with females having higher presence of small airway disease and males presenting more emphysema (Martinez, Curtis et al. 2007, Tam, Churg et al. 2016). Interestingly, animal studies demonstrate that a reduction on the production of female sex hormones (and specifically estrogen) (Bloodworth, Rusznak et al. 2019) via ovariectomy promoted higher levels of emphysema features, similarly to what was observed in male animals. The relation of the outcomes and biological differences may be related with the effect of sex hormone as well as the anatomical differences mentioned above. However, other factors such as immunological differences in response to pathogens as well as sex-specific differences in susceptibility to cigarette smoking may play a role in disease onset and progression (Sin, Cohen et al. 2007, Tam, Churg et al. 2016).

Some previous literature has identified sex-specific differences related to inflammatory responses in mice LPS-induced is influenced by gender, where Card and colleagues showed that male mice have exaggerated airway inflammatory. (Tesfaigzi, Rudolph et al. 2001, Card, Carey et al. 2006, Nguyen, Castro et al. 2019). In general, there is limited information in other larger animal species where the incorporation of clinically relevant interventions can be included (e.g., mechanical ventilation and extracorporeal membrane oxygenation (ECMO)). The general lack of studies examining sex differences in animals, especially large animal-based studies of acute lung injury, is mainly attributed due to the additional cost. This has resulted in a limited amount of literature concerning possible biological sex-specific differences in lung injury responses (e.g. biomarkers, histological differences, etc.) or treatments (Tiba, McCracken et al. 2021).

There is currently no consensus in the literature for the clinical scenario for ARDS. Some studies have found clinical mortality rates to be higher in females with ARDS as well as higher mortality rates in patients with severe ARDS (Heffernan, Dossett et al. 2011, McNicholas, Madotto et al. 2019). However, there is also literature which has found no significant differences with biological sex in acute lung injury (Lemos-Filho, Mikkelsen et al. 2013) or ARDS mortality (Heffernan, Dossett et al. 2011). These discrepancies are likely due to the high heterogeneity of the disease and the challenges with creating well-defined sub-phenotype groups (Bos, Laffey et al. 2022).

Previous work has found that variables such as ventilation parameters may play an important role in the extent of lung injury, where evidence exists for differences in disease severity in smaller females and is associated with ventilatory parameters that

are not always adjusted for size (McNicholas, Madotto et al. 2019). Sex bias in lung diseases in the investigations of the pathogenesis and progression of the diseases needs be addressed with the goal to achieve more equal and a sustainable path forward so that we can better understand features of disease and identify sex or gender specific differences to improve prevention and therapeutic strategies. (Shah and Newcomb 2018)

Treatment for End-stage lung diseases

Despite the advances and the fast major approaches applied to treating ARDS, patients still have a high risk to face severe acute lung injury with life-threatening progression, however the development of techniques to life support and delay the progression of the lung injuries to become end-stage disease have increased in frequency. One example is ECMO which can be performed at highly specialized hospital center. This provides time for the lung and the patient to recover; despite the fact that these techniques are currently in place, some populations do not have access to such technology, due to socioeconomic deprivation (SED) (Beliaev, Alison et al. 2018) and/or the demographic size. ECMO is also used as a bridge to transplantation for some patients and its increasing use might also contribute to increased number of patients on transplantation lists (Quon, Psoter et al. 2012, Lindstedt, Grins et al. 2021).

Especially in cases such as patients with ARDS related to COVID-19, the World Health Organization (WHO) guidelines recommended the eligibility of ECMO (Ramanathan, Antognini et al. 2020). Another controversial aspect connected with lung transplantation is that even that it is already in place well-established for patients with chronic pulmonary diseases with good survival rates, there is ongoing discussion and debates about the use of lung transplantation in acute organ failure (e.g. due to ARDS). There are also discussions about the need of prolongation of postoperative ECMO (Frick, Gan et al. 2022). Due to the fast progression of ARDS and the elevated mortality rate, these patients may not be the ideal candidates for lung transplantation but rather may have a more positive outlook if regenerative techniques can be developed, such as cell therapy or tissue engineered therapies. These may provide alternatives for the current options of treatments and cause a positive outcome to patients.

Animal modelling of lung diseases

Animal Models of COPD

Even though it is widely studied, COPD remains a devastating disease with an enormous negative impact on human quality of life, but due to the fact it remains without an effective treatment, more studies and observations of the disease is needed in different contexts. Due to the complexity of the disease, there are only a limited number of *ex vivo* techniques with human cells and tissue which can be used to study the life cycle of the disease. Therefore, the utilization of animal models allows for the possibility of the progression of features of the diseases and development of new tools that might allow us to investigate mechanisms that may provide deeper understanding of some of the hallmarks of the disease such as inflammatory events, airway remodeling, and development of acute lung injury.

Several distinct species and strains of animals have been used in combination with different damaging agents to study COPD in animals such as intratracheal elastase administration, particle administration and cigarette smoking (Tam, Churg et al. 2016). See (Bölükbas, Da Silva et al. 2019)(*Other publications not included in the thesis*) for a complete discussion of animal models of injury for COPD, as well as acute exacerbations. However, there is no animal model which completely recapitulates human disease. Rather, the models aim to mimic different aspects of both acute and chronic phases of COPD such as exacerbations that are responsible for the worsening of respiratory symptoms presented during the disease's progression (i.e. acute inflammation), bronchitis, and emphysema. Where it can be implied by insults that may to lead to acute lung injuries and in a long term may lead to the development of chronic lung diseases (Sapey, Bafadhel et al. 2019).

Animal modelling of ARDS

While in vitro models can recapitulate some features of ARDS, the disease is multifactorial and therefore small and large animal models remain the main preclinical research tools. The choice of small or large animal model depends on the disease features to be modelled or the type of therapy to be evaluated (Yehya 2019). Each model differs regarding the species selected as well as the injurious agent used to induce acute lung injury. Therefore, each has its own benefits and limitations (Stenlo, Hyllen et al. 2020, Leiphrakpam, Weber et al. 2021). Several different models have been established to mimic the onset and pathology of acute lung injury (ALI) and these differ depending on the injury to be modelled and species used. Some of the commonly used agents shown to be capable of inducing severe lung injury include injurious mechanical ventilation, hyperoxia, viral or bacterial administration, and lipopolysaccharide (LPS) administration (Matute-Bello, Frevert

et al. 2008, Ballard-Croft, Wang et al. 2012, Arora, Ahmad et al. 2019, Tiba, McCracken et al. 2021).

As for many other diseases, murine animal models have been widely explored for their versatility, reproducibility and the ease in standardizing and manipulating their genetic background (Matute-Bello, Downey et al. 2011, Stenlo, Hyllen et al. 2020, Stenlo, Silva et al. 2021). However, for studies that have a main objective to evaluate disease progression and/or potential therapies, incorporation of clinical standards of care, potential therapeutic interventions, such as mechanical ventilation or ECMO, large animal models (typically porcine or ovine) are the most appropriate due to the fact their lung anatomy and injury features are more similar to humans (Moodley, Sturm et al. 2016, Millar, Wildi et al. 2021).

To determine whether an animal model mimics ARDS in patients, clinical parameters described in the Berlin criteria are used (e.g. the ratio between arterial oxygen tension and inspiratory oxygen, appearance of infiltrates in clinical imaging) as well as tissue or molecular signs of injury (Matute-Bello, Downey et al. 2011). ARDS diagnosis is based on the Berlin criteria which encompasses 4 main characteristics. See **Table 2** (Ferguson, Fan et al. 2012, Force, Ranieri et al. 2012).

Table 2 Application of Berlin criteria in the clinic vs animal modeling.

Criteria	Symptoms	Animal models
Timing	Within one week of known injury or new/worsening respiratory symptoms	Timing and type of injury is known
Chest imaging	(X-ray or CT scan) Presence of bilateral opacities not fully justified by lobar/lung collapse or nodules	Not always possible, histological evidence is needed instead
Origin of edema	Respiratory failure not fully justified by heart failure or fluid overload (hydrostatic edema must be excluded), and positive end expiratory pressure (PEEP)≥5 cm H2O	The origin of edema can be confirmed by monitoring cardiac parameters and fluid support intake before injury onset
Oxygenation*		
Mild	(PaO2/FiO2 >200 to ≤ 300 mmHg with PEEP or continuous positive airway pressure (CPAP) ≥5 cm H2O	Graded same as in clinic
Moderate	(PaO2/FiO2 >100 to ≤ 200 mmHg with PEEP ≥5 cm H2O	
Severe	PaO2/FiO2 ≤ 100 mmHg with PEEP ≥5 cm H2O	

* Oxygenation levels can be readily measured through serial blood gases

However, chest images using CT can present a challenge on the incorporation due to the short time of the experiments of injury models utilized for ARDS, scarcity of

equipment, or even difficulties on the transportation logistics. Further, another aspect to that might to be interesting to highlight is the fact that patients in severe states shouldn't be moved and kept in prone position, what also might to be problematic to the imaging (Matute-Bello, Downey et al. 2011, Kulkarni, Lee et al. 2022). Due to this fact, histology is often used as the gold standard selected to serve as a surrogate for the absence of high-resolution chest images and applied to exclude other differential diagnosis by examination of histological tissue; it can also be used to evaluate the level of the injury in animal models (Wang, Bodenstein et al. 2008, Hellbach, Baehr et al. 2018, Leiphrakpam, Weber et al. 2021). Histological assessment of features at different time points can help distinguish ARDS from alternative diagnoses by confirming evidence of lung tissue injury, such as presence of proteinaceous debris in the alveolar space, hemorrhage, hyaline membrane, and accumulation of inflammatory cells (Matute-Bello, Frevert et al. 2008, Matute-Bello, Downey et al. 2011, Lorente, Ballen-Barragan et al. 2015, Kulkarni, Lee et al. 2022).

One advantage of especially large animal models is that biopsies can be taken across different time frames of the disease as it evolves. This is particularly of benefit due to the fact that histological sub-phenotypes have been identified in autopsy cohorts of patients with ARDS (i.e. patients with and without diffuse alveolar damage but clinically diagnosed ARDS according to the Berlin criteria) (Lorente, Cardinal-Fernández et al. 2015, Palakshappa and Meyer 2015). Incorporation of sub-phenotypes based on histological findings is even more challenging, as lung biopsies are not available in all clinical studies as they can be contraindicated clinically (Palakshappa and Meyer 2015).

The incorporation of these finds and moving forward to applications of digital histology and automatization of processes, machine learning may also provide and enormous assistance on the coast of the professional time, been possible to flag areas of interest for the examination of the pathologist, avoiding human variation during studies, when is not possible to provide a good amount of researchers with similar expertise level or time exposed on the material, besides the personal level of criteria, and finally based on that the consistency that it can contribute on the research applications, identification of features that may be missed by human perception (Shinde and Shah 2018).

Histology as a gold standard to assess lung injury

Due to the complexity described above, such as the large number of samples generated in large animal models and different outcomes depending on the injury model selected, histological evidence of lung injury was defined as the most relevant defining feature of ALI (Matute-Bello, Frevert et al. 2008). The pathological

hallmark of ALI in the clinic is DAD and it is characterized by: “Accumulation of neutrophils in the vascular, interstitial, and alveolar spaces, deposition of hyaline membranes, proteinaceous debris, interstitial thickening, evidence of endothelial injury, and intraluminal activation of the coagulation cascade” (Matute-Bello, Frevert et al. 2008, Matute-Bello, Downey et al. 2011).

However, due to physiological differences across species (e.g. variability in innate immune responses to injury (e.g., LPS sensitivity) or size), no animal model fully recapitulates the histological features of DAD observed in humans (Matute-Bello, Frevert et al. 2008). Therefore, the presence of one single feature in an animal model, does not directly denote that the model is reliable and conversely, and the absence of one or more features does not rule out ALI (Matute-Bello, Downey et al. 2011). Despite adopting different approaches, semi-quantitative assessments are indeed fundamental for a reliable analysis of the histologically observed characteristics of ALI in animal models (Meyerholz and Beck 2018).

What is histology?

Histology is the study of microscopic analyses of tissue obtained from biopsy or surgical specimen that is classically performed after some degree of chemical preparation. It is commonly used to study different diseases but can also be used to study developmental processes as well as to evaluate tissue regeneration or even artificially generated tissue in the lab (i.e. bioengineered tissue). The most classical approach for histological evaluation is based on formalin fixation and paraffin embedded (FFPE) samples, followed by placing thin sections of the tissue onto glass slides for staining of specific structures or features and visual observation with a light-based microscope. However, other approaches such as physical fixation (using low temperatures to stabilize the tissue), snap freezing, and embedding in other materials (e.g. optimal cutting temperature (OCT)) are also possible (Suvarna, Layton et al. 2018).

Embedding in OCT followed by cryosection, may preserve the tissue architecture and be used to histological analyses. this approach mainly used in hospitals, when a biopsy collected form a patient needs to be rapidly evaluated possible tumours or material that might to be life threatening to the patient. However, due limitations, such lower optical resolution in the case of OCT, in comparison to FFPE remain as the gold standard to histological evaluation (Da Silva, Gvazava et al. 2023).

The visualization of different components inside the tissue occurs with the use of one or more dyes that stain specific cellular or structural compounds to observe under the microscope. Hematoxylin-Eosin H&E staining has been used for over a hundred years due to its simplicity on the one hand but also that it helps to identify major features such as cell nuclei, cytoplasm, and extracellular matrix with

differential staining pigments. Due to its long history for diagnostic purposes, H&E is still recognized as a state of art technique among pathologists (Fox 2000). However, with new technologies and techniques to prepare the tissue and to observe it have evolved over the years. There remains intense interest to continue to improve and refine this technique (Gurcan, Boucheron et al. 2009). In order to do this, there is a need to understand the steps that leads to an appropriate histological slide to be used directly with light microscope analysis or in a future evolving field, machine learning, are crucial. An overview of histological processing is presented in **Figure 4** can provide some visual of the different approaches relate to the fixation.

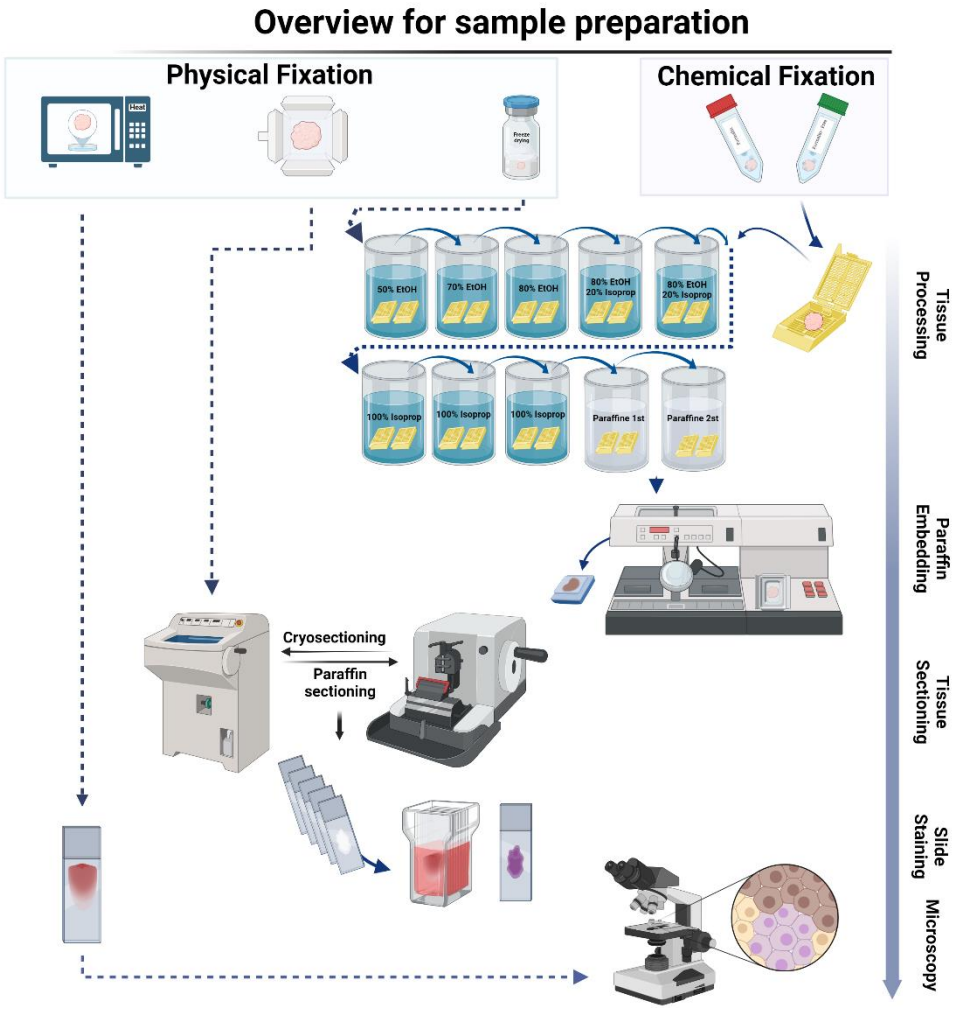


Figure 4 Overview of sample preparation different approaches of fixation.
Created with BioRender.com

Tissue Fixation

As mentioned above, histological examinations depend on different steps that can be executed in several ways, such as, physical, or chemical fixation. The main goal of fixation is prepare the tissue in a way that best helps to promote the understanding of the tissue and cellular architecture of the material. Each tissue and material have different needs for preparation that are very important.

At the start of any histology processes, it is imperative to determine the type of material and its quality. Different types of tissues may have different circumstances such as time from sample collection to fixation in the case of tissue collected during biopsies of patients or bioengineered tissue and polymers. The main goal of tissue fixation is to help preserve the tissue and cellular structure, as well as to minimally change, destroy or remove biological molecules so that later we can observe the histological anatomy of the tissue (e.g., the relationship between cell and the ECM) as well as the cellular nuclei and cytoplasm components so that there can be an assessment of the organization of the compartments of the cells in the tissue (Suvarna, Layton et al. 2018, Da Silva, Gvazava et al. 2023).

The fixation can be achieved using many different approaches which have different underlying mechanisms and principles to preserve the tissue, proteins and molecules (Suvarna, Layton et al. 2018). Tissue fixation approaches fall into different groups or categories, such as chemical fixation through the use of chemicals which impart chemical crosslinks on the tissue. This can be achieved through the addition or spontaneous formation of covalent crosslinks by the addition of reactive groups to form cross-links as well as the formation of weaker chemical bonds such as hydrophobic bonds, electrostatic, hydrogen and metallic bridges (e.g. calcium). This can be achieved through acidification, dehydration by the addition of salts, or temperature control (i.e. heating or freezing, etc), where each has pros and cons. In other words, the major categories of the main fixatives are: a) Aldehydes: formaldehydes, glutaraldehyde (cross-linking proteins), b) oxidizing agents: osmium tetroxide, potassium permanganate (cross-linking proteins), c) Alcohol-based: methyl alcohol, ethyl alcohol, acetic acid (protein denaturants agents) to coagulate proteins and d) metallic-based: mercuric chloride, picric acid (formation of insoluble metallic precipitates)(Thavarajah, Mudimbaimannar et al. 2012).

Despite the fact that there are many different fixatives to choose from, the most commonly used is formaldehyde which has been applied for histological examination practice since the 19th century and is still the fixative of choice for more than 81% of histological laboratories (Suvarna, Layton et al. 2018). Formaldehyde is preferred because of its rapid penetration (diffusion) due to its small size. Penetration distance (d in millimetres) is calculated using the follow function:

$$d = k\sqrt{t}$$

Where *k* is the coefficient of diffusibility (the Medawar constant) times the square root of time (*t*) that the tissues are in the fixative.

Each tissue or subject has a specify *k*, due natural resistance such as, cell membranes, ECM composition and arrangement *(Suvarna, Layton et al. 2018)*.

As the formaldehyde penetrates through the tissue, the formaldehyde stops the process of autolysis which starts as soon as the tissue is removed. After the biopsy is taken (potentially when the organism and specimen still is alive but also potentially *post-mortem* collection) the tissue immediately starts the decomposition process (necrosis). Here, formaldehyde's role is to cross-linking and covalent bonding between proteins to stabilize the tissue (Buesa 2008). This typically results in loss of antigen due to changes in the conformation of proteins in relation to the shape of the antibody formed from proteins in their native state. This can impact during immunohistochemical procedures and antibodies must be tested or the process modified to be compatible with the tissue fixation and processing approach . Tissue fixation can also play a role on the ability to molecularly recuperate DNA/RNA analysis (Jewell, Srinivasan et al. 2002), or also for other examples touched in this thesis, such as bioengineered materials that may be dissociated during fixation processes if incompatible fixative solutions are used (Da Silva, Gvazava et al. 2023). Therefore, it is important to keep this in mind when selecting a fixative. However, despites its utility, a main concern for the use of formaldehyde is its toxicity during professional handling of the chemical, and its negative environmental effect (Buesa 2008, Suvarna, Layton et al. 2018).

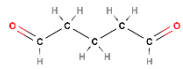
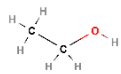
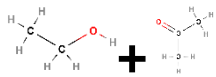
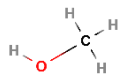
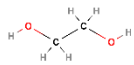
Tissue processing

After fixation, the next step is to further process the tissue so that it can be imaged. The main objective is to protect the tissue (cells and ECM) from distortion and to prevent damage to the tissue. The most common approach for tissue processing is paraffin and the main aim is to remove the water contained in the tissue and replace with a medium (e.g., paraffin, resin) that promotes the necessary rigidity to become sectioned afterwards. Many protocols also include chemicals to remove lipids (called clearing or diaphanization) which helps to improve the optical clarity of sections because lipids refract light.

Depending on the conditions and individual needs of the tissue, different factors can contribute to the increase of the speed and quality of the processing. Some of the known factors that can be implemented and influence the rate of the tissue processing: use of agitation (increase the flow of fresh solutions available to the tissue), heat (increase the rate of penetration and fluidity), viscosity (resistance to the flow of a fluid, where small molecules as a faster, and large slower penetration on the tissue), and vacuum (pressure change to increase the rate of infiltration, decreasing the time necessary to each step) (Spencer, Bancroft et al. 2012).

Several innovations have occurred in the last decades to facilitate these techniques and minimize manual handling involved during the tissue processing. One major innovation is the implementation of automatic tissue processors which help improve the quality the tissue because of the use of a highly standardised protocol which helps to minimize human mistakes, improving reproducibility and reliability. **Table 3** has an overview of the whole tissue processing approach.

Table 3 Summarization of the steps involved during traditional tissue processing.

Steps of tissue procesing	Function	componds	Formula
Fixation	Stabilize and preserve the tissue with minimal cellular distortion	10% neutral buffered formaln (NBF)	
		PFA 4%	
		Glutaraldehyde	
Dehydration	Remove remove residual tissue fixatives and remove water from the tissue	Ethanol	
		Ethanol-acetone	
		Methanol	
		Isopropyl alcohol (2-propanol)	
		Glycol	
Clearing	Remove lipids and remove polar dehydrating solutions to prepare the tissue to receive the infiltrating embedding medium	Xylene	
		Toluene	
		Choroform	
Infiltration / embedding	Inclusion of the tissue in the support medium and orienting prior to solidification.	Paraffin Wax Epox or acrylic resins	

Histology of bioengineered polymers

Bioengineered polymers are being used increasingly in biomedical research for different purposes such as generation of tissue to model diseases or biology and another main effort to support the current gap between the need of transplantation of end-stages patients and the lack of donors available to supply this demand (De Santis, Bolukbas et al. 2018). Another aspect that makes the research on bioengineered tissue using polymers even more needed is the increasing number of patients that do not fully recover their lung function after SARS-COV2 infection. This is generally known as long COVID-19 or post-acute sequelae of SARS-CoV-2 infection (PASC) (Cortinovis, Perico et al. 2021). There are an increasing number of reports investigating this emerging condition and a clear need for the development of different strategies to help these patients. One potential option is regenerative medicine therapies but also bioengineering of transplantable tissue. (Mason and Dunnill 2007, Lindstedt, Grins et al. 2021, Stenlo, Silva et al. 2021, Munblit, Nicholson et al. 2022, Da Silva, Gvazava et al. 2023).

Bioengineered polymers are already used in thoracic surgery (e.g. sutures), surgical implants (e.g. airway stents or ECMO devices), and are also being explored for use artificial tissue (Platine bones, silicone, artificial trachea), and of course as biomaterial scaffolds for tissue engineering. Both regenerative medicine approaches offer promise (i.e. cell therapy and bioengineering tissue) have limitations. In the case of normal clinical lung transplantation, it requires immunosuppression, and it is thought that bioengineered tissue will require some degree of immunosuppression. In addition to the cell associated immune response, biomaterial scaffolds can also promote inflammation. This is an understudied area, and it still needs more research on the topic. The main focus of regenerative medicine so far has been focussing on cell therapy using both somatic and embryonic derived cells which have been differentiated into adult lung cells. More recently, there have been advances in reprogramed cells to become pluripotent cells. Regenerative medicine requires integrating knowledge about cell therapies utilizing gene-based methodologies, molecular medicine, and biomaterials such as, hydrogels or hybrid-hydrogels based in alginate and native tissue (Mason and Dunnill 2007, De Santis, Bolukbas et al. 2018, De Santis, Alsafadi et al. 2021, Da Silva, Gvazava et al. 2023).

There are many different types of materials under investigation for tissue engineering of lung and other epithelial tissues using different manufacturing techniques. One of the most popular is hydrogels due to their network structure. There are different natural and synthetic hydrogels systems which can be used such as alginate (Lee and Mooney 2012), polyethylene glycol (Blakeley, Sharma et al. 2022), chitosan (Shariatinia and Jalali 2018, Kim, Lee et al. 2021), and alginate. This thesis has focused on alginate because of its advantages such as, the possibility to manufacture complex shapes with cells included. Alginate is derived from brown

algae and is currently used in clinical products for wound healing and is under investigation for cell therapy (e.g. pancreatic islets) (Bochenek, Veiseh et al. 2018). Alginate offers the possibility to be manufactured in various approaches (e.g., 3D printing, microspheres, etc.) and applications such as the use of microspheres for drug delivery or 3D constructs for graft investigations. Due to the fast development of these technologies and their proximity to the clinic, the development of validation tools are needed. Histological examinations of potential biomaterials pre and post-implantation allow the observation of this materials and the interactions with the host, at the molecular and cellular and to tissue interaction between the graft and host (Uyen, Hamid et al. 2020, De Santis, Alsafadi et al. 2021, Da Silva, Gvazava et al. 2023). Improper tissue fixation and processing is known to lead to artifacts and can occur during routine handling of even biological tissues. As the interaction of the biomaterials with the host are critical for pre-clinical evaluation, it is important to be able to have reliable techniques to evaluate these materials pre and post-implantation. These evaluations are important for evaluating the compatibility of a biomaterial and cell before going into human studies.

However, bioengineered materials, and particularly alginate, differ from traditional biological materials. Biological materials typically have a higher diversity of chemical groups available for tissue level fixation in comparison to bioengineered polymers which are less diverse. Some of the main challenges to address for reach biomaterials are due to the chemical groups available for crosslinking/fixation, the amount of water that this material contains and even the crosslinking agent used to stabilize the material during manufacturing. For alginate, this can be covalent or ionic crosslinking and the strength of these bonds can affect how the bioengineered tissue behaves during tissue processing. These factors are important to consider, and fixatives and solutions used during the tissue processing need to be compatible with the material to maintain the relevant architecture of the constructs over time. Several approaches have been reported to crosslink cells to biomaterials. Chemical fixation (NBF, PFA, Glutaraldehyde) is most commonly used due to the fact that is commonly used to provide stability during the histological routine (Da Silva, Gvazava et al. 2023). However, physical fixation approaches can also be used using (e.g., snap freezing, lyophilization, and embedding with OCT for cryosection) (De Santis, Alsafadi et al. 2021).

However, the traditional histological routines on the other hand also carry limitations. OCT embedding demands certain temperatures, can cause the risk to the lost of the sample n case of accidental thawing, besides the low optical resolution for histological examination, further, chemical fixations may generate artifacts such as loss of cells and detachment of implanted hydrogels on neighbouring tissue; both of these can make the histological interpretation prone to misinterpretation. Thus, the importance to find reliable techniques to improve the possible utilization of histological examinations are needed. In addition, traditional histological processing is known to contain multiple hazardous chemicals such as formaldehyde and xylene.

There is increased awareness and discussion for the development of green histology and more sustainable approaches; this topic covers not only the environmental aspects, but also the safety of the histopathologist and laboratory technicians who are exposed to these chemicals (De Santis, Bolukbas et al. 2018, De Santis, Alsafadi et al. 2021, Alsafadi, Stegmayr et al. 2022, Silva, Gvazava et al. 2022).

Development of green histology workflows

Common fixatives, such as formalin (formaldehyde) and solvents, such as xylene, used in traditional histological processing can cause dissolution of engineered materials during the processing of polymers such as polypropylene and alginate (Da Silva, Gvazava et al. 2023). Another important aspect to highlight is the fact that specific compounds of interest, such as inhaled materials (e.g. microplastics and particulate matter or pleural anthracosis) are increasingly recognized as a quite important feature which could be best observed on the tissue level with histology. This can help to evaluate pathological changes which accompany respiratory complications due to long-term inhaling of urban pollution, indoor pollution and/or cigarette smoking (Takano, Justo et al. 2019).

The main solvent used in standard tissue processing is xylene. Xylene is known to dissolve many polymers and therefore it may remove some of the compounds above during routine histology. More than 40 years ago, replacements for xylene were discussed and researched but they have been slow to be implemented on a large scale (Wishe, Roy et al. 1980, Falkeholm, Grant et al. 2001). The replacement of the xylene with other chemicals (e.g. isopropanol) has been shown to be suitable (Falkeholm, Grant et al. 2001). This replacement is safer environmentally and occupationally which is especially important when large volumes are used. Therefore, in addition to the environmental and sustainable development the use of isopropanol offers, isopropanol can improve the compatibility of histological techniques using bioengineered hydrogels materials, such bioinks and bioprinted constructs containing ECM and/or alginate (Falkeholm, Grant et al. 2001, De Santis, Alsafadi et al. 2021).

The ethical, legal and societal need to develop green histology workflows

Another issue that is indirectly influenced was concern with the safety of the professional handling the tissue in some of the steps involved in histological preparations, such as formaldehyde, which is responsible traditionally for the tissue fixation. Already in 1981, it was reported that around 1.6 millions of workers were exposed daily and for those, around 4% were exposed daily for more than 4 hrs in

United States (Buesa 2008). However, another chemical traditionally present during the histological pipeline and used for tissue clearing is xylene. It is toxic for both the environment and the end-user may develop serious conditions such as Raynaud's phenomenon due to contact with this solvent; additionally, the facility needs special ventilations and appropriate training for fire as the material is highly flammable. This means that the use of xylene demands a higher energetic cost (Kandyala, Raghavendra et al. 2010, Purdie, Purdie et al. 2011, de Aquino, Zenkner et al. 2016, Silva, Gvazava et al. 2022). Contact with xylene causes eye, skin, mucous membrane, and respiratory tract irritation (Alwahaibi, Aljaradi et al. 2018).

Besides, following the sustainability goals in the Agenda 2030 (**Figure 5**), such as (3) good health and well-being and (12) responsible consumption, the importance to test, validate and implement new strategies on the tissue processing are needed (Silva, Gvazava et al. 2022). In addition, and according to the Declaration of Helsinki – ethical principles for medical research involving human subjects, such as 11. "Medical research should be conducted in a manner that minimises possible harm to the environment". Therefore, there is a high motivation to replace these chemicals.

Figure 5. Sustainable development goals; with permission under a CC BY-NC 4.0.
https://www.un.org/sustainabledevelopment/news/communications-material/

Further, xylene is already on the European Chemical Agency's (ECHA) list of chemicals to be evaluated in its Community Rolling Action Plan (CoRAP) in 2022 for potential stricter regulations. The discussion about the need of the removal xylene of the processing steps are not novel. Since 1980, solutions for replacement of xylene have been discussed and new different approaches have been suggested

(Wishe, Roy et al. 1980, Alwahaibi, Aljaradi et al. 2018). Nowadays, the challenge is well-understood. However, despite all information and possibilities, why the clinic and preclinic research besides pathologic departments at the hospitals are not moving forward with these changes remains unknown.

Principles of green chemistry

Green chemistry is defined by the design of production that aims to reduce and eliminate the generation of hazardous substances. The application of green chemistry is present in all the life cycle of the compounds, since extraction of the initial chemical, manufacturing, and disposal are considered. Green chemistry is differentiated from processes that focus on cleaning pollution that already exists or treating waste streams (remediation) and removing hazardous materials from the environment (Li and Anastas 2012).

Objectively the use of green chemistry is applied in all the areas of the chemistry, and prevents pollution at the molecular level, using innovations and scientific solutions to the environmental problems. This results in the reduction of the human negative impact and use of source consumption and pollution in the end stages of the life cycle of the material (Kidwai and Mohan 2005).

It has emerged due to the trend of ideas and initiatives to develop and find more sustainable applications. The need of rules and guidelines in green chemistry provided the pathway where different laboratories with broader focus could contribute and consolidate what nowadays we recognize as principles of green analytical chemistry. Once that it can be a quite broad topic, laboratories from other expertises contributed later with several other applications of the field. Now, it has evolved to include: Principles of green chemical technologies, principles of green analytical chemistry, and Principles of green engineering (Yilmaz and Soylak 2020).

It focuses especially on awareness of the negative impacts of the utilization of analytical techniques that might not normally consider the environmental and operator risks. The consequences and results that the continuation of the use of certain techniques can cause, such as economical, energy consumption, laboratory waste and emissions, of the analytical steps, are evaluated (Vian, Breil et al. 2017).

However, since the 1970’s, it is not only that there is increased awareness regarding the dangers of chemicals to the environment and to society in general, but there is also increased research on solutions that could be provided for the research community, such as, sample preparation methods with green character as well measurements and data processing to minimize waste (Kurowska-Susdorf, Zwierżdżyński et al. 2019).

Gałuszka and colleagues 2013, suggested a mnemonics SIGNIFICANCE **Table 4** that condenses the principles and the main points of performing green chemistry:

Table 4 Green chemistry principles.

Green chemistry principles	
1. **S**elect direct analytical technique	2. **I**ncrease safety for the operator
3. **I**ntegrate analytical processes and operations	4. **C**arry out in-situ measurements
5. **G**enerate as little waste as possible and treat properly	6. **A**void derivatization
7. **N**ever waste energy	8. **N**ote the sample number and the size should be minimal
9. **I**mplement automatization and miniaturization of methods	10. **C**hoose multianalyte and multiparameter method
11. **F**avor reagents obtained from renewable source	12. **E**liminate or replace toxic reagents

SIGNIFICANCE can help lead us to new insights to production and utilization of chemicals in different sections such as, solvents used or different techniques and the concern with the toxicity for both the environmental and end-user (Gałuszka, Migaszewski et al. 2013).

Pros and cons on the current fixatives available

The choice of the best and most appropriate fixative for the methodological approach planned of course depends on availability and several other considerations and so-called decision-making point of view; however, some fundamental advantages and disadvantages can be summarized and hopefully facilitate this task.

Formalin, the most widely used fixative, has the advantage of having more than 100 years of scientific knowledge accumulated and the use reported in various types of tissue and materials (Fox, Johnson et al. 1985). It is defined as the gold standard for samples fixed in formalin and embedded with paraffin and also used in fixation associated with cryosectioning; most of the histotechniques and manufacturers of antibodies have therefore optimized for this methodology due to its preservation of proteins and lipids, besides the low cost (Buesa 2008). And until this moment, an alternative that completely fulfils all the uses of this compound does not exist yet, but alternatives are emerging.

However, there are also known disadvantages on using formaldehyde. It is widely known that it is reported to have high carcinogenic potential. Histologically, due to the molecular action, formaldehyde is a slow fixative, taking from 1 to 2 days for a complete fixation of all the proteins. Therefore, depending on the size of the tissue, more than 2/3 of the tissue may not be well fixed if larger pieces are used. This may have dire consequences for the follow-up steps of the tissue preparation such as paraffin infiltration and consecutive histology. In addition, it may present challenges with the cross-links formed with the nucleic acids that may be lost or crosslinked with proteins (Buesa 2008).

Alcohols fixatives are an alternative that provide a fast effect. However, they are also known to cause dehydration which promotes tissue shrinkage and hardening caused by the coagulation of proteins and the disruption of nucleic acids. Coagulation can be reversible and this allows the return of the baseline size during rehydration steps. Some alcohol based fixatives also include acetic acid and methanol, to limit shrinkage and perform the fixation of larger pieces of tissue, respectively. Another positive aspect, once that the fixation and the first steps of tissue processing happen simultaneously, are that the processing protocols can be shorter. This can be beneficial for methodologies that needs faster approaches.

Unfortunately, the main disadvantage in choosing to move to formalin substitutes is that techniques are well established in labs and based on using formalin as a fixative. Therefore, switches may need to renew all the controls for proper reliability of the material compared. This is especially important for material scored or that needs be evaluated based on histological examinations. Once that the tissue may appear slightly different, it may prevent a fast transition to formalin substitutes. This is a major challenge concerning immunobiological analysis, where many antibodies may need be revalidated, what can be time-consuming besides the high costs involved in such investment.

Fixative substitutes

The motivation to search for a substitute for formaldehyde is based on two developments. Firstly, the need of a compound less dangerous chemical and more hazard declared by the Occupational Safety and Health Administration (OSHA), and also with the challenge that formalin doesn't warrant a total DNA and mRNA recovery which is essential for biomolecular methodologies and is increasingly important with the development of new methodologies such as spatial transcriptomics and the increase of the sensitivity of certain analyses that may generate artifacts and degradation of sensitive samples (Kok and Boon 1990, Buesa 2008). Therefore, a variety of formalin-free fixatives have been developed and commercialized (**Table 5**).

Most of the alternatives available on the market are alcohol based which also addresses the need of nucleic acid's stabilization. Unfortunately, another aspect generated from the competition among the companies to provide a substitute that can be broadly used was the secrecy of the formulations, which makes the formalin-free substitutes more expensive.

Reflecting off the last point, before the widespread implementation of formalin in tissue fixation protocols, other solutions were used. Some of these solutions may offer inspiration. Looking back to understand what was done and reported before may help us understand how we can implement green methodologies for our current needs. *Carnoy's fixative* dated from 1887, is based on the mixture of 100% or 95% ethanol (6 parts), chloroform (3 parts), and acetic acid (1 part). It has been reported to be the best choice if nuclear fixation is especially required. If the ethanol is substituted for methanol (6 parts), the fixative is called *methacarn* and is a good fixative but not recommended for most antigen recovery techniques (Mitchell, Ibrahim et al. 1985).

100% ethanol (3 parts) plus acetic acid (1 part) is called *modified Carnoy's fixative* or *Clarke's fluid (Suvarna, Layton et al. 2018)*. Based on reports (Cox, Schray et al. 2006) *methcarn modified* prepared with 8 parts of methanol and one of acetic acid, have shown better results in concern with the coefficient of diffusion even higher that the formaldehyde (Suvarna, Layton et al. 2018) and warrant better penetration that the formalin, **Table 5** summarizes the main fixatives substitutes available.

Table 5 Fixatives substitutes available

Fixative	Comercial Product	Active chemical agents (%)	Buffer (if know)	Fixating properties (according to distributor)	Ref of description or comparative
FineFix	Fisher Scientific NC2198603	Ethanol (50-70%)		No provided	(Paavilainen, Edvinsson et al. 2010, Kothmaier, Rohrer et al. 2011, Rahman, Sultana et al. 2022)
HOPE	Polysciences 24823-500	Hepes-Glutamic acid buffer		Amino acids and glucose	(Kothmaier, Rohrer et al. 2011, Nina Hornickel, Kacza et al. 2011)
KinnFix	In-house production	Acetic acid 100% Ethanol 100%	Trehalose (sugar used to stablize proteins)		(Stefanits, Bienkowski et al. 2016)
RCL2	Product descontinued	Alcohol base Acetic acid x- 17,9%		Coagulation	(Kothmaier, Rohrer et al. 2011)
Formalin-Free (Accustain)	Sigma A5472	Ethanol (30-50%)		Coagulation	(Da Silva, Gvazava et al. 2023)
PaxGene	Qiagen 765312	alcohol based (70% ethanol)		Coagulation	(83)
Histochoice	VWR DFU-H112	40% Glyoxal, Ethanol 18% and 9 other components in proprietary amounts		Coagulation	(Vince, Tbakhi et al. 1997, Kacena, Troiano et al. 2004)
Glyoxal	Sigma-Aldrich 128465	Glyoxal 3% v/v		Binds to methyl, amide, and hydroxyl groups.	(Richter, Revelo et al. 2018)

Glyo-Fixx	Epredia 6764262	10%-25% Glyoxal sol., 2-propanol, methanol or ethanol (5%-10%), acetic acid (<5%)		Binds to methyl, amide, and hydroxyl groups.	(Lassalle, Hofman et al. 2009, Paavilainen, Edvinsson et al. 2010)
Prefer	Fisher Scientific NC9053360	Glyoxal in buffer and ethanol	NOT DESCRIBED	Binds to methyl, amide, and hydroxyl groups.	(DeJarnatt and Criswell 2021)
Safe-fix II	Fisher Scientific 23-042600	Glyoxal 8-10% Ethyl alcool 14-16% Methyl alcool 2-4%	1,2-Benzenedicarboxylic acid, monopotassium salt <1%	Binds to methyl, amide, and hydroxyl groups.	(Bussolati, Annaratone et al. 2017)
Natural ones	In-house production	Ethanol based from sugar cane		Coaglation	(Patil, Premalatha et al. 2013, Chittemsetti, Nallamala et al. 2018)
IBF	Leica Biosystems 3800811	3% formaldehyde, methanol, 2-propanol, barium chloride			(Trpkov, Renault et al. 2006)
STF	Streck Laboratories	diazolidinyl urea, 2-bromo-2-nitropropane-1,3 diol and a small amount of formaldehyde as active ingredients	zinc sulfate		(Nace, Steurer et al. 1999)
Pen Fix	Epredia 22-046340	<10% formaldehyde, methanol, ethanol, 2-propanol			(Buesa 2008)
Neo-Fix	Merck Pharmaceuticals	1,2-Propanediol, ethanol, polyvinyl alcohol, water		Coagulation	(Paavilainen, Edvinsson et al. 2010, Zanini, Gerbaudo et al. 2012)
ZBF	In-house production	Zinc salts		Unkwon Mechanism	(Zanini, Gerbaudo et al. 2012)

Eco-friendly use of solvents

Several reports have explored the 'greenification' of analytical sample preparation methodologies. More sustainable and practical solutions have been investigated to utilize more green chemicals in other fields. This increased engagement by diverse researchers into new techniques and fields may help improve our knowledge on the role of the solvent. Solvents are not all equal and need to be chosen for specific sample preparation as well as the methodologies which use it. Solvents which were previously used may have fulfilled more than one purpose, such as xylene in tissue processing. Xylene was used both for de-lipidation (clearing) as well as due to the fact that it is nonpolar and thus can be mixed with paraffin to facilitate infiltration into the tissue, is possible to re-think and find a green solvent option. In our case, the gap on the knowledge initially was to find a solution for the use of xylene during the tissue processing (clearing) (Tobiszewski, Namieśnik et al. 2017) **Table 6.** based on the polarity and recommendation of use (Kokosa 2019) (**Table 4**). Therefore, understanding the properties that are needed can help identify green alternatives. One way to observe and identify solvents which could be substituted is to group the solvents by their polarity (**Table 6**). In the following sections, more details will be given on the potential substitutes.

Table 6 Types of solvents and guidelines for use.

Polarity	Solvent Examples	Recomendation of use
Nonpolar / volatile solvent	pentane, dichloromethane, hexane, benzene, carbon tetrachloride, and chloroform	Highly hazardous
Nonpolar and less volatile solvent	xylene, heptane, toluene, and chlorobenzene	Usable but problematic
Polar solvent	ethanol, 1-propanol, acetone, acetonitrile, 2-propanol, and methanol	Environmentally safe

Xylene substitutes

At the late 1970's, the use of xylene increased and was even promoted as "safer to handle" after reports emerged concerning the carcinogenic potential of chloroform. When xylene also was revealed to have potential health hazards, investigations began on the next substitute with the goal of the health of the technicians that used these compounds. The mains focus was clearing aspects of tissue preparation, but deparaffinization was also an important step that needed be considered for a successful replacement. Furthermore, many mounting medias, applied after the

material been stained, contain xylene (e.g. Pertex). Therefore, xylene is needed throughout histological processing and different substitutes for each of the respective steps involved during the histological sample preparation, (clearing, dewaxing) have been explored.

Using mixtures of isopropanol, ethanol-isopropanol mixtures and mineral oil have been reported as providing a good quality and a possible substitutive for xylene in tissue processing with around 91% score in evaluation. These techniques already provide researchers on the field different options and approaches according with the material studied. Further, it is important to highlight that there are reports on minor process modifications needed when these substitutes are used. These depend on the different types of tissue and common adaptations are related to time of infiltrations and some organ specific information **Table 7.** Some solvents have also been noted to cause variation also among different organs, such as liver tends to become harder with isopropanol on the tissue processing. Therefore, one solvent and protocol may not be able to replace xylene in all situations. Furthermore, for some uses of xylene, such as in deparaffinization, 'homemade' and more sustainable solutions have been reported such as the use of dish washer solution diluted in distilled water (1.7% - 2.0%) and heated to 90° C (Buesa 2000, Buesa and Peshkov 2009).

As mentioned above, various approaches appeared in the literature coinciding with the investigations for finding a "safer" tissue clearing agent. These studies aimed to investigate if the solutions proposed would be suitable in general. Some methods sought to skip clearings steps. For dehydration and paraffin infiltration, terpenes, alkanes (aliphatic, isoparaffinic, naphthenic, and paraffins with larger molecular weight), and vegetal oils have all been explored and will be discussed next.

Tissue clearing using terpenes

Terpenes as known as *terpentine* are polymers obtained from essential oils from plants, such as, cedar, bergamot, wood, oregano, terpineol, etc. This group was the first clearing agent used for histological techniques, more specifically for clearing. Limonene is a subproduct from citric fruits, that is formed from 2 isoprene units, and after the reports about the toxicity of the xylene, several companies started to manufacture d-Limonene compounds as an alternative for the solvent.

However, due to the oxidation when in contact with the air, it can promote allergies, nausea and skin reactions in histology technicians (Foti, Zambonin et al. 2007). Therefore, some companies tried to reformulate the compounds with the goal to keep a safe use. Today, there are several limonene products available and the most common limonene products available on the market are: AmeriClear, HistoClear and Histosolve X. However, these are associated with higher coats in comparison with xylene see **Table 7.**

Table 7 List of xylene substitutes D-limonene based. Modified from (Buesa and Peshkov 2009) with the main terpene substitutes available on the market and evaluations reported.

Xylene Substitutes	Descriptions	Pricetimes the price of Xylene
AmeriClear	60% of the quality of xylene (Wynnchuk 1994); citrus smell in 20-year-old blocks; produces a “metallic taste” in some histologic technicians; requires process modification; does not fade H&E	4.12
CitriSolve-hybrid	aliphHC (50%) + D-limonene + emulsifier + BHA	2.31
CitroClear	better and faster than others, strong smell, tendency to turn yellow and throw out oily deposits	2.22
CitroSolve, CitraSolve, CitriSolve	mostly water and surfactants (91%) + D-limonene (9%) making it water soluble	1.40
Citrus Clearing Solvent	fruitlike odor	1.63
Citrus Natural Solvent	D-limonene; irritant	1.11
Clearene	redistilled d-limonene, contains antioxidant to prevent stains fading	1.27
D-limonene	possible health hazards, nonrecyclable (Wynnchuk 1994); not user friendly (Buesa and Peshkov 2009); can be oxidized with histologic reagents causing dermatitis (Foti, Zambonin et al. 2007); has caused respiratory problems (asthma) in many histologic technicians	1.98
Hemo-De	from less effective (Gubash and Bennett 1989), to 61% (Metzger, Marian-Kfir et al. 2000), to 97% (Miller, Miller et al. 1994), to similar to Xylene (Wynnchuk 1994), (Egleton, Fraser et al. 1986, Aldeen and Hale 1992), contains an unknown amount of BHA	2.44

Histoclear, Histolene	behaves similar to Xylene (Faolain, Hunter et al. 2005, Buesa and Peshkov 2009), poor dewaxing for IHC sections; fades hematoxylin; needs a special mounting medium; hardens brain, liver, and spleen; causes skin problems and headache	1.47
Histoclear II	aliphHC (70%-90%) + D-limonene (30%-10%)	1.46
Histolemon	90% D-limonene, strong odor, results similar to Xylene	2.62
Histosolve X, Bio-Clear	flammable waste, needs special mounting medium, dries tissues, and produces wrinkles difficult to open , some have used it satisfactorily for 17 years, absorbs too much water	2.07
K-Clear	contact dermatitis on histologic technician (Foti, Zambonin et al. 2007) needs to use L-Mount (DDK, Milano, Italy)	N/A
Master Clear Clearing Agent	technical grade d-limonene; strong odor	0.93
Pro-Clear	D-limonene	7.44
Roti-Histol	mandarin fragrance, classified as dangerous to the environment, to be disposed as hazardous waste, skin irritant, needs special mounting medium	1.42
Safety-Solv	mild citrus odor, nontoxic or flammable, biodegradable	1.36

Tissue clearing using alkanes

The group of the alkanes consists of saturated hydrocarbons with a variable number of arrangement and number of carbons, where it can be arranged in three different conformations (aliphatic, isoparaffinic and naphthenic). More simplistically: straight, branched and with one or more carbon rings. Their physical and chemical characteristics are according to the number of carbons and structure (Buesa and Peshkov 2009).

The sustainable advantage of this group of compounds is: low odor (what is very positive for the users), not oily (easier clean-up), potential recycling and re-use, and as well with low hazard level (up to 600ppm). With regard to the properties relevant for tissue processing, it can keep the tissue softer than in comparison with xylene and D-limonene. However, these compounds are known to be less effective on the paraffin removal during the deparaffinization step and are also incompatible with mounting media xylene and toluene based (Wynnchuk 1994). Therefore, these may be best used during tissue clearing steps (see **Table 8**). **Table 8.** Modified from (Buesa and Peshkov 2009) with the main alkene substitutes available on the market and evaluations reported.

Table 8 Clearing with alkane. Modified from (Buesa and Peshkov 2009) with the main alkane substitutes available on the market and evaluations reported.

Xylene substitute	Name and evaluation	Pricetimes the price of xylene
Clear-advantage	(naphhc) low odor level, recyclable.	0.92
Clarify	(naphhc) low hazard.	0.49
Clear-rite 3	(aliphhc) oily, dries and makes tissues brittle, sections wrinkles difficult to open (Quinn, Fuller et al. 2006); fades stains; recyclable; needs processing modifications and changes more frequently; allergen.	0.87
Ems	(isophc) can cause skin, eye, and respiratory tract irritation; absorbs moisture; needs processing modifications; flammable.	N/a
Envirene	(naphhc) methanol and most mounting media incompatible.	2.56
Formula 83	(naphhc) cannot be used in coverslippers, recyclable, irritating.	0.67
Histolab-Clear	Hydrocarbons, C11-C12, isoalkanes, <2% aromatics	1.43
Isopar-l	(naphhc) c_{11-15} hydro heated heavy naphtha.	N/a
K-clear plus	(isophc) needs ecomount-k mounting medium.	N/a
Kerosene	(isophc) combustible; oily (v = 3.4 cs at 20°c).	0.12
Mcs-2806 process fluid	(naphhc) (v = 1.28 cs at 38°c).	N/a
Microclear	(isophc) from 88% - 98% (Miller, Miller et al. 1994) to equal to xylene (Tsiola, Hamzei-Sichani et al. 2003) good for processing and staining, needs microcover mounting medium, xylene required to clean tissue processors, biodegradable.	0.80
Naphtha	(naphhc) causes only minor tissue shrinkage.	1.48
Neo-clear	(stodsol) only for tissue processing.	1.07
P-4	(isophc/naphhc) only for tissue processing (v = 0.8 cs at 25°C).	N/a

Paraclear	(naphhc) only for tissue processing, cannot clean the tissue processor.	0.97
Pro-par	(isophc + pge) from 60% (Buesa and Peshkov 2011) and 88% to equal to xylene; needs an additional container for tissue processing and staining, tissues less dry than with xylene, needs changing more frequently.	0.67
Roticlear	(naphhc) for tissue processing and staining (*v* = 1 cs at 20°c).	0.65
S1-histo	(isophc/naphhc) for dewaxing sections.	N/a
S3-histo	(isophc/naphhc) for clearing stained sections.	N/a
Safeclear	(stodsol) only for tissue processing.	1.72
Safeclear ii	(stodsol + naphhc + isophc) only for tissue processing.	1.91
Shandon xylene substitute	(aliphhc) from 84% to less effective than xylene (Gubash and Bennett 1989); recyclable, less oily, for tissue processing and dewaxing but not to clear stains.	0.99
Shellsol 15	Aliphatic naphtha 85% + light aromatic naphtha 1% + ethyl benzene + tmb; vapors can cause drowsiness and dizziness; causes skin irritation; combustible.	N/a
Shellsol a 100:	(100% naphhc) used satisfactorily since 1980 for tissue processing.	0.24
Slide brite	(aliphhc) from 93% (Wynnchuk 1994) to as good as xylene; somewhat greasy, with ethanol is not transparent; good for tissue processing.	0.84
Slide brite elite	(aliphhc) more compatible with regular mounting media.	0.65
Solvent 100	(light and heavy aromatic naphtha) (*v* = 1 cs at 25°c).	0.47
Sub-x xylene substitute	(naphhc) will not harden tissue, not greasy, slightly water soluble.	0.61
Trican xko	(aromatic and isophc 60%-100% + aromatic naphtha 5%-10% + benzene <1%); skin irritant; contains xylene; flammable.	0.16
Ultraclear	(isophc) with $c_{9\text{-}12}$ replaces xylene, toluene, and D-limonene as clearing agent in tissue processing; dermatologically inert; odorless.	N/a
Waxol	(naphhc) with $c_{11\text{-}15}$ for tissue processing (twa = 171 ppm) (*v* = 1.67 cs at 25°c).	1.27

White spirits	(stodsol) mild odor, noncorrosive, good for tissue processing (*v* = 0.93-2.06 cs at 25°c).	0.54
Xs-3	(naphhc) dewax incompletely; too much water sensitive; slow to dry; tissues less brittle than in xylene; bleeds dab; can cause dermatitis, dizziness, drowsiness, and headache.	0.47
Xylene substitute 2	(aliphhc) as good as xylene (Wynnchuk 1994) biodegradable; all steps except cleaning the tissue processor.	2.44
Xyless ii	(naphhc) only for tissue processing.	0.59

*aliphHC indicates aliphatic hydrocarbon; isopHC, isoparaffinic hydrocarbon; naphHC, naphthenic hydrocarbon; naphtha (TWA = 300 ppm); DAB, diaminobenzidine (chromogen in immunohistochemistry). ν, kinematic viscosity in centi-Stokes (cS); N/A, not available; PGE, propylene glycol ether; StodSol, Stoddard solvent (TWA = 500 ppm); TMB, 1,2,4-try-methylbenzene (CAS no. 95-63-6) a suspected carcinogen.

Challenge of implementation of sustainable methodologies

Reflecting upon the aspects discussed above, despite the description of xylene substitutes since the late 1970's, change has been slow. Identification of the remaining challenges for a complete readaptation is needed. There is increased awareness on the principles of green chemistry, which have been placed to reinforce the need of changes and integrated with Sustainable Development Goals (SDGs) (Li and Anastas 2012, Di Lucia, Slade et al. 2022). With more attention to these aspects, it is hopeful that these green methodologies will be implemented.

There is some research emerging on the main challenges of implementing sustainable approaches. One aspect is that the persons responsible for making the decisions normally prefer to select protocols or methodologies that are simple to apply and simpler to use and understand. As mentioned in the section of the fixatives substitutes, the time needed to revalidate, optimize protocols and methodologies are viewed as limiting factors and barriers to those researchers (Buesa 2008).

In the case of hospitals, more specifically at the pathology department where most of the protocols are well established and critical for diagnostics, a change of the fixatives or tissue processing may generate the need to revalidate all the antibodies as well as investigation if diverse types of tissue can be prepared, fixed, and processed using the same pipelines. In summary, the "decision maker" needs simpler, faster and methodologies that do not demand someone highly skilled to perform the task.

However, in research about implementing sustainable solutions, it has been found that the accuracy, precision and completeness of methodologies are secondary to the sustainable goal. This gap /challenge observed is the lack of clear understanding from the methodologies of producers of needs and priorities from the decision makers to make, practical methodological progress towards sustainable solutions (Di Lucia, Slade et al. 2022).

Score system

With multigroup experimental designs, there is an increased need to quantify the histological differences observed (Crawford and Tykocinski 2005, Da Silva, Gvazava et al. 2023). Classification or scoring systems are tools used to quantify observations of biological systems, such as specific features that can be identified in tissues, bioengineered materials (including deformations) and use observational and or histological examination to score individual samples or regions of samples

(Eaton, Danon et al. 2007, Da Silva, Gvazava et al. 2023). An appropriate scoring system must fundamentally contain the following characteristics: (reproducibility, definable features and produce significant results with underly biological differences) (Crissman, Goodman et al. 2004).

Score system approach

These scoring systems use different approaches to associate numerical values with complex, histological slides. These include categorical disease (i.e. presence or absence of a phenotype), rank (classifies the level of the tissue injury using features that can be correlated within each tissue (i.e. atelectasis, hemorrhage and proteinaceous debris) from least to most affected, or ordinal (histological images are assessed on a pre-determined scale using a progressive scoring system according to the magnitude or distribution of disease in the observed tissue) (Meyerholz and Beck 2018). As all manual based scoring systems rely on humans to classify or categorize the degree of injury, there is an extent of subjectivity which always remains.

While semi-quantitative systems work well in detecting differences between groups, quantitative evaluation of specific features is advantageous as it provides greater robustness. However, quantitative assessment has the potential to produce more rigorous data on a continuous scale that facilitates more accurate correlations with clinical or biological data. Especially combining both, semi-quantitative and quantitative approaches help to improve the application to digital pathology and need to identify and use an appropriate scale and features that may provide robustness to the score system (Bankhead, Fernandez et al. 2018, Meyerholz and Beck 2018).

Features of objective histological score systems

The process of developing a scoring system requires that some parameters are identified as appropriate for the evaluation of histological tissues. Thereafter, one important aspect is the ability to perform **blinding**. Blinding a scoring system requires that reviewers or "scorers" who will perform the task of examining tissue do not know which group or treatment they are looking at. This approach protects the experimental study from accidental bias and enhances the reliability of the experiment (Day and Altman 2000).

Model context, a previous review of similar models can predict possible specific parameters to be observed (Sellers 2012). **Observed lesions**, some types of lesions can be observed in different types of tissue, such as the presence of inflammatory cells. However, lesions that can be detected specifically in the organ or tissue of interest need to be pre-determined. Although it may be difficult to review all injury

parameters for all tissues, there are many sources of specific models that can be found in the literature that can help define these parameters.

Clear categories, provide a clear category of the score, may help on the reproducibility. The use of more general terms such as mild, moderate, or severe in ordinal punctuation can compromise the reviewer's repeatability. The use of specific terminology, and numerical values of affected tissue, can improve the repeatability and sensitivity of the system (Strasak, Zaman et al. 2007). **Consistency**, despite the difficulty of this task, scores should be performed by a small number of people where you try to generate differences, even if the same person performs the score more than once. Ideally, the score is performed by more than 3 professionals (Cross 1998).

Choosing the appropriate measurement of the score

As mentioned above, ordinal methodology allows organization and definition of categories according with the progression of the injury of the tissue, opening the possibility to a vast field of analysis; however, there is much discussion around which score or central tendency of scores from multiple observers to report in order to best represent ordinal data. In the case of the studies included in this thesis, it is possible to observe a slight discrepancy on how the data is presented throughout the different studies.

The challenge with semi-quantitative scoring is that most biological and clinical researchers are used to using mean to represent their data. Means is only appropriate to represent a central tendency in interval and ratio data (see **Table 9)**. Median is the most appropriate measure for central tendency for ordinal data (Gibson-Corley, Olivier et al. 2013). Therefore, within small group of reviewers who are prone to variability of observations, the use of mean can affect the sensitivity of the score system.

To facilitate the observation of how the central tendency may be affected by 1 or 2 outliers. Example: [6,6,6,2,2] → Mean= 4,4 Median=6. A deep discussion for the reasons for these discrepancies are outside the scope of this thesis, but factors such as experience as well as fatigue have been shown to play a major role in histological scoring differences, even among pathologists (Homer 2015).

Table 9 Central tendency measures.

Measure	Formula	Description
Mean	$\sum x/N$	Balance point
Median	$(a + b)/2$	Middle value when ordered

The utilization of an ordinal score system is the most common tool to direct evaluation of tissue using the assessment of reviewers that accomplish this task based on their observations. Although, the variance found in animal-based research and of course the natural variance between people, remains a challenge to be approached by research community (Kulkarni, Lee et al. 2022), part of the work developed in this thesis was try to approach these challenges. As will be described later, we first developed a new semi-quantitative score system based on green-histological preparation and digital slide scanning technology appropriated to the animal model used on the research question. During this project, new methodologies help move us forward towards more reliably assessment of level of tissue damage in porcine models of ARDS.

Range of injury

The appropriate choice of the range applied to categorize the feature(s) observed is crucial to a reliable score system (Hubner, Gitter et al. 2008). Score ranges can vary from "small" range/categories (e.g., three (0-2)), or "large" (e.g., ten (0-10)) or more per system. It is important to reinforce that a small range may reduce the sensitivity of the system, creating the need for large animal/sample numbers to detect differences.

Otherwise, ordinal scores with a larger range can become challenging to distinguish as the distance between the features is less obvious and thereby makes the system prone to have reduced repeatability. The optimal range to increase repeatability and detection has been proposed to be ~4-5 (Schulz, Chalmers et al. 1995). In the case of the score system developed and presented in this thesis **Paper I, II**, the range of the score system is between 0-8, what might be viewed as a too large but has been used successfully in other studies for rodent lung injury (Ashcroft, Simpson et al. 1988). However, in a post-hoc analyses of our data (presented in **Paper I**), we find that our system also adopts a 5-level ranking system for this scoring (**Figure 6**).

Figure 6 Overview of post-hoc analysis conducted in Paper I to determine ordinal score range

Further, appropriate description and clear specification of the features and the range used is important to create as many mechanisms as possible for achieving interobserver agreement. Following the principle of consistence and clarity, it may

help to reduce the agreement challenges faced in several semi-quantitative score systems. However, due to the qualitative character of scoring of visual images, it opens the space for personal perceptions, even among professionals with the same expertise level; and it is still prone to variability (Lidbury, Rodrigues Hoffmann et al. 2017, Ericson Lindquist, Ciornei et al. 2022).

A recent approach to solve the challenges of disagreement and high variability is digital analysis of histological images. In addition to digital analysis removing the amount of time spent by the professional, the digitalization of human tasks and implementation of machine learning approaches is a promising new opportunity to improve sustainable approaches for histological assessments that can also improve accuracy, consistency and agility of the application of score systems using machine learning (Wu, Phang et al. 2020).

Sustainable histology and its assessment

While extremely valuable, current histological pipelines are not sustainable. The use of xylene and formalin may directly affect the environment as well as the end-user. While large amounts of paper are no longer used for histological assessment (e.g. used in some traditional approaches, where pictures of the slides in high magnification was printed and offered for the observers perform the score), whole slide scanning opens up many new opportunities for modern pathology. Automatization of some tasks might save time, energy, and resources in research labs and pathology departments. Another aspect that needs be considered is the time that these experts spend, sometimes looking at routine to slides to confirm a routein diagnosis. The health of the histopathologist that needs to spend hours looking directly on the ocular of the microscope, what might in long term compromise the vision of the professional or cause physical pain in their body due to repetitive tasks..

Taking into account these aspects, digital approaches may help reduce the number of animals needed (if more sensitive quantifications can be performed) and may help fulfil several of the Agenda 2030 SDGs.

Digital histopathological image analysis

Over the past of decades, all the fields of science experienced the process of digitalization. Histopathology is not different. Recent advances such as, automatized tissue processing machines, slide staining and whole slide digital scanners have revolutionized histology and allowed for complex molecular analysis of large clinical cohorts (Mansour, Malmros et al. 2022). Tissue for histopathological slides can now be saved in digital image form which enables the possibility of computational analysis and application of machine learning techniques (Gurcan, Boucheron et al. 2009). However, the transition from the analogous to digital analysis still demands some adaptation from past studies that

were developed on photomicrography using a certain number of fields in higher magnification.

The possibility to navigate on digital slides may improve the experience of the pathologist, such as, digital zoom, wider field of view on the observation of the tissue. Given recent advances, research and pathologists recognized the importance and possibilities of using quantitative analysis of histopathological slides, once that most of the diagnoses are based on subjective analysis from the pathologists. Therefore, research on quantitative digital image-based analysis is urgent.

Furthermore, beyond the diagnostic perspective and the pre-clinical applications, digital analysis can reduce the inter- and intra-observer variations on the observations, reduce the time spent by these professionals and offer them time to spend on other research and work tasks, as well as improve accuracy due to human errors (Gurcan, Boucheron et al. 2009). Histopathological slide analysis provides a comprehensive observation of the changes in the tissue architecture where diagnosis is made based on the presence or absence of hallmarks of diseases specific features. However, despite the progress made in the field, there is still a large gap regarding the variety of imaging methods available for analysis and the identification of disease-specific characteristics and assessment (Gurcan, Boucheron et al. 2009, Kulkarni, Lee et al. 2022).

Different tools are available depending on the need of different research groups. The most basic applications are thresholding to quantify nuclei or other structures of interest on histological slides, morphometric analysis, or segmentation. Digital sources of the imaging metadata, such as European Bioinformatics Institute (EMBL-EBI) provides the possibility of collaboration across different laboratories, increasing the throughput and minimizes batch and inter-laboratory differences on the development of different technologies to data analysis.

Machine learning based on scoring, provides a great opportunity to observe, detect and quantify specific features that the human may have difficult to connect due to our subjective capacity of visualization, besides the opportunity of automatization of the human task (Webster and Dunstan 2014).

Machine learning

With the emerging amount of data generated by the digitalization of our histological routines, such as the digitalization of histological slides, both for research and teaching there is also the need to automate the process of classification of these images As discussed above, the manual semi-quantitative score system has several good qualities, however unfortunately, variation due to the subjective character of

the scoring approach and time that it consumes still are not optimal for the current advances of the field (Banerji and Mitra 2022).

Histological application remain as the gold standard, especially in analysis for clinical and animal studies where it is not possible to use different tools. Digital scanned slides are increasingly used in modern histology for both research and clinical diagnostics. Emerging new fields such as, digital pathology and machine learning benefit from a large amount of information previously generated in machine learning image detection. This has helped these tools evolve for image retrieval, archiving or recording, but also can be employed as computational tools for decision making on precision medicine (Banerji and Mitra 2021, Silva, Gvazava et al. 2022).

The machine learning process can be divided in two major categories. Supervised (class labels) and unsupervised (clustering/segmentation). Architectural features such as arrangement, spatial topography of the histological sample, or nuclei are used to teach the machine to detect histological features. Convolutional neural networks (CNN) are the most common approach for the implementation of machine learning analysis of histological slides. However, employing this tool may be challenging due to the variability of the tissue architecture, variation on the colour of the stains, and depending on the size both from the samples and the data set used for each system (Chikontwe, Nam et al. 2022). CNN's combine efficient use of graphic processing units, rectified linear units, dropout regularization, and data augmentation (Krizhevsky, Sutskever et al. 2017, Banerji and Mitra 2021).

The automatization of these processes such as, extraction of information from images, benefits professionals reducing the time spent on analysis and data processing but also can help reduce possible bias and the accessibility to exchange information across different laboratories. All of this can serve to benefit the patients. Due to the complexity of the images applied on the machine, one of most reported approaches for dealing with the volume of information follows a classification network, where the input is in general a square image and convolution layers create filters to detect features and recognize patterns. Polling layers down sample data, connecting the features that were identified, and generating domain-specific classification based on the probability of the output belonging to each class **Figure 7.**

The classification can be based in rank of features identified such as, score, presence (yes or no), spectrum of amount of the feature, and probability to be a part of a certain group (Banerji and Mitra 2021).

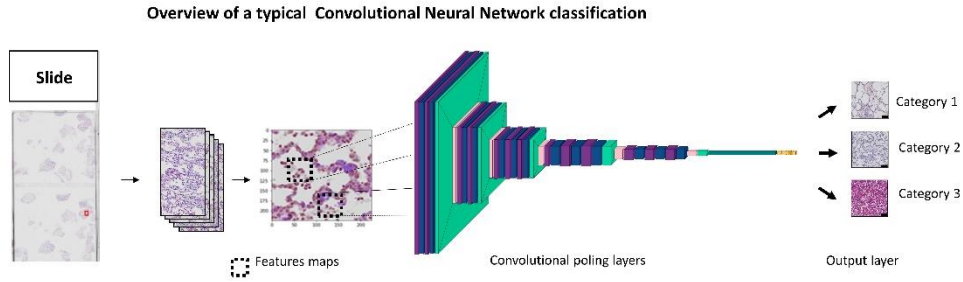


Figure 7 The structure of a typical convolutional neural network used for classification.

Challenges on the implementation of digital histological images

Based on computerized analyses of images provides a substantial extent of morphologic analysis of histological architecture and anatomy in large-scale datasets. Observing not only cell compartments and architecture of tissue, it can provide scales and extent of the presence of determined characteristics, such as injury on the tissue. Further, due to the natural complexity and morphological heterogenicity, the machine or inter-observer variability, data small size, staining variations, the machine needs algorithms of validation to deal with and provides qualitative analysis **Table 10.** Summarizes the main challenges of digital histological evaluations and possible solutions (Komura and Ishikawa 2018).

Table 10 Challenges of digital histological applied to machine learning.

Challenge	Solution	Reference
Image size	Aggregation of pixel / patch / object level classification considering tissue as texture	(Rexhepaj, Agnarsdottir et al. 2013, Chaofeng, Jun et al. 2017, Roy, Banik et al. 2019)
Insufficient data annotation	Active learning / Tracking observer behaviours / Multiple instance learning	(Brunye, Carney et al. 2014, BenTaieb, Li-Chang et al. 2017, Jia, Huang et al. 2017, Nalisnik, Amgad et al. 2017)
Variance of magnification of histological image	Multiscale analysis	(Song, Zhang et al. 2015)
Staining and artefacts variations	Normalize colour variation / Artefact detection	(Kothari, Phan et al. 2013, Hang, Phan et al. 2015, Ciompi, Geessink et al. 2017, Lafarge, Pluim et al. 2017)

Table modified from (Banerji and Mitra 2022)

FAIR Data principles in Artificial Intelligence Research

Machine learning has greatly benefited from the emergence of Findable, Accessible, Interoperable and Reusable Data (FAIR). FAIR is the association of principles that have a goal to provide as a guidance for the emerging field of data analysis and how it can be applied in research and even for teaching. Similarly with the SDGs, it may help researchers that have the goal to recycle and reutilize their or others data (Wilkinson, Dumontier et al. 2016, Ravi, Chaturvedi et al. 2022). **Table 11.** Relates the use of these principles for how the machine can access, find and reutilize data automatically.

Table 11 The FAIR guiding Principles

FAIR principles	
Findable	(meta)data are assigned a globally unique and persistent identifier;
	data are described with rich metadata;
	metadata clearly and explicitly include the identifier of the data it describe;
	(meta)data are registered or indexed in a searchable resource.
Accessible	(meta)data are retrievable by their identifier using a standardized communications protocol;
	the protocol is open, free, and universally implementable;
	the protocol allows for an authentication and authorization procedure, where necessary;
	metadata are accessible, even when the data are no longer available.
Interoperable	(meta)data use a formal, accessible, shared, and broadly applicable language for knowledge representation;
	(meta)data use vocabularies that follow FAIR principles;
	(meta)data include qualified references to other (meta)data.
Reusable	meta(data) are richly described with a plurality of accurate and relevant attributes;
	(meta)data are released with a clear and accessible data usage license;
	(meta)data are associated with detailed provenance;
	(meta)data meet domain-relevant community standards.

Table modified from (Wilkinson, Dumontier et al. 2016)

Application of machine learning on digital histology represents a step forward on this centenary technique that is the gold standard for tissue examination in clinical pathology and life science research, with interesting possibilities since it can improve examinations, be able to detect interesting features and classifying in groups. It can also help perform virtual staining using tissue autofluorescence. This

advance may not only improve histological staining with regard to time and consistency from one staining batch to the next, but it also falls under the sustainability point of view to reducing the consumption of hazardous chemicals, allowing the direct collaborations of researchers what in retrospectively will also make the digital system even stronger (Bai, Yang et al. 2023).

However, this fascinating tool needs a large amount of data to become efficient. Applications utilizing score systems and well-annotated datasets will help the machine be able to identify features and classify based on the human performance. This reinforces the importance of a reliable score system to be used in machine learning based analysis of histological slides.

The research in this thesis

Gap in knowledge

Lack of semi-quantitative methods adapted for virtual slide scanning to evaluate large amounts of histological changes in large animal models of lung injury

Our understanding of lung diseases is improving, and we have been able to expand the investigations in different approaches; one area of research that has gained prominence is the study of the progression of acute lung injury in large animals such as porcine and ovine models, allowing the observation and utilization of tools that before only was possible in the clinic, such as mechanical ventilation and ECMO. These models help the understanding of the progression of diseases, especially in more severe disease which can be sustained while the animal is sedated to allow longer-term induction of ARDS (Matute-Bello, Frevert et al. 2008, Matute-Bello, Downey et al. 2011, Ballard-Croft, Wang et al. 2012, Stenlo, Hyllen et al. 2020, Kulkarni, Lee et al. 2022).

However, the approximation of the investigations with the clinical scenario also creates the need to establish reliable readouts that allows the translation or comparison with the clinic, such as the utilization of the Berlin criteria (Ferguson, Fan et al. 2012). One of the most important features to be observed is the CT scans to ensure that loss of blood gases is due to changes in the lung and not another reason (e.g. embolism or hear failure). CT may not be possible in some investigations for several reasons, e.g., lack of resources, because this equipment is very expensive, as well as challenges to transport the animal.

In this case the histological analysis is applied as the gold standard tool to assess lung injury. However, this also presents challenges, such as the differences between the pathological hallmarks of acute lung injury in porcines and humans. The main pathological hallmark is DAD; in humans, this is characterized by the early exudative and followed by the proliferative phase (Lorente, Ballen-Barragan et al. 2015, Lorente, Cardinal-Fernández et al. 2015). However, no animal model reflects completely the histological features observed in DAD in humans; nonetheless, the absence of one or more features also doesn't indicate that ALI was not induced (Matute-Bello, Frevert et al. 2008, Matute-Bello, Downey et al. 2011).

In addition to qualitative analysis, the utilization of an appropriate semi-quantitative or quantitative assessment of histological evidence of lung injury has become a

critical tool to evaluate the extent and progression of ALI. This is a technically challenging and time-consuming task due to the large number of samples generated from each experiment with large animals, where serial biopsies are possible. Further, the most well-described score systems have been developed for rodent models which can have different histological features of injury.

Lack of sustainable techniques for histological evaluation using green chemistry for natural and bioengineered tissues

In parallel to this scientific need for improved scoring systems and methods to objectively assess the extent of lung injury, there is also a need of the development of sustainable approaches of these techniques. Sustainability is an emerging topic, and the importance of implementation of solutions using green chemistry is a motivation for new technologies that also offers opportunities to evaluate new biomaterials and achieve more sustainable research practice (102).

On the clinical perspective for patients with acute and chronic lung diseases, there is a need of solutions for end-stage diseases patients for transplantation. There is also a potential, pending increase in the waiting list in a post pandemic scenario where the gap between demand and need for lungs will growth even more. Alternative solutions to improve the feasibility of lung transplantation or other regenerative medicine approaches is an emergent gap, that bioengineered tissue may be a potential solution (De Santis, Bolukbas et al. 2018). However, there have thus far been limited research into determining optimal ways of evaluating these materials using histology pre-and post-implantation and implementation of reproducible and technically accurate histological techniques have been limited and challenging.

Aim of this thesis

This thesis work consisted of three main scientific aims which were connected as well to the following SDGs 2030 **Figure 8**:

1) Establish and implement a green histology approach (i.e., without xylene or other hazardous chemicals, such as formaldehyde) for use in processing biological and bioengineered tissues. (Paper I, III, IV).
2) Develop and validate a semi-quantitative histological scoring system to determine the extent of lung injury in a porcine model of ARDS using administration of LPS through intratracheal and intravenous routes as implemented in collaboration. (Paper I).
3) Develop a machine learning approach for digitized whole slide scanning technology to expedite and remove human bias from the analysis of histological specimens, such as ARDS lung tissue. (Paper II).

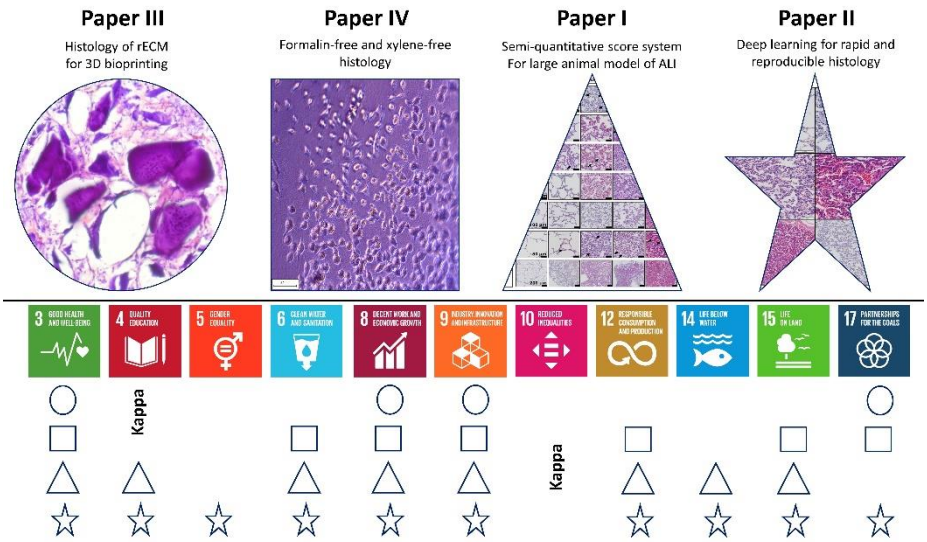


Figure 8 Schematic representing the SDGs addressed in this research

Approach and Methodology

Automatization of tissue processing and xylene substitution

The automatization of protocols is fundamental for improving efficiency. In addition, they help save time and resources that in a long term might cause more cost if not used with caution. Specifically in the histological pipeline, the utilization of automatization may achieve several important aspects described in this thesis, such as sustainability (saving time, resources, that may affect the environment if not properly disposed), safety (end -user of the material, that can be exposed on hazardous fumes that can be carcinogenic, or even other conditions e.g., Raynaud phenomenon), better use of samples and if there is the possibility of application of green chemistry approaches. As the example below shows **Figure 9**, experiments in large animals may generate a considerable sample size to be processed which uses a large number of resources (person-hours, energy and chemicals) Further, the use an automatized improve the standardization of protocols and efficiency on the tissue preparation **Figure 10**.

Figure 9 Example of samples that can be generated from just one experiment round

Figure 10 Spin Tissue Processor STP 120 showing individual containers 1-12 and the programmable interface.
Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

It was clear that only the inclusion of the automatized tissue processor as a way to reduce to the exposure of chemicals to the histopathologist was not enough to improve our routine and fulfil sustainable goals. Even that automatized and not need manual tissue processing, the use of toxic substances such as xylene, still demands appropriate ventilation and the issue with their disposal remains. The substitution of the xylene therefore become not only a goal, but also a need, where after long investigation the utilization of isopropanol on the clearing step was chosen to help address our scientific research questions. Of course, the use of isopropanol still has pros and cons, and depending on the material it may need revision **(Paper I).** The application of this protocol substituting xylene with isopropanol is applied in both **(Paper II and IV** and (Lindstedt, Grins et al. 2021, Alsafadi, Stegmayr et al. 2022, Gvazava, Konings et al. 2023) -*Other papers not included in the thesis***)**.

Manual Scoring System Development

The development of a manual score system demanded an iterative approach until the "final" version that was applied for training a machine learning algorithm and for use in validating its use. Here I will try to summarize the main aspects that was important to take in consideration during this project. The very first challenge was how to help observers from different expertise levels to come from the same starting point. Even though observers had different expertise levels, the objective was to give some "handbook" with background: why we are doing it and most important a guide that could help the observers to have a reference of the features, once that part of this observers never had the chance to look in a microscope before (i.e. novices)

Digitalization of the slides

The digitalization of the slides with a whole slide scanner, provide the possibility to navigate on the tissue slide digitally. This allowed for fast access to the whole slide from anywhere. This was especially important during the pandemic when the number of persons in a room was restricted and constant study of slides at a microscope was challenging. It also provided us a library of material that could be used for training persons without showing them the exact slides to be scored. The images were selected by one expert experimental histology to be based on the representative features identified on the whole slide. Such images were used to develop the instructions handbook and later for scoring.

One challenge that we faced during this process as well, is the fact that some whole slide scanning companies do not provide the raw images generated as they are captured and only provide stitched images. Even when the whole slide is scanned, in the reality the camera collects several neighbour images and digitally stitches

them together. Other companies capture and save the individual images which you can then use later. Unfortunately, the microscope software that we used for the scanning of the whole slides had this limitation and we only had access to stitched images which are not necessarily representing the same data on the microscope slide.

Instruction handbook

The training guidebook was developed with the goal to provide information and a resource of examples for the observers. Persons were initially given a background of what a score system is, why we use and some details about the score system that gave the inspiration for our use of a larger score range than previously used in other studies. This methodology of using a larger score range has been previously applied to estimate severity in fibrotic murine lungs, utilizing histological figures from A-I highlighting specific features and this should be scored a range from 0-8 (Ashcroft, Simpson et al. 1988). Further, we also provided background of acute lung injury and why the score system could help our research once that histological assessment remain as the gold standard for pre-clinical research (Matute-Bello, Frevert et al. 2008, Matute-Bello, Downey et al. 2011). We also provided an overview from the main porcine lung architecture **(Figure 11)**.

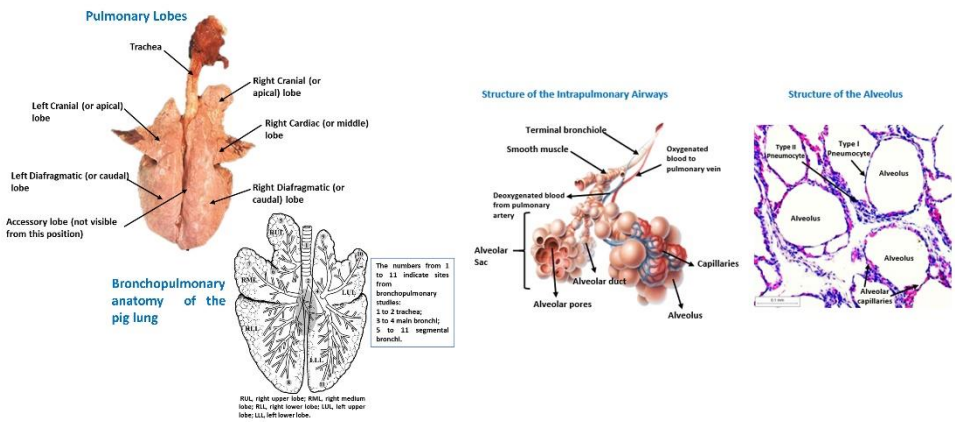


Figure 11 Overview general porcine lung structures

Pilot testing

In order to confirm that the training and the score sheets were ready for scoring of our actual samples of interest and with persons of suitable expertise, we performed a pilot test with three members of the laboratory (one of them without knowledge in histology, and the other 2 members with moderate knowledge of lung histology). Using the feedback received from this member, a final version was developed and used with all the observer in all the studies. See example of the sheet that the observers utilized to score and as well the score that one of then provided to this respective sheet **(Figure 12)**.

These members only participated on the pilot test, and their score was never used in any formal analysis due to the fact that they received different background and information from the others. Their main task was to give feedback on the provided material: content of the training material and how the score sheet was presented.

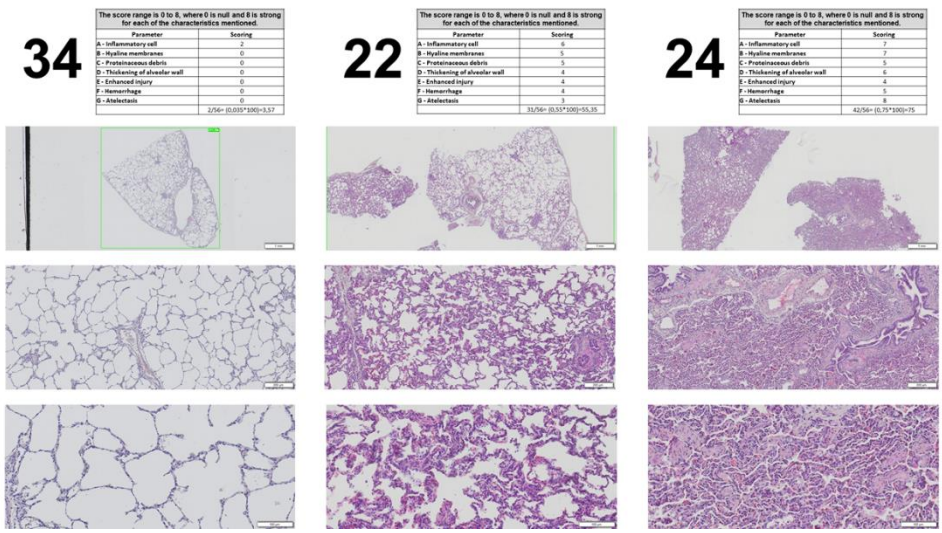


Figure 12 Representative examples of three different scoring sheets supplied to reviewers for the histological scoring system.

Three different examples of the photomicrographs at three digital magnifications (4×, 10×, and 20× to provide representative views across magnifications). Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

Data Handling

In the first step of this project, we decided to work with the data on the form of a total score, due to the complexity of the data and the interest for exploring it in that moment. For the publication in which the score system was used with our collaborator, the purpose of the data was organized by the experimental groups where the animals received or not different disease models and therapeutic or treatments (Stenlo, Silva et al. 2021, Ghaidan, Stenlo et al. 2022). However, the interesting aspect to us, was on the observation of the observer's behaviours, among their groups (novices, moderate and preclinical experts) **Figure 13**. Here, the question came up of how it was possible to have such disagreement levels, even belonging to the same category of expertise.

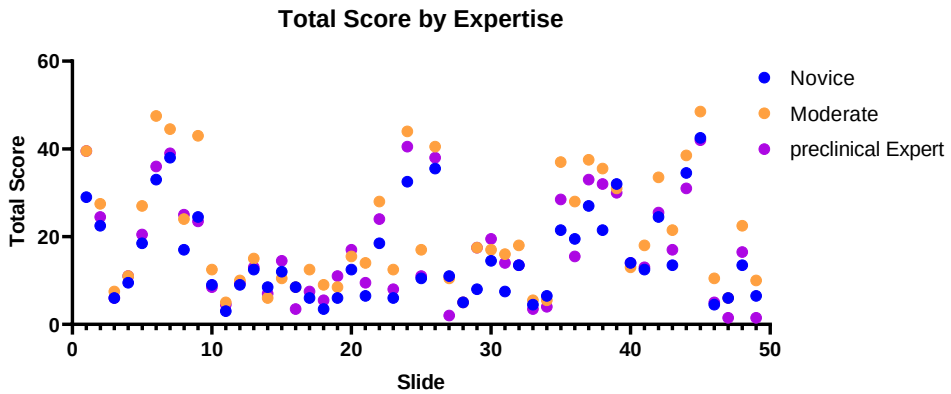


Figure 13 Representative examples of scoring by expertise across multiple slides (data derived from S-BIAD419)

Based on this, we developed the (**Paper I and II**) where we explore the aspect that we developed a digital system to collect histological images and we further used the total score of the observers as the gold standard to teach the machine to recognize and classify acute lung injury.

Data validation

In order to verify if the score system developed was appropriate for teaching machine learning, we had to verify the reliability and consistency of the system. To be able to check if the system was reliable and consistent, multiple comparisons were done. First, an entirely new cohort of persons performed the scoring and 2

board certified pathologists were added. Then, we performed a comparison with the traditional score system and finally, we further compared among the observers for their self-agreement by repeat testing with more than one month difference on the same slides.

First, using the same slides to make sure that we are comparing from the same source, we modified the score sheet and utilized the same features as described in the Matute-Bello publication (Matute-Bello, Downey et al. 2011) **Table 12**.

Table 12 Score system utilizing Matute-Bello range

The score range is 0 to 2, where 0 is null and 2 is strong for each of the characteristics mentioned.

Parameter	Points			Score
	0	1	2	
A - Inflammatory cell	None	1-5	>5	
B - Hyaline membranes	None	1	>1	
C - Proteinaceous debris	None	1	>1	
D - Thickening of alveolar wall	<2x	2x-4x	>4x	
E - Enhanced injury	None	1-2	>2	
F – Evidence of Hemorrhage	None	1-5	>5	
G - Atelectasis	None	1	>2	

The total score was then calculated using the recommended weighted formula (9): Score = [(20 × A) + (14 × B) + (7 × C) + (7 × D) + (2 × E)]/ (number of fields × 100). This yields a value between 0-1. Therefore, for comparison, scores were normalized to be in a range of 0-100 by multiplying the total score with 100. This scoring was performed by six observers (N3, N4, N5, M3, pE3 and pE4) and the data referred to as the "C" cohort.

The second aspect that was important to include was the perception of two board certificate pathologist *versus* two the pre-clinal experts (with extensive expertise in evaluating porcine lung histology and other animal models of lung injury) that performed the validation scores. The inclusion of the board certificate pathologists was important to reinforce the common preclinical scenario where normally one or two pathologists perform the score and as well once that this type of assessment

required understanding of the features it could give to us some light on the disagreement observed **Figure 14**.

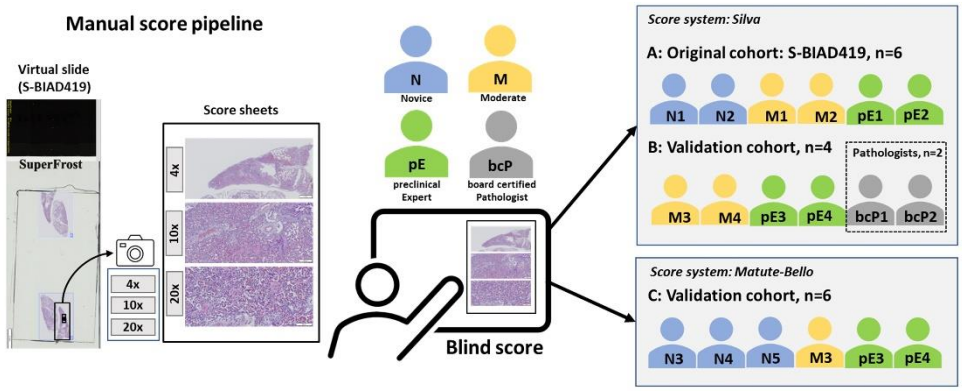


Figure 14 Detailed manual pipeline of the manual score approuch
The lettering signifying the original (A), validation (B) and Matute-Bello (C) cohorts is used throughout the other figure panels

Another approach that we decided to take in consideration was the aspect of reliability that is very important to make sure that the score observed is really suitable for machine learning teaching. This is important because the machine learns from human subjective observation. Therefore, we thought that it would be important also in the validation cohort to have replication of the same observers after around 1 year where we included 1 moderate (M3) and 2 pre-clinical experts (pE3 and pE4).

Data transference

The images used from the digitally scanned hematoxylin and eosin (H&E) were deposited in the Biostudies archive of the European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/biostudies/studies/S-BIAD419) as part of **Paper I** in accordance with the journal requirements, FAIR principles and open access/science mandates. In order, to generate samples for the machine learning training, 10 images from the digital slides scored previously from the observers were collected and transferred for data pre-processing. Large images were separated into tiles of 224 X 224. Next, steps of augmentation were performed to mimic human and/or microscope variations (focus, brightness, contrast, distortion, and rotations)

in order to train models of convolutional neural networks where a 3-fold validation strategy was used. This means that the dataset is broken into 3 parts to account for histological score assigned to the slide and number of tiles to be balanced. Then, the machine is trained 3 separate times and one part of the data is hidden each time so it can be used as the 'test' set. This approach allows the use of a single dataset for initial proof of concept machine learning. Finally, it provides a prediction based on the classification separated for the machine; in our case this classification was levels of lung injury (mild, moderate, and severe) and afterwards the performance of the system is compared with the human assessment **Figure 15**.

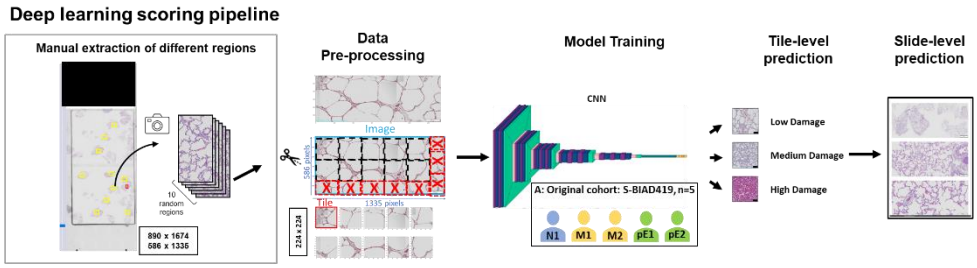


Figure 15 Overview of the peline of deep learning scoring

Formalin-free and Xylene-free histology

In papers I, II, and IV, we implemented and utilized a xylene-free tissue processing methodology by replacing xylene with isopropanol. This also required optimization of the paraffin infiltration time for each type of sample. While not included in the formal papers included in this thesis, we applied this xylene-free approach within several different projects during my PhD and for different types of native tissues and biomaterials which included cells. The different types of tissues were human, mice, porcine, chicken and salamander and the different type of biomaterials were calcium alginate, reinforced extracellular matrix hydrogel (rECM).

The protocol utilized for histological examination of alginate and rECM scaffolds containing cells - prior to optimization of fixation protocols described in Paper (**Paper III**) was as follows: the samples were washed in PBS three times and OCT embedded to facilitate processing of 5 µm thin cryocuts, mounted on superfrost plus adhesion microscopic slides (Fisher Scientific), Haematoxylin and eosin staining utilizing the same protocol presented in detail at (**Paper I**). Images were acquired on a Nikon H600L brightfield microscope.

Comparison of different chemical fixatives

While removing xylene is an important step towards achieving the SDG goals 3 – good health and well being and 12 – responsible consumption and production, other chemicals remain in the histological process which are known carcinogens (i.e., cancer causing) and can have negative environmental effects if disposed of incorrectly.

Aldehyde-based chemicals are commonly used in histology and processing for medical diagnosis. Formaldehyde is the most used chemical for histology while glutaraldehyde-based fixatives (e.g., Carnovsky's fixative comprised of both glutaraldehyde and formaldehyde) is the most common for electron microscopy. Efforts to replace formaldehyde in pathological analysis have been ongoing for several decades and while several fixatives have been identified, they each need to be tested in the application to ensure that they work for that experiment.

Preparation of Different Fixative Solutions

We compared the differences for different fixatives with both native mouse tissues as well as a bioengineered material (**Paper IV**).

Tissue harvest and precision tissue slice preparation

All experiments involving murine tissue was done with approval from the Malmö/Lund Ethics Committee on Animal Testing (M11908-19). Once that histological examination of disease was not the primary aim of the study, we utilized excess tissue available from mice scheduled to be euthanized for other studies [APP/PS1 mice (Jackson Labs, United States)]. Animals were anaesthetized via an intraperitoneal injection of sodium pentobarbital (40–50 mg/kg) followed by transcardial perfusion with ∼20 mL of oxygenated (carbogen, 5% CO2, 95% O2) artificial cerebrospinal fluid (aCSF) as we described previously (Gvazava, Konings et al. 2023). aCSF was comprised of (in mM): 92 N-methyl-D-glucamine (NMDG), 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2·2H2O, and 10 MgSO4·7H2O. pH was titrated to 7.3–7.4 with 37% hydrochloric acid (∼7 ± 0.2 mL).

All the tissues were sectioned to 200 µm thickness using a 7000SMZ-2 Vibrotome (Campden Instruments, Ltd.). Tissue was then fixed overnight with one of 4 fixatives: 10% neutral-buffered formalin (NBF) (HT501128-4L Sigma-Aldrich, Sweden), formalin free fixative (A5472-1GAL Sigma-Aldrich, United States), 4% PFA in 0.1 M phosphate buffer (HL96753, Histolab AB, Sweden), or 2.0% electron microscopy grade glutaraldehyde (G5882-100 ML Sigma-Aldrich) in 0.42x PBS

(1x PBS, 10 mM PO43-, 18912014 Thermo Scientific, Sweden), 0.5X NBF and then transferred to 1x PBS until tissue processing.

Prior to the embedding of the tissue, pieces were organized as “four spot” square tissue arrays into molten paraffin and allowed to cool as a single block. Paraffin-embedded tissue was cut into 5 µm tissue sections using a microtome (Model: 1516 Automated Leitz, Germany) and mounted on Super Frost adhesive microscope slides (Epredia, United States). Prior to chemical de-paraffinization, slides were placed vertically (lengthwise) overnight in a 65°C oven (ED56, Binder, Germany), followed by de-paraffinization using Histo-Clear (Ted Pella, United States).Text taken from (Da Silva, Gvazava et al. 2023).

Native tissue autofluorescence, staining and imaging

Prior to chemical-deparaffinization and rehydration, autofluorescence (λex = 469 nm, λem = 525 nm) was acquired using the manual imaging mode in a Cytation 5 multimode reader with a wide field camera (BioTek, Agilent Technologies, United States). Montage images were collected with the ×4 objective. GFP imaging filter cubes were used using the following acquisition settings for the liver, kidney, and heart: LED intensity: 2; integration time: 30 ms; camera gain: 24.

After de-paraffinization of TMA sections, microscopic slides were stained with hematoxylin and eosin (H&E) (Merck Millipore, Germany), dehydrated, and mounted with Pertex mounting media (Histolab, Sweden). Images were then captured with an inverted Nikon Ts-2R microscope using a 20X objective. Slide scanning was performed with the Cytation 5 multimode reader using the 4X objective and a wide field camera using the montage approach. With the selected area around the region of interest, the image was processed using the stitching tool image (linear blend) parameter. Bright field color images of H&E-stained slides (i.e., following de-paraffinization and staining) were acquired at same time with identical settings using a 4X objective. Text taken from (Da Silva, Gvazava et al. 2023).

Cell culture

Human alveolar epithelial cells, A-549 (American Type Culture Collection, CCL-185, RRID:CVCL_0023) were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM F12) (11554546, Fisher Scientific, Sweden) supplemented with 10% fetal bovine serum (FBS) (11573397, Fisher Scientific, Sweden), 1% penicillin and 1% streptomycin (15140122, Thermo Fisher Scientific) in a humidified incubator with air supplemented with 5.0% CO2 at 37.0°C. Text taken from (Da Silva, Gvazava et al. 2023).

Manufacture of calcium alginate hydrogels using diffusion through dialysis tubing

2% (w/v) solutions of alginate were placed in dialysis tubing (D9527-100FT, Sigma-Aldrich, Sweden) with a typical molecular weight cut-off = 12–14 kDa and completely submerged in 50 mM CaCl2 in de-ionized water for 0.5, 1, 2, and 18 h. After each timepoint, crosslinked hydrogels were removed from the dialysis tubing and a 4 mm biopsy core was obtained and placed in a polystyrene Petri dish 60 × 15 mm (82.1194.500, Sarstedt AB, Sweden). An initial photo was collected using a Dino-Lite Edge Digital Microscope camera using the in-built DinoCapture Software V2.0 followed by addition of each fixative or 50 mM CaCl2 as a control. Sequential images were acquired over time with each fixative. Images were blinded for semi-quantitative scoring with ordinal scoring of −1 (dissolved), 0 (deformed), 1 (preserved). Contour maps were generated in R using the filled. contour function in the "ggplots" R package. Full code can be found on https://github.com/Lung-bioengineering-regeneration-lab/green_histology. Text taken from (Da Silva, Gvazava et al. 2023).

Manufacturing of hollow tubes containing cells using a 3D printed mold

A two-part polylactic acid (PLA, Eco White PLA 2.85 mm filaments, Dustin) mold was rendered using Shapr3D app for iPad and processed through Ultimaker Cura software prior to fabrication using a conventional extrusion 3D printer (Ultimaker 3, Creative Tools). Theoretical wall thickness between the inner and outer mold was 1 mm while molds with inner diameters of 6, 8, 10 mm were used in this study and a well-depth of approximately 5 mm .STL files are available at https://github.com/Lung-bioengineering-regeneration-lab/green_histology.

Alginate (4% w/v) was diluted 1:1 in complete DMEM/F12 media containing 1 × 106 cells/mL. 50 mM CaCl2 was first applied to the well and inserts of the 3D printed molds and aspirated off immediately prior to adding the alginate/cell solution. The inserts of the 3D printed molds were placed into the center of each well and the alginate/cell solution was slowly applied until the entire tube portion of the well was filled. Then, 50 mM CaCl2 was applied on top of all tubes, filling the upper chamber of the molds (i.e., solution reservoir). After 30 min, the excess CaCl2 was removed, and the inner portion of the mold was removed. Tubes were carefully removed from the insert and moved to a 24-well plate. Some tubes were immediately processed for fixation while others were covered with 2 mL complete media and cultured overnight and fixed on the following day with either FF or PFA_Ca. The tubes cultured overnight and fixed both with FF or PFA_Ca were subjected to tissue processing and histologically processed for blinded scoring as described below. Text taken from (Da Silva, Gvazava et al. 2023).

Manufacture of alginate and alginate-gelatin films

Alginate and alginate-gelatin thin films were fabricated via casting. 4 mL of either 2% (w/v) alginate or 2% (w/v) alginate with 5 mg/mL of gelatin (410875000, Acros, Fisher Scientific) were poured into 60 mm diameter polystyrene Petri dishes (Sarstedt) and placed into an oven at 65 °C (ED56, Binder) for at least 2 h until all liquid had evaporated. Films were then carefully removed from the Petri dish and subsequently manufactured into discs with an 8 mm diameter biopsy punch. Dry disks were then weighed using a precision scale (Mettler Toledo) and their radius was measured optically using a DinoLite camera and the in-built three-point circle function in the DinoCapture software version 2.0 to calculate the radius. Next, disks were rehydrated and crosslinked using 50, 200 or 500 mM of CaCl2 solution for 1 h followed by incubation overnight (i.e., 18 h) with complete cell culture and finally fixation in either PFA_Ca or FF. Mass swelling ratios were calculated according to the following (Fazljou, Torbati et al. 2020) formula:

$$swelling\ ratio\ (\%) = \frac{crosslinked\ weight\ (g) - dry\ film\ weight\ (g)}{dry\ film\ weight\ (g)}\ x\ 100\%$$

Text taken from (Da Silva, Gvazava et al. 2023).

Cell culture on alginate or alginate-gelatin films for cross-sectional histology

Thin films were manufactured as above using 200 mM of CaCl2 crosslinking. Prior to cell seeding, calcium alginate membranes were placed into the center of a 24-well tissue culture plate (83.3922, Sarstedt AB, Sweden) and allowed to dry. 100,000 cells suspended in 25 µL of media were placed as a single droplet onto the center of each disk and allowed to attach in the incubator for 30 min. Next, 500 µL of media was slowly added to each well and disks were cultured for 36 h prior to fixation or metabolic assessment. At the completion of cell culture, disks were imaged using embossed contrast (EC2) with a 20X objective on a Nikon Ts-2R inverted microscope followed by fixation in FF or PFA_Ca using the same method as described above for tubes. Samples for histological processing were stored in 70% ethanol in 24-well plates until placing in a tissue processor or proceeding to processing for scanning electron microscopy as described below. All experiments were done at least three times (N = 3) with at least n = 4 technical replicates. Text taken from (Da Silva, Gvazava et al. 2023).

Fixation of 3D tissue engineered tubes and membranes for tissue processing

Media was carefully removed followed by the addition of 5 mL of either FF or PFA_Ca fixative directly into the well for 30 min. Tissue processing commenced thereafter by performing the first dehydration step in 70% ethanol in the well and then carefully placing the tubes into cassettes with foam pads to stabilize them during tissue processing. Cassettes were then loaded into an automatic tissue processor (Automated Spin Tissue Processor, model: STP 120, Myr) in an ascending series of ethanol solutions 70% and 80% ethanol, 2% × 80% ethanol and 20% isopropanol, followed by 3% × 100% 2-propanol (15,518,744, Fisher Scientific, Sweden). Each step was 1 h long and was conducted without stirring. Initial paraffin infiltration occurred for 3 h and was followed by a second incubation in paraffin for at least 6 h (Histowax (00403), Histolab, Sweden). Tubes were oriented in paraffin with the lumen parallel to the cutting surface. Membranes were oriented in paraffin blocks with their cross section parallel to the cutting surface. This was accomplished by first cutting paraffin infiltrated membranes in half with a scalpel. Next, membranes were dipped into molten paraffin to increase their thickness to allow for orienting in the metal molds prior to filling with liquid paraffin. Paraffin blocks were allowed to cool and then 5 µm thick slices were generated with a microtome and placed on Super Frost Microscopic Slides and allowed to dry prior to staining. Staining was performed as previously described, but with reduced hematoxylin staining of 5 min due to the large uptake of hematoxylin in alginate hydrogels we observed in pilot experiments. Text taken from (Da Silva, Gvazava et al. 2023).

Cell counting

Calcium alginate tubes containing cells were either fixed with PFA_Ca or FF and subjected to xylene free histological processing, paraffin embedding and sectioning for H&E staining. 20 random brightfield pictures were taken from transverse histological sections of tubes with a 10X objective using an inverted Nikon TS-2R microscope. Images were then blindly scored by an independent observer who had not previously seen the images and quantified using the ImageJ tool “Cell counter”. The number of normal appearing cells per field, cells with visible separation from the biomaterial and empty spaces were quantified. Next, the total number of cells was calculated by adding the number of normal cells and cells exhibiting detachment from the biomaterial. The percentage of cells exhibiting detachment from the biomaterial to the total number of cells present in the field was then calculated. Text taken from (Da Silva, Gvazava et al. 2023).

Water-soluble tetrazolium-1 viability assay (WST-1)

Water-soluble tetrazolium salt reagent (WST-1) was used to assess metabolic activity of tissue engineered constructs. Crosslinked calcium alginate and calcium alginate-gelatin films seeded with cells, as described above, were incubated with 1 mL of complete cell culture medium and 100 µL WST-1 (Roche, Sigma-Aldrich, United States) for 1 h in a humidified incubator with 5% CO2 in air at 37°C. Then 3 replicates of 100 µL of the supernatants from each well were removed and pipetted into a new 96 well plate. Optical density was measured at 440 and 650 nm (Epoch plate reader, BioTek, Winooski, Vermont, United States). Cell free calcium alginate and cell free calcium alginate-gelatin films manufactured and cultured in parallel served as negative controls. Absorbance at 650 nm was subtracted from all 440 nm absorptions to account for non-specific absorbance. Text taken from (Da Silva, Gvazava et al. 2023).

Scanning electron microscopy (SEM)

For SEM preparation, calcium crosslinked hydrogel disks were fixed in FF or PFA_Ca for 30 min, then dehydrated in serial solutions of 80% and 90% ethanol in de-ionized water, for 3 minutes each, and followed by 100% ethanol for 3 × 3 min for each dehydration step. The samples were then stored in 100% ethanol until chemical dehydration treatment with hexamethyldisilazane (HMDS, Ted Pella, United States) in serial grading 33%, 50%, and 66% HMDS in ethanol for 5 min each, followed by 100% HMDS for 5 min. The samples were then left to dry in a chemical fume hood for 30 min, then fixed on SEM stubs with a conductive silver paint (Ted Pella, United States) and were sputter coated with platinum-palladium at 80:20 ratio (Q150T ES, Quorum Technologies, United Kingdom) at 10 nm thickness with 40 mA sputter current before being mounted and examined in a Jeol JSM-7800F FEG-SEM under secondary electron imaging mode at 3.0 kV accelerating voltage. Text taken from (Da Silva, Gvazava et al. 2023).

Statistical analysis related to the PhD work (Paper I)

The use of the median total score of each feature or slide across all scorers also helps to reduce the potential impact of outliers. Data is then visualized and analysed using GraphPad Prism 9 (GraphPad Software Inc, La Jolla, CA, USA). Values can be compared between all the features or scores and between experimental groups using one- or two-way ANOVA for repeated measures with Kruskal–Wallis test or Mann–Whitney U, with p-values of ≤ 0.05 considered significant. Non-parametric testing and presentation of median for central tendency is the most appropriate statistical analysis and representation because semi-quantitative scoring systems are ordinal,

and therefore the differences between different scores cannot be assumed to be linear.

Statistical analysis related to the PhD work (Paper II)

Aggregated scoring results are shown as median with standard deviation as error bars in plots generated in GraphPad Prism 9 (GraphPad Software Inc, La Jolla, CA, USA). Median total scores for each slide were compared between cohorts using simple linear regression. To assess the reliability or internal consistency of the score system, the Spearman rank correlation was calculated between all reviewers and across all cohorts. The range of scores per slide were calculated for each expertise level and for each cohort. Linear regression analysis was performed to assess the intra-observer variation. P-values <0.05 indicate a significant difference. Plots related to the deep learning pipeline were generated with Matplotlib (version 3.5.9) and seaborn (version 0.11.2) python packages.

Power and statistical analysis related to the PhD work (Paper IV)

Statistical analyses were performed using GraphPad Prism 9 unless otherwise stated. Owing to the low sample numbers as well as variability in manual measurements and manufacturing approaches utilized here, all measured populations were assumed to have non-parametric distributions. The statistical test used, and central tendency measures used are listed in the corresponding figure legends. Power analyses to determine the number of n's needed for determining the swelling ratio and change in dimensions for cast films was done using pilot data. Power analyses were conducted in R using the "pwr2" package. Text taken from (Da Silva, Gvazava et al. 2023).

Key results and specific contribution to the field

A novel semi-quantitative score system developed based on specific features of acute lung injury in porcine model (Paper I)

Acute lung injury induced in animal models is characterized especially for histopathological examination as a gold standard to confirm lung injury; however different animal models of experimental lung injury model progression of the diseases differently. The main aspect to be considered beforehand is the selection of the injury type and choice of the animal used to the development of the mechanism that will be utilized to induce lung injury. Despite the fact that mice are widely used, and their morphological characteristics are very well reported, it is very difficult to recapitulate clinical approaches such as mechanical ventilation and ECMO due to their size. In this case, the utilization of large animal models such as the porcine model has emerged as a potential good strategy due to the fact that it is possible to utilize different mechanisms to induce lung injury. In previous research, **Figure 16** we collaborated in a project which utilized LPS to drive the onset of ALI/ARDS (Stenlo, Hyllen et al. 2020).

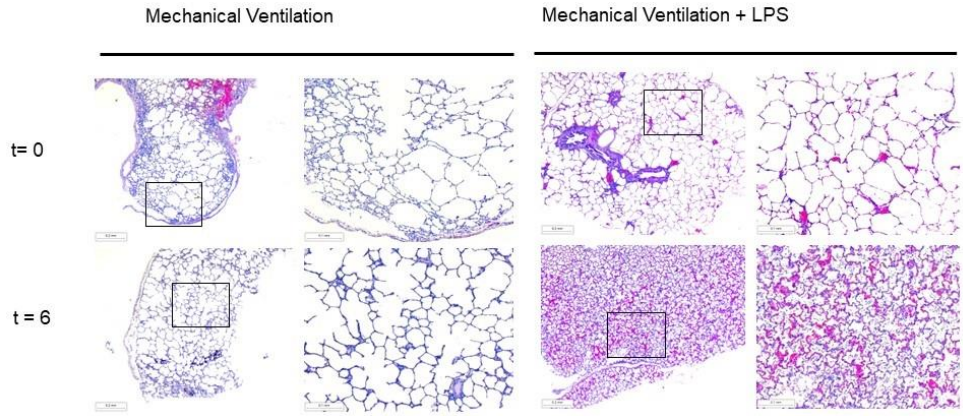


Figure 16 hematoxylin and eosin-stained lung sections from an individual pig before (baseline) and 6 h after LPS administration.
(6 h post-LPS) and also after 6 h of sham treatment (6 h post-sham). Scale bars: left, 0.2 mm; right, 0.1 mm. Figure modified from (Stenlo, Hyllen et al. 2020) with permission.

One of the challenges that accompanies large animal models of ALI/ARDS is the challenge of confirming that the injury model induces lung specific injury which is the cause of the reduced blood oxygenation detected. Clinically, chest x-rays and CT scans are used but this is not available in all preclinical large animal facilities. Therefore, histology has been a substitute gold standard to confirm acute lung injury. While binary classification of lung injury is possible (yes/no), it became clear in the first collaborative project that there was a need to somehow grade the extent of the injury as it was heterogenous across lobes, animals and regions of the biopsy (Stenlo, Hyllen et al. 2020). The implementation of a score system to provide a total lung injury score has already been reported as a promising approach to be explored but was optimized for small rodents (Matute-Bello, Downey et al. 2011).

Based on our observations in this first project (Stenlo, Hyllen et al. 2020) (*Other papers, not included in the* thesis) we also noted the large number of samples generated for this analysis due to the size of the animal model and that biopsies were taken over time. These samples were processed by hand and using toxic chemicals (formaldehyde and xylene). (Buesa and Peshkov 2009, Kandyala, Raghavendra et al. 2010, Purdie, Purdie et al. 2011). In addition to the potential and importance of developing a consistent, reproducible workflow using an automated tissue processor for future studies, the use of hazardous chemicals in an automated tissue processor in our laboratory would only have been possible by redesigning the ventilation system. The alternative was to continue processing these samples by hand which would result in long term exposition to hazardous chemicals, such as xylene, during the steps of tissue processing.

Following the green chemistry principles, we investigated solutions that might to facilitate a sustainable approach of research without affect the occupationally safety, environment and/or compromise the research. Besides the promotion of a green pipeline of tissue processing (clearing step), the automatization of the process utilizing an automatic tissue processor helps to avoiding the direct manipulation of chemicals by users and also improves the time consumed during this task (Falkeholm, Grant et al. 2001, Kandyala, Raghavendra et al. 2010). We developed a method to score acute lung injury driven by the administration of LPS in a porcine animal model, utilizing a green histological approach removing xylene of the clearing steps (**Paper I, IV)** and **(Stenlo, Silva et al. 2021, Ghaidan, Stenlo et al. 2022, Silva, Gvazava et al. 2022)**. In addition to the porcine models, we have also successfully applied xylene-free tissue processing (clearing step) to several types of classical histology staining and immunofluorescence across different species **(Figure 17)**.

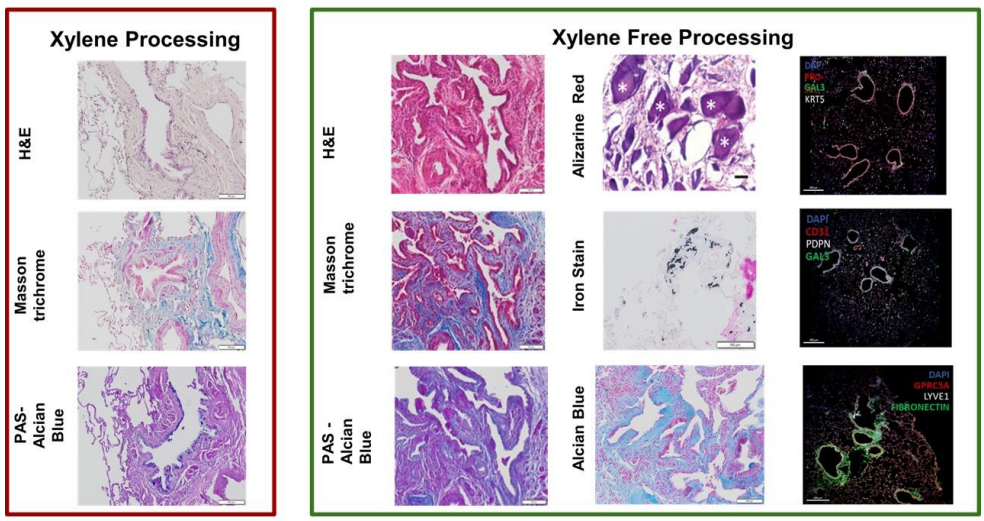


Figure 17 Ilustration of different examples of tissue and material from different groups can be processed using xylene-free pricessing (Clearing) protocol.

Furthermore, another sustainable approach implemented was the repurposing of equipment discarded for other groups, such as the manual microtome and the refurbished block heater. In addition, we initially constructed our own paraffin tank dispenser utilizing a water boiler retrofitted with a stain-less stell spigot. While the commercial paraffin embedding machine cost around 50.000 – 60.000 SEK while the boiler water tank coast 300 SEK (Colglazier 2015) **(Figure 18)**.

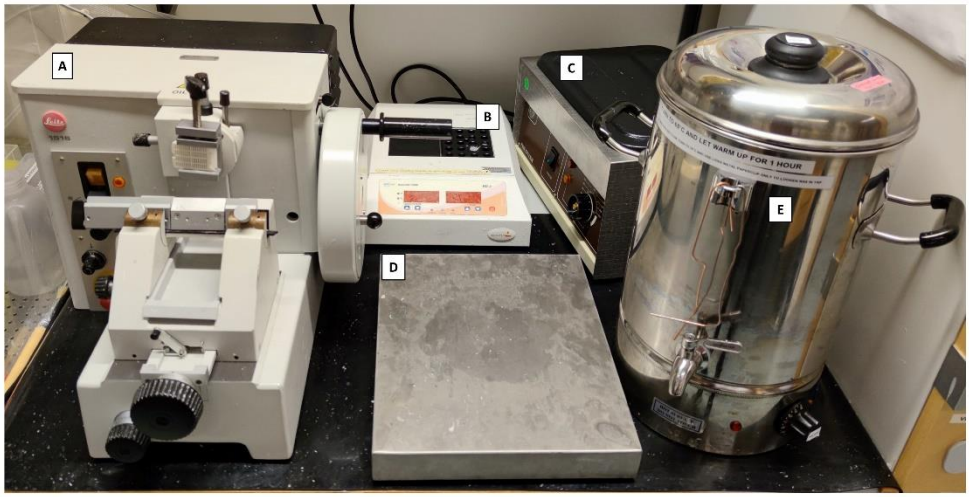


Figure 18 Histopathology area, developed with revitalized, refurbished, and repurposed equipment.
A) Manual microtome (revitalized); B) Block heater (refurbished); C) Water bath (revitalized); D) Coolingtray (repurposed); E) Paraffin tank dispenser (water boiler retrofitted with a stainless-steel spigot). Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

After having implemented our xylene-free histological approach, we focused on the need to develop a new scoring system as the traditional approach already broadly used was developed for mice and may not recapitulate very well the histological features we observed in a porcine model (Matute-Bello, Frevert et al. 2008).

Based in our experience with the model, we selected features that could better correlate with our findings in the porcine model in order to develop a score system that adheres to the criteria of the principles of a valid histopathological score system. We selected seven histological features for our porcine scoring system after evaluating the initial histological patterns present in the initial paper describing the model (Stenlo, Hyllen et al. 2020) (inflammatory cells, hyaline membranes, proteinaceous debris, thickening of alveolar wall, hemorrhage, atelectasis, and a general injury score entitled ‘enhanced injury’) (Gibson-Corley, Olivier et al. 2013, Meyerholz and Beck 2018) **(Figure 19)**. The choice of these main features has been recently and independently confirmed by an international panel of experts to be present and relevant in animal models of ARDS (Matute-Bello, Downey et al. 2011, Kulkarni, Lee et al. 2022).

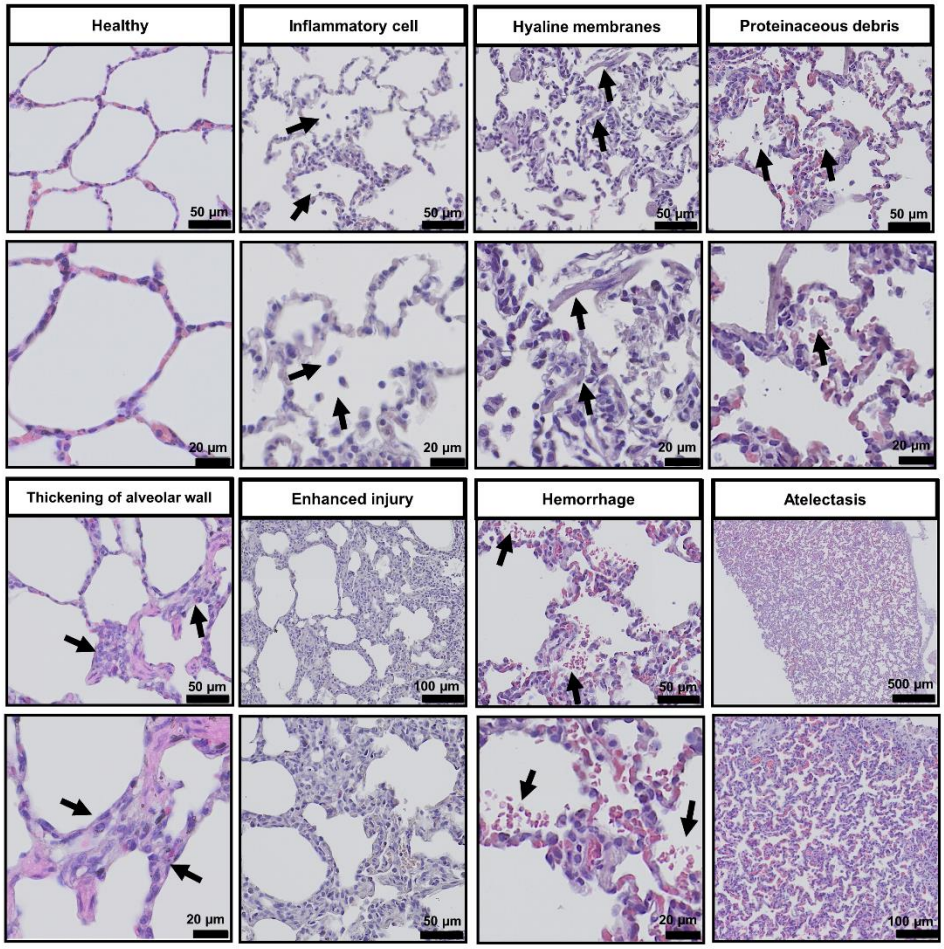


Figure 19 Representative photomicrographs of H&E stained sections demonstrating the different features selected for development of the lung tissue injury score system in low and high magnification.

Scale bar for healthy: 50 µm and 20 µm. Scale bar for Inflammatory cell: 50 µm and 20 µm, Scale bar for Hyaline membranes: 50 µm and 20 µm, Scale bar for Proteinaceous debris: 50 µm and 20 µm, Scale bar for Thickening of alveolar wall: 50 µm and 20 µm, Scale bar for Enhanced injury: 100 µm and 50 µm, Scale bar for Hemorrhage: 50 µm and 20 µm, Scale bar for Atelectasis: 500 µm and 100 µm. Black arrowheads demonstrate examples of features. Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

Further, we felt that the traditional score system doesn't reflect the character of the heterogenicity observed in our studies. In the original score system, the range varies from 0-2 (none presence of the feature and, 1 medium or low and 2 high presence of the severity); however as the authors also discuss, there exists a large variability on the tissue. This is especially true in our case, where the sample amount is very big. In addition, we discussed that the traditional score system might be too heavily

biased towards inflammatory cells and other features, such as evidence of hemorrhage, might be a more important feature in porcine models and may also be possible to observe reductions in if the animals receive treatment (Matute-Bello, Downey et al. 2011). Such as with the example bellow **(Figure 20)**.

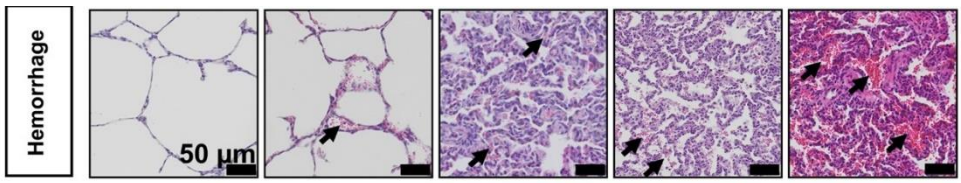


Figure 20 Example of variability amoung same feature (Hemorrhage).
Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

Using this example of hemorrhage how would it be possible to perform a score between 0-2 observing such variability? Based on our observations, we developed a score system using a range from 0-8 that allowed us to manually cluster in five classification levels. We developed this guide table (**Table 13**) which includes the features and the correspondent description, besides the total score formula.

Table 13 Features of acute lung injury to score in H&E stained histological sections of porcine lung tissue.

Features	Scoring	Feature Description
A – Inflammatory cell		Visible inflammatory cells in air and interstitial spaces
B – Hyaline membranes		Acellular deposit (i.e. devoid of hematoxylin staining) in the alveolar region and stained with eosin
C – Proteinaceous debris		Acellular debris in airspaces
D – Thickening of alveolar wall		Alveolar wall thickening (i.e. at least >1 cell layer thick)
E – Enhanced injury		Overall impression of tissue level injury
F – Hemorrhage		Visible red blood cells in the interstitium or airspaces
G – Atelectasis		Complete or partial collapse of distal airspaces
	Total Score = (Sum / 56) x 100	

The score range is 0 (null) to 8 (severe) for each of the features mentioned. Table taken from (Silva, Gvazava et al. 2022) with permission.

Examples of slides showing the 5 classification of injury levels for each feature: no damage (Minimum), mild damage (2/3), moderate damage (4), pronounced damage (5/6) and extensive damage (Maximum) for each feature is shown in **(Figure 21).**

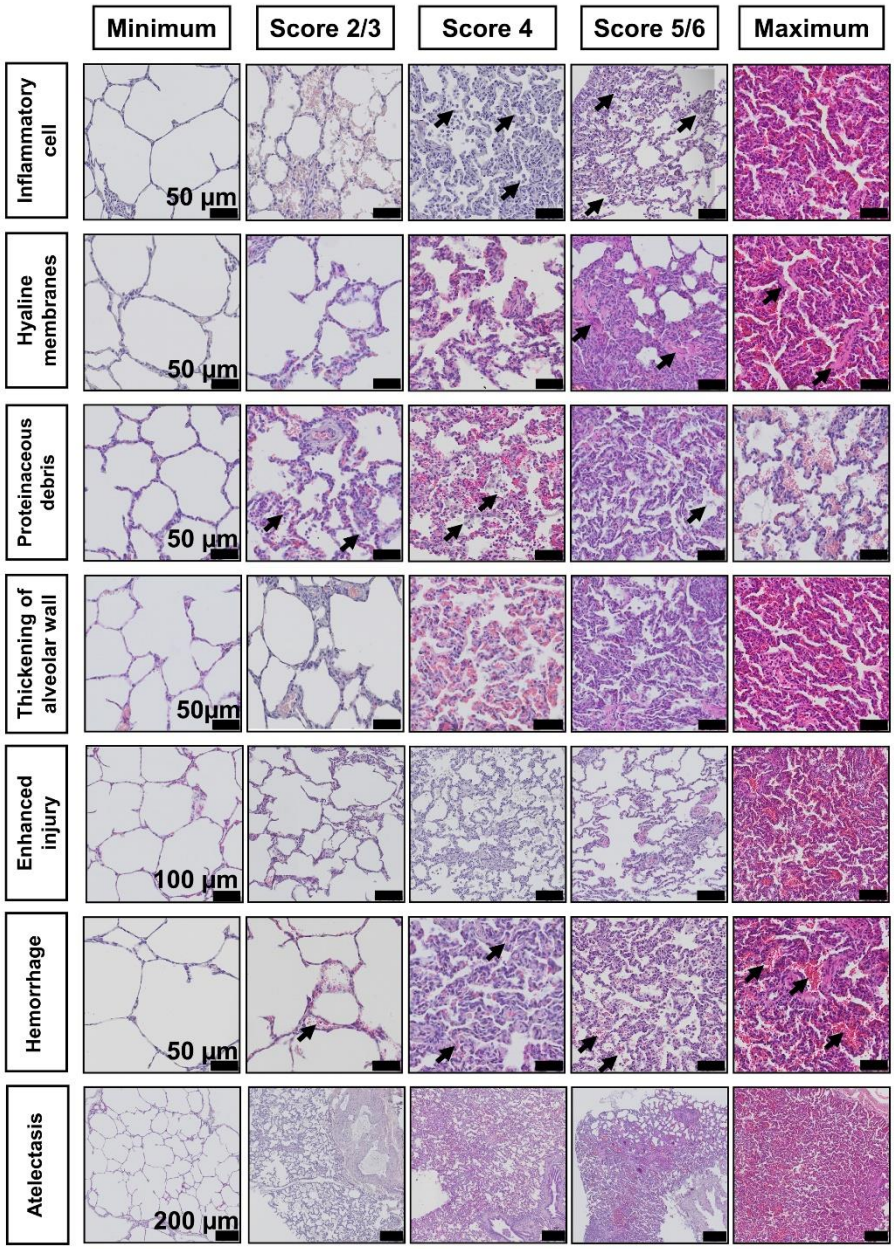


Figure 21 Representative photomicrographs of sections stained with H&E which demonstrate a range of scores across all features.

Score ‘Minimum: absence of injury’; ‘Score 2/3’: mild injury; ‘Score 4’: moderate injury; ‘Score 5/6’: pronounced injury and highest score given. Scale bar for inflammatory cell, hyaline membranes, proteinaceous debris, thickening of alveolar wall, and hemorrhage: 50 µm; scale bar for enhanced injury: 100 µm; scale bar for atelectasis: 200 µm. Arrows demonstrate examples of features. Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

In addition, we started to implement xylene-free deparaffinization before the staining and final clearing in **Paper I (Figure 22)**.

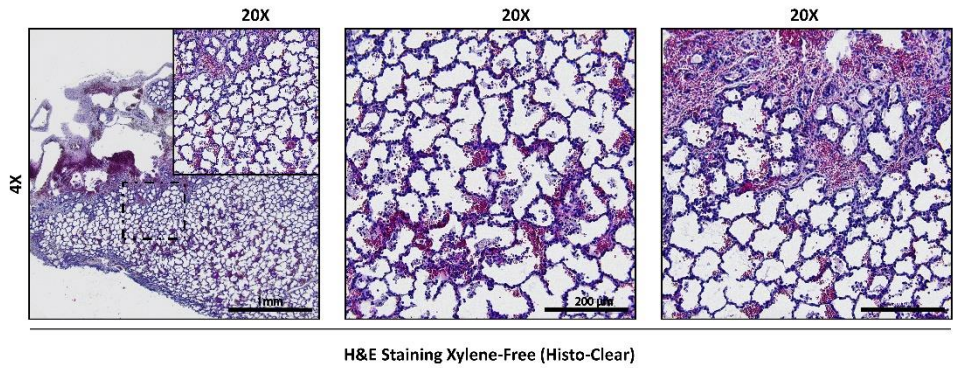


Figure 22 Example of H&E staining of porcine lung tissue using xylene-free tissue processing described and xylene-free deparaffinization and final clearing protocols.
Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

Next, we examined whether the semi-quantitative scoring system for green histopathological evaluation of large animal models of acute lung injury we developed was robust and could identify lung injury. However, we interestingly observed that there was variation among the observers which seemed to correspond to their expertise level **(Figure 23)**.

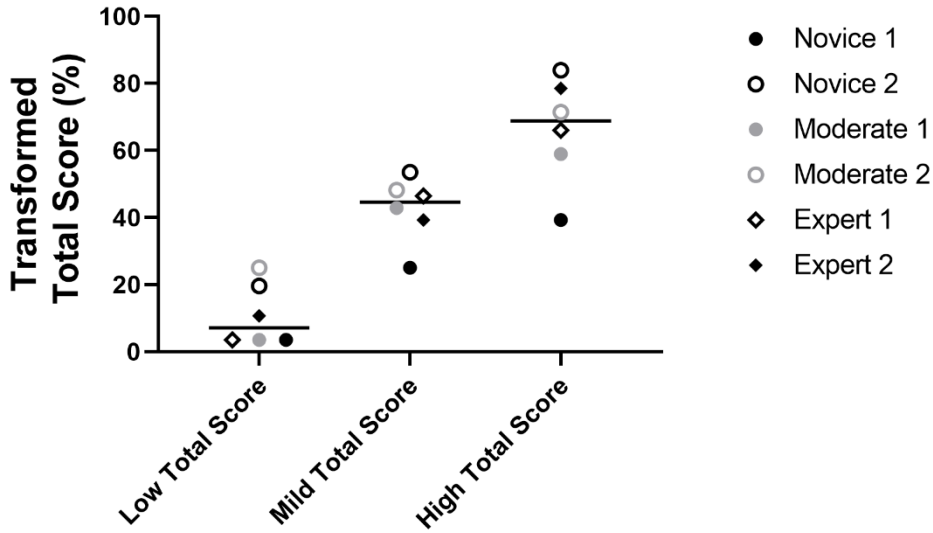


Figure 23 Example scoring outcomes and relative reproducibility across different independent scorers with different levels of experience (novice, moderate and expert).

Representative total scores from the 3 example of scores and transformed to a scale of 0-100. The total score and the ability of users to distinguish between different severities of injuries did not differ dramatically based on experience. These samples were selected to represent a range of the scores our system detected (low, mild and extensive level of lung injury) as judged by the blinded observers. The horizonal line represents the median of all scorers for each slide. Figure and text taken from (Silva, Gvazava et al. 2022) with permission.

One interesting aspect observed afterwards, was to further analyze why or how the observer level of disagreement happened. We could initially identify features where we have more potential of disagreement. We observed that some features were problematic for some observers. One example is blood (hemorrhage). We noticed that there was high disagreement on how some observers scored hemorrhage. In later discussing with them, we discovered that they were simply scoring based on the observation of the colour red and not whether that red was associated with an object resembling/having the shape of an erythrocyte. Therefore, this feature was miss characterized on some slides where proteinaceous debris (i.e. eosin stained debris) was categorized as hemhorrage. This further points to the importance of having well-characterized features (both in pictures and words) to help harmonize scorers.

Another example we observed was the identification of hyaline membranes. As our animal model was still acute, there is limited development of hyaline membranes. In addition, due to staining variation, some observers expressed uncertainty (especially pathologists who expressed their hesitancy to score this feature without better resolution/staining due to its importance diagnostically). Therefore,

sometimes the same slide was observed as having a high score in one slide but low in others **(Figure 24)**. This reinforced the need for working towards a machine learning approach, to achieve more consistency on the identification and score of features that can be challenging for humans. These informal interviews and post-scoring interviews were also a reminder that machines only can learn from human subjective experience. Therefore, the machine will initially be dependent on the source and quality of the training data. Many laboratories cannot afford the time of a highly experienced researcher or pathologist for every study. Therefore, the possibility to utilize machine learning can provide more reliable results and standardization across papers from different labs.

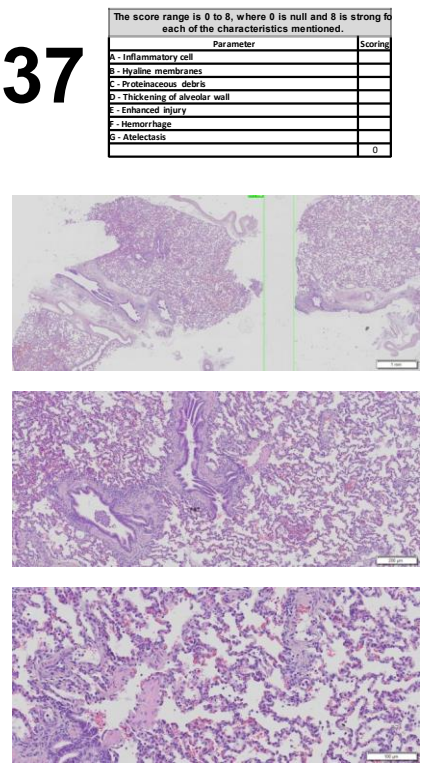


Figure 24 Example of slide that may be miss classified by human observers
Figure and text taken from (Silva, Gvazava et al. 2022) with permission.Data transference, using score system developed to teach machine learning (Paper II).

The data set was generated and deposited at the Biostudies archive of the European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/biostudies/studies/S-BIAD419) (**Paper I)** and the publications where the *in vivo* model system was reported and the score system was first applied (Stenlo, Silva et al. 2021, Ghaidan,

Stenlo et al. 2022) (*Other papers not included in the thesis*). However, in all of these papers, it was clear that the human based evaluations are accompanied with a certain subjectivity which may affect the reproducibility. Further, due to the intra- and inter-observer variability, the sensitivity of the semi-quantitative score system to detect meaningful biological differences was still somewhat limited among digital histological slides (True 1988, Lidbury, Rodrigues Hoffmann et al. 2017, Lindquist, Ciornei et al. 2022).

In **(PAPER II)** we therefore more deeply analysed if the semiquantitative score system was robust, reproducible and reliable to teach a system of machine learning with the objective to reduce variations due to human subjectivity and especially differences which are prevalent in most papers with preclinical histological scoring which arises from the different expertise levels**.**

A recently developed semi-quantitative score system for acute lung injury is suited to generate ground truth for training deep neural networks.

High quality ground-truth labels are needed to implement deep learning models such as CNNs to detect and examine histological slides of lung injury in a supervised manner. In order to make sure that the machine will receive a good source of information, we examined two different score systems using the same histological slides. They were scored independently for different expertise levels, as would occur in a traditional pre-clinical research, to compare which score system would be potentially more suitable for ground truth labels (**Figure 14)**.

We observed a high similarity of the median scores between the original "A" cohort and the validation "B" cohort (**Figure 25 a-c**). We then compared these scores to those assessed with the Matute-Bello system modified for use with digital slides ("C" cohort), normalizing the scores from both scoring systems to a common range. We found that the "C" cohort scores were overall higher and distributed in a bimodal pattern (**Figure 25 a-d**). To further assess how much the scores from the "B" and the "C" cohort differed from our originally reported scores, we plotted the scores for the "B" and "C" cohorts versus the "A" cohort and calculated the slope of the linear regression line and the correlation metric, R-squared **(Figure 25 d)**. While the "B" cohort showed a high correlation with the "A" cohort (slope of 0.91 and an R-squared value of 0.93), the "C" cohort had a slope of 1.146 which indicates that the Matute-Bello score system skewed the total score towards higher values compared to the two cohorts using the Silva et al. system **(Figure 25d)**.

Overall, our findings indicated that our recently developed semi-quantitative scoring system provides similar results when comparing median values from two cohorts of observers (Silva, Gvazava et al. 2022). This, together with a large scoring

range that allows for nuanced scoring, suggested that it would be well-suited to generate ground-truth labels for supervised training of deep neural networks.

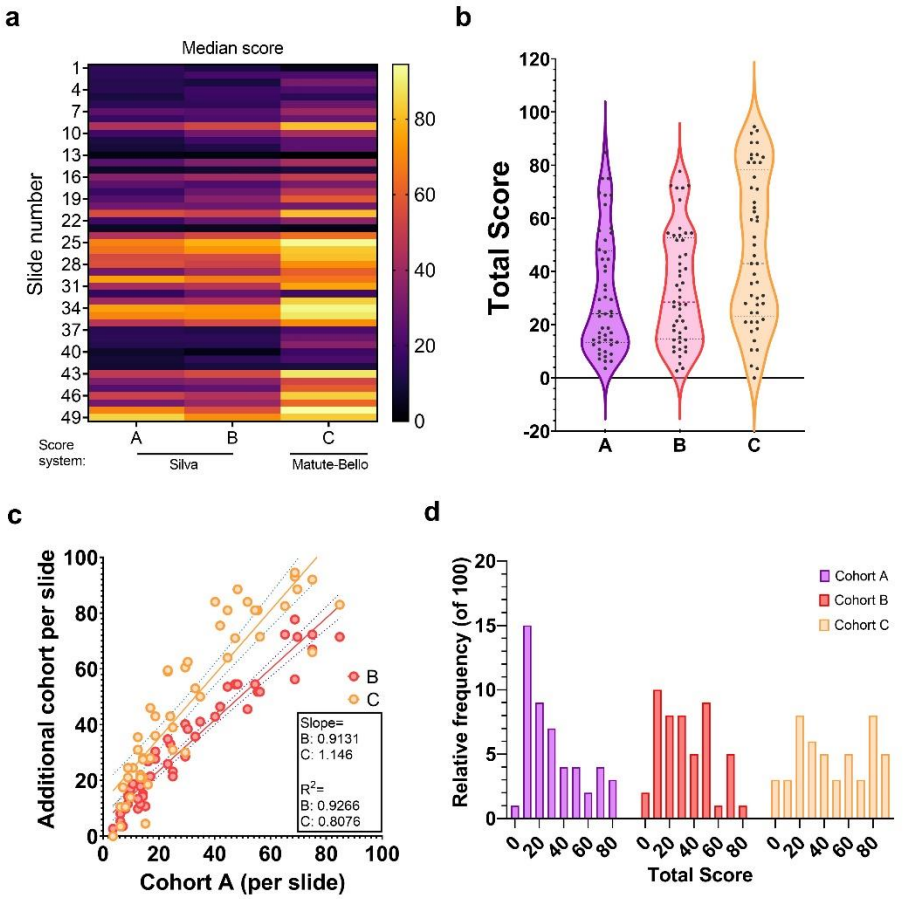


Figure 25 Assesment of semi-quantitative score systems reveal that our score system is suitable to generate ground-truth labels for supervised training of deep newural network.
(a) Heatmap of median total score per slide for the selected cohorts. Scores were normalized to lie in a range of 0-100 for this and the following figure panels. (b) Violin plot comparing the distributions of the normalized median total scores from the three cohorts. (c) Scatter plot comparing the normalized median total scores of the two new cohorts with the original Silva cohort. N refers to the number of scorers. Slope refers to the slope of the regression line. (d) Histogram demonstrating the distribution of the normalized median total scores of the selected cohorts. Figure and text taken from the manuscript (da Silva and Rashed, et al 2023) with permission.

Variability between human scorers reinforces the need for automatization of the scoring with deep neural networks.

Observing the differences among the distribution of the total score systems, we next needed to assess if the variation across the various expertise levels would affect the sensitivity of either scoring system **(Figure 26)**. Observing individually both score systems performance, we can initially visualize that Matute-Bello system has higher heterogenicity on the distribution of total score, or in other words higher inter-observer variability. On the other hand, there was a generally higher rank correlation between observers with the Silva et al. System.

We detected lower rank correlation based on experience level in both score systems **(Figure 26c, f, and g-i).** Interestingly, we observed higher inter-observer variability between board-certified pathologists with extensive clinical training as compared to the inter-observer variability between observers with a high level of expertise in histological scoring for preclinical models **(Figure 26h)**. Except for one of the board-certified pathologists and one of the novice observers (N1), all other observers had at least 82% rank correlation with one another **(Figure 26 c).**

In general, the modified Matute-Bello system (modified for digitally scanned slides and scoring at the slide level) resulted in higher inter-observer variability **(Figure 26 f, 2i)** and we observed tendencies towards higher scores with a bi-modal distribution between high and low scores for all observers **(Figure 26 e)**. One limitation of these experiments was that different observers scored the slides using the two different scoring systems, and only a small subset of observers scored using both scoring systems.

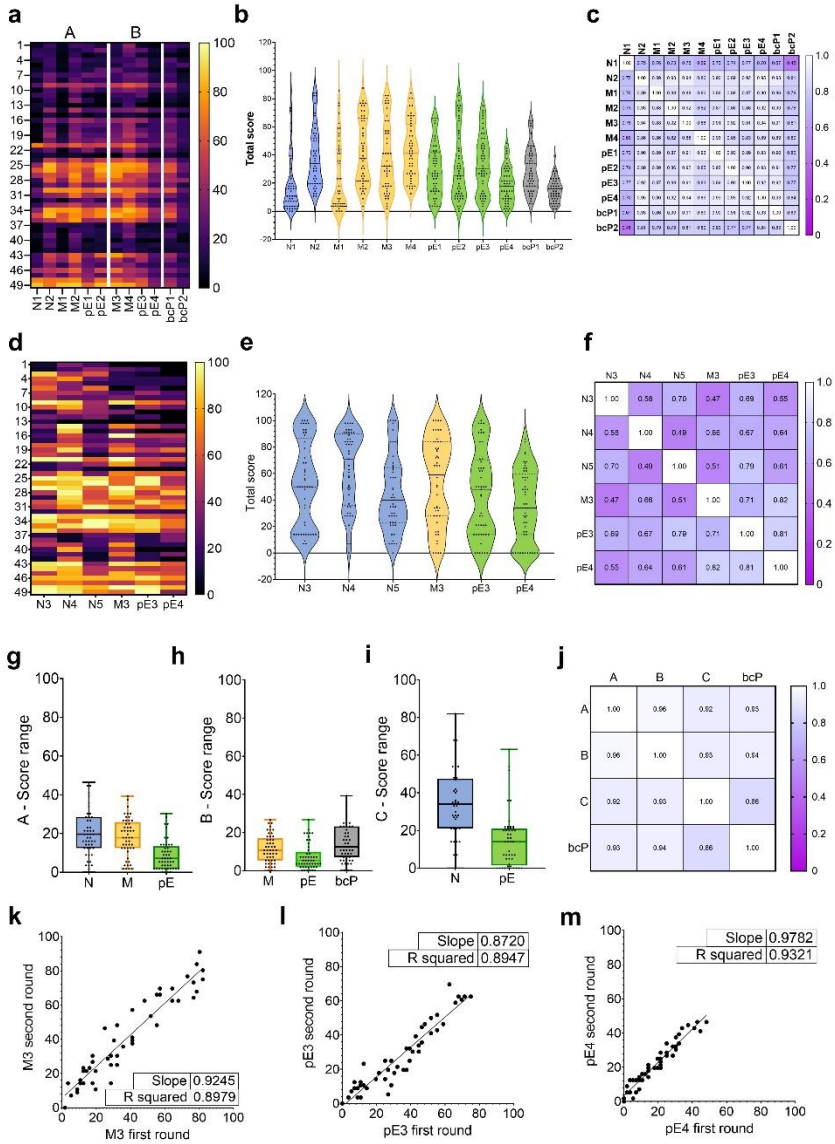


Figure 26 Variability between human scorers reinforces the need for automatization of the scoring with deep neural networks.

(A) Heatmap of normalized total scores from the reviewers in different cohorts that performed scoring based on the Silva score system. The lettering on top signifies the original (A) and validation cohort (B). (B) Violin plot of the normalized total scores from the reviewers in different cohorts that performed scoring based on the Silva score system. (C) Correlation matrix indicating the Spearman rank correlation between the total scores from the reviewers in different cohorts that performed scoring based on the Silva score system. (D) Heatmap of normalized total scores from the reviewers that performed scoring based on the Matute-Bello score system. (E) Violin plot of the normalized total scores from the reviewers

that performed scoring based on the Matute-Bello score system. (F) Correlation matrix indicating the Spearmann rank correlation between the total scores from the reviewers that performed scoring based on the Matute-Bello score system. (G) Range of the total normalized scores from the Silva score system given by scorers of the original cohort with the same level of expertise. For this and panels H and I, the range was calculated for each slide by subtracting the lowest score given by a reviewer in the respective expertise group from the highest score given by a reviewer in the same expertise group. (H) Range of the total normalized scores from the Silva score system given by scorers of the validation cohort with the same level of expertise. (I) Range of the total normalized scores from the Matute-Bello score system given by scorers with the same level of expertise. (J) Correlation Matrix indicating the correlation between the total normalized median scores from each cohort as well as the group of two certified pathologists the . (K) Scatter plot comparing the self-agreement for the total normalized scores from selected reviewer with moderate expertise level (M3), (L) pre-clinical expert (pE3) and (M) second pre-clinical expert (pE4) for scoring performed on identical slides but over an at least one month time period. Slope refers to the slope of the regression lines. Figure and text taken from the manuscript (da Silva and Rashed, et al 2023) with permission.

While low inter-observer variability is important to consider when selecting a scoring system for training CNNs, low intra-observer variability is also desired (i.e. self-agreement). Therefore, we asked a subset of observers with moderate to high levels of expertise in evaluating pre-clinical models of acute lung injury to repeat their scoring after at least one month using the same set of images.

We found that all three observers **(Figure 26 k-m)** exhibited high correlation (>87%) between their first and second round of scoring, thus indicating that the Silva et al. system has low inter and intra-observer variability making it a promising candidate for training CNNs using a slide-level scoring approach (**Figure 27)**.

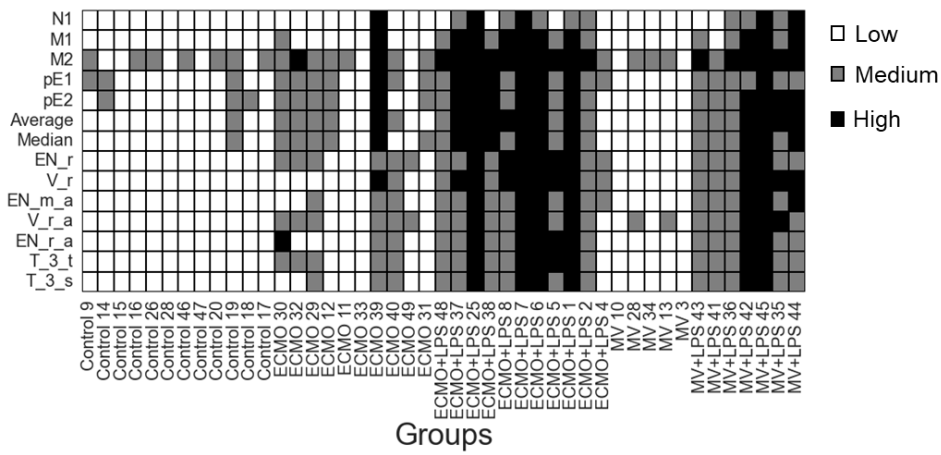


Figure 27 Development of deep neural networks for automated histology scoring.
Slide scores assigned by the reviewers from the original cohort in comparison with predictions by CNNs. Individual reviewer scores, their average and median (used as ground truth label for training the CNNs) and predicted scores by the 2 individual CNN models trained on raw dataset (EN_r and V_r), 3 best individual CNN models trained with augmentation (EN_m_a,V_r_a, and EN_r_a) and two ensemble predictions obtained by combining predictions from the three models on tile level (T_3_t) and slide level (T_3_s). Slides are identified by treatment group and slide number. Figure and text taken from the manuscript (da Silva and Rashed, et al 2023) with permission.

In summary, we could identify that the use of semi-quantitative score system can be used as a strong candidate for training deep neural network to automate the human task. However, there are limitations with different aspects to be reflected upon. The first aspect that is important to discuss and considerer the importance of an appropriate semi-quantitative score system. Using the system, we described in Paper I, with lower intra- and inter-observer disagreement observed in Paper II, this is important to help the machine on the definition of what is the truth, as well as the reliability and consistency. We also observed good reproducibility when the same observers repeated the scoring task.

Is important as well to remember that the machine never be better than a human, due to that fact that it needs a human to teach it to recognize the features. The promising aspect of the machine is mainly to avoid the human bias, consistency, and our natural subjectivity which can vary from day to day. Further we can correlate the use of machine learning applied to identification of histological scoring to the sustainability approaches where the machine can reduce the time and cost, especially with the time of the highly specialized professionals, simplifying and accelerating research.

OCT can be used for H&E to examine rECM hydrogels (Paper III)

In **(Paper III)** The main objective was on the development of a 3D printed construct using a hybrid bioink comprised of ECM derived from either human or mice. In order to develop this ECM based bioink, the ECM needed reinforcement from another material. We chose to use alginate as it is one of the most commonly used bioinks in 3D bioprinting. This new material was therefore comprised of the polymer alginate and ECM - developing then the rECM (reinforced extracellular matrix bioinks). In addition to its cellular compatibility and printability, we also checked its biocompatibility in comparison with the non-clinical grade alginate alone in an immunodeficient animal mimicking clinical immunosuppression of transplant recipients (i.e. T-cell deficient animals, *FoxN1* nude mice) **(Figure 28)**.

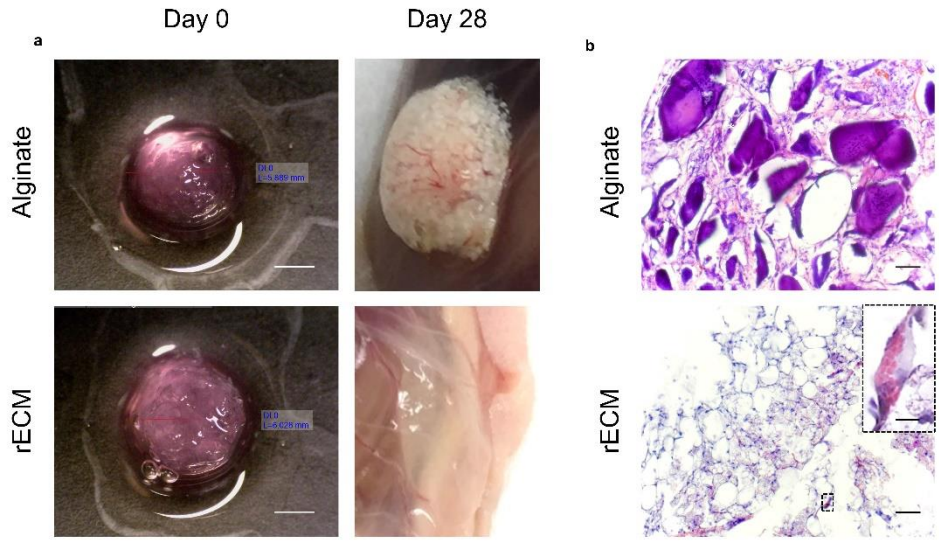


Figure 28 Biocompatibility and angiogenic potential of rECM hydrogels.
a) 3D printed alginate and rECM hydrogels in disk shape before subcutaneous implantation and when explanted on day 28. Scale bars: 2 mm. b) H&E staining of subcutaneously implanted alginate and rECM hydrogels after 28 days. White asterisks * indicate large, non-proteinaceous debris. Inset showing red blood cells in the inner lumen of a blood vessel. Scale bars: 50 and 10 µm (inner panel).

Using a short formalin fixation time and OCT embedding approach, we were able to generate histological sections. H&E could be performed for histological examination of the architecture, although the quality was low due to challenges in sectioning **(Figure 28 b)**. Nonetheless, we noted some interesting features already at this time. The first thing we noticed is that in the rECM, the alginate portion seems to be missing from the tissue or is not stained with hematoxylin (as we have observed previously). It was therefore unclear if it was totally remodeled/degraded after the 28 day implantation, or if some step on the histological examination dissociated it. On the other hand, a foreign body response was clear in the non-clinical grad alginate alone. However, again, we did not observe any alginate staining as we have seen in pre-implantation evaluations. At the time, we evaluated that this may have been due to several challenges described on the methodology. The performance of the H&E staining was only possible utilizing a short formalin fixation time and OCT for post cryosection. This initiated the discussion about re-evaluating part of the protocol to allow for paraffin embedding in the future. This would also remove the utilization of OCT which demands the use of energy long term to keep the tissue at -20°C.

Furthermore, the optical resolution resulted from the OCT cryosections are not optimal in comparison with traditional fixation and processing tissue methodologies. This limits the ability to accurately examine details that may be

misidentified. In particular, evaluating cellular differentiation of cells by their morphology on H&E staining when seeded on biomaterials demands a higher level of detail. The utilization of cross-sections, in particular for evaluating air-liquid-interface cultures to mimic the airway epithelium provide a more clear visibility to the epithelial tissue. OCT was used for **Figure 29** and . A short formalin fixation time and OCT embedding for cryosections was used for **Figure 29** which was generated from cells grown on alginate and hybrid (rECM) scaffolds at air-liquid interface. We also used the xylene free approach to process murine tracheal epithelial cells grown on a commercial polyester membrane (Transwell) where we could see clear retention of the membrane which allows careful evaluation of cell-Transwell membrane interaction which is otherwise challenging **Figure 30** (Alsafadi, Stegmayr et al. 2022) (*Other publications not included in the thesis*).

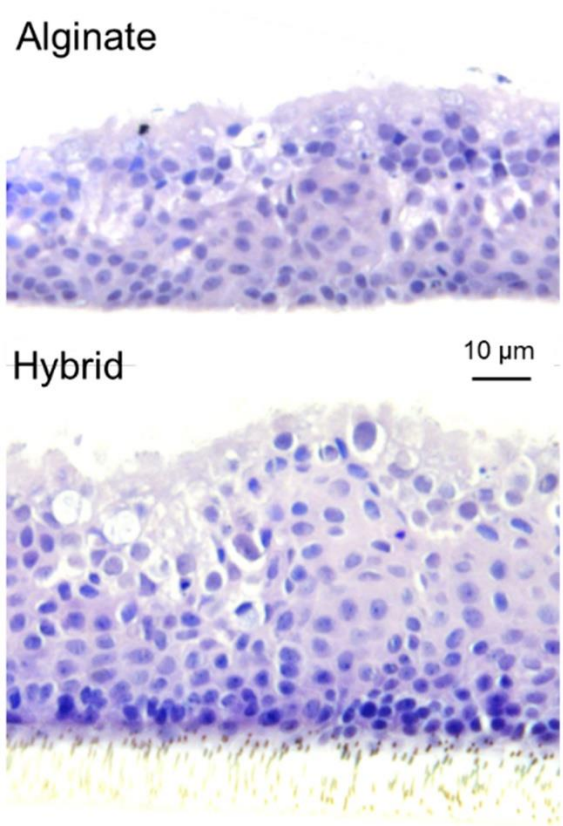


Figure 29 Human-derived rECM hydrogels as bioinks for airways
Cross section H&E staining of HBECs 28 days after seeding on top of alginate and rECM hydrogels. Scale bar: 10 µm.

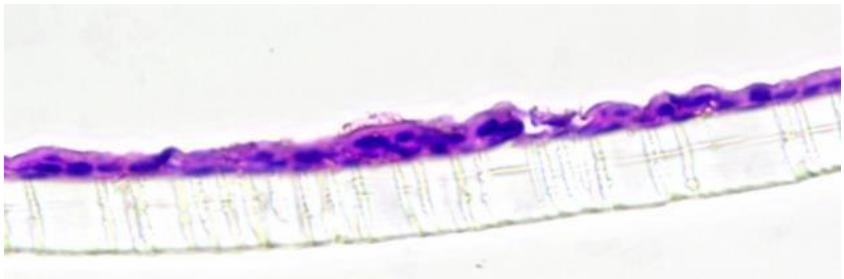


Figure 30 Differentiation of 3DLD isolated proximal epithelial cells into a pseudostratified epithelium with normal apical-basal polarity.
Cross section Hematoxylin and eosin staining and bright field imaging of differentiated proximal epithelial cells cultured in ALI at day 28 confirming the presence of a pseudostratified monolayer. Imaged at 40X magnification.

Development of Formalin-free fixation and xylene-free tissue processing protocols which allows histological examination of both of tissue and 3D calcium alginate tissue engineered construct (PAPER IV)

As mentioned previously, different fixatives have different and specific mechanisms to stabilize the tissue of interest (as already described in this thesis). However, histological examination utilizing alginate hydrogels is known to be quite challenging. Therefore, our goal was to identify the best candidate for tissue fixation of alginate based bioinks that are ionically crosslinked with calcium which can be compatible with our already implemented xylene-free tissue process. The development of such a pipeline is important in order to improve histological examination and if formalin-free solutions can be implemented, can help make the whole histological process more sustainable for research. Our first goal was to evaluate and initially confirm that both aldehyde containing and aldehyde-free fixative methodologies are compatible with native tissue architecture across organs with different compositions, such as lipid vs protein ratio due to the diversity of molecular groups available for crosslinking **(Figure 31)**.

Commercial and laboratory made fixatives comparably preserve histological architecture of diverse native tissue types.

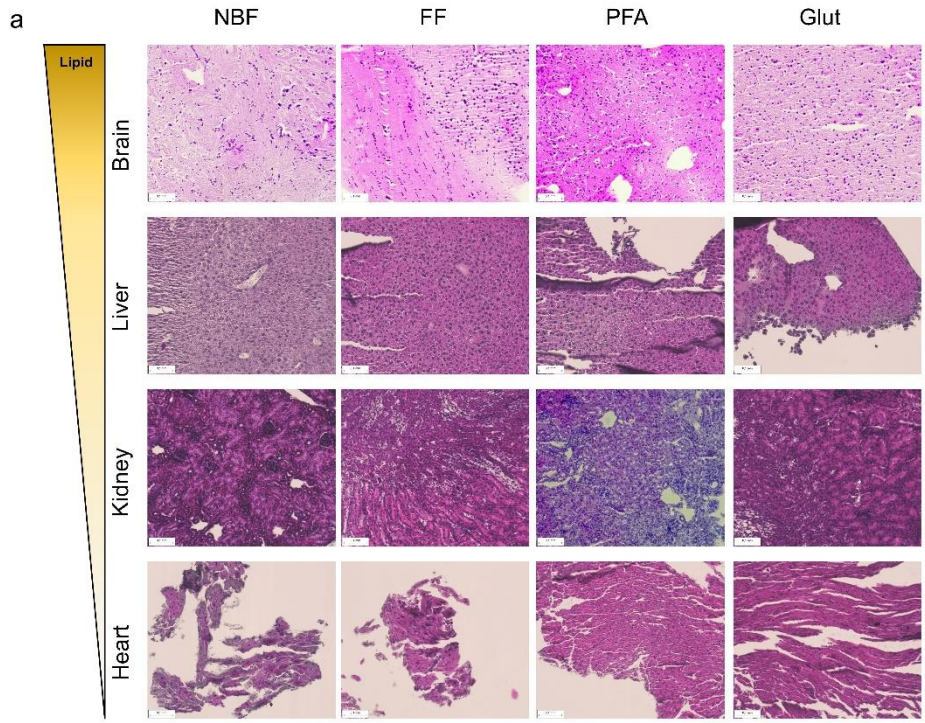


Figure 31 Commercial and laboratory made fixatives comparably preserve histological architecture of diverse native tissue types.
Fixation of native murine tissues of varying lipid-protein composition with different commercially available fixatives. (A) Overview of tissue harvest (brain, heart, kidney and liver) from a single animal followed by vibratome slicing and then fixation of neighboring sections using one of four commercially available fixatives (detailed in Table 1). (B) Overview of histological processing workflow to generate tissue microarrays (TMAs) for assessing tissue autofluorescence with different fixatives and simultaneous staining with hematoxylin and eosin (H&E) and imaging. (C) Organs are arranged from highest to lowest lipid content. H&E images acquired using a ×20 objective on a Nikon Ts-2R inverted microscope with constant acquisition settings for all images. Scale bar is 100 µm for all images. Schematic in (A) and (B) made using Microsoft Icons by the authors. Figure and text taken from (Da Silva, Gvazava et al. 2023) with permission.

All four fixatives (NBF, Formalin-Free, PFA and Glutaraldehyde) were able to comparably retain the tissue architecture during xylene-free tissue processing and paraffin embedding. One aspect that we noted, and as reported in the literature, is that we observed variations on H&E staining intensity depending on the fixative. Glutaraldehyde and NBF showed weaker eosin staining **(Figure 31)** which has been previously observed in studies comparing aldehyde-based crosslinking to alcohol based fixatives (Perry, Chung et al. 2016). Taken together, this indicates that all four

of the chosen commercial fixatives can be used in combination with xylene-free tissue processing protocols for native biological tissues and could potentially be used to histologically evaluate alginate-based scaffolds.

Ethanol based, formalin-free fixative and calcium containing fixatives preserve hydrogel structure during standard fixation times

Based on our observations in **Figure 31**, we next wanted to make sure that the fixatives could macroscopically keep the architecture of ionically crosslinked calcium alginate hydrogels. This is a manufacturing approach which is being used for tissue engineering (e.g. 3D bioprinting in **Paper III**) and cell therapy. Scaffolds and microspheres are generated using different amounts and time duration of calcium exposure and this is known to change the stability of the formed calcium alginate scaffold. In general, we found that increased time of calcium chloride exposure led to the formation of hydrogels which could withstand longer fixation times **(Figure 32)**. However, fixatives which stabilize tissue via chemical crosslinks and high amounts of phosphate and contained no added calcium ions (i.e., NBF and PFA) caused physical disintegration of the gels upon macroscopic observation as we had observed in the preparation of **Paper III** (data not shown).

We could observe that both the ethanol-based and laboratory-made PFA fixative which we enriched with calcium (PFA_Ca) kept the macroscopl architecture of the construct, within both manufacturing approaches. Independent of the calcium concentration used to crosslink the alginate, we found that the formalin-free fixative was a promising candidate to evaluate for retaining alginate-cell interaction in a 3D construct.

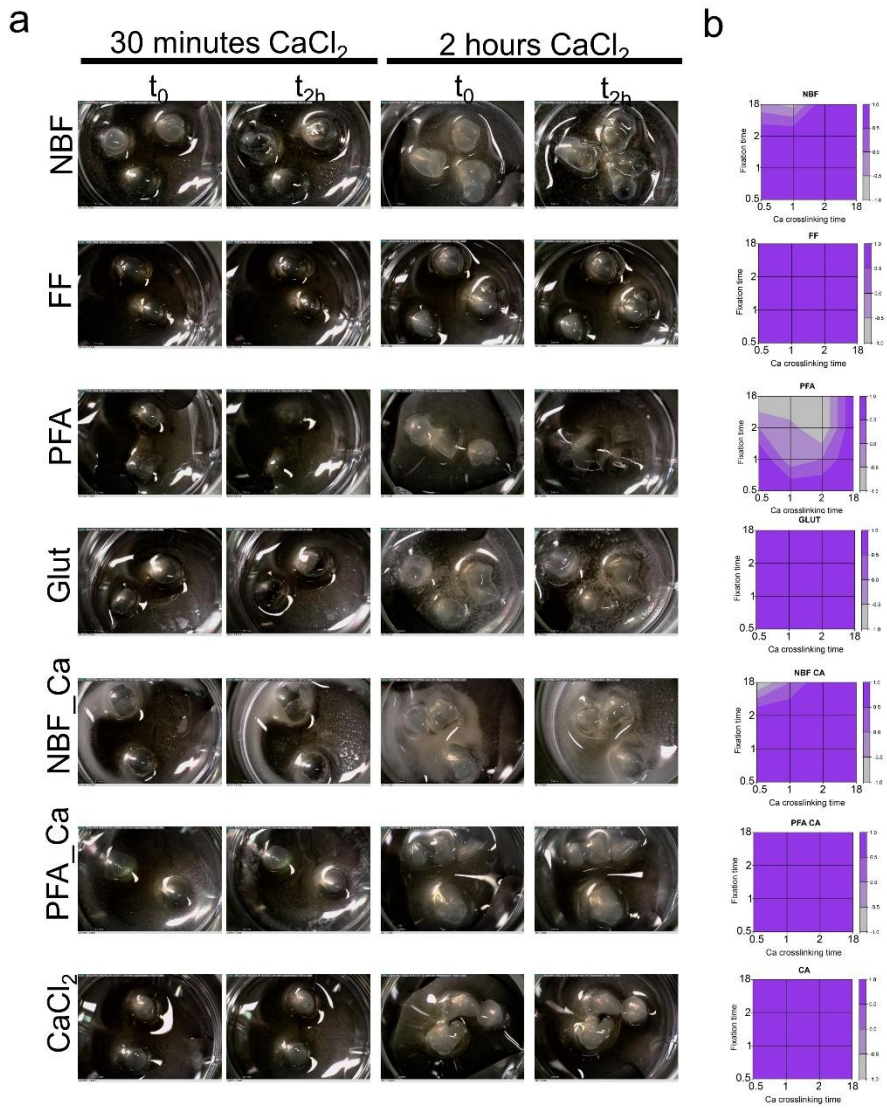


Figure 32 Macroscopic examination of calcium alginate hydrogel

(a) Representative image for 30 min and 2 h of calcium crosslinking time and immediately after fixation and after 2 h of fixation for various fixatives as listed. (b) Contour plots of blinded scoring. Figure and text taken from (Da Silva, Gvazava et al. 2023) with permission.

Ethanol based, formalin-free fixation preserves calcium alginate-cell interactions in 3D cast tubes and allows for histological processing using xylene free approaches

We manufactured 3D calcium alginate tubes containing cells utilizing a 2-part 3D printed mold and cell culture overnight. We found that ethanol-based, formalin-free fixative provided good architecture stability on the calcium alginate-cell 3D construct in comparison with the enriched PFA_Ca. Even though both could be used for histological evaluation, separation of the alginate-cell interactions were obvious as well as total detachment of the cells from the matrix in some locations, promoting empty spaces. This observation matches what has already been described previously for calcium alginate scaffolds (Khatab, Leijs et al. 2020).

This is likely due to the swelling which occurs upon exposure to PO_4^{3-} ions which can result in loss of calcium from the hydrogel and thus relaxation of the ionically crosslinked alginate hydrogel network allowing for water uptake before chemical fixation occurs (Segale, Giovannelli et al. 2016). Finally, we can conclude that the ethanol-based formalin-free fixative is compatible with the xylene-free tissue processing, allowing a better cell adhesion for histological examination **(Figure 33 a-e)**.

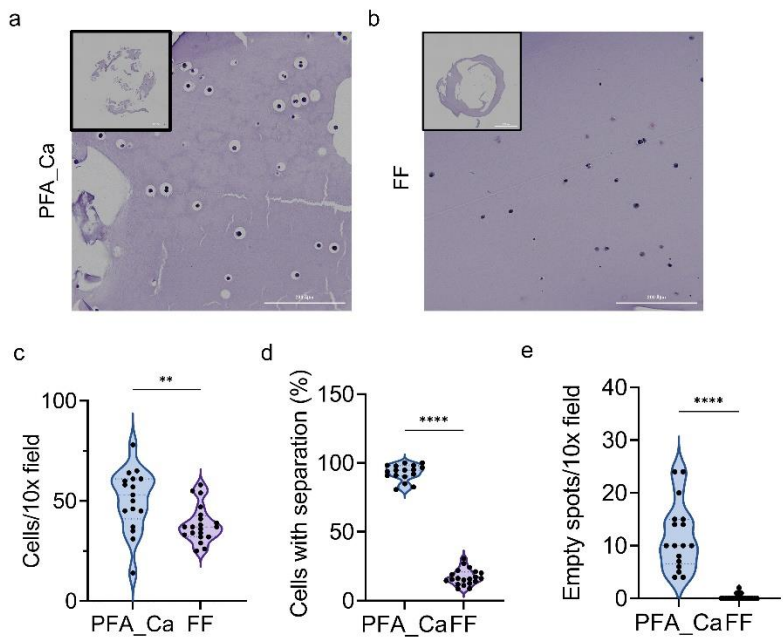


Figure 33 3D contructs of calcium alginate-cell

(a) PFA_Ca and (b) FF fixed calcium alginate tubes. (c) Number of cells per field captures with a 10X objective shows a significant reduction in the number of cells detected per field with FF. (d) Percentage of cells with separation between cell and biomaterial. (e) Number of empty spots per field of view captured with a 10X objective shows a significantly increased number of empty spots in PFA_CA as compared to

FF. Central tendency measures on violin plots: long dashed lines is the median while shorter dashed lines indicated quartiles. * indicates p-value<0.05. All statistical analyses were performed using Mann-Whitney Rank Test due to the non-normality of the data. Experiments are representative of two independent experiments (N = 2) with at least three technical replicates each time (n = 3). Figure and text taken from (Da Silva, Gvazava et al. 2023) with permission.

Ethanol based, formalin-free fixation permits histological evaluation of thin alginate and alginate-gelatin constructs for histological and SEM visualization of epithelial monolayers.

As reported in the past, alginate alone contains no binding sites for cell adhesion (Rowley, Madlambayan et al. 1999). We corroborated this and found that the immortalized cell-line which we seeded onto the alginate-only scaffolds were only lightly adherent to the scaffolds after formalin-free fixation (**Figure 34a)**. On the other hand, alginate-gelatin hybrid scaffolds contained more cells and with subjectively more cells displaying a flattened or adherent morphology.

As well, we also could use the formalin-free fixative for histological cross sections showed that FF could be used to preserve cell-biomaterial interactions of cells growing on the alginate-gelatin membranes. These microscopic observations correlated with statistically significant increases in metabolic activity between alginate and alginate-gelatin scaffolds according to metabolic assessment with WST-1 **(Figure 34b)**. And finally, the most promising is that formalin-free fixative may be a candidate for substitution of glutaraldehyde-based chemical fixation for evaluating alginate hydrogels. Glutaraldehyde is broadly used as a state of art for tissue preparation for electronic microscopy (**Figure 34c)**.

Evaluation of hydrogels are known to be challenging in SEM due to their high water content. One of the other more commonly used approaches is Cryo-SEM due to the fact that it prevents architectural deformations in the tissue. However, it still demands lower temperatures (-137°C) that require higher energy costs to prevent formation of artifacts in the crystalline form with cracks appearing on the surface of samples already in early stages of sublimation with increase of temperatures from (-145°C) to (-105°C) (Aston, Sewell et al. 2016). Therefore, the formalin-free substitute we evaluated seems to be a very promising alternative to reduce the usage of both formaldehyde and glutaraldehyde in evaluating tissue engineering constructs and may also prevent unique advantages for use with conventional approaches such as paraffin embedding for histology and scanning electron microscopy.

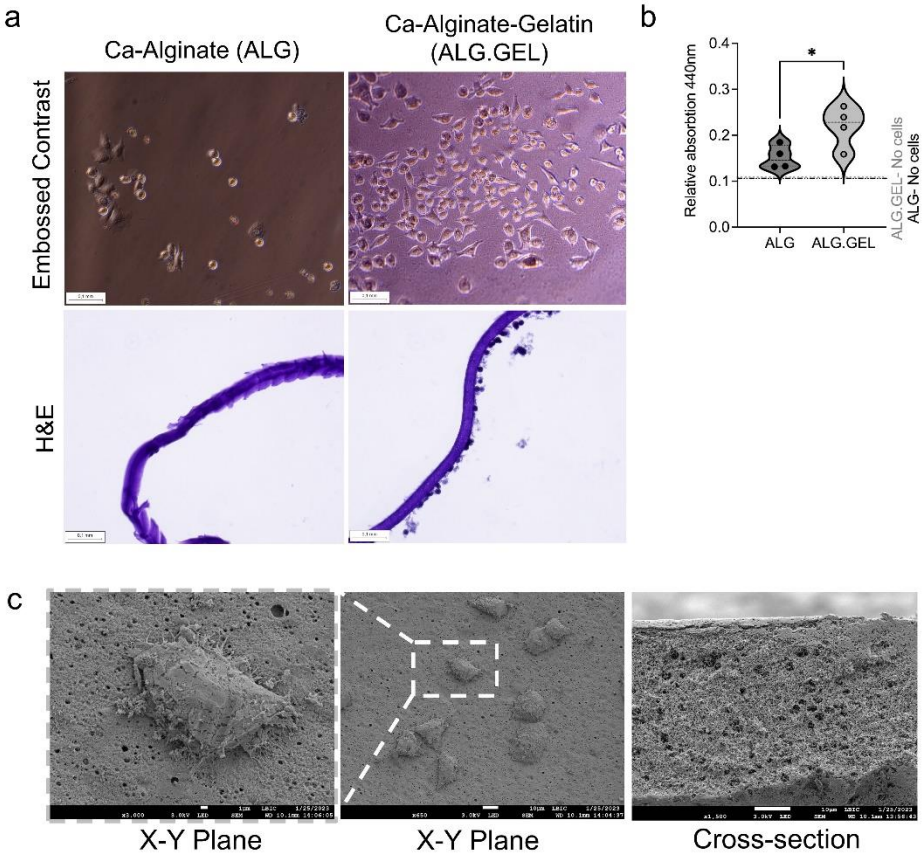


Figure 34 Fabrication and characterization of epithelial cells grown on alginate and hybrid alginate-gelatin membranes for histological cross section and scanning electron microscopy evaluation.

(a) Embossed contrast imaging (representing x-y) imaging of cells grown on alginate versus hybrid alginate-gelatin membranes. H&E cross sectional (x–z) plane imaging showing monolayers of epithelial cells growing on only one side of a membrane. Both membranes were processed using FF fixation. (b) Increased metabolic activity as assessed by WST-1 assay. Individual dots are technical replicates (n = 4) from the same manufacturing and cell seeding experiment. Representative of 3 independent experiments (N = 3). Calcium alginate and hybrid calcium alginate-gelatin membranes without cells but cultured under identical conditions serve as controls. All WST-1 values are above background levels. *p-value<0.05 is considered statistically significant. Non-parametric, Mann-Whitney t-test. Central tendency data is shown with a darker dashed line to indicate the median while lighter dashed lines indicate quartiles. (c) SEM micrographs of the apical and cross sectional surface of a hybrid alginate-gelatin membrane fixed using FF and chemically dried with HMDS. Retention of fine structures, such as individual lamellipodia are still visible, demonstrating cell-biomaterial interactions. Cross sectional view of hybrid alginate-gelatin membrane demonstrating retention of porous structure during preparation from fixed FF samples. Figure and text taken from (Da Silva, Gvazava et al. 2023) with permission.

Concluding remarks and outlook

The development of techniques that allow the understanding of lung disease progression both in the acute and chronic stages as well as the evaluation of bioengineered materials is needed. These topics are even more imperative due to the increased number of patients predicted to be affected by respiratory conditions, especially in the post-pandemic scenario where post-acute sequelae of SARS-CoV-2 infection still requires more data exploration. However, a crucial aspect on the development of techniques is the choice of the model; of course, no model will be able to fully recapitulate the natural scenario, and that is the reason it is important the reflection upon the features that is aimed to be observed and studied.

The choice of large animal models, such as porcine, to investigate respiratory diseases, is important to model clinical approaches such as the use of the Berlin criteria which is traditionally used for confirmation of the diagnose of ARDS. Furthermore, tools and therapies applied for humans that may be very challenge to recapitulate it in murine model can be used, e.g., mechanical ventilation and ECMO and particle flow rate to detect early signs of ARDS.

The use of power calculation for example is an interesting planning step, where the researcher has the chance to calculate and prepare beforehand the number of samples that will be needed for generation of clear results, maximizing findings and the contribution for the field.

Taking in consideration these crucial aspects, emerges the need to implement sustainable strategies, that allows the optimization of methodologies with a conscious use of reagents and materials. Reinforcing that the automatization and or digitalization of some approaches may be not only more sustainable within different aspects (e.g., cheaper, faster, reliable), but more environmentally and health-conscious and efficient.

In this current research, we developed and optimized techniques to study and understand the role of remodelling of the architecture of the lung along acute and chronic stages of lung diseases. First, we identified histopathological features of DAD that could be identified in large animal model of ALI/ARDS. Based on this, we developed a semi-quantitative score system to assess the extent of lung injury. In addition, we utilized green chemistry principles and employed the strategy of green tissue processing by removing the utilization of xylene in the final dehydration and clearing steps. We also utilized digitalization the histological slides for

examination (Paper I). In this study, we found that the green histology methodology allows the assessment of the tissue with H&E staining without damage in the architecture of the material, utilizing a more sustainable approach. Further, the identification of features in the porcine model for ALI/ARDS may improve future findings. And finally, the semi-quantitative score system that we developed, showed to be able to detect and quantify the extent of lung injury in a porcine model of ALI/ARDS induced with LPS.

Furthermore, parallell with these findings, we could observe that the expertise level of the observers may influence on the reliability of the score system, and possibly the automatization of the human task could be improved with a more reproducible score system. In order to evaluate if this hypothesis would be applicable, in (Paper II) we first, validated the consistency of the semi-quantitative score system in comparison with the traditionally used score system reported for Matute-Bello; secondly, based on our findings, we tested whether the total score of the extend of lung injury generated would be enough to train a machine learning algorithm. This allowed us to evaluate the feasibility of the automatization of the human task.

In this study we found that our semi-quantitative score system seems more reliable. This might possibly be explained based on the range of the score, where Matute-Bello system showed to be too narrow, not allowing the observe to express more variable extents of lung injury. Moreover, we could observe that even with our score system showing to be consistent, humans still struggle agreeing with other, especially among the observers with lower expertise level. This may be explained due to the lack of self-confidence assessing histological features, particularly features such as hemorrhage where more frequently novices tend to score based on the visual presence of red color instead of distinguishing erythrocytes.

Another interesting observation was, when the board-certified pathologists were included, we could detect as well, disagreement. This was somewhat surprising, but it can be explained due to the lesser expertise level on preclinical analysis and their attempt to recapitulate the clinical scenario. Animal models differ in the level and time of damage that brings the patient sample to be analysed by a pathologist and this time is much higher. These factors may decrease the perceived degree of artificially induced lung injury, which is only done for a short amount of time. Therefore, even though they are no doubt experts in clinical pathology, it indicates the challenges of quickly transferring those skills and knowledge to a new task.

Finally, our score system was deemed to be the best candidate to be used to teach machine-based learning due to its reliability and reproducibility. We found that we could train and test a machine learning algorithm, even with low amount of sample needed to teach the machine as a proof of concept. We found that our score system could be used, with predictions of 83% with only 7-9 misclassifications. Therefore, this will help to reduce the time and cost, especially when we think on the time that a expert would have to spend assessing this number of images. This should also help

reduce the variability of the total scores. However, one main limitation of the approach we took was that we assigned scores on the slide level and not on the individual biopsy piece or tile level. This is a major limitation which would need further annotation by humans in the future to refine the training. We also only trained using the LPS model of acute lung injury which is not representative of the clinical scenario with active infection. Other groups have developed models of activity bacterial and viral infections, sepsis as well as gastric acid aspiration models, have higher clinical relevance. This reinforces the need of future studies on the possibility of using more datasets to help improve the detection potential of the machine model.

Further, new research questions emerge on the possibility of the machine be used to detect isolated features instead of classifying the extent of lung injury. This will also require collaborations with other researchers that use another mechanism to induce ALI/ARDS to evaluate the capacity of the machine detect other features that we are not aware yet. In particular, machine learning might be especially of interest to apply to clinical cohorts where patients are diagnosed with ARDS but histologically differ: DAD features that appear only in half of the patients on the clinic. The application of machine learning could be a tool to allow us to investigate different methodologies and optimization of features in the clinical scenario.

Another future direction, is the utilization of artificial staining, based in machine learning (Bai, Yang et al. 2023), where the same slide could be examined with different stainings and then be used to providing a score. This could dramatically helping the pathologist and serve as a tool for fast detection of features of interest to be explored by the professional.

During the investigation on (Paper III), we aimed to evaluate if tissue specific rECM 3D hydrogel constructs would be compatible for transplantation. We found that the material not only was compatible, but angiogenic. However, the histological evaluation at that time presented challenges. During tissue fixation and processing, we were forced to use an OCT-based approach due to the lack of techniques to preserve the tissue for paraffin processing. This approach does not provide the best optical resolution and therefore, it may be important to repeat these experiments with the new fixation approach shown in Paper IV.

In order, to solve the challenge of tissue preparation of calcium alginate-based 3D constructs, in order to investigate new methodologies to elucidate and find out a possible solution, in (Paper IV) we investigated the interaction between different fixatives and tissue and calcium alginate-based hydrogels. In this study we found that aldehyde-based fixative, even with lower concentration of phosphate buffers in the formula, may not favour aldehyde-based fixatives of larger samples. Therefore, we searched for an optimal candidate for fixation of calcium alginate-based materials. We found that an ethanol-based fixative, Accustain, can be presented as a better candidate, once that was not observed deformation on the constructs.

However, the composition of the solution is unknown and therefore further optimizations will be challenging.

Nonetheless, this ethanol-based fixative allowed the performance of light histological examination using H&E staining, as well for scanning electron microscopy of the 3D constructs. Whether or not it can be used for light sheet fluorescence microscopy is currently unknown, but this will be an important direction moving forward. The ability for it to be used in diverse applications highlights the versatility of the fixative, in addition to it being a sustainable strategy. This helps connect what we learned in (Paper I) removing both aldehyde-based fixatives during fixation as well xylene-base tissue processing.

This PhD thesis promotes an exciting future for histological techniques and application of other sustainable applications, such as removal of toxic chemicals across the tissue preparation steps; however despite the optimization of the protocol, there is also the need of revalidating of anti-bodies focused on immunohistochemistry and immunofluorescence, once that different fixative interacts different with tissue components. Utilization of other types of solvents during the clearing steps as well may interfere with the tissue architecture what may make the tissue stiffer in some cases, reinforcing the need of material or tissue specific adaptations.

Certainly, more investigations are required on the utilization of animal models to mimics clinical scenarios and the choice of features that can relate with all the profile of patients (sex and gender aspects, race and ethnicity, socioeconomic status, etc). It will also be especially important to see if the machine learning approach can be adapted to be used clinically and it may be interested to investigate the different clinical phenotypes, especially in those patients presenting or not DAD for example. This is both an opportunity but also is a challenge to accomplish. Finally, the use of machine learning may help on the detection of features and groups, however, the machine may not be more capable than the expert that provides the standards for examination. Therefore, there will remain an need for the contribution of the researchers and pathologists along the way to may provide more information of automatization of this human task. It will be important to continue to apply the SDGs, with the conscience and opportunity to the creation of sources such as open dataset libraries generated with the FAIR principles, which may benefit and achieve on the exchange of information across geographical boundaries and ultimately improve of our approaches.

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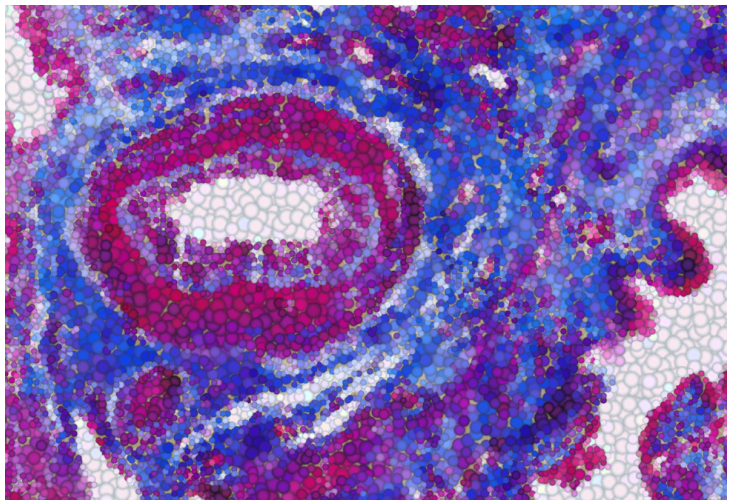

Development of techniques to determine extracellular matrix alterations in acute and chronic lung diseases and bioengineered tissues

This thesis summarizes the journey and the years of research that culminate in findings and contributions for a more sustainable development of techniques, systems, and methodologies for further optimization of histopathological examination of alterations on the architecture in acute and chronic lung diseases and bioengineered tissue.

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