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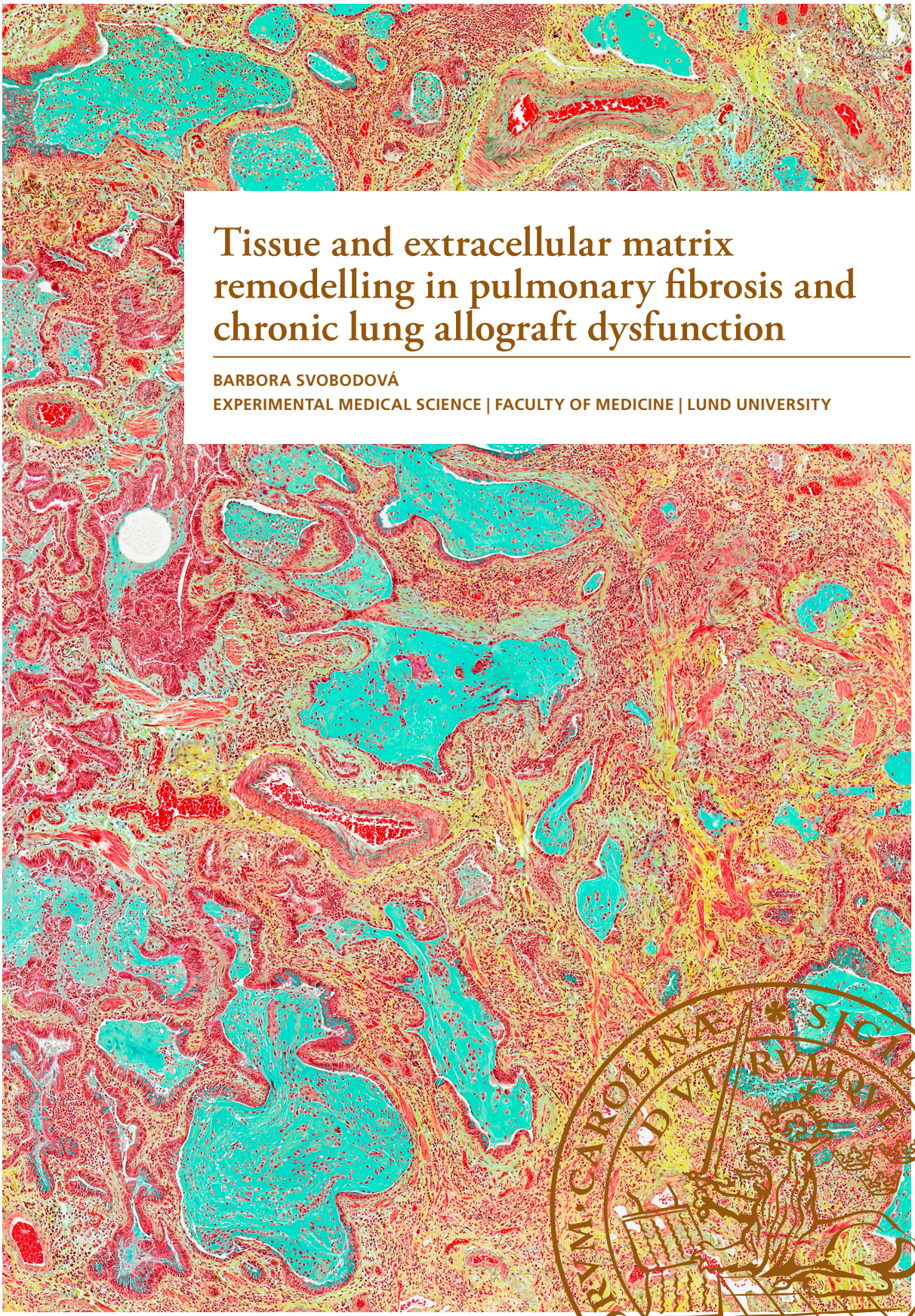

Tissue and extracellular matrix remodelling in pulmonary fibrosis and chronic lung allograft dysfunction

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EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

Tissue and extracellular matrix remodelling in pulmonary fibrosis and chronic lung allograft dysfunction

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LUND
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DOCTORAL DISSERTATION

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Abstract:

In recent years, the prevalence of idiopathic pulmonary fibrosis (IPF) and post-COVID-19 pulmonary fibrosis has risen. Nevertheless, effective diagnostic and prognostic biomarkers alongside targeted treatments are lacking, making lung transplantation the last resort for many patients with end-stage disease. However, bronchiolitis obliterans syndrome (BOS), a manifestation of chronic lung allograft dysfunction, limits long-term survival post-transplantation. Despite the clinical significance of IPF, post-COVID-19 lung fibrosis, and BOS, the underlying molecular mechanisms driving these conditions remain incompletely understood.

This research aimed to deepen our understanding of tissue remodelling and ECM alterations, particularly within the lung epithelial-mesenchymal niches, that contribute to pulmonary fibrosis and CLAD/BOS. Using histological and molecular analyses at both RNA and protein levels in human lung tissue, we identified previously unrecognized ECM and ECM-associated molecules that appear to play a role in end-stage IPF and post-COVID-19 fibrosis, as well as airway remodelling in BOS.

Notably, increased collagen VII expression was observed in pathologically remodelled alveolar and airway structures in IPF, with basal cells and aberrant basaloid cells identified as the primary cell types expressing collagen VII. In BOS-affected small airways, altered levels of periostin, known for its role in fibroblast activation and collagen fibrillogenesis, as well as changed levels of LOX and LOXL1, enzymes involved in the covalent cross-linking of structural ECM components such as collagen and elastin, were found. Although IPF and post-COVID-19 fibrosis are triggered by different factors, shared patterns of in situ lung remodelling were identified, including altered periostin and LAMP3 expression, alongside systemic protein alterations. These findings highlight potential molecular targets for further investigations.

This thesis provides novel insights into the complex molecular landscape of pathological lung microenvironments driving pulmonary and airway fibrosis. Expanding this knowledge may pave the way for improved understanding of disease pathogenesis, ultimately leading to better diagnostic and prognostic tools and novel therapeutic strategies.

Key words:
Idiopathic pulmonary fibrosis, Post-COVID-19, Chronic lung allograft dysfunction, Bronchiolitis obliterans syndrome, lung fibrosis, remodelling, extracellular matrix, collagen, basal cells, fibroblasts

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For all those dedicated to uncovering the mechanisms of fibrosis
- in the lung, the airways, and beyond

To all patients fighting fibrotic diseases every day

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Papers included in the thesis

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Paper II

Svobodová B, Löfdahl A, Nybom A, Wigén J, Hirdman G, Olm F, Brunnström H, Lindstedt S, Westergren-Thorsson G, Elowsson L. **Overlapping Systemic Proteins in COVID-19 and Lung Fibrosis Associated with Tissue Remodeling and Inflammation.** Biomedicines. 2024 Dec 19;12(12):2893.

Paper III

Kalafatis D*, Björnson M*, Svobodová B, Kistner A, Nygren-Bonnier M, Runold M, Bruchfeld J, Wheelock A, Elowsson L, Westergren-Thorsson G, Sköld M. **Protein profiling in ICU-treated COVID-19 patients identifies biomarkers of residual lung abnormalities.** ERJ Open Research. 2025; in press

*The authors contributed equally

Paper IV

Svobodová B*, van der Ploeg EA*, Borghuis T, Timens W, Brunnström H, Gan TC, Löfdahl A, Akbarshahi H, Magnusson J, Elowsson L, Burgess JK, Westergren-Thorsson G. **Small airway remodelling and matrisome protein expression in bronchiolitis obliterans syndrome**; manuscript in preparation

*The authors contributed equally

Additional peer-reviewed papers not included in the thesis

Falcones B, Kahnt M, Johansson U, Svobodová B, von Wachenfelt KA, Brunmark C, Dellgren G, Elowsson L, Thånell K, Westergren-Thorsson G. **Nano-XRF of lung fibrotic tissue reveals unexplored Ca, Zn, S and Fe metabolism: a novel approach to chronic lung diseases**. Cell Communication and Signalling. 2025 Feb 7;23(1):67.

Kadefors M, Rolandsson Enes S, Åhrman E, Michaliková B[#], Löfdahl A, Dellgren G, Scheding S, Westergren-Thorsson G. **CD105+CD90+CD13+ identifies a clonogenic subset of adventitial lung fibroblasts.** Scientific Reports. 2021; 11(1), 24417.

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Wijk SC, Prabhala P, Michaliková B[#], Sommarin M, Doyle A, Lang S, Kanzenbach K, Tufvesson E, Lindstedt S, Leigh ND, Karlsson G, Bjermer L, Westergren-Thorsson G, Magnusson M. **Human Primary Airway Basal Cells Display a Continuum of Molecular Phases from Health to Disease in Chronic Obstructive Pulmonary Disease**. American Journal of Respiratory Cell and Molecular Biology. 2021 Jul;65(1):103-113

[#]Michaliková – maiden name, Svobodová – married name

Selected abbreviations

ARDS	Acute respiratory distress syndrome
AT2	Alveolar type II (cell)
BOS	Bronchiolitis obliterans syndrome
CLAD	Chronic lung allograft dysfunction
COL7	Collagen VII
COVID-19	Coronavirus disease of 2019
DCN	Decorin
DLCO	Diffusing capacity for carbon monoxide
ECM	Extracellular matrix
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
HRCT	High resolution computed tomography
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
KRT17	Keratin 17
KRT5	Keratin 5
LAMP3	Lysosome-associated membrane protein 3
LOX	Lysyl oxidase
LOXL1	Lysyl oxidase like 1
MMP7	Matrix metalloproteinase 7
NPX	Normalized protein expression
TLC	Total lung capacity
UIP	Usual interstitial pneumonia

Preface

The lung is a highly complex organ with dynamic microenvironments and a dormant yet remarkable regenerative capacity. However, when repetitive and persistent damage at the epithelial-mesenchymal interface occurs — in conditions such as pulmonary fibrosis of unknown origin (IPF) or triggered by COVID-19 (post-COVID-19 pulmonary fibrosis) — the delicate balance of normal tissue repair shifts toward aberrant wound healing. This shift results in excessive extracellular matrix (ECM) deposition in the lung parenchyma, leading to altered cellular behaviour, progressive scarring remodelling the lung architecture, respiratory decline, and ultimately, lung failure. Lung transplantation may extend the lives of patients affected by these diseases; however, their long-term survival is often compromised by chronic lung rejection. The most common manifestation of this is bronchiolitis obliterans syndrome (BOS), which is also characterized by scarring but primarily in the small airways and results in a lung function decline, progressing eventually to recurring lung failure.

Considerable research on animal models of lung fibrosis has provided valuable insights into its pathological mechanisms. However, these models do not fully replicate all aspects of human lung biology and disease, particularly the chronic nature of the condition. Additionally, significant progress has been made in understanding the hierarchy of lung stem and progenitor cells that contribute to lung regeneration. Despite the notable ECM accumulation in the diseases studied here, its molecular spectrum co-creating the microenvironment for lung cells and shaping their behaviour, in turn affecting the ECM itself, is largely unexplored.

The work in this thesis is a result of the joint effort of researchers from both Sweden and the Netherlands, combining basic, translational, and clinical research approaches. It explores various aspects of tissue remodelling, along with cell and primarily ECM alterations in human tissue, that potentially contribute to the progression of IPF, post-COVID-19 pulmonary fibrosis, and BOS. Additionally, the research aimed to identify blood proteins that could mirror early changes in diseased lungs, which may serve as potential biomarkers for future clinical use. The knowledge gained in this thesis contributes with relevant insights into the complex pathological puzzles underlying the diseases studied herein.

Introduction

Lung architecture and cellular components

The lung is a highly complex organ with hierarchical branching resembling a tree that begins with a single trachea and undergoes, on average, 23 generations of dichotomous dividing [1]. This process results in hundreds of airways that eventually terminate in 300 million alveoli - tiny sac-like structures where gas exchange occurs (Figure 1). Moreover, each of these structures is intertwined with an extensive network of pulmonary blood vessels.

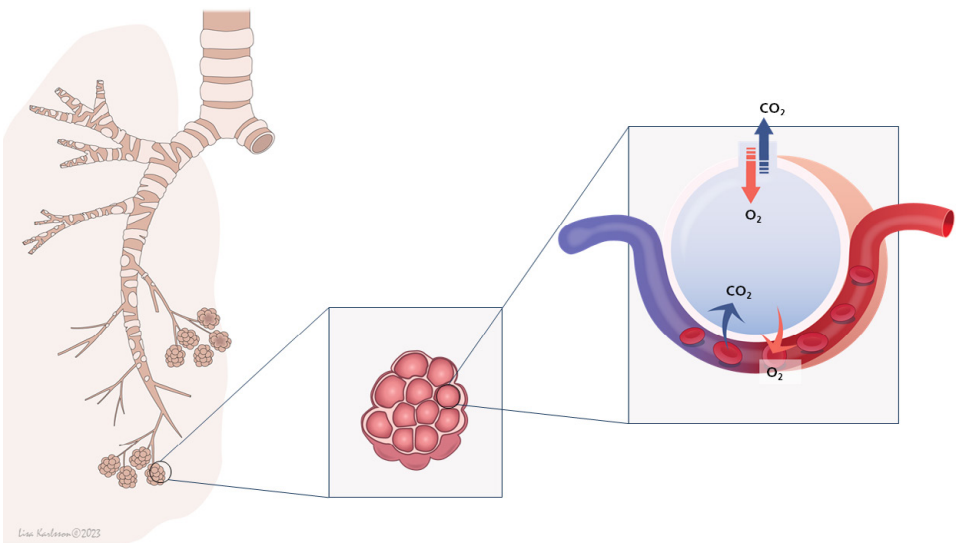


Figure 1 The lung structure. The lung starts with a single trachea, branching into bronchi and bronchioles and terminating in pulmonary sacs—alveoli. Here, the exchange of oxygen and carbon dioxide occurs between the air from the external environment and blood circulation and vice versa. Illustration made by Lisa Karlsson (Lung Biology group, Lund University, Sweden)

Conducting zone

The respiratory system can be divided into two sections of conducting airways: upper and lower airways [2]. When air is inhaled, it first passes through the upper conducting airways, which include nasal cavity, pharynx and larynx. The air then moves into the lower conducting airways, consisting of the trachea, bronchi, bronchioles and terminal bronchioles. Finally, the air reaches the respiratory zone, the primary site of gas exchange, which includes the respiratory bronchioles, alveolar ducts, and alveoli.

The bronchi, which contain cartilage, are some of the stiffest structures in the otherwise soft, highly expandable lungs. The cartilage plates reinforce the airway walls to prevent collapse during airflow. The walls also contain submucosal seromucous glands that secrete mucus and antimicrobial proteins into the airway lumen to moisten and warm the inspired air and trap the pathogens. Together with the mucosa lining the airways, these features form an essential first line of defence against inhaled pathogens and foreign particles [2].

The bronchial mucosa is lined with pseudostratified, ciliated epithelium that consists of a large range of specialized cells (Figure 2). These include ciliated columnar epithelial cells, mucus-producing goblet cells, basal cells and other less common cell types such as brush/tuft cells, ionocytes, pulmonary neuroendocrine cells and others [2]. Basal cells, marked by expression of tumour protein p63, keratin 5 (KRT5) and keratin 17 (KRT17) [3], are considered the primary progenitor population for human proximal airways [4]. These cells possess the ability to self-renew and differentiate into ciliated and secretory cells both during homeostasis and in response to injury.

As the bronchi branch into bronchioles and further into terminal bronchioles, the structure of the airway wall becomes less complex, and the composition of epithelium changes. In bronchioles, the cartilage and submucosal glands disappear, and the epithelium transitions from pseudostratified to simple columnar and ultimately to simple cuboidal epithelium in the terminal bronchioles. The basal and goblet cells gradually decline in numbers with the decreasing size of the airway, while non-cilliated secretory Club cells (formerly Clara cells) increase in numbers in small airways [5]. Characterized by SCGB1A1 expression, these cells contribute as progenitors to the regeneration of human distal bronchioles [6].

The airway wall also encompasses other crucial cell types within the interstitium, a space under the epithelium enriched with extracellular matrix (ECM). These cell types include immune cells, and neuronal cells, along with mesenchymal cells such as fibroblasts, smooth muscle cells, and pericytes. The resident fibroblasts are the primary cell type responsible for synthesizing and remodelling the interstitial ECM and co-creating the immunoregulatory niche [7,8]. Their functional heterogeneity is influenced by their origin, activation state, and the local microenvironment.

Respiratory zone

The respiratory bronchioles, following terminal bronchioles, branch into alveolar ducts, which open into alveoli. The key epithelial cells in the parenchymal region are alveolar type I (AT1) cells and alveolar type II (AT2) cells (Figure 2). The squamous and extremely thin, specialized cell bodies of ATI cells cover around 95% of the lung surface [9]. These terminally differentiated cells lose their ability to proliferate, and their basement membrane fuses with the basement membrane of the endothelial cells in capillaries. Together, they form a functional alveolar-capillary barrier that facilitates the effective diffusion of oxygen and carbon dioxide.

In contrast, the cuboidal ATII cells are multifunctional cells that are approximately in double numbers than ATI cells [10]. They serve as the primary progenitor population for alveoli upon lung injury, enabling them to replace ATI cells or replicate themselves [11]. Additionally, ATII cells are significant producers of surfactant proteins (SP), including SP-A, SP-B, SP-C, and SP-D [9]. These proteins in the mixture with surfactant phospholipids reduce surface tension to prevent alveolar collapse during expiration. Furthermore, SP-A and SP-D contribute to innate immunity. The surfactant proteins are stored and secreted by lamellar bodies, specialized organelles within alveolar type 2 (AT2) cells. These organelles are characterized by the expression of lysosome-associated membrane protein 3 (LAMP3) and ATP-binding cassette transporter A3 (ABCA3), both of which are established markers of AT2 cells [12].

Alveolar septa, lined by alveolar epithelial cells, contain an extensive network of pulmonary capillaries. Within the septal interstitium, additional resident supportive cell populations, including fibroblasts and macrophages, are embedded. Alveolar macrophages reside within the alveolar lumen and play a critical role in immune surveillance, surfactant homeostasis, and clearance of inhaled pathogens and debris [13,14].

Pulmonary blood circulation

Endothelial cells line the pulmonary capillaries and form an integral component of the alveolar-capillary barrier, regulating gas exchange, vascular permeability, and immune responses [15].

When oxygenated air is transported to the alveolar lumen, it diffuses through the alveolar-capillary barrier into the pulmonary circuit. From there, the oxygen-rich blood is carried to the left side of the heart, through which it is further distributed throughout the rest of the body via systemic circulation. Simultaneously, the pulmonary circuit returns deoxygenated blood from the right side of the heart to the alveoli for gas exchange. The tissues of the lungs, including the bronchi, lymph nodes, and visceral pleura, receive their blood supply from the bronchial arteries, which are part of the systemic bronchial circulation [16].

Visceral pleura

The visceral pleura is a delicate, serous membrane that envelops the entire lung parenchyma, extending into the fissures between lobes (Figure 2). It is composed of a layer of mesothelial cells supported by a thin connective tissue matrix, which together help maintain lung structure. Additionally, it produces serous fluid reducing frictional forces during respiration.

Throughout the thesis, the bronchi are occasionally referred to as proximal airways, while the bronchioles and alveoli are considered distal airways and distal lung tissue, respectively, located near the visceral pleura.

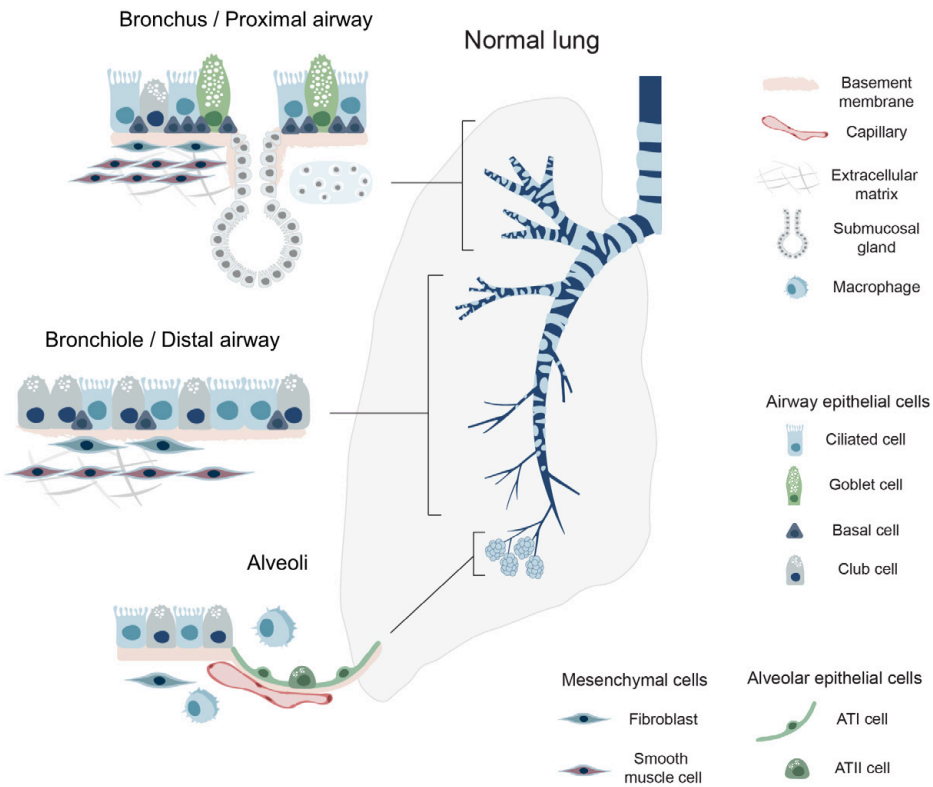


Figure 2 The simplified cellular spectrum of the lung. The whole lung is covered by visceral pleura. Illustration made by Lisa Karlsson (Lung Biology group, Lund University, Sweden)

Extracellular matrix in the lung and beyond

Much like cells, the ECM is essential to all organ tissues, including the lung, providing crucial biophysical and biochemical signals that promote cell attachment, survival, and function [17]. The ECM is a dynamic and complex scaffold - a meshwork of macromolecules and crosslinked proteins - collectively defined as the "matrisome" by Naba et al. The matrisome comprises:

- **Core matrisome proteins:** Including collagens, glycoproteins, and proteoglycans.
- **Matrisome-associated proteins:** Including ECM-affiliated proteins, ECM regulators, and secreted factors that modulate cell behaviour and ECM remodelling.

Through these components, the ECM controls key cellular processes like migration, proliferation, differentiation, senescence, and apoptosis. In most cases, cells and their surrounding matrix are interdependent, with the ECM being continually synthesized and degraded by cells along with the influence of other regulatory factors. This dynamic remodelling can be utilized in assays detecting fragments of newly synthesized or degrading proteins in the body fluids in the context of pulmonary diseases[18–21].

Spatially and functionally, the ECM is divided into two main compartments: the **basement membrane** and the **interstitial matrix**. Both the basement membrane and interstitial matrix, along with cellular elements, co-create specialized microenvironments - niches - that play an important role in maintaining the self-renewal properties and directing the differentiation of progenitor cells, as well as supporting the function of fully differentiated, tissue-specific cells.

Basement membrane

The basement membrane is a thin, highly specialized barrier that separates epithelial cells, which interact directly with external environmental cues from the underlying interstitial matrix or when fused, constitutes the alveolar-capillary barrier [22]. It also underlies vascular endothelium and pericellularly surrounds other cell types such as neurons, muscle, and adipose tissue[22]. Composed mainly of collagens, glycoproteins, and proteoglycans, its most abundant components are collagen IV and laminins, with additional stabilization provided by perlecan, agrin, and collagen XVIII.

Interstitial matrix

The interstitial matrix is the intricate network of extracellular components that fills the space between cells in the stroma. It is primarily formed by core fibrous proteins like collagen I and III and elastin, as well as glycoproteins and proteoglycans [23]. In the lung, the interstitial matrix is crucial for maintaining tissue elasticity and mechanical stability.

ECM-producing cells

While epithelial and endothelial cells are generally believed to be the main producers of basement membrane proteins, fibroblasts are considered the primary cell type producing interstitial ECM. Nevertheless, other mesenchymal cells, such as smooth muscle cells and pericytes, also express interstitial collagen [8,24]. Notably, epithelial ATII cells have been shown to express many interstitial fibrillar collagen types and other ECM components, which become additionally upregulated upon TGF-β1 treatment [25]. Conversely, fibroblasts can compensate for epithelial cells by depositing some proteins in the basement membrane zone such as collagen IV, collagen VII and perlecan [26–28]. This illustrates the plasticity of various cell lineages in producing different matrix components in both homeostasis and pathological conditions.

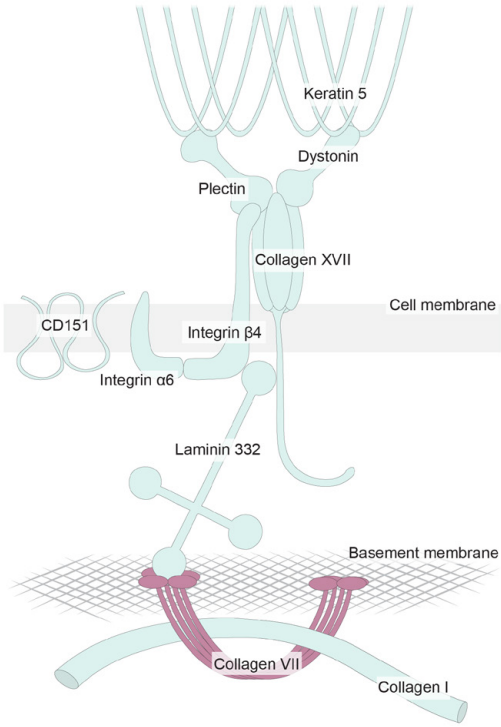
Collagens

Collagens are the most abundant ECM proteins, providing structural support essential for cell attachment and growth, as well as the tensile strength of lung tissue. Beyond their mechanical role, collagens are integral to cell signalling within the interstitial matrix and basement membrane of epithelial tissues[29–31]. Moreover, several other functions have been described for these macromolecules and their derived fragments, e.g. anti-angiogenic, anti-tumour and anti-fibrotic [32–34].

The collagen superfamily includes, to date, 28 discovered types of collagen, which, based on their domain structure and supramolecular assembly, fall into different groups: 1) fibril-forming collagens (I, II, III, V, XI, XXIV, XXVII); 2) fibril-associated collagens with interrupted triple helices (FACITs, IX, XII, XIV, XVI, XIX, XX, XXI, XXII); 3) network-forming collagens (IV, VIII, X); 4) transmembrane collagens (XIII, XVII, XXIII, XXV); 5) endostatin-producing collagens or multiplexins (XV, XVIII); 6) anchoring fibrils (VII); 7) beaded-filament forming collagen (VI); and 8) uncategorized collagens (XXVI, XXVIII) [35]. Collagen molecules are formed as triple helices composed of either three identical alpha chains (homotrimers) or a combination of different alpha chains (heterotrimers), depending on the collagen type. Collagen VII has been studied in the context of IPF/usual interstitial pneumonia in this thesis.

Collagen VII

Collagen VII constitutes the main component of supramolecular structures, so-called anchoring fibrils, which have been identified using immunohistochemistry and transmission electron microscopy in several normal tissues with stratified and pseudostratified epithelia, including trachea, bronchi and bronchioles [36,37]. However, collagen VII was absent beneath simple epithelia and the blood endothelium in comparison to collagen IV and laminin, which are consistently found in all basement membranes, including those of blood vessels [36,37].



Collagen VII is a homotrimer with each of the alpha-1 chains encoded by the *COL7A1*. In the form of anchoring fibrils, collagen VII stabilizes the epithelium attachment to the underlying ECM via binding to interstitial collagen I and/or to basement membrane components such as laminin-332 (α3, β3 and γ2 chains) and collagen IV [38,39]. Laminin 332 and transmembrane collagen XVII are connected to keratin intermediate filaments 5 and 14 via components of hemidesmosomes, including integrin α6β4, plectin (PLEC), tetraspanin (CD151), and dystonin (DST) [40]. Together, these proteins establish a structural anchoring complex of epithelial cells, anchoring them to the basement membrane and the interstitial matrix (Figure 3).

Figure 3 Schematic illustration of the hemidesmosomal and the anchoring complex of epithelial cells in their basement membrane zone, based on studies in skin. Illustration was made by Lisa Karlsson (Lung Biology group, Lund University, Sweden)

The lack of collagen VII in the skin, resulting either from loss-of-function mutations in the COL7A1 gene or from autoantibodies targeting collagen VII epitopes, has been associated with a group of skin disorders known as epidermolysis bullosa. These conditions lead to severe clinical symptoms, including skin blistering, finger and tooth abnormalities, fibrosis of the skin and mucous membranes, and, eventually, an increased risk of developing cutaneous squamous cell carcinoma [41,42].

Glycoproteins and proteoglycans

Core matrisome glycoproteins play pivotal roles in mediating cell-matrix interactions, influencing processes such as cell adhesion, migration, and signalling. This group includes molecules such as periostin, fibronectin, laminins and tenascins.

Periostin has been identified as an important player in profibrogenic processes such as fibroblast activation, collagen fibrillogenesis, and ECM production, and it has been of interest as a potential therapeutic target in IPF [43,44].

In addition to fibrous collagens and glycoproteins, proteoglycans are key core matrisome components, consisting of a core protein covalently bound to sulphated polysaccharides or glycosaminoglycans [23]. Due to their high polysaccharide content, proteoglycans are hydrophilic, facilitating hydrogel formation and contributing to the lung viscoelastic properties. They include basement membrane components such as perlecan and agrin, as well as hyalectans/lecticants like versican, aggrecan. Additionally, proteoglycans encompass small leucine-rich proteoglycans such as decorin, biglycan, and lumican.

Elastic fibres

Elastic fibers are integral structural ECM components, providing the lung with elasticity and elastic recoil. These fibers primarily comprise the core crosslinked protein elastin, surrounded by microfibrillar-associated proteins such as fibrillin.

ECM-associated proteins

Beyond structural ECM proteins, various other proteins interact with core matrisome components, shaping the architecture, mechanical properties, and signalling dynamics of the lung ECM. Key regulators of ECM remodelling include proteins of the lysyl oxidase (LOX) family, modulating ECM crosslinking, and metalloproteinases (MMPs), responsible for ECM degradation, and turnover.

Tissue remodelling during normal lung repair

Wound healing and the associated tissue remodelling is an essential part of homeostasis mechanisms to regenerate tissue after injury. This complex repair process is a well-coordinated interplay of several cell types, factors and ECM components. The process, mostly based on studies in the skin, can be extrapolated to the lung and divided into these phases [45–47]:

1) **Injury**
   Initially, lung injury results from various factors, such as infection, allergens, environmental pollutants, or mechanical damage, disrupting airway and alveolar epithelial and endothelial barriers along with their basement membranes. Damage stimulates the activation of platelets and coagulation pathways to protect injured areas temporarily through fibrin-rich clot formation, which is additionally supported by airway epithelial cells [48,49].
2) **Inflammation**
   The subsequent inflammation phase involves recruiting leukocytes through chemokine gradients, clearing debris and secreting cytokines and growth factors essential for initiating pulmonary tissue remodelling and repair.
3) **Proliferation and remodelling**
   The inflammation stage transitions into the proliferation phase, characterized by fibroblast activation, differentiation into collagen-producing myofibroblasts, extracellular matrix (ECM) synthesis, neovascularization and re-epithelialization, which are essential for re-establishing pulmonary tissue integrity.
4) **Resolution**
   Finally, resolution occurs through apoptosis and clearance of inflammatory cells and myofibroblasts by phagocytes, coupled with epithelial regeneration, to restore normal lung architecture and function.

A critical balance between these phases ensures efficient pulmonary repair without pathological fibrosis. Regulatory mechanisms involving cytokines, MMPs and their inhibitors critically control ECM production, maintaining transient and reversible matrix deposition. Thus, tight regulation of these factors and cell players is crucial for restoring tissue structure and function without excessive fibrotic scarring.

Aberrant remodelling in pulmonary and airway fibrosis

Dysregulated pulmonary wound healing can arise due to persistent or repetitive injury, unresolved inflammation, or genetic susceptibility, shifting the balance towards chronic fibrosis. In pulmonary fibrosis, it is believed that ongoing injury to alveolar and airway epithelial cells drives aberrant remodelling, whereas in airway fibrosis, the bronchiolar mucosa is primarily affected [50–54]. In both cases, dysfunctional epithelial cells release excessive cytokines, chemokines, and growth factors - most notably transforming growth factor β (TGF-β) - perpetuating chronic mesenchymal activation and pathological ECM synthesis and deposition [55]. The mesenchymal activation includes the recruitment and proliferation of resident fibroblasts, circulating fibrocytes and potentially other cell types, followed by their differentiation into pathological fibroblasts and myofibroblasts.

A key feature of this pathological remodelling is the disruption of the basement membrane [56], which forms the critical interface between epithelial and mesenchymal compartments. Basement membrane degradation, driven by excessive MMPs activity and impaired repair mechanisms, facilitates epithelial dysfunction and allows for aberrant cell-matrix crosstalk that promotes fibrosis. Furthermore, despite still being under debate, partial epithelial-mesenchymal transition (EMT) - a process in which epithelial cells lose polarity and acquire mesenchymal characteristics - may contribute to the pool of aberrant cells depositing ECM [57].

The formation and persistence of fibroblast and myofibroblast foci lead to excessive ECM deposition, disrupting tissue architecture and lung function. Altered ECM dynamics, including excessive collagen crosslinking, matrix stiffening, and impaired degradation, further enhance fibrotic progression by promoting aberrant cell behaviour. Additionally, aberrant reactivation of developmental signalling pathways, such as WNT, Sonic hedgehog, Notch, Hippo, along with reduced myofibroblast apoptosis, contributes to the persistence of pathological remodelling [58].

Ultimately, this uncontrolled fibrotic response results in irreversible pulmonary and airway fibrosis, severe impairment of gas exchange, and progressive respiratory failure. These processes are associated with several distinct clinical conditions studied in this thesis, such as idiopathic pulmonary fibrosis (IPF), post-COVID-19 pulmonary fibrosis and bronchiolitis obliterans syndrome (BOS) as the primary phenotype of chronic lung allograft dysfunction (CLAD). Each of them presents with different aetiology, disease courses, and clinical implications, yet they share similarities in the mechanisms mentioned above. The following sections provide more details on the epidemiology, diagnosis, treatment and pathology of these fibrotic disorders.

Idiopathic pulmonary fibrosis

Epidemiology

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disease affecting approximately 3 million individuals worldwide [59]. If left untreated, the median survival is approximately 3–5 years after diagnosis. A worse prognosis has been linked to advanced age (over 70 years), male sex, low body mass index, extensive impairment on HRCT, and pulmonary hypertension [60]. Additional factors and conditions have also been associated with a higher risk of IPF development, including smoking history, farming and occupational exposures, and infections caused by viruses and bacteria [61–65]. The disease is characterized by progressive deterioration in lung function, increasing difficulty breathing (dyspnea) and chronic cough.

IPF is one of the most prevalent forms within a diverse group of interstitial lung diseases (ILDs), which encompasses over 200 disorders with mostly known causes (e.g., autoimmune diseases, occupational or environmental exposures, drug-induced injury, radiation therapy) [66]. Additionally, ILDs are hallmarked by inflammation, fibrotic remodelling and abnormalities within the lung interstitium. When all identifiable ILD causes have been excluded, the condition is termed "idiopathic" pulmonary fibrosis, indicating that the origin is unknown.

Diagnosis and treatment

The diagnosis is established through a multidisciplinary approach and relies on identifying usual interstitial pneumonia (UIP) pattern using high-resolution computed tomography (HRCT) and histopathological assessment [67]. On HRCT, the UIP is identified by traction bronchiectasis/bronchiolectasis (irregular dilatation of bronchi and bronchioles due to the contraction of surrounding fibrotic tissue), and/or honeycombing predominantly located in the subpleural regions and lower lobes [68]. Additionally, irregular thickening of interlobular septa, reticular pattern and mild ground-glass opacities are typically present.

Histologically, UIP is characterized by patchy dense collagen-rich fibrosis distorting the alveolar architecture, the presence of fibroblast foci, and honeycomb cysts. Fibroblast foci are considered active sites of ongoing fibrosis, as they are spatial clusters of activated fibroblasts and myofibroblasts within lung interstitium that pathologically deposit ECM. Their presence in lung biopsies was found to correlate with lung function decline and patient survival [69,70].

Microscopic honeycombing or honeycomb cysts are cystic airspaces within dense fibrotic tissue that are frequently lined by single-layered or hyperplastic basal cells and/or bronchiolar-like epithelium, additionally filled with mucus and inflammatory cells [3,67,71].

In addition to the symptom management, IPF patients react poorly to anti-inflammatory treatment [72], and to date, only two antifibrotic drugs, pirfenidone and nintenanib, have been FDA-approved for the treatment of IPF. However, they are not able to halt nor reverse the disease. As a result, lung transplantation is often the only option for selected IPF patients.

Pathology

Despite the above-described hypothesis of the pathological tissue remodelling in pulmonary fibrosis, the precise molecular and cellular mechanisms initiating and driving the IPF are not completely known. One of the hypothesized mechanisms is premature aging and cellular senescence [73].

In the past years, the loss of alveolar type II cells and the parallel increase of various abnormal epithelial cell populations in the IPF peripheral lung regions have been highlighted [3,74,75]. These cells share similarities with epithelial cells found in the large proximal airways but exhibit distinct transcriptomic changes influencing progenitor characteristics, mucus secretion, ECM deposition, senescence, and other cellular functions [74–77].

Post-COVID-19 pulmonary abnormalities and fibrosis

Epidemiology

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the coronavirus strain responsible for the global COVID-19 pandemic since its emergence in 2019. It has resulted in over 700 million confirmed cases and nearly 7 million deaths worldwide[78]. While the majority of individuals infected with SARS-CoV-2 experienced mild symptoms and recovered without requiring hospitalization, a significant number developed moderate to severe COVID-19 pneumonia frequently accompanied by acute respiratory syndrome (ARDS) necessitating hospitalization, including admission to intensive care unit (ICU). Furthermore, beyond the acute phase of the COVID-19 disease (≥4 weeks post-infection), a portion of recovered patients experience a range of persistent health post-COVID-19 sequelae, with lung abnormalities and pulmonary fibrosis (or ILD) being some of the most significant long-term complications [79]. Reported prevalence of post-COVID-19 lung abnormalities and fibrotic changes varies widely, ranging from as low as 0.50% in a nation-wide study [80] and 4.8% in all hospitalized COVID-19 patients post-discharge [81], and 35% of survivors of severe COVID-19 pneumonia [82], with rates reaching as high as 72% among patients who required mechanical ventilation [83]. In light of the magnitude of the COVID-19 pandemic, the concerns of increasing future burden on the health care system have been raising, despite the risk has been lowered by vaccination [80].

Several risk factors for this entity have been identified, such as severe acute COVID-19 illness requiring ICU care or mechanical ventilation, prolonged mechanical ventilation, and extensive lung damage on HRCT in the acute phase [81,82]. Additionally, some of the risk factors for post-COVID-19 interstitial changes overlap with risks associated with IPF, encompassing advanced age, male sex, smoking history and short telomere length [79,82,83].

Diagnosis and treatment

Post-COVID-19 lung abnormalities, fibrosis or ILD is diagnosed in a similar manner as other ILDs based on a combination of persistent respiratory symptoms, characteristic HRCT findings, pulmonary function impairment, and the exclusion of other causes. Lung function tests typically include forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), diffusing capacity for carbon monoxide (DLCO), and total lung capacity (TLC). Additionally, the 6-minute walk test and the modified Medical Research Council (mMRC) dyspnea scale are commonly performed.

The predominant HRCT pattern observed post-COVID-19 is organizing pneumonia, which is primarily treated with immunosuppressive therapy, particularly corticosteroids, leading to lung function improvement [81,84,85]. An alternative pattern found was UIP, along with ground-glass opacities with interstitial thickening or peripheral reticulations with bronchiectasis on CT [86]. For patients with progressive fibrotic disease unresponsive to immunosuppressive therapy, antifibrotic agents may be considered. Similar to IPF, lung transplantation remains the ultimate therapeutic option for selected patients with end-stage disease.

Pathology

Similarly as for IPF, the pathogenesis of post-COVID-19 pulmonary fibrosis is not entirely understood, however it involves several postulated mechanisms, such as persistent and dysregulated inflammation, epithelial and microvascular injury, and fibrotic remodelling [87–89]. Furthermore, directly fibrotic toxic effect of the virus has been proposed. SARS-CoV-2 uses its spike protein to attach to the angiotensin-converting enzyme II (ACE2) receptor, which is present on epithelial cells in the respiratory zone and various other tissues, to enter host cells. This interaction increases the activity of angiotensin II, known as potent profibrotic agent

The primary cause of death in severe COVID-19 cases is respiratory failure resulting from diffuse alveolar damage (DAD). This histopathological pattern of ARDS is initiated with an injury to AT2 and endothelial cells, followed by exudative (acute) phase marked by edema, hyaline membrane formation (mixture of cellular debris, plasma proteins such as albumin, fibrinogen, and immunoglobulins, and surfactant components), and interstitial acute inflammation [90]. The subsequent organizing (subacute) phase is characterized by AT2 cell hyperplasia (proliferation) and loose organizing fibrosis mostly within the alveolar septa. DAD can also be seen in other

lung conditions, such as acute exacerbations of IPF and primary graft dysfunction in lung transplant recipients.

A study on 28 patients that died of COVID-19 ARDS compared with 27 fatal ARDS cases of other causes than COVID-19 showed increased deposition of collagen, fibronectin, versican, and TGF-β in the lungs of patient with COVID-19 ARDS [88].

DAD accompanied with inflammatory response, including a cytokine storm, activates regulatory pathways for tissue repair. If the tissue repair is prolonged or dysregulated, it can lead to fibrotic remodelling of the lung detected at HRCT as interstitial thickening, ground-glass opacities, irregular interface, coarse reticular pattern, and parenchymal bands. The irregular interface and presence of parenchymal bands may be early indicators of developing pulmonary fibrosis, contributing to the disease severity and mortality [91].

Chronic lung allograft dysfunction

Epidemiology

Lung transplantation remains the only therapeutic option for many patients with end-stage chronic lung disease. However, it is among the most challenging transplantable organs due to high graft failure rates. Chronic lung allograft dysfunction (CLAD) is the primary cause of chronic lung function decline (beyond three weeks) and a major barrier to long-term survival, with survival rates of 54% at 5 years and 32% at 10 years [92,93].

Several risks have been associated with CLAD such as acute rejections, lymphocytic bronchiolitis, infection with *Pseudomonas*, aspiration, donor and recipient genetic differences, primary graft dysfunction, presence of HLA antibodies, or antibodies to self-antigens [93–96].

Bronchiolitis obliterans syndrome (BOS) is the most common form of CLAD, accounting for 65-75% of cases. Patients diagnosed with BOS have a median survival period of 3 to 5 years, which is similar to that of IPF [93]. In contrast, restrictive allograft syndrome (RAS) is another phenotype of CLAD with a worse prognosis, found in 25-35% of patients and with a median survival of only 6 to 18 months after diagnosis. In addition, it is known that CLAD patients may transition between these phenotypes, which is referred to as a mixed phenotype [97,98].

Diagnosis and treatment

CLAD is clinically defined as a persistent, obstructive decline in FEV1 of at least 20% compared to the average of the two best post-transplant FEV1 values, after excluding other potential pulmonary and extrapulmonary causes of FEV1 decrease, such as acute rejection or infection [99]. Transbronchial biopsies show poor sensitivity for BOS diagnosis due to the patchy presence of affected airways [100].

RAS is further characterized by a TLC decline of at least 10% compared with the mean of the two best post-operative values and the presence of persistent opacities on chest imaging [99].

Apart from the management of infections, inflammation and gastroesophageal reflux by immunosuppressive and immunomodulatory therapies, CLAD presents limited treatment options; in advanced cases, lung re-transplantation may be the only treatment alternative for carefully selected patients [99].

Pathology

CLAD encompasses a wide range of fibrotic changes affecting the airways, lung parenchyma, and pleura, along with pathological alterations in the epithelium, vasculature, and inflammatory processes [101]. BOS and RAS exhibit considerable overlap within this spectrum.

Bronchiolitis obliterans - a formation of fibrotic obliterative lesion in small airways - is considered the main histopathological correlate of lung function decline in the clinical syndrome BOS [93]. Interestingly, bronchiolitis obliterans also occurs in other conditions, such as pulmonary graft-versus-host disease following hematopoietic stem cell transplantation and autoimmune disorders like rheumatoid arthritis. Furthermore, it is also one of the histopathologic features of RAS, although this phenotype is primarily characterized by pleuroparenchymal fibroelastosis and diffuse alveolar damage [102].

In a study conducted by Kerckhof et al. [103], BOS and mixed phenotype explants showed obstruction in 76% and 84% of bronchioles, respectively, predominantly in proximal airways (generation >5). In contrast, RAS explants exhibited obstruction in 22% of airways, primarily in the most distal bronchioles (generation >12). The airways were primarily obstructed by lymphocytic inflammation or the fibrotic thickening of the airway wall.

It is hypothesized that airway obstruction results from persistent damage to the bronchial and bronchiolar epithelium induced by bacteria, viruses, gastroesophageal reflux, or air pollution [51] This damage leads to a chronic inflammatory response, the recruitment and activation of local fibroblasts and circulating fibrocytes, epithelial-mesenchymal transition, excessive ECM deposition, and, ultimately, complete occlusion of the airway lumen by fibrotic tissue.

Aims

The overall purpose of this thesis was to gain deeper insight into tissue remodelling and ECM alterations, particularly in the lung epithelial-mesenchymal niches, that may contribute to the progression of different forms of pulmonary fibrosis and chronic lung allograft dysfunction and/or be reflected at the systemic level.

The specific aims of the studies included in this thesis were:

- To characterize collagen VII in the normal lung and idiopathic pulmonary fibrosis in terms of its localization, cellular expression, and potential regulatory mechanism (Paper I)
- To identify systemic proteins related to tissue remodelling and inflammation that are shared between COVID-19 or post-COVID-19 patients and IPF patients and could potentially identify individuals at risk of developing pulmonary fibrosis (Paper II and III)
- To investigate the involvement of tissue remodelling proteins periostin, LOX and LOXL1 in the progressive small airway obstruction in bronchiolitis obliterans syndrome (Paper IV)

Methodology

This thesis explores cell and ECM alterations associated with pulmonary fibrosis and bronchiolitis obliterans syndrome across multiple levels, from gene and protein expression to histopathological changes and clinical outcomes.

The following subchapters provide a brief conceptual overview of the patient material and the methods used in the studies included in this thesis. More detailed information regarding the materials and methods can be found in the individual papers I-IV.

Patient material and ethical considerations

Lung tissue

In all studies of this thesis, explanted human lungs were the primary source for histopathological analyses. These lungs were obtained through collaborations with Sahlgrenska University Hospital in Gothenburg, Skåne University Hospital in Lund, Sweden, and The University Medical Center Groningen, The Netherlands. For the investigation of disease-related changes, the lungs were obtained from patients with end-stage disease (IPF, post-COVID-19 fibrosis, BOS) who underwent lung transplantation (ltx). The BOS patients underwent re-transplantation due to chronic lung rejection of their first transplant. This provided a valuable purpose for the tissue that would have otherwise been discarded.

As control tissue for the IPF and COVID-19 groups in **Papers I, II, and III**, lungs from organ donors that were not found suitable for lung transplantation (for instance, due to immune incompatibility, size mismatch, advanced donor age or smoking history or other factors) were used. These donors had no prior history of lung disease. Clinical information, including sex, age, smoking status, and major comorbidities, was collected from both patients and lung donors. For the experiments, the control group was approximately matched to the disease groups in terms of sex, age, and smoking status.

In the case of BOS patients in **Paper IV**, unobstructed airways served as controls to partially and completely obstructed airways despite their presence in the same lungs.

Additionally, extensive clinical information such as recipient sex, age at ltx, smoking status and underlying disease, as well as donor sex, age, smoking status, immunosuppression after ltx, acute rejection, primary graft dysfunction, time to development BOS and lung function measurements was documented for these patients.

Given the spatial and temporal heterogeneity of these diseases, capturing the pathological region of interest may be challenging. The end-stage tissue used in this thesis represents valuable material due to the large tissue area for examination. Despite many of the features representing the terminal stage of the pathology, partially preserved alveolar regions and airways can also be found in the tissue, which was utilized in **Papers III, IV**, and partially in **Paper I**. The observations from comparing these areas with extensively remodelled regions can provide insight into potential dynamics between normal and abnormal tissue.

For studying temporal tissue changes, central and transbronchial biopsies obtained during bronchoscopy may be more suitable. Although these biopsies are crucial for diagnosing various pulmonary diseases, including ILDs, the gained tissue is very small, and the procedure is invasive, causing significant discomfort to already seriously ill patients along with a risk of post-procedural bleeding [104].

All studies were approved by local ethical committees and conducted in accordance with the ethical permissions specified in the individual papers. Written informed consent was obtained from all subjects involved in the studies or the donor's closest relative.

Blood samples

In **Paper II**, plasma samples were collected from a small cohort of hospitalized pre-vaccine COVID-19 patients two weeks after hospital admission. Patients with severe COVID-19 and acute respiratory distress syndrome (ARDS, n = 8) receiving mechanical ventilation at the intensive care unit (ICU) were defined as the “Severe” group, while those with moderate symptoms (n = 8) treated with supplemental oxygen were defined as the “Moderate” group. Samples were collected in EDTA tubes to prevent coagulation.

In **Paper III**, serum samples from patients who were previously treated at ICU, were collected at two visits - 4 and 10 months after discharge. Comprehensive clinical information, including sex, age, tobacco consumption, comorbidities, ventilatory support, lung function measurements, 6-minute walk test (6MWT), evaluations of dyspnea, and HRCT scans was gathered from these COVID-19 patients.

Cell culture

In **Paper I**, primary fibroblasts for cell culture experiments were derived from small tissue fragments isolated from distal parenchymal regions of the explanted IPF and control lungs as previously described [105]. The lung fibroblasts were further expanded in cell culture and treated with 10 ng/ml of the pro-fibrotic agent transforming growth factor beta (TGF-β1) for 48 hours. Following the treatment, cell lysates were collected for gene expression analysis.

Compared to established cancer cell lines, primary cells have a limited number of cell divisions before undergoing senescence. To minimize this effect on gene expression in the experiments, only cells up to passage 8 were used. While primary fibroblasts better reflect individual patient variability, their heterogeneity in behaviour and response to stimuli can be challenging, especially compared to the relative consistency of cancer cell lines or immortalized cells, which we observed in our *in vitro* experiments.

Histology

Tissue processing

In **Papers I, II** and **III**, lung tissue was dissected upon arrival to samples from distal parenchymal regions of the explanted lung adjacent to the visceral pleura and from proximal lung regions containing large airways/bronchi. All samples were fixated in 10% formalin solution, dehydrated and embedded in paraffin (FFPE). In **Paper IV**, similarly processed archival BOS lung tissue was retrieved from pathology biobanks at Sahlgrenska Hospital in Gothenburg, Sweden, and University Medical Center Groningen, The Netherlands.

Fixation in formalin was introduced in pathology in the late 19th century and has since become the gold standard for archiving patient tissue in pathology laboratories [106]. This method provides excellent long-term preservation of tissue morphology compared to frozen sections. However, formalin fixation also leads to masking protein epitopes by forming methylene bridges, which requires antigen/epitope retrieval for effective protein detection in immunohistochemistry. The most common antigen retrieval methods are **heat-induced epitope retrieval (HIER)** and **enzymatic digestion**.

When using HIER, the pH of the antigen retrieval solution plays a critical role, with higher pH solutions (e.g., Tris-HCl buffer with pH 7.2-9.0) typically resulting in the best staining outcome for most antigens [107]. Extracellular matrix proteins, such as collagens, are often complex and highly crosslinked structures. Therefore,

enzymatic antigen retrieval using agents, e.g. proteinase K or pepsin, is commonly used to digest these crosslinks and enhance antigen accessibility, improving the detection of these proteins [108].

Histological stains

Histological stains provide relatively simple yet effective protocols for visualizing a wide range of structures, from individual cells to components of connective tissue, both specifically and non-specifically. The following stains were applied in this thesis:

- **Hematoxylin and Eosin (H&E)** – A fundamental stain in pathology used to evaluate tissue morphology and major tissue structures by contrasting nuclei, cytoplasm, extracellular matrix (ECM), and muscle cells. H&E staining was performed across all **Papers I-IV**.
- **Verhoeff-Van Gieson (VVG)** – A widely used histological stain in pathology that highlights elastic fibers, providing essential contrast for connective tissue evaluation. This stain was applied in **Paper IV**.
- **Modified Russell-Movat pentachrome** (hereafter referred to as pentachrome) – A specialized stain particularly valuable for studying connective tissue. This method combines multiple chemicals that highlight collagen, elastic fibres, fibrin, muscle, and glycosaminoglycans, and was used in **Paper I, II and IV**.

RNA detection

Genetic information in humans is encoded in DNA. When a cell is activated to produce a specific protein, for instance, an extracellular matrix protein, a segment of the DNA corresponding to that gene is transcribed into messenger RNA (mRNA). The mRNA can be detected *in situ* or *ex vivo* using various bioanalytical methods such as **RNA in situ hybridization**, **RT-qPCR** or **RNA sequencing.** However, these approaches do not reveal whether the RNA is ultimately translated into a functional protein or is prevented by degradation, silencing or other mechanisms [109].

RNA in situ hybridization

RNAscope in situ hybridization, developed by the Advanced Cell Diagnostics company, offers a highly sensitive technique to detect and localize RNA molecules

within tissue and cells [110]. The principle is based on a unique Z probe design with multiple short oligonucleotides that hybridize in pairs to the target RNA and are further amplified, allowing for detecting even low-abundance transcripts. The approach enables visualization of gene expression at the single-cell level, providing spatial information about RNA distribution within tissue sections. One advantage of detecting RNA for matrix proteins compared to IHC (visualizing their mostly extracellular localization) is the identification of the specific cell type responsible for their production.

In **Paper I,** this RNAscope in situ hybridization was performed to detect COL7A1 mRNA in control and IPF lung tissue sections.

Mining public single-cell and bulk RNA sequencing datasets

RNA sequencing represents a powerful technique for the analysis and quantification of mRNA in biological samples. It provides a comprehensive, in-depth view of the cell transcriptome, enabling the study of gene expression dynamics and biological patterns. Based on analysis, two main types of RNA sequencing exist – **bulk RNA sequencing** and **single-cell RNA sequencing**.

Bulk RNA sequencing analyses gene expression from a typically homogeneous cell population, such as a particular cell type isolated from tissue or cells cultured under different conditions. This method is not as cost-demanding; however, since the cells are mixed, the information on individual cell variability is lost. In contrast, single-cell RNA sequencing provides information on gene expression from individual cells, making it particularly useful for studying heterogeneous cell populations. However, this method is more complex and expensive and requires advanced bioinformatics tools to manage high-dimensional data.

Publicly deposited RNA sequencing datasets have provided an invaluable opportunity for data sharing across research laboratories worldwide, accelerating the progress of studies that might otherwise be limited by the lack of expertise or funding. Another advantage is that the datasets can be utilized to validate internal data. In **Paper I**, two publicly available datasets, GSE134692 [111] and GSE135893 [75], downloaded from the Gene Expression Omnibus repository, were used to explore COL7A1 expression in larger cohorts and identify the specific cell types expressing COL7A1 and the related genes.

RT-qPCR

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is a highly sensitive method used to detect and quantify gene expression at the mRNA level. Isolated mRNA is first reverse-transcribed into complementary DNA (cDNA), which then serves as a template for quantitative PCR (qPCR). The

amplification of the target gene is measured in real-time through fluorescence, and the cycle threshold (Cq) value is used to quantify relative expression levels using $2^{-\Delta\Delta Cq}$ method.

RT-qPCR has been a standard tool in molecular biology for analyzing mRNA expression for many decades. Despite the rise of RNA sequencing technologies, RT-qPCR remains a preferred approach for certain applications due to its simplicity, lower costs, and sensitivity. It is often used for expression analysis of single genes or to validate RNA sequencing results.

In **Paper I**, RT-qPCR was used to assess the expression of COL7A1, COL1A1, ACTA2, LAMB3 and LAMC2 genes in both control and IPF fibroblasts in response to TGF-β1 treatment, as well as in untreated cells.

Protein detection

To produce a functional protein, mRNA is transported from the cell nucleus to ribosomes in the cytoplasm, where mRNA translation is initiated. Ribosomes facilitate this process by reading the mRNA sequence and guiding transfer RNA molecules that deliver the corresponding amino acids. The resulting protein chain undergoes folding and post-translational modifications such as glycosylation, phosphorylation, hydroxylation and others in the cytoplasm, endoplasmic reticulum, and Golgi apparatus to become a fully functional protein [35].

While some proteomic methods, such as **proximity extension assay,** detect intact protein forms, **mass spectrometry** requires protein digestion into peptides – a process that is particularly challenging for complex ECM proteins due to their extensive crosslinking or other modifications. **Immunohistochemistry** and **immunofluorescence** enable detecting both forms, depending on the tissue processing, antigen retrieval method and the availability of a specific antibody targeting the whole protein or a related fragment.

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) allows for spatial detection of a protein within specific cell compartments or the extracellular matrix in tissue sections. The method utilizes formation of a complex between a target protein (antigen) and a specific primary antibody. The antigen-antibody complex is then visualized by a fluorophore directly labelling the primary antibody or by secondary antibody binding the primary antibody. The secondary antibody can be directly conjugated to a fluorophore resulting in fluorescent signal, or enzyme (e.g. horseradish peroxidase (HRP), alkaline phosphatase (AP)) reacting with a chromogenic substrate (e.g.

diaminobenzidine (DAB), substrate for alkaline phosphatase) and producing a colour precipitate.

Both detection methods have advantages and limitations, depending on the used tissue, desired readout and type of analysis. IHC with chromogen detection is often preferred for studying the general localization of proteins within tissues, as it avoids the issues associated with autofluorescence. This can be particularly challenging in IF staining, especially in lung tissue, which exhibits much autofluorescence due to its high collagen and elastin content, among other factors. However, IF is suitable for the co-staining of two and more markers, as the resulting signals can be easily separated later on. Overall, optimizing antibodies can be difficult and time-consuming, depending on the quality of the antibody, whether it is commercially available or produced in-house.

In **Paper I,** both IHC and IF were used to detect collagen IV, VII and its co-localization with KRT17, which was additionally co-stained with KRT5, all in control and IPF lungs.

In **Paper II**, IHC was performed to visualize decorin and periostin in control, post-COVID-19 and IPF lung tissue.

In **Paper III**, LAMP3, KRT5 and KRT17 were targeted using IF to investigate their spatial relationship in the in control, post-COVID-19 and IPF lung tissue.

In **Paper IV**, IHC was utilized to investigate patterns of periostin, LOX and LOXL1 proteins, primarily in small airways of BOS lungs.

Mass spectrometry data analysis

Mass spectrometry (MS) is a well-established analytical method for identifying and quantifying molecules based on their mass-to-charge ratio (m/z). For proteomic analysis using MS, samples must first be digested enzymatically into peptides, which are subsequently analysed by MS for quantification. The identified peptides are then computationally assembled into proteins by using bioinformatic tools. Advantages of this method include high sensitivity, specificity, as well as ability to analyse complex protein mixtures. Furthermore, a quantitative proteomic approach to studying the dynamics of cell-repopulated systems and quantifying newly synthesized versus preexisting proteins can be performed using stable isotope labelling with amino acids (SILAC) [56,112,113].

In **Paper I**, we utilized and re-examined the proteomic dataset PXD012322 generated with LC-MS/MS in our previously published study using Maxquant software [56].

Proximity extension assay

In **Papers II and III**, an alternative technique for the simultaneous exploration and quantification of multiple proteins was used - proximity extension assay (PEA) developed by the company Olink Proteomics AB (Uppsala, Sweden). This technology utilizes two DNA oligonucleotide-conjugated antibodies that both bind to a specific protein target. Following the binding, oligonucleotides in proximity hybridize, the pairs are extended by DNA polymerase and amplified through qPCR, allowing quantification of the target protein. This mechanism enables high sensitivity while reducing unspecific binding, and it is suitable for the analysis of liquid biological samples such as plasma, serum, bronchoalveolar lavage and others.

A multiplex 92 protein immunoassay panel (immune-oncology panel) was selected for the proteomic analysis of blood samples due to the inclusion of multiple proteins of interest involved in tissue remodelling and inflammation. This allowed us to compare several studies investigating healthy subjects, IPF patients and COVID-19 and post-COVID-19 patients. Results were normalized using standard Olink workflows to obtain relative protein concentrations on a log2 scale, expressed as Normalized Protein eXpression (NPX). A difference of 1 NPX equals an approximate doubling of protein concentration, with higher values representing greater protein abundance.

Image analysis

Whole slide imaging is a method used in digital pathology to scan and digitize entire microscope slides with high resolution. This allows for the later viewing of tissue samples at various magnifications and enables the selection of representative areas for subsequent image analysis. Many parameters can be quantified in tissue sections, including the area stained for specific proteins and the quantities of different cell types that support descriptive observations. However, image analysis relies heavily on the quality of the input data. Furthermore, setting up the analysis workflow may be challenging, and the quantification process of the large data files may require a significant amount of time or high computational power.

In all **Papers I-IV**, glass slides with stained tissue sections were scanned with VS120 virtual microscopy slide scanning system (Olympus, Tokyo, Japan) or Hamamatsu NanoZoomer 2.0HT digital slide scanner (Hamamatsu Photonic K.K., Hamamatsu, Japan). The images were then viewed using the software Olyvia (v4.1, Olympus, Tokyo, Japan), Qupath (v 0.3.0; 0.4.3), or NDP.view2 (Hamamatsu Photonics K.K., Japan).

In **Paper I**, the frequencies of basal cell populations were calculated as a percentage of all cells detected in the whole tissue sections. Collagen VII (COL7) was

quantified as the COL7-positive area relative to the entire tissue area. Additionally, the frequencies of KRT17+ cells located near COL7-positive area were quantified. All quantitative analyses were performed using Qupath (v0.3.0).

In **Paper IV**, regions of interest (ROI) capturing the whole airway area were manually annotated in Qupath (v0.4.3), in all small airways identified. The ROI was defined as the outer borderline of the airway adventitia to the inner borderline of the airway lumen. In the case of fully obstructed airways, only the adventitial borderline was used, as no lumen was found. Following this, the positive area for periostin, LOXL1, or LOX was calculated as a percentage of the total ROI area using Fiji software (v1.53t).

AI tools

This thesis has, in part, been produced with the assistance of the generative AI models ChatGPT and Grammarly to translate and improve language and correct grammar and spelling. I have processed the generated text and image and take full responsibility for the content.

Summary of included papers

Paper I

Collagen VII is associated with airway remodelling, honeycombing and fibroblast foci in UIP/IPF

Increased collagen deposition and changes in the expression of various collagen types have been previously observed in idiopathic pulmonary fibrosis [114–118]. However, the turnover and role of some less abundant collagen types in pulmonary fibrosis remain poorly understood. This study aimed to shed light on collagen VII - a well-characterized collagen type in the skin but relatively unrecognized in the lung - by describing its localization in the lung, cellular expression, and potential regulatory mechanisms.

After initial findings of collagen VII increased levels in lung tissue of IPF patients compared to controls, we aimed to investigate its spatial distribution in both distal and proximal lung regions. IPF lungs are hallmarked by subpleural honeycombing and airway remodelling, along with the associated striking increase of epithelial cell types normally found proximally in larger airways, in distal parts of the lung [74,75,77,119,120]. In the control lungs, collagen VII was only localized in the basement membrane area beneath bronchial and bronchiolar epithelia, with its levels gradually decreasing along the airway tree toward the alveoli. In contrast, in the distal lung tissue, collagen VII deposition accompanied the increase of airway and honeycomb structures as part of their basement membrane zone. No expression was detected in the basement membrane zone of blood vessels, indicating epithelial specificity, consistent with observations in the skin [37].

Since collagen VII was primarily detected at the interface of the epithelium and the interstitium with mesenchymal cells, we sought to determine which cell type is the primary collagen VII producer. RNA in situ hybridization revealed high mRNA levels in epithelial cells, mainly at the base of pseudostratified epithelia. Publicly available RNA sequencing data corroborated the collagen VII expression in the epithelial cells and revealed different epithelial cell types, pointing primarily to basal cells and KRT5-/KRT17+ alias aberrant basaloid cells as the main expressing cell type. Furthermore, in the IPF distal lungs, the quantification of different basal cell populations demonstrated a significant increase of KRT5+ populations, along with more abundant KRT17+ cells in close proximity to collagen VII positive area.

Additionally, collagen VII mRNA and protein were detected in pathological fibroblast foci in the IPF lung tissue, supported by gene expression in mesenchymal subsets in the RNA sequencing data. When primary lung fibroblasts were treated with strong profibrotic agent TGF-β1, control fibroblasts significantly upregulated COL7A1 expression, suggesting TGF-β1 involvement in collagen VII regulation. Interestingly, at the interface of many fibroblast foci and abnormal epithelium above, the inner epithelial cells with intracellular collagen VII expression were observed. These cells, often with an impaired extracellular collagen VII underneath, showed similarities to abnormal epithelial cells in sandwich fibroblast foci found in other studies, co-expressing basal cell and mesenchymal cell markers [121,122].

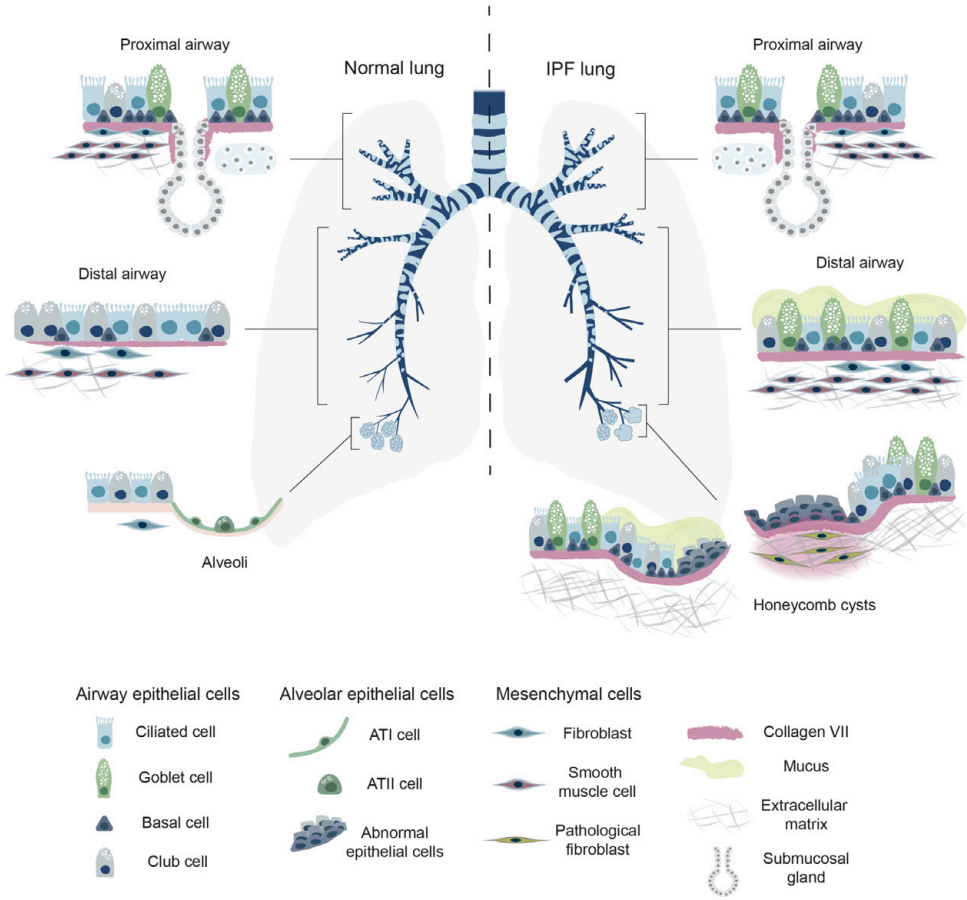


Figure 4. Summary of results in this study.
While collagen VII is expressed to a similar extent in the basement membrane zone of bronchi in the control and the IPF proximal lungs, the IPF distal fibrotic lungs display abundant pathologic structures such as enlarged and remodelled bronchioles and honeycomb cysts, which are surrounded by collagen VII in their basement membrane zone. In contrast, in the control distal lungs, collagen VII was localized only in larger bronchioles. Furthermore, collagen VII was found in many IPF fibroblast foci and intracellularly in epithelial cells overlying these structures. Illustration made by Lisa Karlsson

In conclusion, our results showed that collagen VII is increased in the distal lungs of IPF patients, where it is situated at the pathologically remodelled sites containing abnormal airways, honeycomb cysts, and fibroblast foci (Figure 4), associated with increased basal and basal-like cell populations and regulated by TGF-β1 *in vitro.* These findings motivate the investigation of collagen VII's functional role in IPF pathogenesis.

Paper II

Overlapping systemic proteins in COVID-19 and lung fibrosis associated with tissue remodelling and inflammation

Concerns about the potential increasing incidence of pulmonary fibrosis induced by SARS-CoV-2 emerged during the COVID-19 pandemic. A nationwide study done by Kim et al. found that COVID-19 was linked to an increased incidence of newly diagnosed ILD; however, this risk could be reduced through COVID-19 vaccination [80].

In this small-scale study, we aimed to identify plasma proteins in hospitalized COVID-19 patients with both moderate and severe disease, recruited before initiating the vaccination program, that overlap with the blood profile of IPF patients from a previous study [123]. Thus, these proteins could potentially serve as early biomarkers for the risk of developing post-COVID-19 pulmonary fibrosis.

Among the hospitalized COVID-19 patients, men were the majority in both the severe and moderate groups. PEA analysis on plasma samples showed 42 significantly changed proteins (>1 NPX mean difference) in the severe COVID-19 patients compared to the healthy controls, with 23 of these proteins also being increased in the moderate cases.

Significantly elevated proteins in the severe cases compared to the healthy controls revealed decorin (DCN) among other proteins. DCN and periostin (POSTN), are proteins known to be implicated in collagen fibrillogenesis and tissue remodelling [124–126].

Furthermore, DCN along with proteins such as chemokine (C-X-C motif) ligand 9 (CXCL9), monocyte chemotactic protein-3 (MCP-3), chemokine (C-X-C motif) ligand 13 (CXCL13), hepatocyte growth factor (HGF), pleiotrophin (PTN) and TNF receptor superfamily member 12A (TNFRSF12A) were significantly higher in the severe group compared to the moderate group (>1 NPX mean difference), with TNFRSF12A exhibiting the highest levels. Furthermore, MCP-3 and HGF were significantly elevated in the moderate COVID-19 group compared to the healthy controls, with increasing levels from the control group to the severe group.

Histological analysis of end-stage post-COVID-19 and IPF lungs compared to healthy controls revealed striking lung parenchyma remodelling in both groups. This remodelling was characterized by increased ECM deposition, abnormal mucus production, and inflammatory infiltrates, including macrophages. Furthermore, accumulated spindle-shaped fibroblasts in post-COVID-19 tissue resembling fibroblast foci in IPF were observed (Figure 5).

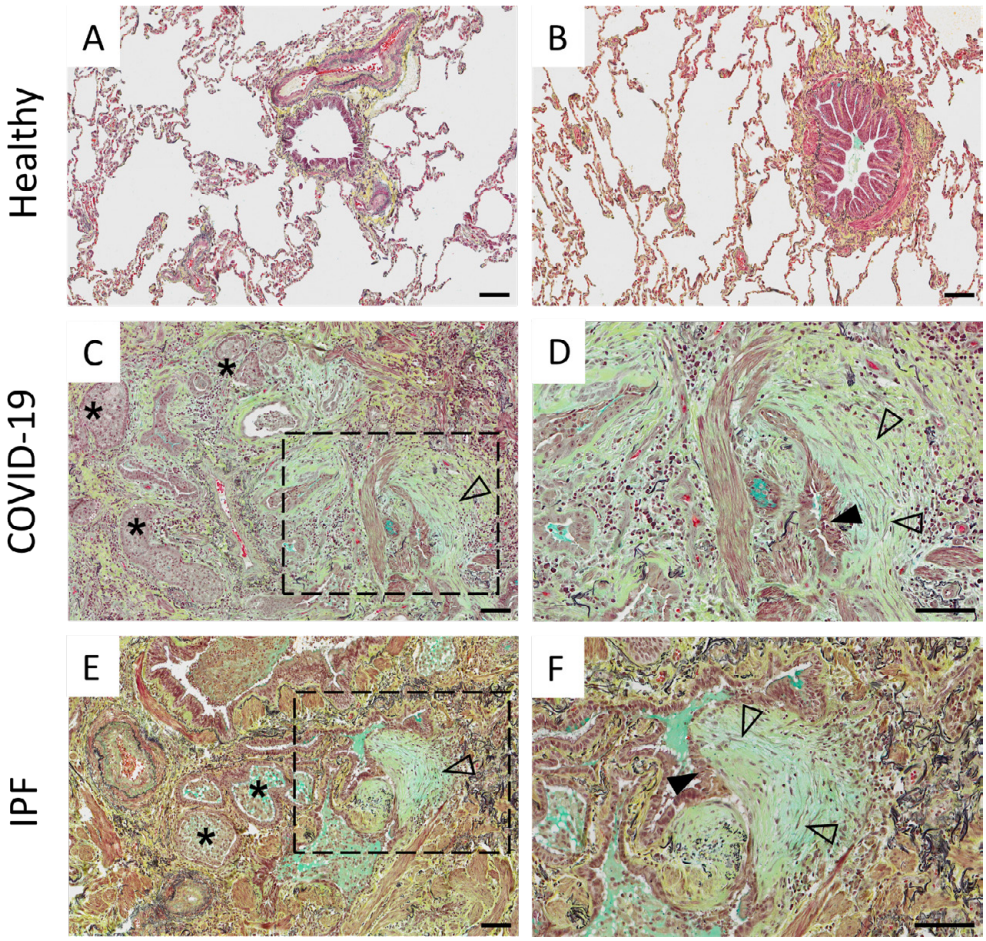


Figure 5. Lung tissue remodelling and shared histopathological features in post-COVID-19 and IPF.
A-B) Normal lung tissue stained with pentachrome, highlighting collagen and elastic fibres within thin alveolar walls. C-D) Lung parenchyma in post-COVID-10 was disrupted and remodelled by increased deposition of collagens and proteoglycans, formation of mucus-filled abnormal epithelial structures, smooth muscle cell hyperplasia and cell infiltration, similar to lung tissue in IPF (E-F). Structures with spindle-shaped myofibroblasts (C, empty arrowheads) resembling fibroblastic foci in IPF tissue (E, empty arrowheads). Accumulations of foam macrophages in post-COVID-19 (C, black arrowheads) and IPF lung tissue (E arterisks). Scale bars for all images are 100 µm. Legend for the pentachrome staining: yellow – collagens; blue – mucins/glycosaminoglycans/proteoglycans; black - elastic fibres; bright red – red blood cells; red - SMCs/fibrin; dark red/brown – other cell types. Various shades of green can also result from the colocalization of collagens (yellow) and proteoglycans (blue).

HRCT imaging of one of the post-COVID-19 patients demonstrated parenchymal changes consistent with ARDS and fibrosis. DCN and POSTN exhibited increased expression in the post-COVID-19 fibrotic tissue proportional to the increased amount of tissue in some regions, with distinct expression in bronchioles,

fibroblastic foci, blood vessel adventitia and visceral pleura, aligning with findings in IPF lung tissue.

In conclusion, we identified shared histopathological features in explanted lungs of post-COVID-19 and IPF patients associated with tissue and ECM remodelling, along with similar DCN and periostin patterns. Among the analyzed systemic factors in the circulation of COVID-19 patients, DCN, TNFRSF12A, CXCL13, CXCL9, HGF and MCP-3 emerged as the most promising candidates for early biomarkers of pulmonary fibrosis. These findings warrant further investigation in larger, more diverse cohorts of post-COVID-19 patients to investigate the utility of the identified proteins as biomarkers of pulmonary fibrosis.

Paper III

Protein profiling in ICU-treated COVID-19 patients identifies biomarkers of residual lung abnormalities

Similarly to Paper II, this longitudinal follow-up study aimed to identify potential biomarkers that may indicate patients at risk of developing persistent lung complications and fibrosis following COVID-19.

We investigated serum samples from recovered COVID-19 patients who were previously hospitalized and treated at ICU due to severe COVID-19 pneumonia. The samples were collected four and ten months after discharge, together with extensive clinical data (Figure 6).

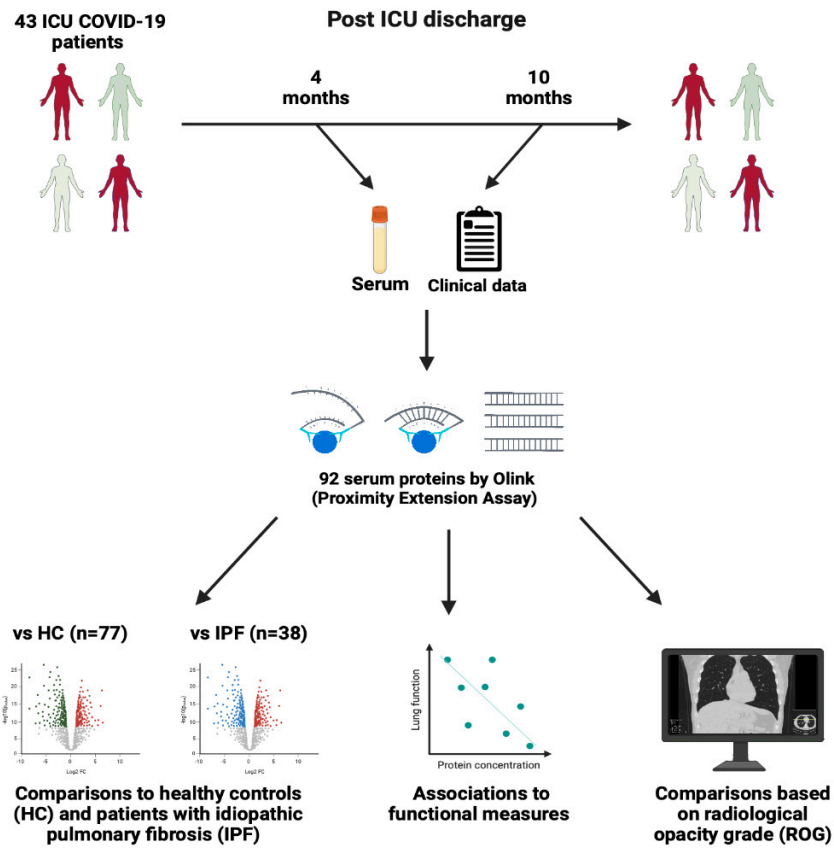


Figure 6. Schematic of study outline.

A total of 43 ICU-treated COVID-19 patients were included in this study, with the majority experiencing ARDS (72%) and approximately two-thirds (65%) requiring ventilatory support during hospitalization. Four months post-discharge, COVID-19 patients showed reduced FVC% and FEV1% compared to healthy controls but showed no significant differences in lung function when compared to IPF patients. Ten months post-discharge, these parameters had improved; however, all lung function measures, including DLCO% and TLC%, remained lower than those of the control group. In contrast, the IPF patients exhibited lower DLCO% than COVID-19 patients.

PEA analysis of differentially expressed proteins in the serum samples four months after discharge revealed upregulation of 60 and 20 proteins in COVID-19 patients compared to the controls and IPF patients, respectively. ADGRG1, CXCL13, CCL19, HGF and MMP7 were increased in COVID-19 compared to the controls among other proteins. In contrast, 2 and 14 proteins were elevated in the controls and IPF patients compared to COVID-19 patients, respectively, with PTN, LAMP3, CXCL9 and MCP-3 being higher in IPF. Despite a modest decline of serum levels ten months post-discharge, the majority of the proteins were persistently upregulated.

Four months after discharge, elevated levels of MMP7, LAMP3, and MCP-3 were correlated to reduced FVC% and shorter 6MWD. MMP7 showed a negative correlation with FVC%, TLC%, and DLCO%, while LAMP3 was inversely associated with TLC% and DLCO%. At 10 months, these associations persisted.

Radiologic analysis revealed that 73% of COVID-19 patients with available scans at four months had residual lung involvement >5% (high radiology opacity grade, ROG). The mean total score was 20.4 ± 12.6% in the high ROG group versus 2.18 ± 1.70% in the low ROG group, with the lower lobes most affected. High ROG patients were older, had longer hospital and ICU stays, lower FVC%, and more dyspnea (defined as mMRC $\geq$ 2). Proteomic analysis showed elevated serum levels of LAMP3, an alveolar type 2 cell marker, in high ROG patients.

Ten months after discharge, the number of patients in the high ROG group was reduced to 53%, though lung involvement remained higher than in low ROG patients (13.3 ± 6.34% vs. 2.17 ± 1.65%, $p < 0.0001$). Persisting high ROG was associated with more ventilatory support days, lower TLC% and DLCO%, and a greater proportion of patients with DLCO% <80 compared to low ROG. Elevated LAMP3 persisted, with MMP7 also increasing in high ROG patients.

Parenchymal involvement at four months correlated inversely with FVC%, while at ten months, it correlated with TLC% and DLCO% but not FVC%. LAMP3 and MMP7 levels strongly correlated with parenchymal changes at both time points.

Immunofluorescent staining of distal lung tissue from healthy controls, end-stage post-COVID-19 patients and IPF patients revealed LAMP3 localization to alveolar type 2 (AT2) cells, supported by its overlapping pattern with pro-SPC in normal alveoli of controls and partially preserved alveolar regions of the diseased lungs (Figure 7). This overlap was even more prominent in parenchymal areas with hyperplastic AT2 cells. However, these regions lacked basal cells, as indicated by KRT17 and KRT5 staining, which were restricted to larger airways. In contrast, the post-COVID-19 and IPF lungs exhibited extensively remodelled areas enriched with honeycomb cysts, pathological airways, and hyperplastic basal and basal-like cells while showing reduced LAMP3 and pro-SPC expression in these areas.

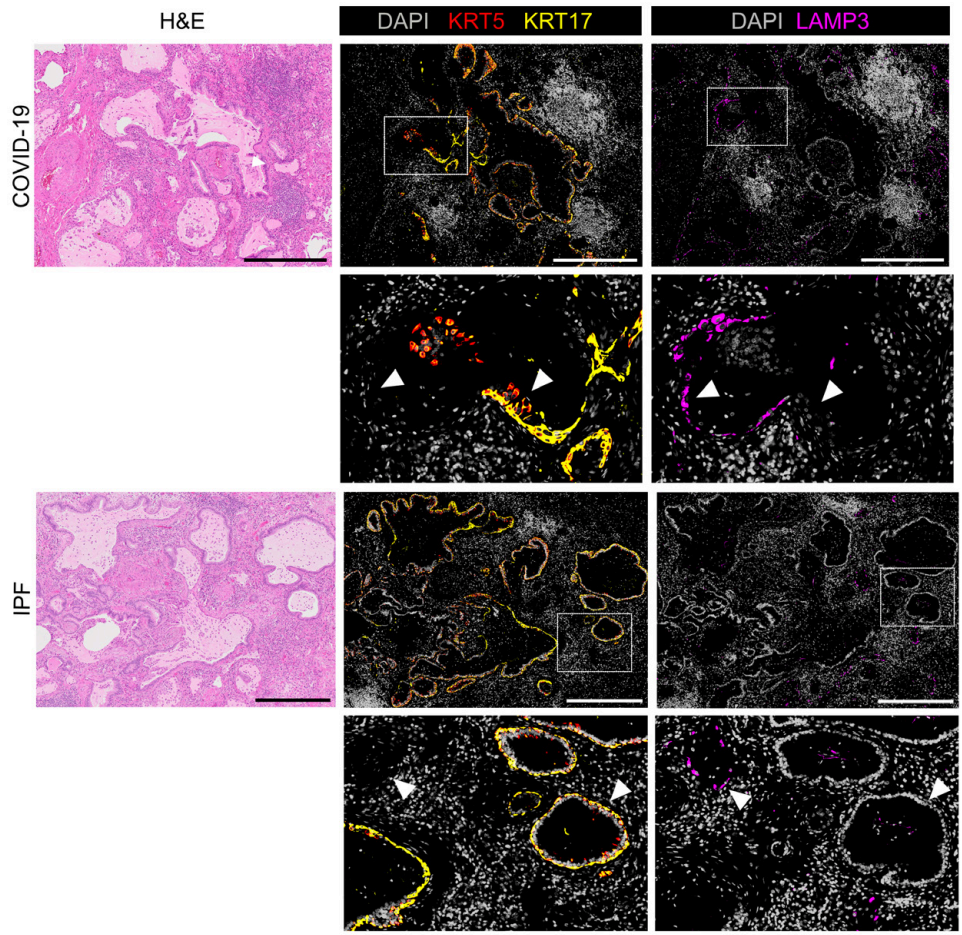


Figure 7. KRT5, KRT17 and LAMP3 protein expression in fibrotic distal lung tissue of IPF and COVID-19 patients. Left panel: H&E staining; middle panel: IF co-staining of KRT5 and KRT17; right panel: IF staining of LAMP3. Magnified areas from images of lower magnification are indicated by the dashed boxes. Arrowheads point approximately to the same location with non-overlapping pattern of KRTs and LAMP3. Scale bars in all images are 500 µm.

In conclusion, our findings show that despite functional and radiological improvements following COVID-19, inflammation and tissue remodelling factors remain persistently upregulated in the serum even ten months post-hospitalization discharge, including LAMP3 and MMP7. The additional similar expression patterns of LAMP3 in both post-COVID-19 and IPF lung tissue may indicate its potential as a biomarker for chronic lung damage, motivating further validation and mechanistic investigation in an independent cohort.

Paper IV

Small airway remodelling and matrisome protein expression in bronchiolitis obliterans syndrome

Bronchiolitis obliterans syndrome (BOS) is characterized by enhanced ECM deposition in small airways progressively narrowing the airway lumen, leading to a progressive decline in lung function and, ultimately, lung allograft rejection. However, knowledge of the precise composition of the pathological microenvironment in the BOS-affected airways driving the progressive airway obliteration is lacking. This ongoing multi-centre study aims to unravel the contribution of tissue remodelling proteins periostin, lysyl oxidase (LOX) and lysyl oxidase-like protein 1 (LOXL1) to the pathological niche.

Explanted lung tissue from two cohorts of end-stage BOS patients (n=15) was stained using histological stains such as hematoxylin-eosin, Verhoeff-Van Gieson and pentachrome stains to systematically identify all airways and classify them based on the ratio of fibrosis and immune cell infiltration in the subepithelial layer.

The airways exhibited large heterogeneity in the degree of obstruction and remodelling. We identified a total of 309 small airways, which we classified into five groups: (A1) unobstructed small airways, (A2) partially obstructed, immune-active small airways, (A3) partially obstructed, fibrotic small airways, (A4) partially obstructed, mixed small airways, and (A5) completely obstructed small airways (Figure 8 A). The most abundant groups were the partially obstructed, fibrotic airways (A3, n=168) and the completely obstructed airways (A5, n=78), whereas the least frequent were the mixed phenotype airways (A4, n=19). Pentachrome stain highlighted significant submucosal thickening with collagen- and cell-rich tissue (Figure 8 A).

Periostin, LOXL1 and LOX were expressed across all examined groups of airways. Periostin (Figure 8 B) showed the highest median positive stained area (42-43%) in all groups of partially obstructed airways (A2-4), with spread-out pattern within the ECM of the subepithelial layer. In contrast, in unobstructed airways (A1), periostin was more confined near the basement membrane, with a lower median positive area (31%). Its expression was lowest in completely obstructed airways (A5), with a median positive area of 19%.

Similarly, LOXL1 (Figure 8 C) displayed a consistent median positively stained area (42–44%) across all partially obstructed airways (A2–A4). Conversely, its expression was highest in unobstructed airways (A1, 61%) and second highest in completely obstructed airways (A5, 56%).

LOX (Figure 8 D) exhibited a spatial distribution similar to LOXL1, though its expression varied across the airway groups. The partially obstructed airways with mixed phenotype (A4) had the lowest positively stained area (39%), whereas the

partially obstructed, immune-active (A2) displayed the highest (67%) positively stained area.

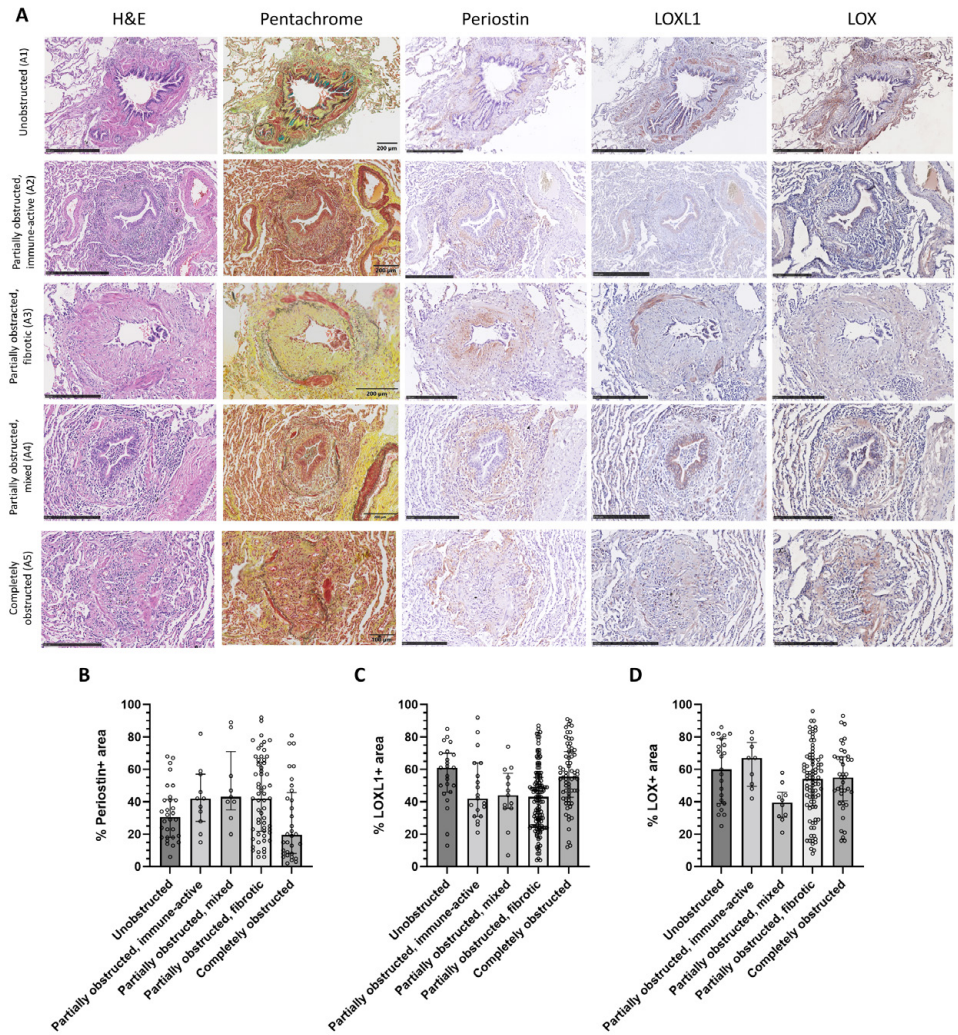


Figure 8. Periostin, LOXL1 and LOX spatial expression in differentially remodeled small airways in lung explants of BOS patients.

A) Images of serial sections shown are stained for H&E, pentachrome, periostin, LOXL1 and LOX proteins. Pentachrome highlights elements of connective tissue (collagen – yellow, elastic fibres – black, mucins – green to blue). B-D) Quantification of percentage of protein-positive area is calculated in relation to the whole tissue area within ROI of each airway. Median and interquartile range are shown for each graph.

In conclusion, our findings support the involvement of periostin, LOX and LOXL1 in the pathological remodelling of small airways in BOS. This involvement could contribute to enhanced collagen fibrillogenesis and crosslinking, leading to local stiffening of the tissue. This process may further perpetuate a profibrotic feedback loop, underscoring the need for additional research in this area.

General discussion and conclusions

Over the last decades, the prevalence and incidence of various forms of pulmonary fibrosis have been on the rise. Despite this increase, no cure currently exists for these fibrotic diseases. The antifibrotic medications nintedanib and pirfenidone, which are used in the treatment of IPF, have only succeeded in slowing the disease progression and are often associated with severe adverse effects [127]. Moreover, the precise therapeutic mechanism of pirfenidone, as well as the overall molecular pathways underlying the pathogenesis of these diseases, are still not fully uncovered.

The lung epithelial barriers in COVID-19, IPF and BOS are thought to be critical initial sites of disease development [128]. Epithelial cells within these barriers undergo repetitive microinjuries caused by inhaled toxins, pollutants, and pathogens, as well as by intrinsic factors related to ageing, genetics and the immune system, or unknown factors in the case of IPF. This damage triggers a dysregulated tissue repair response at the epithelial-mesenchymal interface, including disrupting the basement membrane and activating immune players that drive inflammation and mesenchymal cells that produce excessive amounts of extracellular matrix. This process leads to increased tissue stiffness, which aberrantly alters the alveolar and airway epithelial-mesenchymal niches including the normal cells, further enhancing tissue remodelling and the profibrotic feedback loop.

Research in the pulmonary fibrosis field has long been centred around fibroblasts, the primary cells responsible for producing ECM under normal conditions and exaggerated ECM deposition in pulmonary and airway fibrosis. Interestingly, in the past years, other cell types have also been recognized as important players in disease pathogenesis [53,129,130]. Furthermore, the diseased lung microenvironment, along with changed biomechanical properties have a crucial impact on directing the cellular behaviour towards abnormal responses [56]. However, there are still significant gaps in our knowledge of the ECM composition and properties of the pathological niches in IPF, BOS and COVID-19, promoting the abnormal activity of not only fibroblasts but also the other cell types.

This thesis focused on characterizing some of the cellular or ECM components of the complex pathological microenvironment driving the lung tissue remodelling to improve our understanding of the progression of pulmonary and airway fibrosis. Furthermore, the thesis sought to identify proteins in the systemic circulation that

would mirror early remodelling and inflammation processes in the lung and could thus serve as potential biomarkers for better diagnostics and prognostics.

By using a combination of histological and molecular techniques exploring both RNA and protein levels solely in human material, we found substantial lung tissue remodelling in end-stage IPF and post-COVID-19 lung fibrosis, in addition to heterogeneous small airway remodelling in BOS. Increased expression of minor collagen type VII in histopathological features of IPF and altered expression of periostin, LOX and LOXL1 in small airway obliteration indicate their involvement in pathological tissue rebuilding. Despite differences in the trigger and disease manifestation between IPF and COVID-19/post-COVID-19 exist, they also exhibit several similarities in terms of the *in situ* lung and ECM remodelling and altered levels of systemic proteins, with LAMP3 and MMP7 showing correlations to the lung function decline.

Animal models, particularly rodents, remain the gold standard for preclinical research in lung diseases, yet they fail to accurately replicate the chronic and progressive nature of IPF [131]. The most commonly used model involves bleomycin (BLM)-induced lung injury, which triggers acute inflammation followed by the development of fibrosis but lacks the gradual ECM remodelling, persistent fibrosis, and long-term disease progression seen in human patients. These limitations, along with significant interspecies differences in lung anatomy, immune responses, and fibrosis resolution, contribute to the high failure rate of potential therapeutics in clinical trials [132,133]. Given these challenges, studying human explanted lung tissue provides a clinically relevant approach to understanding disease mechanisms, as it allows direct analysis of end-stage fibrosis, ECM alterations, and cell interactions in human lungs, offering valuable insights that cannot be captured in animal models.

It should be acknowledged that despite not being a primary focus in this study, the immune system is significantly involved in pulmonary and airway fibrosis, especially in the bronchiolitis obliterans syndrome [134]. Notably, several immune cells release pro-inflammatory cytokines such as TGF-β1 that activate fibroblasts, driving the excessive ECM deposition [135]. On the other hand, ECM fragments such as fibronectin, vitronectin, fibrinogen, and collagen are known to activate immune cells [136].

Perspectives on future development

This thesis contributes relevant insights into the complex pathological puzzles underlying the diseases studied herein. However, many aspects remain undiscovered, and a complete understanding of disease pathogenesis is still needed to ultimately improve patients' quality of life. Building on the findings in this thesis, the following proposed experiments could further expand our knowledge of the identified molecules and, ultimately, the fibrotic diseases.

In **Paper I**, collagen VII was characterized in detail regarding its localization, cellular expression and, partially, the possible regulatory mechanism by TGF-β1 in both normal and IPF lungs. It would be interesting to investigate the role of collagen VII alongside other hemidesmosomal proteins to examine how they mechanistically influence basal cell behaviour, potentially contributing to the aberrant re-epithelialization of the fibrotic tissue. As the non-functional gene for Col7a1 in mice is lethal [137], Col7a1-hypomorphic or inducible Col7a1 knockout mouse models could be used to study the effects of the absence of functional collagen VII [138]. If even feasible, this model could be combined with the instillation of IPF patient-derived basal cells and BLM treatment – a combination that has been shown to better replicate honeycombing formation compared to traditional BLM models [3,139].

As an alternative to *in vivo* studies, coating of tissue plates with the individual proteins might be used to study, for instance, how these proteins affect the proliferation and migration of primary human bronchial epithelial cells (HBECs) or immortalized basal cells *in vitro*. Furthermore, more advanced culture systems, such as air-liquid interface (ALI) culture and organoids, are available. The ALI culture exposes the apical surface of cells to air while keeping the basal surface immersed in culture medium to mimic the lung environment, and it has been applied to study the differentiation of basal cells into pseudostratified epithelium [140]. This method, along with culturing organoids might be used to study the temporal aspect of the gene and protein expression of collagen VII and other related genes. For instance, to determine whether they are needed for the differentiation into the pseudostratified epithelium or are already expressed earlier and how this process would be affected with silenced COL7A1 gene. These culture conditions could also be further tweaked with an IPF-relevant cocktail of cytokines to simulate a profibrotic environment [141].

Moreover, as UIP is not the histopathological pattern only for IPF, but it is also present in other ILDs such as the ones associated with rheumatoid arthritis and systemic sclerosis, it is plausible that lung collagen VII is altered in these chronic conditions as well. Notably, increased collagen VII levels were detected in serum and skin of patients with systemic sclerosis, regulated by TGF-β1 [142,143].

Similarly, in **Paper IV**, periostin, LOX and LOXL1 might be further studied mechanistically, whether they truly contribute to collagen fibrillogenesis and crosslinking of the fibrotic obstruction in the small airways, therefore enhancing stiffening of the tissue and perpetuating the fibrotic positive feedback loop. Also, it remains unclear if there is any spatial-temporal relationship between periostin and LOX(L) proteins in the BOS-affected airways that contribute to the remodelling. Notably, we identified four types of obstructed airways, distinguished by the presence or absence of subepithelial immune cell infiltrate and fibrosis, along by the extent of obstruction. Investigating which airway type predisposes patients to rapid lung rejection progression and whether this correlates with altered protein profiles in serum could provide valuable prognostic insights. Periostin has been previously detected in the serum of BOS patients at significantly higher levels compared to stable BOS patients and controls, with a positive correlation to declining FEV_1% [144]. Additionally, periostin levels were significantly elevated at the onset of BOS compared to those measured one-year post-transplantation in the BOS group. Thus, it would be of interest to study serum periostin levels along with LOX and LOXL1, whether they could stratify patients with fast or slow progression.

In relation to **Paper II** and **III,** as the primary goal of these studies was to identify early systemic markers of lung damage or remodelling, the serum proteins found in these studies should be validated in larger, independent studies. We identified DCN and LAMP3 as potential biomarkers for early development of lung fibrosis. It might be of interest to further study their relevance and role in the lung tissue using *in vitro* and *in vivo* approaches. For instance, LAMP3 has been widely used as an established marker of AT2 cells in the RNA-seq data, yet the data on the function of this lysosome-associated protein and how it is affected in pulmonary fibrosis is scarce.

Taken together, by further characterizing the cellular and ECM proteins identified in this study and many other currently unrecognized proteins involved in tissue remodelling in the pathological microenvironment including the basement membrane, we can build a solid foundation for understanding the molecular mechanisms driving pulmonary and airway fibrosis. In the long run, this may help us develop better diagnostic and prognostic tools and new patient-tailored treatments.

Popular scientific summary

In the past decades, the number of individuals suffering from chronic lung diseases such as idiopathic pulmonary fibrosis (IPF) has increased worldwide. Pulmonary fibrosis is a condition characterized by scarring of the lung tissue (fibrosis), leading to breathing difficulties and reduced oxygen uptake. This condition, after experiencing COVID-19 infection, has also emerged as one of the most severe long-term health complications after the COVID-19 pandemic. For patients with end-stage disease, a lung transplant may be the only option, but even then, long-term survival can be compromised by bronchiolitis obliterans syndrome (BOS). This severe form of chronic lung rejection, clinically termed chronic lung allograft dysfunction, is marked by the formation of scar tissue inside airways, causing their progressive closure and the deteriorating function of the transplanted lung over time. A major challenge in tackling pulmonary fibrosis and BOS is the lack of effective treatments and reliable biomarkers for early diagnosis.

Our lungs are very complex organs with branching airway and vascular trees terminating in pulmonary sacs, alias alveoli, which are the primary site of vital gas exchange between inhaled air and body blood circulation. The air-blood barrier surface area is roughly equivalent to a badminton court and is roughly 100 times thinner than a human hair to enable the efficient diffusion of oxygen and carbon dioxide. The lungs are formed by a large spectrum of specialized cells and intertwined meshwork of molecules of extracellular matrix (ECM) that provide cells with the essential structural support and critical signals to survive and function. Under normal circumstances, the lungs have a relatively great ability to repair themselves. However, repeated damage to the airway mucosal barrier and epithelial cells lining pulmonary sacs - such as from bacteria, viruses, pollution, or immune reactions – can disrupt normal healing processes. Instead of normal repair, the lung tissue starts forming excessive scar tissue, which leads to stiffening of the lungs, making it harder to breathe and resulting in gradual lung failure. However, the molecular mechanisms behind these abnormal processes, especially in diseases like IPF, post-COVID-19 pulmonary fibrosis, and BOS, are not fully elucidated.

The research in my thesis aimed to improve our understanding of tissue remodelling and ECM structural and molecular changes, particularly at the interface of mucosal and epithelial barriers and the underlying tissue that contribute to the progression of these fatal diseases. By studying human lung tissue samples at the gene and protein levels, we found significant structural changes in end-stage IPF and post-COVID-

19 pulmonary fibrosis, as well as in the small airways in BOS. Specifically, we found increased levels of collagen VII in remodelled lungs of IPF patients and altered levels of proteins like periostin, LOX, and LOXL1 in obstructed small airways in the lungs of BOS patients, suggesting they may play a role in lung scarring by promoting the abnormal remodelling.

Diseased lungs release various proteins into the bloodstream and identifying these could provide valuable biomarkers for early detection. Although IPF and post-COVID-19 fibrosis have different causes and outcomes, they show some similar patterns of lung damage, including altered localization or levels of proteins such as LAMP3, periostin and decorin and changed blood proteins such as LAMP3, MMP-7, DCN, TNFRSF12A, CXCL13, CXCL9, HGF and MCP-3 pointing to potential common targets for future investigations and clinical use.

The knowledge from this thesis and uncovering other molecular changes associated with disease progression may bring us one step closer to better diagnostic tools, improved patient monitoring, and new treatment strategies to combat lung fibrosis and transplant-related lung remodelling.

Populärvetenskaplig sammanfattning

Under de senaste decennierna har antalet individer som lider av kroniska lungsjukdomar såsom idiopatisk lungfibros (IPF) ökat globalt. Lungfibros är ett tillstånd som kännetecknas av ärrbildning i lungvävnaden (fibros), vilket leder till andningssvårigheter och nedsatt syreupptag. Detta tillstånd har, efter genomgången COVID-19-infektion, också uppmärksammats som en av de allvarligaste långsiktiga hälsokomplikationerna efter COVID-19-pandemin. För patienter med långt framskriden sjukdom kan en lungtransplantation vara det enda alternativet, men även då kan den långsiktiga överlevnaden påverkas negativt av bronkiolit obliteranssyndrom (BOS). Denna allvarliga form av kronisk lungavstötning, kliniskt benämnd som kronisk lungallograftdysfunktion, kännetecknas av ärrbildning inne i luftvägarna, vilket leder till att de gradvis stängs igen och att funktionen i den transplanterade lungan försämras över tid. En stor utmaning i arbetet med att behandla lungfibros och BOS är bristen på effektiva behandlingar och tillförlitliga biomarkörer för tidig diagnos.

Våra lungor är mycket komplexa organ med förgrenade luftvägar och blodkärlsträd som mynnar ut i lungblåsor, så kallade alveoler, vilka är den primära platsen för det livsnödvändiga gasutbytet mellan inandad luft och kroppens blodcirkulation. Luft-blodbarriärens yta motsvarar ungefär en badmintonbana och är ungefär 100 gånger tunnare än ett människohår för att möjliggöra effektiv överföring av syre och koldioxid. Lungorna är uppbyggda av ett stort spektrum av specialiserade celler och ett sammanvävt nätverk av extracellulär matrix (ECM)-molekyler som ger cellerna nödvändigt strukturellt stöd och viktiga signaler för att överleva och fungera. Under normala förhållanden har lungorna en relativt god förmåga att reparera sig själva. Men upprepad skada på slemhinnans barriär och de epitelceller som täcker lungblåsorna – till exempel från bakterier, virus, föroreningar eller immunreaktioner – kan störa de normala läkningsprocesserna. Istället för normal reparation börjar lungvävnaden bilda överdriven ärrvävnad, vilket leder till att lungorna stelnar, gör det svårare att andas och slutligen orsakar gradvis lungsvikt. De molekylära mekanismerna bakom dessa oreglerade processer, särskilt vid sjukdomar som IPF, post-COVID-19-lungfibros och BOS, är dock ännu inte helt klarlagda.

Denna forskning syftade till att förbättra vår förståelse för vävnadsombyggnad och förändringar i ECM, särskilt vid gränssnittet mellan slemhinnebarriärer och epitel samt underliggande vävnad som bidrar till dessa dödliga sjukdomar. Genom att studera mänskliga lungvävnadsprover på gen- och proteinnivå fann vi betydande

strukturella förändringar vid slutskede IPF och post-COVID-19-lungfibros, liksom i de små luftvägarna vid BOS. Specifikt fann vi ökade nivåer av kollagen VII i lungorna hos IPF-patienter och förändrade nivåer av proteiner såsom periostin, LOX och LOXL1 i de blockerade små luftvägarna hos BOS-patienter, vilket tyder på att dessa kan spela en roll i ärrbildning, genom att främja onormal vävnadsombyggnad.

Sjuka lungor frisätter också olika proteiner i blodomloppet, och identifiering av dessa skulle kunna ge värdefulla biomarkörer för tidig upptäckt och diagnos av sjukdom. Även om IPF och post-COVID-19-fibros har olika orsaker och förlopp, uppvisar de vissa liknande mönster av lungskada, inklusive förändrad lokalisering eller nivåer av proteiner som LAMP3, periostin och DCN samt förändrade blodproteiner såsom LAMP3, MMP-7, DCN, TNFRSF12A, CXCL13, CXCL9, HGF och MCP-3, vilket pekar på potentiella gemensamma målmarkörer för dessa sjukdomar och för i framtida klinisk användning.

Kunskapen från denna avhandling och upptäckten av molekylära förändringar som är förknippade med sjukdomsprogression kan föra oss ett steg närmare bättre diagnostiska verktyg, förbättrad patientövervakning och nya behandlingsstrategier för att bekämpa lungfibros och transplantationsrelaterad lungombyggnad och förändringar.

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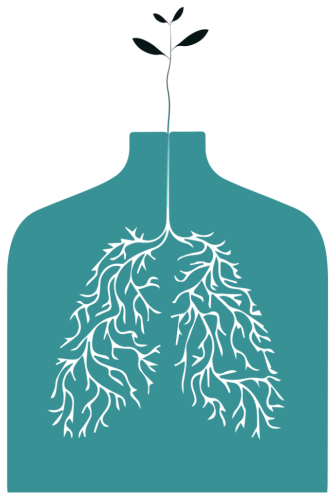

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