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IDIOPATHIC PULMONARY FIBROSIS: DISEASE PRESENTATION, CLINICAL COURSE AND POTENTIAL BIOMARKERS

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Idiopathic pulmonary fibrosis: disease presentation, clinical course and potential biomarkers

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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For the patients living with IPF

To my beloved family

POPULAR SCIENCE SUMMARY OF THE THESIS

Idiopathic pulmonary fibrosis (IPF) is a detrimental, chronic lung disease of unknown cause with an average life expectancy of approximately 3-5 years after diagnosis if no treatment is initiated. IPF is characterized by an altered lung architecture as a result of accumulation of scar tissue. The last decade has been crucial in the management of IPF with refinements of diagnostic criteria and approval of the first antifibrotic treatments nintedanib and pirfenidone. These drugs slow the progression of the disease but neither of them can improve lung function or quality of life.

Despite these advances, many challenges and questions remain. Aside from the elusive underlying mechanisms, we do not know why some patients are worse off than others, and what impact comorbidities have. Thus, clinicians have no tools to predict the course of the disease and the long-term prognosis. In order to obtain real world data in broader patient populations which can be a complement to clinical trials, several patient registries have been initiated around the world. The Swedish IPF registry was launched in 2014 and collects clinical data from individuals diagnosed with IPF from more than 20 hospitals in Sweden. In addition to the registry, a biobank collecting biological material such as plasma, serum and blood from included patients was started in 2016, enabling linkage of clinical features and outcomes to the pathobiology of IPF.

This thesis is based on data and biological samples from the Swedish IPF registry in order to investigate various aspects of the disease. Studies across different populations have established that IPF is a disease of male predominance. In addition, the prognosis is worse in males. These differences are insufficiently characterized and may have implications on diagnosis, prognosis and treatment. In the first project we used registry data to cross-sectionally investigate potential gender differences. Results showed that female patients with IPF had a more preserved lung function than males while males had a increased burden of cardiovascular comorbidities.

In the second project we studied data accumulated in the registry during the years 2014-2020 with the aim to provide a comprehensive view of baseline characteristics and long-term disease behavior. We also took a first-time look at data related to treatment with antifibrotics. Furthermore, we wanted to explore if patients could be assigned to specific groups, *phenotypes*, according to their similarities and differences in clinical characteristics and mortality. With this comprehensive approach, we were able to reiterate and reinforce established information with regards to clinical characteristics, prognostic measures,

treatment effects and survival. Importantly, we presented new information on disease severity beyond the established ones and how certain clinical and demographic characteristics and comorbidities may discern patients with distinct outcomes which may require particular attention.

Together with colleagues from Lund University we set up a two-part study; one experimental, and one clinical. In the experimental part, we used a model to study secreted proteins from fibroblasts in response to the aberrant environment seen in the lung in patients with IPF and compared it to fibroblasts' response in healthy lung tissue. In the clinical part, we set out to verify the experimental results in serum from patients with IPF and healthy controls, and explored both established and new biomarkers of diagnostic and prognostic potential. Proteins involved in inflammation and the build-up of the stiff environment seen in IPF were elevated both in the experimental model and in serum from IPF, supporting the experimental model as a novel method of studying mechanisms in IPF. Further, we found several proteins in serum that were associated with clinical measures of disease severity and progression, contributing with further information in an area where there is an unmet need of biomarkers that can aid in the assessment of the disease.

The type of lung damage seen in IPF has also been observed in patients with rheumatoid arthritis (RA) and have raised questions on potential mechanistic similarities. In collaboration with a research group at Karolinska Institutet, we investigated the occurrence of specific proteins related to RA in serum from patients with IPF and compared it to RA-patients and healthy controls. We found that almost half of patients with IPF had presence of self-recognizing antibodies (autoantibodies) indicating that autoimmunity may play a role in a subset of patients with IPF.

By using registry data and results from analyses of biological samples from patients with IPF, this thesis has strived to contribute with further knowledge regarding the patient population, disease behavior and biological mechanisms that are of importance in the development and course of the disease. We were able to provide information that demonstrates the complexity and heterogeneity observed in the clinic and increased our knowledge of disease mechanisms. Taken together, our results may contribute to improved diagnostic and prognostic procedures, aid in the clinical management of patients with IPF but also lay ground for further research on this patient cohort.

ABSTRACT

The interest and research within the area of the fatal lung disease idiopathic pulmonary fibrosis (IPF) has increased exponentially over the past decades. This has resulted in important insights into the nature and pathogenesis of the disease, which ultimately constitute the knowledge base upon which diagnostic, management, treatment decisions and guidelines are shaped. With a prognosis worse than many cancers and a complete absence of curative treatments apart from lung transplantation, the introduction of the first approved treatments, although not curative but disease modifying, has been a major leap forward for patients and the research community.

Despite the increasing interest, many questions still remain unanswered. Observational studies based on registry data can provide important complementary information to data generated in clinical trials about the patient population, disease behavior and treatment. Combining clinical data with biological samples such as serum and lung tissue from patients with IPF, enables studies where associations between biological processes and the clinical behavior can be explored.

This thesis attempts to address open questions in IPF by using data from patients enrolled in the Swedish IPF registry and samples from its biobank. The studies can be divided in two parts: In the first part, we have explored potential gender differences. Further, we have taken a comprehensive look into the patient population and explored patient characteristics, disease severity, evaluated antifibrotic treatment and discerned potential disease phenotypes with distinct disease trajectories. The second part comprises studies where we have leveraged register data with results generated from analyzes of serum samples. In these studies, we have taken different approaches in order to profile the repertoire of autoantibodies and proteins present. We demonstrate, both in an *in vitro* cell culture model and in patient serum, how proteins related to remodeling, inflammation and cell recruitment are upregulated in IPF and we describe their associations to disease severity and progression. Investigating the presence of antibodies related to rheumatoid arthritis in IPF revealed how autoimmune mechanisms are active and might play a role in a subgroup of IPF patients. Taken together, these discoveries contribute to the field by expanding established observations while also generating results and hypotheses that warrants further studies to refine and improve our understanding of IPF.

LIST OF SCIENTIFIC PAPERS

I. **Kalafatis D**, Gao J, Pesonen I, Carlson L, Sköld M, Ferrara G. *Gender differences at presentation of idiopathic pulmonary fibrosis in Sweden.* BMC Pulmonary Medicine 2019, 19, 222

II. Gao J, **Kalafatis D**, Carlson L, Pesonen I, Li CX, Wheelock Å, Magnusson JM, Sköld M, *Baseline characteristics and survival of patients of idiopathic pulmonary fibrosis:a longitudinal analysis of the Swedish IPF registry*. Respiratory Research 2021, 22:40

III. **Kalafatis D***, Löfdahl A*, Näsman P, Dellgren G, Wheelock ÅM, Elowsson Rendin L, Sköld M †, Westergren-Thorsson G †. *Distal Lung Microenvironment Triggers Release of Mediators Recognized as Potential Systemic Biomarkers for Idiopathic Pulmonary Fibrosis.* International Journal of Molecular Sciences 2021, 22, 13421

IV. **Kalafatis D**, Joshua V, Hansson M, Mathsson-Alm L, Hensvold A, Sköld M. *Rheumatoid Arthritis Related Antibodies in Idiopathic Pulmonary Fibrosis. Manuscript*

* These authors share first authorship

† These authors share senior authorship

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Pesonen I, Gao J, **Kalafatis D**, Carlson L, Sköld M, Ferrara G. *Six-minute walking test outweighs other predictors of mortality in idiopathic pulmonary fibrosis. A real-life study from the Swedish IPF registry.* Respiratory Medicine:***X*** 2020, 2, 100017

II. Durheim MT, Bendstrup E, Carlson L, Sutinen EM, Hyldgaard C, **Kalafatis D**, Myllärniemi M, Sköld C.M, Sjåheim T. *Outcomes of patients with advanced idiopathic pulmonary fibrosis treated with nintedanib or pirfenidone in a real-world multicentre cohort*. Respirology 2021,26:982–988

CONTENTS

LIST OF ABBREVIATIONS

AE	Acute exacerbation
ACPA	Anti-citrullinated protein antibody
Anti-CCP	Anti-cyclic citrullinated peptide
BAL	Bronchoalveolar lavage
CPI	Composite physiological index
DLCO%	Diffusing capacity of carbon monoxide, % predicted
ECM	Extracellular matrix
FDR	False discovery rate
FEV_1%	Forced expiratory volume during 1 second, % predicted
FVC%	Forced vital capacity, % predicted
GAP	Gender-Age-Physiology
HC	Healthy control
HRCT	High resolution computed tomography
IIP	Idiopathic interstitial pneumonia
IQR	Interquartile range
IL	Interleukin
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
KBILD	King's brief interstitial lung disease questionnaire
NPX	Normalized protein expression
PEA	Proximity extension assay
QoL	Quality of life
RA	Rheumatoid arthritis
TLC%	Total lung capacity, % predicted
UIP	Usual interstitial pneumonia
6MWT	6-minute walk test

1 INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of the idiopathic interstitial pneumonias (IIP), a subgroup of disorders belonging to a heterogenous and large group of lung diseases termed interstitial lung diseases (ILD) [1]. IPF is by definition, a fatal chronic progressive fibrosing ILD of unknown etiology, characterized anatomically by scarring of the lung parenchyma due to an excessive deposition of extracellular matrix components such as collagen. Patients with IPF usually present with exertional dyspnea, chronic dry cough and a deteriorated lung function. Over time, the progressive impairment of lung function results in restricted ventilation, hypoxemia, decreased quality of life, respiratory failure and death, usually within 3-5 years from diagnosis [2]. Epidemiological studies have identified several environmental and occupational risk factors that may increase the risk of developing IPF. These include smoking, metal or wooden dust, pollution, gastric aspiration and infections [3]. In addition, approximately 10-20% of patients experience acute exacerbations, i.e. episodes of acute, clinically significant respiratory deterioration of unknown cause. Exacerbations may result in death in patients within months after symptom onset [4]. Despite its incompletely understood etiology and pathogenesis, two antifibrotic therapies, pirfenidone and nintedanib, have proven to be effective in slowing the disease progression and are now approved [5, 6].

Precise epidemiological estimates of the prevalence and incidence globally have been difficult to compare due to differences in methodologies and disease classifications. The estimated incidence in Europe and North America, ranges between 2.8-19 cases/100 000 people per year [7, 8]. A recent study investigating the incidence and prevalence in Sweden between the years 2001- 2015, reported an increasing prevalence from 15.4 to 68.0 /100 000 and a stable incidence of 10.4-15.4/100 000 per year [9]. There is evidence to suggest that the incidence of IPF is increasing [7] and with life expectancy increasing worldwide, the impact of IPF is expected to rise with increased demand on economic and healthcare resources [10].

In spite of the introduction of new treatments, IPF is still considered an incurable disease requiring a complex and comprehensive approach. The disease mainly affects elderly men with a history of smoking and a significant burden of comorbidities which have been reported in up to 89% of IPF patients [11]. Studies further demonstrate comorbidities' increased prevalence compared to matched controls and the general population [12, 13] as well as their negative influence on mortality and quality of life [13–15]. This means that optimal management includes not only identification and treatment of comorbidities, but also non-pharmacological interventions such as pulmonary rehabilitation, supplemental oxygen, lung transplantation and palliative care [16]. IPF is a male predominant disease with worse prognosis in males [17, 18], which raises questions on sex-dependent differences in biology, disease severity and clinical presentation.

Furthermore, as IPF is a heterogenous disease with significant variability in disease course [19], clinicians are faced with great challenges in making accurate predictions of future disease behavior with the currently available clinical measures. Biomarkers that could improve diagnostic accuracy, treatment allocation and predict future disease behavior would constitute a big leap forward for both patients and clinicians.

Patient registries enables collection of longitudinal data where demographics, clinical characteristics and disease severity can be studied. These data are complementary to clinical trials and offer an opportunity to validate findings from other cohorts and discover new aspects of the disease. When a biobank is included within the framework of a registry it extends the possibilities by allowing linkage of pathobiological data to clinical information.

2 BACKGROUND

2.1 THE RESPIRATORY SYSTEM – STRUCTURE AND FUNCTION

The lung can do many things. However, its main function is to allow oxygen to enter the circulation and remove carbon dioxide. To reach the gas exchange area of the lung, air is transported through the airways which is composed of branched tubes that become more and more narrow the deeper we reach into the lung [20]. Thus, air is transported through the nasal cavity to the trachea which divides into the left and right main bronchi, followed by the terminal bronchioles which divide into respiratory bronchioles and finally the alveolar ducts.

The exchange between blood and air occurs in these most distal parts of the lung by a process termed passive diffusion, which means that gases move across areas of high to low pressure without requiring energy [20]. The part of the lung responsible for this exchange, the blood-gas barrier is located in small air sacs called alveoli. This area works tremendously well with its exceptional thinness, covering a surface area of approximately 50-100 m^2. The human lung contains approximately 500 million alveoli, all surrounded by pulmonary capillaries that facilitate the transportation of oxygenated and deoxygenated blood.

A basement membrane is lining the airways, alveoli and capillaries where epithelial and endothelial cells are attached [21–23]. The basement membrane is made of a thin sheet of extracellular matrix (ECM) which is a complex network made up of collagens, glycoproteins, proteoglycans and different proteins which depending on their configuration, create specific microenvironments for cells. Having an important role as provider of structural support, the basement membrane also functions as a binding site for cytokines and growth factors with the ability of modulating cellular activity.

The respiratory epithelium forms a physical barrier and constitute the first line of defense against pathogens. Lining the trachea, bronchi and bronchioles are multiple cell types, dominated by ciliated cells followed by the secretory goblet and club cells [24]. Together they cooperate in response to environmental insults and pathogens by synthesizing and secreting antimicrobial proteins and mucins into the airway lumen, facilitating the mucociliary clearance of pathogens. Beneath the epithelial layer are basal cells which serve as progenitors to both ciliated and secretory cells and have a crucial role in regenerating the epithelium.

Meanwhile, only two cell types line the alveoli, the alveolar epithelial type 1 and type 2 cells. Alveolar epithelial type 1 cells cover the majority of the alveolar surface, localized in close proximity to the pulmonary capillaries, and constitutes the area for gas exchange. Alveolar epithelial type 2 cells produce lipid rich surfactant that reduces surface tension and prevents collapse of the lung, while also having an important role in homeostasis as progenitor cells to the type 1 cells [25]. Between the alveoli and the capillaries lies the interstitium, consisting of ECM. This specific space is the location of the deposition of ECM proteins resulting in the remodeled, thickened interstitium and impaired gas diffusion observed in IPF.

2.2 IPF PATHOGENESIS

For a long time, the pathophysiological concept in IPF was that of chronic inflammation being the precursor to the progressive fibrotic remodeling of lung tissue. Over the last decade and after failed clinical trials of anti- inflammatory and immunosuppressive agents [26–28], this view has shifted. In the current paradigm, persistent and recurrent microinjuries (caused by e.g. smoking, gastroesophageal reflux, infections) to the alveolar epithelium in genetically susceptible individuals causes increases in cell death, abnormal epithelial repair and dysregulated crosstalk between epithelium and fibroblasts promoting persisting activation of mesenchymal cells and ECM deposition [29]. The dysfunctional alveolar epithelium is a key factor in the initiation of disease with the type 2 alveolar epithelial cell having a central role due to its regenerative ability of the alveolar epithelium. Activation of cells within the alveoli and the epithelium leads to release of a plethora of mediators that stimulate the migration, proliferation and differentiation of lung fibroblasts into myofibroblasts, a proliferative, contractile and secretory cell type regarded as the main effector and the major source of collagen and ECM components.

2.2.1 Genetic susceptibility to IPF

Early reports from genetic studies in patients with pulmonary fibrosis in other diseases such as familial pulmonary fibrosis [30, 31] and a subset of patients with dyskeratosis congenita (a disease of premature aging) who have developed UIP [32], led to important advances in the understanding of genetic susceptibilities in the development of pulmonary fibrosis. A dominant underlying cause of IPF has not been identified yet, but several genetic factors have been established. The most prominent one is a single nucleotide polymorphism in the gene coding for the secreted mucin *MUC5B*, important in the mucociliary clearance of pathogens [33]. Considered the strongest risk factor for the development of IPF, overexpression of *MUC5B* is found in bronchoalveolar epithelium and while its role in the pathogenesis of IPF is still unclear, it has been hypothesized that it either enhances injuries due to reduced clearance or inhibits repair mechanisms arising as a consequence of the damaged epithelium in the distal parts of the lung [33, 34]. Paradoxically, although carriers of the allele have an increased risk of developing IPF, patients with the allelic variant have a later onset of disease and decreased mortality [35]. Of interest, the mutation has also been observed in patients with rheumatoid arthritis (RA) that have developed RA-ILD with a HRCT-determined UIP-pattern [36]. This finding provides evidence for shared genetic background and raises the question of shared pathogenic mechanisms.

Other genetic variants found in IPF and other ILD include mutations affecting telomere maintenance (*TERT, TERC, RTEL, DKC1, PARN*) [37–39], leading to accelerated aging and activation of cell senescence mechanisms; mutations affecting surfactant proteins' composition and metabolism (*SFTPC, SFTPA2, ABCA3*), leading to among others endoplasmatic reticulum (ER) stress and activation of the unfolded protein response and apoptosis of alveolar epithelial type 2 cells [31, 40–44]; mutations related to innate immunity (*TOLLIP*) and age and smoking caused defects in proteostasis (i.e. processes handling protein folding, unfolding, degradation) resulting in ER-stress, mitochondrial dysfunction (*PINK1, DIO2*) and aberrant autophagy and mitophagy in alveolar epithelial type 2 cells [45–47].

2.2.2 Influence of the local microenvironment and immune system

While the dysfunctional alveolar epithelium is proposed to be key in the initiation of disease, factors that determine the persistent and progressive fibrotic deposition remain poorly delineated, although studies of the local microenvironment, the immune system and cell intrinsic factors have provided important clues. Previously, it was thought that the ECM only served as structural support of end-stage fibrosis. Now, there is evidence that deposition of collagens and other components into the ECM, with subsequent changes in the mechanical characteristics, act as powerful regulators of cell behavior and amplifiers of profibrotic feedback loops [48, 49]. Understanding these mechanosignalling pathways and how they influence the migration and activation of fibroblasts might provide us with new treatment targets.

Despite its questioned role in disease initiation, studies on inflammation in IPF has evolved the role from causal to disease modulating that either promote or suppress fibrogenesis [50]. For example, macrophages and macrophage-driven pathways is one of the most studied immunopathogenic cell types with reports of fibrosis-stimulating properties since several decades [51]. As the most abundant immune cell in the lung, macrophages play important roles in the remodeling processes seen in pulmonary fibrosis [52–54]. Similar to the dysfunctional epithelial cells, macrophages release profibrotic mediators such as transforming growth factor-beta (TGF-β) and platelet derived growth factor (PDGF) which have direct effects on fibroblasts by inducing fibrocyte and fibroblast migration, proliferation and their differentiation into myofibroblasts.

T- and B-lymphocytes are increased in IPF lung tissue and BAL-fluid compared to healthy individuals [55–57]. Overexpression of genes related to B-cells have been demonstrated in lungs from IPF patients as well as aggregates of B-cells close to fibroproliferative lesions [57–59]. Accumulating B-cells in diseased tissue areas are signs of ongoing immune activity and exert pathogenic effects [60]. The breakdown of immune tolerance within the adaptive immune system, termed autoimmunity, is characterized by the secretion of antibodies by B-cells (“autoantibodies”) against self-antigens. Although IPF patients by definition do not fulfill criteria of autoimmune diseases, immunoglobulins towards self-antigens can be found in a substantial proportion of patients and target mostly epithelial antigens [61–65]. Altogether, this proposes humoral autoimmunity against autoantigens’ activity and role in promoting inflammatory mechanisms and aberrant repair mechanisms in IPF.

2.3 DIAGNOSING IPF

An official statement on the approach to IPF diagnosis was first described in international guidelines in 2002 [66]. Since then, guidelines have been updated in 2013 [1] and 2018 [2]. A couple of weeks ago, an updated version was published which included progressive pulmonary fibrosis [67]. The guidelines provide precise criteria on diagnosis based on clinical, radiological and histopathological features. In addition, the guidelines established the multidisciplinary discussions as the gold standard for deciding on an IPF diagnosis, i.e. an active discussion of accumulated clinical information among experienced pulmonologists, pathologists and radiologists.

Establishing a diagnosis of IPF requires the exclusion of known causes of interstitial lung disease (ILD) such as environmental exposures, medications and systemic inflammatory disorders. Also, an usual interstitial pneumonia (UIP) pattern on high resolution computed

tomography (HRCT) or surgical lung biopsy is required[2]. HRCT play an essential role in the evaluation of patients with ILD, and can be diagnostic in the appropriate clinical context. Typical HRCT-features of UIP are basal, subpleural distribution of honeycombing (i.e. clustered cystic air spaces), with or without traction bronchiectasis (i.e dilated bronchi due to retractive forces caused by fibrosis of the lung parenchyma) and bilateral reticular patterns (i.e. thickening of inter-and intralobular septa)[2].

Surgical procedures such as lung biopsies are warranted when the combined clinical and imaging data is discordant of a diagnosis of IPF [2]. However, such invasive procedures are not indicated in older patients with high burden of comorbidities or patients presenting with advanced disease due to increased morbidity and mortality risks. Complications of surgical lung biopsies include pneumonia, pneumothorax, infections and acute exacerbations [68, 69]. Therefore, the benefit of the additional information gained from surgery must be weighed against the expected risks. In selected cases, transbronchial cryobiopsy may be an alternative [67]. Key pathological features of UIP include non-uniform distribution of subpleural and/or paraseptal fibrosis with intervening areas of normal lung parenchyma and presence of fibroblast foci (i.e. small clusters of active fibroblasts and myofibroblasts) adjacent to areas of dense fibrosis [2].

Spirometry play an important part both in diagnosis and the follow up of IPF. The accumulation of excessive collagen in the lung leads to increased stiffness, alterations of the mechanical properties of the lung and restrictive lung function [70]. Patients with IPF usually show a restrictive pattern, recognized by a low total lung capacity (TLC), forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO). However, lung volumes can be normal in IPF. In contrast, DLCO is usually affected in early stages of disease. Emphysema may also be present since smoking is a common risk factor for both emphysema and fibrosis. In these cases, which have a broad reported prevalence ranging from 8-51% of patients [71, 72], lung volumes are often normal or slightly decreased, while DLCO is usually severely affected.

2.4 TREATMENT OF IPF

The introduction of the antifibrotic therapies nintedanib and pirfenidone, meant a paradigm shift in the pharmacological treatment of IPF since they were the first drugs with proven evidence in slowing down lung function decline. Studies *in vitro* have shown that nintedanib have inhibitory effects on essential processes of fibrosis such as recruitment, proliferation and differentiation of fibrocytes and fibroblasts and extracellular matrix deposition, by inhibiting tyrosine kinase mediated signaling [73]. Pirfenidone's mode of action is less well defined as its specific target remains unknown, but studies in vitro suggest inhibition of profibrotic mechanisms (e.g. TGF-β signaling) in fibroblasts and fibrocytes [74, 75]. Both treatments have demonstrated a reduced decline in lung function measured by FVC by approximately 50% over a 52 week period in clinical trials [5, 6] and sustained effects across different values of FVC and in different subgroups of age, gender, race and concomitant medication [76–80]. Although clinical trials of these treatments have not been powered to show effects on acute exacerbations and mortality, increasing studies are emerging showing that antifibrotic treatments reduce the risk of acute declines in lung function [81, 82] and mortality [83–87].

Antifibrotic treatments are associated with a number of adverse events [88–90]. Nintedanib's most common side-effect is diarrhea, reported in 62% of treated patients in the INPULSIS trials [6] leading to permanent treatment discontinuation in approximately 4% of nintedanib treated patients. Pirfenidone is more associated with nausea, decreased appetite and skin-related events (rashes, photosensitivity reactions) [88, 90]. Management of side-effects is crucial to help patients stay on treatment. Dose adjustments (interruption, reductions of dose), symptomatic relief of e.g. gastrointestinal events through loperamide and hydration, minimization of sun exposure and use of high-factor sun block, are recommended to manage side-effects [91, 92].

The increasing knowledge of macrophages and their progenitor cell, monocytes, have resulted in molecules, currently in clinical trials, directly targeting these cells. Pentraxin 2, or serum amyloid P (SAP), is an acute phase reactant protein produced by hepatocytes involved in inflammation and innate immunity [93]. SAP inhibits neutrophil recruitment and monocyte differentiation into profibrotic macrophages and fibrocytes, i.e. the circulating progenitor cells of fibroblasts and myofibroblasts, capable of producing ECM and differentiating into myofibroblasts [94, 95]. Compared with controls, patients with pulmonary fibrosis, renal fibrosis, scleroderma and RA have demonstrated lower levels of SAP [93]. In patients with IPF, SAP levels are lower than in controls and positively correlated with FVC [53]. In a phase 2 trial of recombinant human SAP in IPF patients [96], SAP showed promising results by improvements in lung function, which has resulted in initiation of phase 3 trials. Inhibition of the matricellular protein connective tissue growth factor (CTGF) in patients with IPF have shown promising results in a clinical phase 2 trial [97] by demonstrating reduction in lung function decline. CTGF is secreted by various cell types including fibroblasts and myofibroblasts and interacts with important regulators of fibrosis such as TGF-β [98]. By binding to various cell surface receptors, CTGF can regulate cell signaling, cell-matrix interaction and cell adhesion. Building on the encouraging data from the phase 2 trial, the antibody inhibiting CTGF is currently tested in phase 3 trials.

Non-pharmacological interventions such as pulmonary rehabilitation, lung transplantation and supplemental oxygen, are important in the management of IPF-patients. The purpose with these interventions is to help IPF-patients live as normal as possible. Lung transplantation is the only intervention with survival benefits [99]. Long term oxygen treatment is recommended for patients with clinically relevant hypoxemia at rest using same indications as in COPD. Evidence for ambulatory oxygen treatment in fibrotic lung disease are scarce. Pulmonary rehabilitation, i.e. structured exercise programs designed for patients with IPF, has been shown to increase exercise capacity, quality of life, walking distance and reduce dyspnea in patients with IPF and is strongly recommended. [100–102].

2.5 AUTOIMMUNITY IN IPF

An important part of the diagnostic work-up of IPF is to exclude other causes of interstitial lung disease, in particular connective tissue diseases such as, rheumatoid arthritis (RA), systemic sclerosis, myositis and Sjogren's syndrome. In the case of RA, the prevalence of ILD related to RA range from 1- 50% depending on methodology and criteria used [103–105]. While the radiological pattern of ILD in connective tissue diseases (CTD) is predominated by nonspecific interstitial pneumonia pattern (NSIP), studies in patients with RA-ILD suggest that UIP pattern is more prevalent, occurring in between 40-60 % of patients with RA-ILD [106, 107], and is indicative for worse prognosis [108, 109]. Interestingly, besides the prevalence of UIP in RA, RA-ILD have several common characteristics with IPF.

These include shared genetic background through mutations in telomere maintenance genes (e.g. *TERT, PARN*) and the surfactant gene SFTPC [110], and most recently and prominently, *MUC5B [36]*. Moreover, RA-ILD shares some phenotypic risk factors including smoking [104, 111], progressive lung fibrosis and predictors for poor prognosis (age, male gender, disease severity measured by DLCO and FVC) [104, 111–113].

Exclusion of CTD are particularly important since the pulmonary manifestations can dominate the clinical picture or even present before joint symptoms appear. [114–116]. Evaluation of signs and symptoms of autoimmune disease and routine testing of serological markers such as antinuclear antibodies (ANA), antibodies against cyclic citrullinated peptides (anti-CCP), antineutrophilic cytoplasmic antibodies (ANCA) and rheumatoid factor (RF) is recommended to improve diagnostic accuracy [2]. Over the years, a number of different nomenclatures for ILD-patients with unclear systemic inflammatory disease which do not meet all criteria for being diagnosed as a CTD has been proposed. Currently, this group of patients are suggested of having interstitial pneumonia with autoimmune features (IPAF). Patients with IPAF must fulfill at least one criteria across three diagnostic domains: serologic (autoantibodies), clinical (specific extrapulmonary symptoms) and morphological (radiology and/or histopathology) [117].

Despite UIP's existence in systemic autoimmune disease, a UIP pattern alone is not a part of the criteria for a diagnosis of IPAF due to its lower prevalence in CTDs in contrast to NSIP [118] and thus does not increase the likelihood of having a CTD. Therefore, an IPF/UIP with the presence of autoantibodies does not fulfill the criteria for IPAF and will still be diagnosed and managed as IPF if the patient does not have at least one feature from the other domains.

Presence of autoantibodies in IPF patients without showing evidence of a defined CTD have been described before [119–123], using different set ups and reference values of the clinically utilized serological markers ANA, RF, ACPA and ANCA. Presence of the non-specific autoantibodies ANA and RF has been identified in 1-53% and 6-17% respectively [124], while the more specific antibodies ANCA and anti-CCP has been detected in 0-32% and 1-13%, respectively [122, 124]. Studies on their clinical significance in patients in IPF are inconclusive, although a majority of studies suggests no differences in clinical characteristics and mortality between patients with or without these autoantibodies. Great emphasis is, however, laid on the substantial heterogeneity in study design and the limited number of patients which warrants careful interpretation. Anti-CCP which is the most specific test for RA utilized in the clinic with a specificity of 98-99% [125], measures antibodies against posttranslationally modified citrullinated protein/peptides. Analyses of anti-CCP, their prevalence and clinical significance in IPF has been limited by small patient cohorts and there are few studies on their prognostic importance [119–122].

2.5.1 Citrullination and other posttranslational modifications

A majority of patients with RA are characterized by the presence of antibodies reactive to proteins that have been subjects to various posttranslational modifications [126]. These antibodies include mainly antibodies against citrullinated proteins (ACPA) but also other modifications, termed anti-modified protein antibodies (AMPA). These include, among others, acetylation [127] and carbamylation/homo-citrullination [128]. Recent studies have demonstrated cross-reactivity with ACPAs against acetylated and carbamylated proteins, suggesting a different "flavor" of ACPA reactivity [129, 130]. Autoimmunity and ACPAs are very specific for RA, and studies have shown increased levels of anti-CCP in patients with RA-ILD [131–134].

Citrullination is a posttranslational conversion facilitated by the enzyme peptidyl arginine deiminase (PAD) [135], of the positively charged amino acid arginine into citrulline which has a neutral charge. The shift in electrostatic properties makes them immunogenic in genetically susceptible individuals (HLA-DRB1 shared epitope alleles) in RA and thus targets of ACPA [126]. Citrullinated proteins have been found in BAL and lung tissue in IPF, RA-ILD and RA [136–138].

The anti-CCP test used in the clinic utilizes synthetic cyclic citrullinated peptides as a surrogate for the citrullinated proteins generated *in vivo.* Advances in RA serology techniques have demonstrated that the anti-CCP test does not capture all autoreactivities seen in RA, with multiplex methods enabling detection of multiple ACPA fine-specificities, i.e. the repertoire of reactivity against different modified epitopes on proteins, which can vary among patients [139, 140]. Studies of RA patients with ILD have demonstrated associations between the presence and number of ACPA specificities with the parenchymal abnormalities seen in ILD [141, 142].

2.6 BIOMARKERS IN IPF

Biomarkers are defined as "*characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention*" [143]. Several multidimensional prognostic models that integrate clinical, physiological and radiological variables have been validated in IPF, such as the composite physiological index (CPI) [144] and Gender-Age-Physiology (GAP) index [145]. These composite values are more accurate in predicting baseline and longitudinal mortality than individual physiological variables, while allowing stratification of patients. However, more powerful measures that are able to predict future behavior (decline in lung function or acute exacerbations) or treatment response are needed [146]. Molecular biomarkers provide insights into the underlying pathobiology of disease and allows identification of potential disease phenotypes in patients who may have different biological mechanisms causing their disease. Integrating these dynamic parameters with biological signatures of disease is needed to provide accurate measures on disease progression, stratification of disease and guide treatment. In addition, utilization of biomarkers could result in more efficient clinical trials, with biomarkers used as surrogate endpoints. Currently, no biomarkers have been implemented in the clinic, and studies are limited by small sample sizes, differences in analytical and statistical methodology and usage of different outcomes which yields inconsistent results that are difficult to generalize. Nevertheless, considerable progress has been made in detecting and proposing biomarkers with the ability to distinguish patients from healthy, reflect disease severity and predict disease progression [147, 148]. Apart from molecular biomarkers, i.e. soluble factors or cell types found in serum or plasma, biomarkers may also be genetic, with encouraging results generated in studies of genetic signatures predicting the risk of developing disease [149, 150], or image-based, using radiological procedures to predict prognosis or patients with increased risk of developing IPF [151–153].

Given the different pathological pathways and active cell types involved in IPF, the most promising molecular biomarkers are related to alveolar epithelial cell dysfunction, extracellular matrix remodeling and fibroproliferation and immune dysregulation [147]. Serum levels of markers of epithelial cell dysfunction, surfactant protein A (SP-A), D (SP-D) are elevated in ILD and IPF, and have been shown to be important predictors for outcome in IPF across different cohorts [154–159].

Similarly, Krebs von den Lungen 6 (KL-6), expressed on alveolar epithelial type 2 cells and released during cell proliferation, activation or injury, stimulates fibroblast migration, proliferation and survival and has been shown to be elevated in IPF and associated with acute exacerbations and mortality [159–162]. Biomarkers related to ECM remodeling, and the matrix metalloproteinase (MMP) family of proteins in particular, has long been implicated in IPF pathogenesis and studied extensively. Metalloproteinases are a collection of proteases responsible for the remodeling of ECM through breakdown of matrix components [163]. Further, these proteins can promote epithelial-to-mesenchymal transition, i.e. a process where epithelial cells acquire molecular features associated with mesenchymal cells with enhanced abilities to produce ECM components, recruit profibrotic mediators and promote abnormal wound healing. MMP7 is one of the most studied biomarkers in IPF and has been linked to differential outcomes. MMP7 holds the largest promise as a viable diagnostic and prognostic biomarker in IPF [164, 165, 159, 166–168]. CXCL13, a chemokine responsible for B-cell migration and activation in inflammatory lesions, is found elevated in blood and lung tissue in IPF [169], is associated with increased risk of progression in IPF, but also in other groups of ILD such as CTD-ILD, chronic hypersensitivity pneumonitis and unclassifiable ILD[169–171]. Several other cytokines, interleukins and immune related mediators have been found in IPF and in other progressive fibrosing ILD [50].

The multitude of pathways involved in IPF highlights the complexity but perhaps also the unlikeliness of finding a single biomarker specific enough to IPF that also predicts disease behavior. A “group-signature” approach to biomarkers in IPF is likely to be more logical. Recent studies using proteomic approaches have highlighted the multitude of ongoing processes, with simultaneous activity of proteins related to immune regulation and activation, inflammation and remodeling [172–174].

3 RESEARCH AIMS

The overarching purpose of this thesis is to increase our understanding of idiopathic pulmonary fibrosis (IPF) both in a clinical and biological context. By using the Swedish IPF registry and biobank, we have approached different aspects of the disease where there is a need for further research and where we can contribute by reinforcing accumulating evidence that have been found in other IPF cohorts and generate hypotheses for further studies.

The specific aims of the studies were to:

- Determine if clinical characteristics differ between males and females with IPF
- Provide a comprehensive overview of patients diagnosed with IPF in Sweden and evaluate measures of disease severity, treatment and potential patient phenotypes
- Identify biomarkers associated with remodeling, inflammation and chemotaxis in a novel *ex vivo* model and evaluate these findings in IPF
- Identify autoimmunity with specific focus on citrullinated antigens and other posttranslational modifications and their potential implications in IPF

4 MATERIALS AND METHODS

This section summarizes the data sources, biological material and methods used for the different analyses. More detailed descriptions of the methodologies can be found in the corresponding papers.

4.1 ETHICAL APPROVALS AND CONSIDERATIONS

The Swedish IPF-registry and its associated biobank are approved by Stockholm's Regional Ethical Committee (RefNo. 2014/1202-31/4). The use of data and samples from the biobank for the projects described in this thesis have been approved in a separate application (RefNo. 2018/1449-31/1).

In order to be included in the registry, the patient must read and sign an informed consent. Patients are informed that only relevant data concerning their care is collected and that their consent can be withdrawn at any time without explanation or it affecting future management and care. An inclusion in the registry does not mean any additional test or visit. However, if patients donate samples to the biobank they might experience mild pain when drawing blood, a consequence inherent to every blood test.

The registry is based on a web-based platform only accessed on computers approved centrally by the registry coordinator. Patients' social security number is used to register the patient in the registry, but absent at data extraction where patients are pseudonymized and their social security number replaced by a patient-ID number.

4.2 STUDY SUBJECTS, STUDY DESIGN AND ANALYTICAL METHODS

4.2.1 The Swedish IPF registry

The Swedish IPF registry is a nationwide registry launched in 2014 that enrolls patients with a diagnosis of IPF. The registry collects comprehensive data from more than 20 respiratory medicine units across the country. In order to be eligible for inclusion in the registry, the patient is required to have a confirmed diagnosis of IPF in accordance with the international and national guidelines [2, 16] by a specialist in respiratory medicine. Patients diagnosed with IPF before 2014 are also included. No other exclusion criteria for inclusion are considered, but the patient have to understand oral and written Swedish. The registry collects diagnostic and longitudinal follow-up patient data which is registered manually from medical records by nurses and/or physicians at each site. In 2016, a biobank was connected where blood, plasma and serum from two hospitals are collected at different time points. Biobank samples are drawn from patients, aliquoted and stored in -70°C within two hours of sampling. Table 1 provides an overview of study recruitment from the IPF registry and baseline characteristics in respective paper.

Table 1. Overview of study cohorts enrolled in respective paper

	Paper I	Paper II	Paper III	Paper IV
Number of patients	348	662	38	120
Age (median, range)	72.0 (46–88)	72.7 (68.0–78.0)	73.8 ± 7.83 [a]	75 (70-80)
Male/female, n (%)	250 (72) / 98 (28)	490 (74) / 172 (26)	29 (76) / 9 (24)	83 (69) / 37 (31)
Smoking history				
- Never smokers (n, %)	103 (30)	177 (27)	8 (21)	28 (23)
- Ex-smokers (n, %)	222 (64)	405 (61)	29 (76)	89 (74)
- Current smokers (n, %)	12 (3)	24 (4)	1 (3)	3 (3)
- Missing data (n, %)	11 (3)	56 (8)	-	-
FVC%	70.2 ± 15.6 [a] (N= 287)	71 (61–85) [b] (N= 507)	80.8 ± 20.2 [a] (N= 38)	77.5 (66-89) [b] (N= 114)
FEV_1%	76.1 ± 16.6 [a] (N= 307)	78 (66–90) [b] (N= 540)	81.2 ± 17.9 [a] (N= 38)	80 (68-92) [b] (N= 114)
DLCO%	46.2 ± 13.9 [a] (N= 221)	47 (37–56) [b] (N= 394)	50.4 ± 11.8 [a] (N= 38)	47 (39-57) [b] (N= 112)
TLC%	63.9 ± 12.0 [a] (N= 193)	66 (57–74) [b] (N= 361)	64.3 ± 11.2 [a] (N= 38)	64 (55-72) [b] (N= 113)
GAP-stage (n,%)				
1	NA	157 (41)	21 (55)	43 (36)
2		196 (51)	17 (45)	58 (48)
3		31 (8)	0 (0)	18 (15)
Missing data		-	-	1 (1)

[a] = Mean±SD; [b] = Median (Q1-Q3); **FVC%**:forced vital capacity,% predicted; **FEV_1%**: forced expiratory volume in 1 s,% predicted; **DLCO%**: diffusing capacity of carbon monoxide,% predicted; **TLC%**: total lung capacity,% predicted; **GAP:** gender-age-physiology stage for IPF

Paper I - A cross-sectional analysis of data accumulated at time of inclusion in the Swedish IPF registry between September 2014 and December 2017 was performed. Data on demographics, comorbidities, lung function, 6-minute walking test (6MWT) and quality of life (QoL). The latter was evaluated with the health status questionnaire K-BILD, which comprises 15 items across three domains: psychological, breathlessness and activities and chest symptoms, with domain and total points ranges from 0-100, with 100 representing best status. Data was extracted and analyzed from a cohort comprising 348 patients.

Paper II – This study included all patients enrolled in the registry from September 2014 to April 2020 (n=662). In the longitudinal analyses of survival, we decided to calculate survival both from diagnosis and inclusion. As a result, the registry includes both "prevalent" and "incident" cases (defined as patients included in the registry <6 months from diagnosis). Also, patients needed to have a minimum follow up of at least 6 months. The number of patients fulfilling these criteria was 540, while survival from time of inclusion was based on data from 480 patients. Patients were followed until death or transplantation, while patients who were still alive were censored at the last registered date of follow up visit. Variables extracted from the registry database encompassed demographics, basis for diagnosis, exposures, comorbidities, lung function, 6MWT, KBILD and outcome status. Spirometry and 6MWT performed within 6 months prior to or after registry inclusion were considered as baseline values. Patients were considered treated with antifibrotics if they received treatment for 6 months or more, while untreated patients were patients who either remained untreated throughout the observation period or received antifibrotic treatment for less than 6 months. Two established severity and risk prediction measures were calculated, presented and verified; the Gender-Age-Physiology (GAP) index, developed by Ley et. al [145], is a model that provides an average risk of mortality in IPF patients by assigning points depending on patients' sex, age and lung function (FVC and DLCO). The composite physiological index (CPI) developed by Wells et. al [144], is a score derived to account for the morphological

extent of fibrosis seen on HRCT while considering the potential confounding effects of emphysema. CPI was calculated using the following formula: CPI = 91.0 - (0.65 × DLco%) - (0.53 × FVC%) + (0.34 × FEV1%).

The cluster analysis required complete data in every individual covering 15 variables in addition to a minimum follow up time of 6 months. Applying these criteria restricted the cohort to 164 patients.

Paper III - To study the interaction between fibroblasts and extracellular matrix (ECM), we used an *ex vivo* model developed by the collaborating group, where fibroblasts were cultured within scaffolds*,* i.e. lung tissue slices with maintained ECM structure and composition but devoid of cells. Refer to [175, 176] for detailed description of the model. Lung tissue from distal localizations was obtained from four patients with IPF and four healthy donor lungs. Primary lung fibroblasts from one healthy donor were cultured on each type (healthy and IPF) of scaffold for up to nine days, with the medium used in the model changed at day 1, 3 and 6. The proteomic analysis was performed on the cell culture medium collected at day 1 and day 9.

IPF patients were selected based on the availability of baseline and follow up samples with associated clinical data performed maximum 6 months before or after sampling. This to enable linkage and correlations of protein concentrations to established measures of disease severity and progression. Serum samples from 38 patients from the registry enrolled from Karolinska University Hospital were analyzed. Baseline samples were collected at a median time of 2 months (IQR: 12 months) from diagnosis, while follow samples were collected at different time points with a median time of 16 months (IQR: 9.5 months) from baseline. Data on demographics, lung function and treatment status were extracted from the registry. Patients were considered to have progressive disease if a decline in FVC% of at least 10% and/or decline in DLCO% of 15% or more was observed at the time of follow up sample. In addition to this, we further evaluated disease progression over an observation period of 36 months from baseline, considering all available lung function tests performed.

Single serum samples from a control cohort including 77 healthy subjects of whom 37 were never smokers and 40 current smokers, were obtained from the COpd and Smoking from an oMIC perspective (COSMIC) cohort [177].

Paper IV – This project included serum samples and data from patients and individuals from three separate cohorts: 1) 120 patients diagnosed with IPF between 2007-2020 and enrolled in the registry; 2) 120 sex- and smoking matched healthy controls from the Epidemiological Investigations for Rheumatoid arthritis (EIRA) cohort [178] and 3) 104 patients with rheumatoid arthritis from the LUng investigation in newly diagnosed Rheumatoid Arthritis (LURA) cohort [179]. Serum from the IPF patients were collected at a median time of 11 months (IQR: 1-28 months) from diagnosis with no exclusion of patients with regards to clinical characteristics, comorbidities or treatment.

Demographic data, lung function, 6MWT, results from autoantibody tests performed as part of the diagnostic work and outcome status was extracted from the IPF registry. Clinical characteristics from the LURA cohort included demographics and lung function, while only information on age and smoking was available in controls from EIRA. Follow up data was not available in the LURA and EIRA cohort, whereas IPF patients were followed from serum collection to death or transplantation or were censored at Jan 1st 2022. Moreover, analyses of progression-free survival, with progression defined as death, transplantation or decline of at least 10% in FVC%, and/or a decline of 15% or more in DLCO% over a time period of 36 months (+3months) from time of serum collection were also performed.

Furthermore, as the Swedish IPF-registry enrolls patients regardless of diagnosis date and the biobank was started in 2016, we performed the same analyses on a cohort of patients (n=67) where serum was collected <15 months from diagnosis. This in order to minimize potential biases that may arise when including prevalent cases which may lead to underestimation of survival.

Analysis of proteins in serum (Paper III)

The cell culture medium from the *ex-vivo* model and the serum samples from IPF patients and controls were analysed through a panel of 92 proteins provided by Olink Proteomics AB (Uppsala, Sweden). Proteins were measured and quantified via multiplex proximity extension assay (PEA) as described in previous work [180]. The PEA is a dual recognition immunoassay which uses antibody pairs labelled with unique DNA-oligonucleotides functioning as probes that bind to each other upon the pairwise antibody-binding to the target proteins. Binding brings the antibodies into proximity and the oligonucleotides on each antibody hybridize and function as a template for extension and quantified through polymerase chain reaction (PCR). Results are reported as normalized protein expression (NPX) value which is an arbitrary unit on a log2-transformed scale, where a high value corresponds to high protein concentrations. Proteins with a detectability lower than 67% (i.e. >33% of samples below limit of detection per protein) were excluded.

Analysis of autoantibodies in serum (Paper IV)

Detection of ACPA and AMPA fine specificities against modified peptide antigens in serum from healthy controls, IPF patients and patients with early RA were performed using a custom multiplex peptide microarray platform based on the Immuno solid-phase Allergen chip (ISAC) (Thermo Fisher Scientific, ImmunoDiagnostics, Uppsala, Sweden) [139].

Peptides in both citrullinated and native form were printed in spots onto a glass slide. Serum samples, diluted 1:40 in buffer (Phadia, Uppsala, Sweden), were incubated on the slides and bound autoantibodies were detected using a Cy3 conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Newmarket, UK). Fluorescence-intensity was measured and converted to arbitrary units (Au/ml) based on an internal calibrator present in each chip. Cut-off values for the different ACPA and AMPA reactivities were set at the 98th percentile of the reactivities observed in the healthy controls' serum samples.

4.2.2 STATISTICAL ANALYSES

A number of statistical analyses were used with selection based on the hypothesis tests and underlying statistical requirements of the datasets. Comparisons of continuous variables between two groups were performed using Student's t-test (normal distributed data), Mann-Whitney U test (skewed distribution) or analysis of variance (ANOVA). Differences or associations in categorical variables were analyzed using Chi-square test (or Fischer's exact test as appropriate).

In **paper I,** differences in comorbidities stratified by gender and smoking status were analyzed and presented with odds ratios (OR; i.e. odds for having certain comorbidity compared with the odds of not having comorbidity). Multivariate statistical analysis was used to evaluate the correlation between quality of life, with KBILD total score as the dependent variable and clinical variables as independent variables.

In **paper II,** agreements in the classification of "mild" disease severity defined by GAP stage 1 with different levels of composite measures of severity (CPI) and measures of lung function, was calculated and compared using Cohen's kappa coefficient. Kappa values are categorized as: ≤ 0 = poor; 0.01-0.20 = slight; 0.21-0.40 = fair; 0.41-0.60 = moderate; 0.61-0.80 = substantial and 0.81-1.0 = excellent/perfect. Kaplan-Meier curves and log-rank tests were estimated to evaluate median survival times and comparisons between groups. Univariate and multivariate Cox analysis was used to discern the significance of clinical variables, treatment, the severity classifications and clusters' associations with survival.

The number of clusters was evaluated using Ward hierarchical cluster analysis and factor analysis. Kaiser-Meyer-Olkin (KMO) measure and Bartlett test of sphericity were derived to check for appropriateness. KMO calculates the proportion of variance among variables and tells if there is enough variance to compute a factor analysis. The Bartlett test evaluates if there are relationships between variables and the data is enough to be compressed in a factor analysis. A KMO value of >0.6 and $p<0.05$ in Bartlett test means that data are likely to factor well and a factor analysis is suitable. K-means cluster analysis was performed to classify clusters using the results from the hierarchical clustering. A stepwise discriminant analysis was used to identify variables that distinguishes each cluster. In order to validate the robustness of the clusters, the leave-one-out method was performed to ensure the stability and precision of the cluster model.

In **paper III** where a large number of proteins were analyzed and compared to controls in the *ex-vivo* model and in serum from patients with IPF, multiple hypothesis testing was performed using Sidak's adjustment for the two-way ANOVA in the experimental part. In the clinical part, Benjamini-Hochberg procedure controlling for a false discovery rate (FDR) of 5% was utilized. Further, given that a clear age discrepancy was evident between IPF patients and controls, with IPF patients being older, the one-way ANOVA performed was adjusted for age.

In **paper IV**, survival time estimates were calculated from time of serum collection to transplant, death or censoring at Jan 1st 2022. Progression-free survival was defined as time from serum collection to death or transplant or a decline of at least 10% in FVC% and/or a decline of at least 15% in DLco% over a time period of 36 months (+3months).

Statistical analyses were performed using the statistical softwares Wizard Pro (version 1.9.22 (240), Evan Miller), SPSS 25.0 (IBM Corporation, Armonk, NY USA), STATA 13.1 (StataCorp LP, College Station, TX, USA), SAS (SAS system for Windows 9.4, SAS Institute INC., Cary, NC, USA), R (R Core Team, 2020, version 3.6.3) and Graphpad Prism (version 9). Results were considered statistically significant if $p<0.05$.

5 RESULTS AND DISCUSSION

5.1 *I. GENDER DIFFERENCES AT PRESENTATION OF IDIOPATHIC PULMONARY FIBROSIS IN SWEDEN*

Knowledge on gender related differences was limited when we initiated these studies. The male predominance in IPF was well established and studies investigating new prognostic tools such as the multidimensional GAP-index had shown that males had an increased mortality compared to females [145]. However, the underlying causes or contributors to these differences remain unclear. Further, the prevalence of comorbidities in IPF patients had not been studied in Sweden.

A total number of 348 patients was included, reflecting the typical IPF population summarized in Table 1, paper I. The male predominance was evident (70%), the median age was 72 years and a majority of patients were ex-smokers (63.8%). Arterial hypertension was the most common comorbidity, reported in 33% of patients. Gastroesophageal reflux, other cardiovascular disease (including atrial fibrillation and heart failure) and coronary heart disease followed, with 32.5%, 24.7% and 19.3%, respectively. Quality of life, assessed with King's Brief Interstitial Lung Disease (KBILD) questionnaire demonstrated that patients' perception of quality of life was impaired with a mean (± standard deviation) KBILD score of 53.7 ± 10.7, on a 0-100 scale (poor to good).

Gender-stratified characteristics for the cohort are summarized in Table 2. No differences in age and BMI was observed, but men more frequently reported a history of smoking and a higher tobacco consumption reflected by pack-years (24.8 ± 15.1 vs 17.8 ± 13.3). Additional stratification by smoking status, showed that physiological variables including FVC% and TLC% were lower in males compared to females in general (FVC%: 68.9 ± 14.4 vs 73.0 ± 17.7, $p<0.05$; TLC%: 62.2 ± 11.8 vs 68.6 ± 11.3, $p<0.05$) and in ex-smokers (FVC%: 68.7 ± 12.7 vs 75.8 ± 16.3, $p<0.01$; TLC%: 62.0 ± 11.7 vs 71.3 ± 11.0, $p<0.001$), where a lower DLCO% (43.8 ± 13.3 vs 49.3 ± 13.3, $p<0.05$) was observed among males as well. KBILD, its total score and three domains covering psychological, breathlessness and activity and chest symptoms were similar between the genders with the exception in never smoking individuals, where the domain covering chest symptoms was lower in females compared to males (71.4 ± 21.4 vs 60.6 ± 20.4, $p<0.05$).

Table 2. Gender-stratified characteristics of the cohort

	Male	Female
IPF-patients (N, % in total)	250 (71.8)	98 (28.1)
Age (Median, range)	72 (46–88)	72 (56–84)
BMI (M±SD)	27.0 ± 3.74	27.3 ± 5.18
Smoking status		
Never smoker (N, % in gender)	64 (25.6)	39 (39.8)
Ex-smoker (N, % in gender)	169 (67.6)*	53 (54.1)
Smoker (N, % in gender)	7 (2.8)	5 (5.1)
Missing info (N, % in gender)	10 (4.0)	1 (1.0)
Packyear in ex-smokers (M±SD)	24.8 ± 15.1*	17.8 ± 13.3

N number(s); **BMI** body mass index; *$p < 0.05$, male vs. female

Evaluation of comorbidities showed, overall, that coronary heart disease (OR: 3.48 (95% CI: 1.59–7.58)) and other cardiovascular diseases (such as atrial fibrillation and heart failure) (OR: 3.84 (1.89–7.80)), where more prevalent among males. Meanwhile, females were more likely to have thyroid diseases (OR: 0.36 (0.15–0.87)), asthma (OR: 0.18 (0.06–0.54)) and osteoporosis (OR: 0.10 (0.01–0.65)). Further separation of the group by smoking status revealed that only coronary heart disease remained consistently more prevalent in males vs females (never smoking males vs females: OR: 10.6 (1.34–84.5).

We further set out to investigate potential differences in factors that impact the quality of life by multivariate analysis with KBILD total score as dependent variable and age, BMI, gender, FVC%, FEV_1% and number of comorbidities as independent variables. FVC% was the only variable that impacted the KBILD total score in the entire cohort (beta: 0.225, 95% CI: 0.060-0.389, p=0.008) and in males (beta: 0.246, 95% CI: 0.034-0.458, p=0.023), with higher FVC% associated with increased KBILD.

Discussion of paper I

Studies of gender differences with regard to the clinical presentation of the disease are lacking. We aimed to characterize and investigate potential gender differences across several clinical characteristics. We were able to confirm the male predominance [83, 84, 181–183], and provided new information on increased burden of cardiovascular disease in males and also differences in other comorbidities, such as asthma and thyroid diseases in females. The differences likely reflect the imbalance in prevalence observed in the general population, but they may also be an important prognostic factor that require further studies in larger populations.

We showed that males had lower lung volumes, particularly in ex-smokers. Despite this, quality of life did not differ between genders in general, although never smoking females reported worse on the KBILD domain of chest symptoms. A possible explanation could be that the perception of the disease is different due to other causes, but our study was unable to provide any evidence for this.

Although our study was only cross-sectional, more recent studies on larger cohorts in other countries have been able to confirm our results of increased prevalence of coronary heart disease among males and thyroid diseases in females, along with lower FVC% in males at presentation [184, 185]. In general, research into different aspects of gender differences in IPF has expanded and highlighted other important perspectives on the management of patients with IPF as well. Recent studies have shown that male and females perceive their disease differently with females being more affected psychosocially than males [185]. Evaluation of HRCT features have demonstrated that honeycombing is more prevalent in males [184, 185], while analysis of differences in outcomes in more than 1200 IPF patients have shown that males have a 40% higher risk of death or lung transplantation compared to females [18]. However, information on comorbidities and their contribution to the survival disadvantage were not studied. Other important perspectives, such as gender bias in the diagnosis of IPF have also been brought to attention. Males are more likely to be diagnosed with IPF compared to females after adjustments for age, smoking, exposures and autoantibodies [186]. This was especially apparent in males with HRCT patterns other than definite UIP. Our understanding of the impact of gender is increasing and greater consideration should be taken in both basic and clinical research as well as in the management of patients.

The study constituted, for the first time, a compilation of large number of registry variables. Hence, it posed an opportunity to get an insight in to the challenges, such as missing data limiting the statistical power and awareness of interpreting data from small groups. In addition, it also displayed the inconsistencies in registration between centers which is inherent to registries covering several hospitals. Nevertheless, the study generated important information that were confirmed in other studies and gave us a broader picture of the opportunities and areas of improvement in the registry, which laid ground for the subsequent studies presented.

5.2 II. BASELINE CHARACTERISTICS AND SURVIVAL OF PATIENTS OF IDIOPATHIC PULMONARY FIBROSIS: A LONGITUDINAL ANALYSIS OF THE SWEDISH IPF REGISTRY

Several registries have been initiated around the world in recent years, collecting a plethora of information from thousands of patients which provide us with important knowledge on the clinical course and management of IPF. In this study, we set out to provide a comprehensive overview of the patient population in Sweden, including treatment with antifibrotics, disease severity classifications and potential phenotypes with differences in mortality.

A total of 662 patients had been enrolled in the registry between September 2014 and April 2020. Enrolled patients reflected in large parts what is established in IPF with a majority being males, ex-smokers, with a median age of 72 years (IQR: 67-77) (Table 1, paper II). Diagnoses were decided based on clinical and radiological information in 88.7%, while approximately 10% of patients had undergone surgical biopsy (thoracoscopic or open lung biopsy). Diagnosis after a multidisciplinary conference was reported in 41.5% of patients. Classification by GAP-stage was feasible in 384 patients and showed that half of patients (51%) belonged to GAP-stage 1, 40.9% to GAP-stage 2 and 8.1% as GAP-stage 3. Prevalence of comorbidities were in line with our previous study, with hypertension (35.6%), acid reflux (31.6%), other cardiovascular disease (20.2%), coronary heart disease (19.8%) and diabetes (15.4%) being the most frequent ones. Multimorbidity (≥1 comorbidity) was reported in more than 40% of patients at baseline. During the observation period, 195 patients had died and 23 had undergone lung transplantation (33% in total). The year one, two, three, four and five cumulative mortality rates from diagnosis (n=540) was 7%, 16%, 30%, 39% and 48%, respectively (Figure 1).

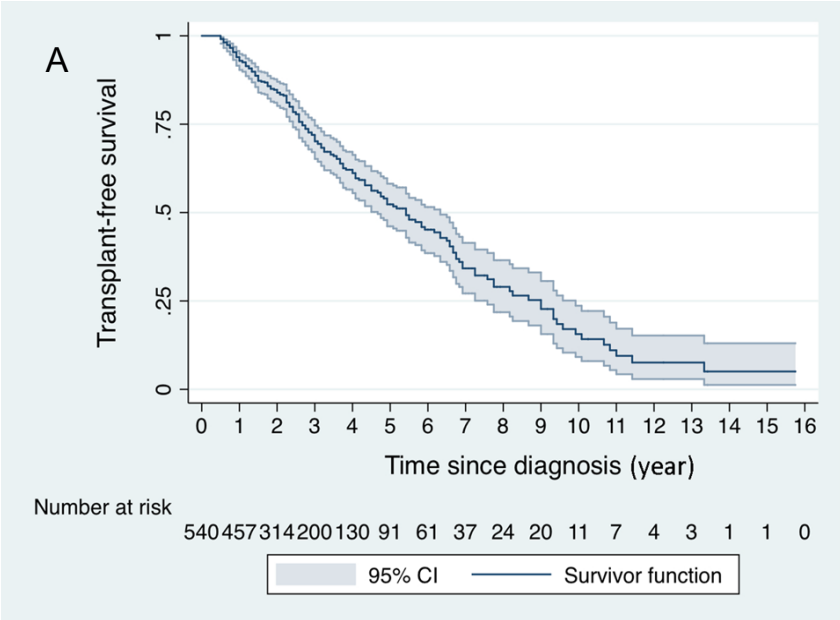

Figure 1. Kaplan-Meier analysis for survival in IPF-patients registered in the Swedish IPF-registry

Approximately two thirds (64%) of patients were followed for at least 6 months from diagnosis (n=540) had received anti-fibrotic therapy. The distribution of treatment assignment was similar, with 33.9% receiving pirfenidone, 26.3% nintedanib, followed by a small group of patients (4.1%) who had switched treatment. Treated patients were slightly younger at diagnosis, had higher CPI and lower DLCO% compared to untreated patients (Table 4, paper III). Stratification by treatment revealed no differences between the groups, including demographics, lung function and composite measures as GAP and CPI. Treated patients lived longer than untreated patients both in the whole cohort (Figure 2A) (log rank p=0.037) and in patients with GAP-stage ≥2 (Figure 2B) (log rank p=0.034). Multivariate Cox analysis adjusted for age, gender, BMI, BMI, smoking status, FVC% and DLCO%, showed that this prolonged survival remained significant in patients taking antifibrotic treatment (hazard ratio: 1.797, 95% CI 1.173-2.753, p=0.007).

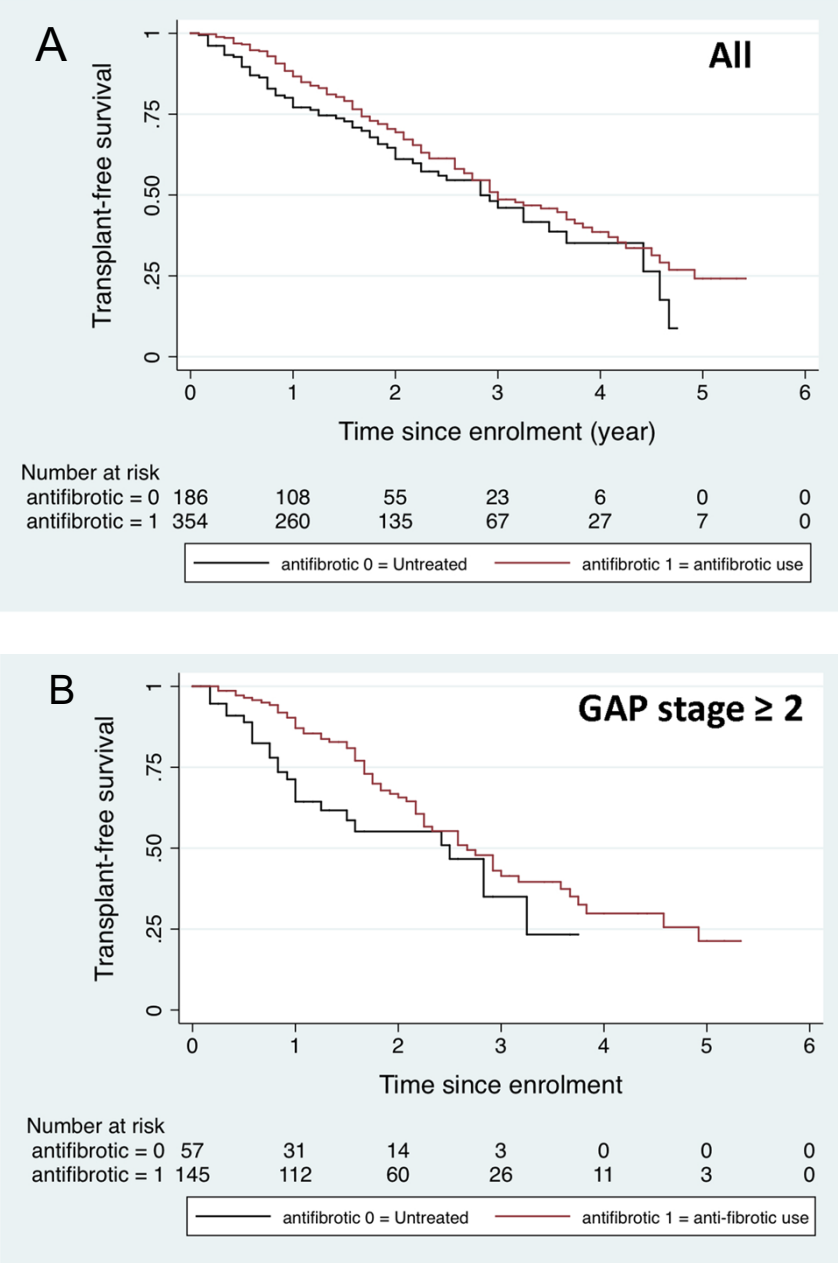

Figure 2A-B. A) Kaplan-Meier analysis for survival in IPF-patients by antifibrotic treatment use; **B)** survival in IPF-patients with GAP-stage ≥2 by antifibrotic treatment use. **Red** line indicates treated patients; **Black** line indicates untreated patients.

We further investigated the agreement of individual lung function measures including FVC%, DLCO%, TLC% and CPI, with the GAP stage 1 classification which has been regarded as a "mild" disease. GAP stage 1 had a good agreement with CPI ≤ 45 (kappa value (k)=0.62), a moderate agreement with DLCO ≥ 55% (k=0.58), FVC ≥ 75% (k=0.50) and TLC ≥ 65% (k=0.47). With this "new" classification of mild disease for each measure we evaluated their ability to distinguish differences in mortality. Patients with GAP stage 1, CPI ≤ 45, DLCO ≥ 55%, FVC ≥ 75% and TLC ≥ 65%, was predictive of better survival as opposed to patients with moderate to severe disease in univariate and multivariate Cox model adjusted for age, gender, BMI, smoking and antifibrotic treatment (Table 5, paper II).

By integrating registry variables, we sought to explore and identify distinct patient phenotypes. Three clusters were identified with differences in clinical characteristics and mortality. Table 6, paper II and Figure 3a-d provides a summary of each cluster. Cluster 1 (n=55) comprised of mostly older, male patients (87.3%) with heart diseases (96.4%, includes hypertension, other cardiovascular diseases and coronary heart disease) and moderate to severe disease. Cluster 2 (n=70) were the cluster with most females (52.9%) and with mild disease severity. Cluster 3 (n=39) were younger patients with moderate to severe disease.

The factor analysis discriminated two factors with clusters distributed differently (Figure 3e). Function 1 included mainly disease severity measures and function 2 mostly variables related to comorbidities. Kaplan-Meier analysis showed that patients in cluster 1 had worst survival compared to patients belonging to cluster 2 (log rank $p<0.001$) and cluster 3 (log-rank $p=0.036$) (Figure 3f). Meanwhile, patients in cluster 2 survived longer than patients in cluster 3 (log-rank $p=0.017$). Investigating survival in clusters adjusting for antifibrotic treatment in multivariable Cox analysis demonstrated that cluster 1 (HR: 3.154, 95% CI 1.855–5.364, $p<0.001$) and cluster 2 (HR: 0.291, 95% CI 0.160–0.528, $p<0.001$) were predictors of survival.

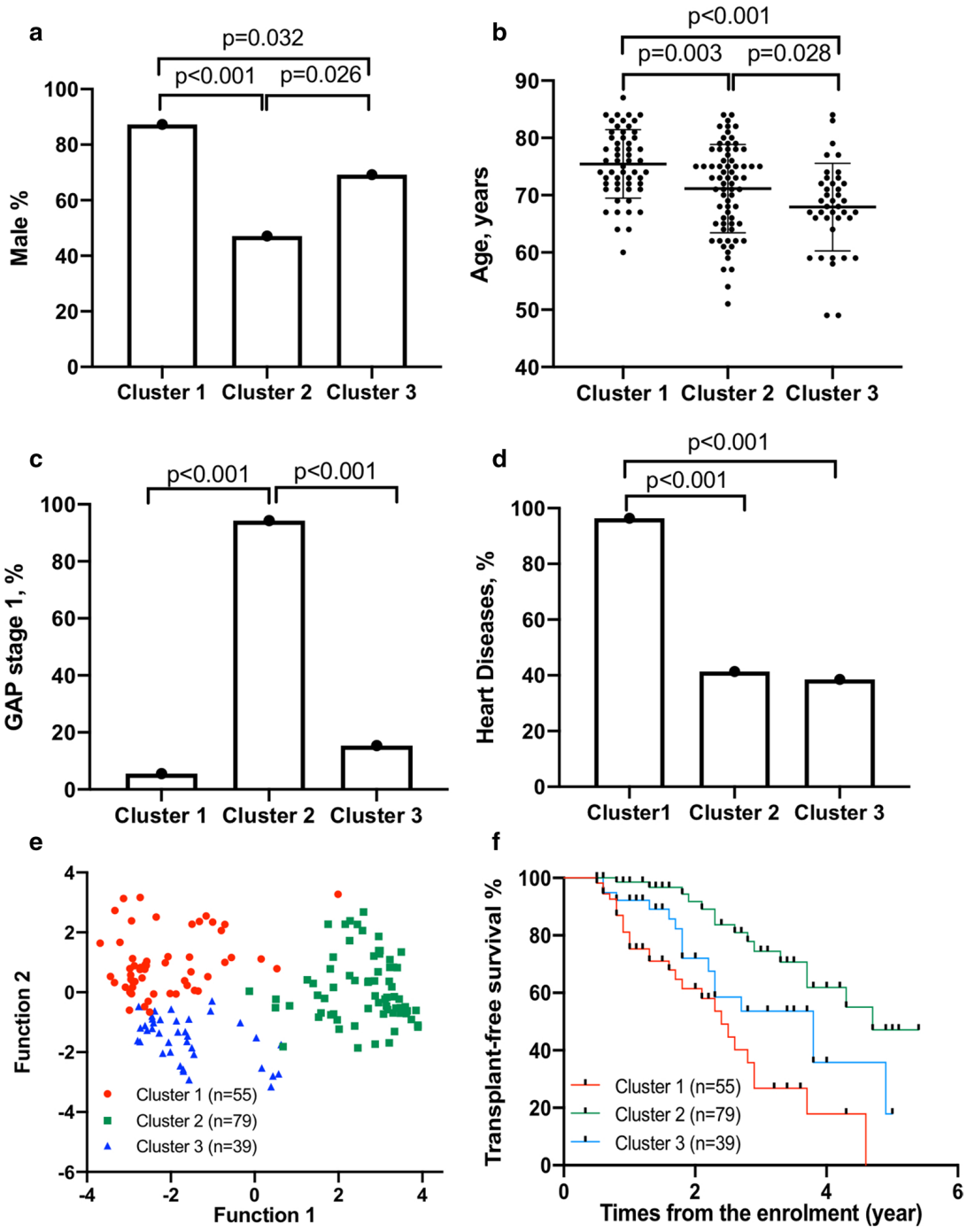

Figure 3a-f. Patient characteristics in respective cluster and survival. **A-D)** Distribution and differences in clinical characteristics between clusters. **E)** Differences between clusters by factor analysis. **F)** Kaplan-Meier analysis for survival by clusters

Discussion of paper II

Patient registries offer opportunities to provide data that may complement those generated in clinical trials. Furthermore, results from registry studies can be validated and confirmed in other registries, although such comparisons should be done with caution due to differences in patient populations and methodologies. Register data can also be used to achieve new insights on disease behavior. The patient cohort presented in this study was similar to cohorts presented in other registries with regards to clinical characteristics, comorbidity burden and survival [83, 84, 187, 188]. In addition, our results reinforce established knowledge by validating the prognostic importance of individual physiological variables and composite measures such as GAP and CPI.

As clinical trials only study treatment effects over a restricted time period, registries can provide insights in pharmacological treatment patterns and their long-term efficacy. Our analyses of treatment effects on mortality, albeit relatively superficial on a methodological plane, was able to contribute to the expanding number of studies based on real world data and meta-analyses demonstrating the beneficial effects of antifibrotic treatment on survival [83, 85–87, 189–191]. Prospective, randomized controlled trials remain the gold standard to evaluate treatment effects as the design is superior in accounting for biases and minimizes the effects of unmeasured variables.

We further set out to leverage the range of variables and long-term data to explore potential patient phenotypes. Three clusters were identified with different patient characteristics, disease severity, comorbidity profiles and survival. Moreover, the cluster analysis highlighted the complexity of disease severity as individual variables that were non-significant in univariate Cox analysis were important predictive factors in the cluster analysis. Cluster analysis in IPF is a concept that has gained increasing attention, and our data is one of the first to explore this. Subsequent studies exploring clusters have included a number of variables including clinical, laboratory and radiological, which may confine comparisons between studies. Notwithstanding, results have demonstrated how e.g. comorbidities [192], gender and disease severity [193] are able to distinguish patients with IPF. Common for the cluster analyses published to date, ours included, is the limited number of patients available for analysis, which requires further studies in larger patient numbers or the addition of other cohorts. Altogether, analyses of phenotypes offer a new approach which may increase our knowledge of the heterogeneity of the disease. Through improvements of patient recruitment, for example through collaborations, we may advance into more personalized patient management in the future.

The opportunity to corroborate and refute findings in large cohorts collected in registries is certainly an advantage. However, the real-world environment in which patient registries operate, constitute a challenge in terms of data collection and analysis. This is truly evident in our study both with respect to the number of data points and the number of individuals available in respective analyses. This can also be viewed as an opportunity such as variability between hospitals regarding the comprehensiveness of testing or differences in management.

Missing data limits statistical power and becomes an increasing issue as loss or inconsistencies in follow up becomes more apparent. This can be due to worsening of disease or death which may not be registered, or that patients with milder disease may return to the hospitals less frequently. Ensuring the completeness and quality of data requires administrative and economic resources but is vital to secure the reliability of results. Having said that, it is important to remember that registration of data is a time-consuming process performed in the clinic with the everyday commitments health care personnel have. Another limitation is the absence of data on acute exacerbations, which significantly affects mortality

in IPF. Numbers on the incidence in this cohort and knowledge of which patients developed AE would open for more refined analysis on mortality and potential effects of treatment. Current data in the registry on AEs are limited and needs further characterization to discern hospitalizations due to AE or comorbidities.

Taken together, this paper set out to provide an overview of the patient population enrolled in the Swedish IPF-registry. The results strengthened previous observations with regards to clinical characteristics, disease severity, survival and treatment, and provided insight into phenotypes of patients with IPF with unique characteristics and prognosis.

5.3 III. DISTAL LUNG MICROENVIRONMENT TRIGGERS RELEASE OF MEDIATORS RECOGNIZED AS POTENTIAL SYSTEMIC BIOMARKERS FOR IDIOPATHIC PULMONARY FIBROSIS

This two-part study explored the proteomic profile in 1) healthy primary fibroblasts repopulated in a novel *ex-vivo* model that recapitulates the compositional and biomechanical properties of the lung tissue and the extracellular matrix (ECM) observed in IPF, and 2) in serum from patients with IPF. The second, clinical part of the study was expanded by exploring proteins' relationship with measures of disease severity and progression.

Fibroblasts seeded in lung matrix ("scaffolds") from healthy individuals, differed from fibroblasts grown in end-stage IPF matrix. The latter demonstrated an altered response by showing increased release of 12 proteins related to either remodeling, inflammation and chemotaxis at two time points, either day 1 or day 9 or both (Table 1, paper III). Matrix metalloproteinase-7 (MMP7) and C-X-C motif chemokine 13 (CXCL13) displayed the largest differences between IPF and controls at day 1. Meanwhile, matrix metalloproteinase-12 (MMP12), galectin-9 (Gal9) and CXCL13 were the only proteins elevated at both day 1 and day 9 in culture. The proteins decorin (DCN) and cluster of differentiation 40 (CD40) were shown to be increased compared to controls at day 9 only. An example of these differences seen in protein concentrations in the ex-vivo model, with specific focus on proteins related to remodeling are presented in Figure 1, paper III.

Age corrected analysis of differences in protein concentrations between IPF patients (n=38) at baseline and controls (n=77) revealed elevated concentrations of 44 proteins (Figure 4). A majority of proteins elevated in IPF (30 out of 44) regulated inflammation and chemotaxis, while remaining proteins were associated to remodeling (7 proteins) or had overlapping functions (remaining 7 proteins). Evaluation of the proteins elevated in the *ex-vivo* model showed that ten out of the twelve proteins were increased in patient serum. Table 3 displays a selection of the top differentially increased proteins found in IPF serum compared to controls together with the significant proteins observed in the *ex-vivo* model.

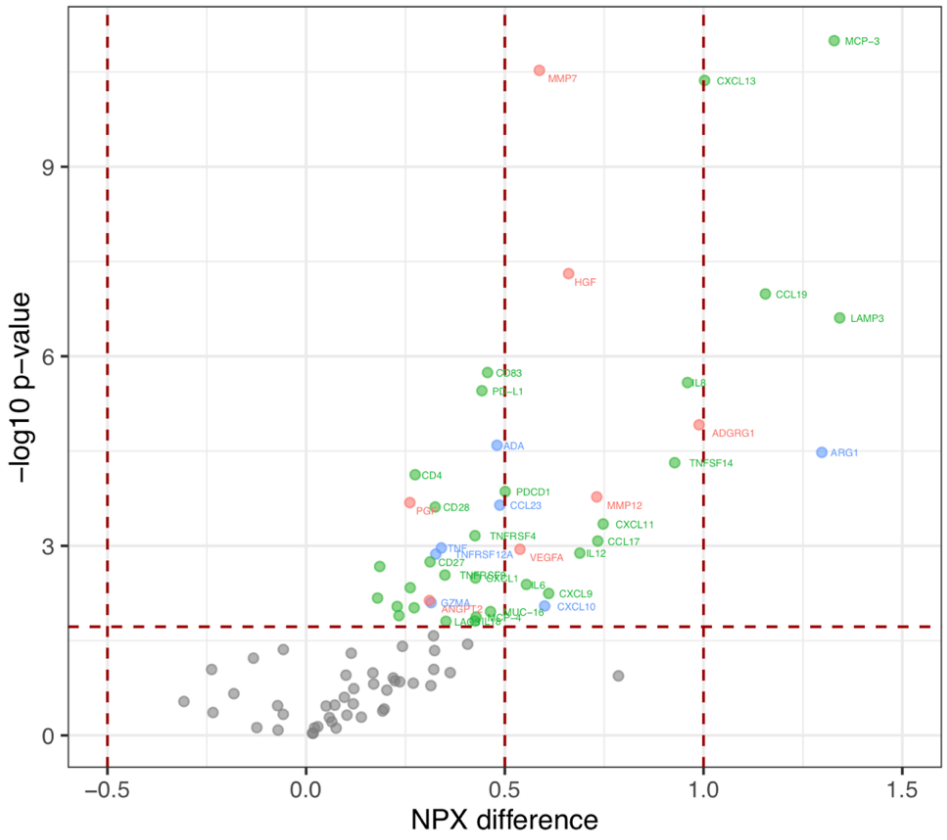

Figure 4. Levels of circulating proteins in IPF patients versus controls. The difference in relative protein amount (NPX) (x-axis) by log10 of p-value. **Red**: proteins associated with tissue remodeling; **Green**: proteins associated with inflammation/chemotaxis; **Blue**: proteins with overlapping functions.

Table 3. Top proteins found increased in IPF serum at baseline and in the *ex vivo* model. Proteins are categorized by biological function.

Protein	NPX-difference	p-value	FDR adjusted p-value
Tissue remodeling			
ADGRG1	0.99	1.22E-05	5.75E-03
MMP12*	0.73	1.68E-04	9.20E-03
HGF	0.66	4.91E-08	2.30E-03
MMP7*	0.59	2.98E-11	1.15E-03
VEGFA	0.54	1.13E-03	1.38E-02
PGF*	0.26	2.07E-04	9.77E-03
Inflammation/Chemotaxis			
LAMP3	1.34	2.47E-07	3.45E-03
MCP-3	1.33	1.01E-11	5.75E-04
CCL19*	1.16	1.03E-07	2.87E-03
CXCL13*	1.00	4.31E-11	1.72E-03
IL8	0.96	2.61E-06	4.60E-03
TNFSF14	0.93	4.84E-05	7.47E-03
TNFRSF9*	0.35	2.90E-03	1.67E-02
CD4*	0.27	7.48E-05	8.05E-03
CD40*	0.26	4.61E-03	1.84E-02
Gal-9*	0.23	1.27E-02	2.36E-02
Overlapping functions			
ARG1	1.30	3.32E-05	6.90E-03
CXCL10	0.60	8.96E-03	2.13E-02
CCL23	0.49	2.27E-04	1.03E-02
ADA	0.48	2.56E-05	6.32E-03
TNFRSF12A	0.33	1.35E-03	1.49E-02
GZMA*	0.31	7.92E-03	2.07E-02

* indicate proteins elevated in the ex-vivo model; **NPX:** normalized protein expression; **FDR:** false discovery rate

Examination of protein concentrations in follow up samples from IPF patients showed that 24 of the initial 44 proteins found in the baseline samples were persistently increased at follow up. These included among others, the remodeling proteins MMP7, MMP12, hepatocyte growth factor (HGF), vascular endothelial growth factor A (VEGFA), the inflammation/chemotaxis related proteins CXCL13, monocyte chemoattractant protein -3 (MCP-3) and lysosomal associated membrane protein 3 (LAMP3). Concentration of 21 proteins were statistically lower in follow-up samples compared to baseline (Table 4, paper III). These included the remodeling proteins epidermal growth factor, fibroblast growth factor-2 and the protein caspase-8 with overlapping function.

Next, we investigated the elevated proteins' associations to disease severity. Several proteins demonstrated correlations with at least one measure of disease severity (FVC%, TLC%, DLCO% and CPI) at both baseline and follow up. Concentrations of MMP7 were negatively correlated with FVC% (Spearman's ρ: -0.51, p=0.006; ρ: -0.52, p=0.001) and TLC% (ρ: -0.47, p=0.003; ρ: -0.39, p=0.017) at baseline and follow up respectively, and showed a positive correlation with CPI (i.e. increased fibrosis) at follow up (ρ: -0.51, p=0.006). HGF was negatively correlated with FVC% (ρ: -0.48, p=0.003) TLC% (ρ: -0.37, p=0.022) and CPI (ρ: 0.40, p=0.013) at baseline, but only TLC% (ρ: -0.35, p=0.036) at follow up. Inflammatory/chemotaxis proteins with promising correlations included MCP-3 with its negative correlations to FVC% (ρ: -0.53, p=0.0006; ρ: -0.49, p=0.002) and TLC% (ρ: -0.50, p=0.001; ρ: -0.46, p=0.004) at baseline and follow up, in addition to a positive correlation with CPI (ρ: 0.42, p=0.01) at follow up.

The consecutive serum sampling and associated clinical data enabled analysis of associations between changes in protein concentrations and changes in lung function. For instance, increasing MMP7 concentration at follow up correlated with decline in FVC% (Figure 5A) and DLCO% (ρ: -0.35, p=0.035). Increasing levels of VEGFA and HGF correlated with decreased FVC% and DLCO%, respectively (Figure 5B-C). Meanwhile, while no correlations between concentrations of the protein tumor necrosis factor receptor superfamily member 12A (TNFRSF12A) and disease severity variables were observed neither at baseline and follow up, increasing levels of TNFRSF12A showed a strong negative correlation with declining DLCO% (Figure 5D).

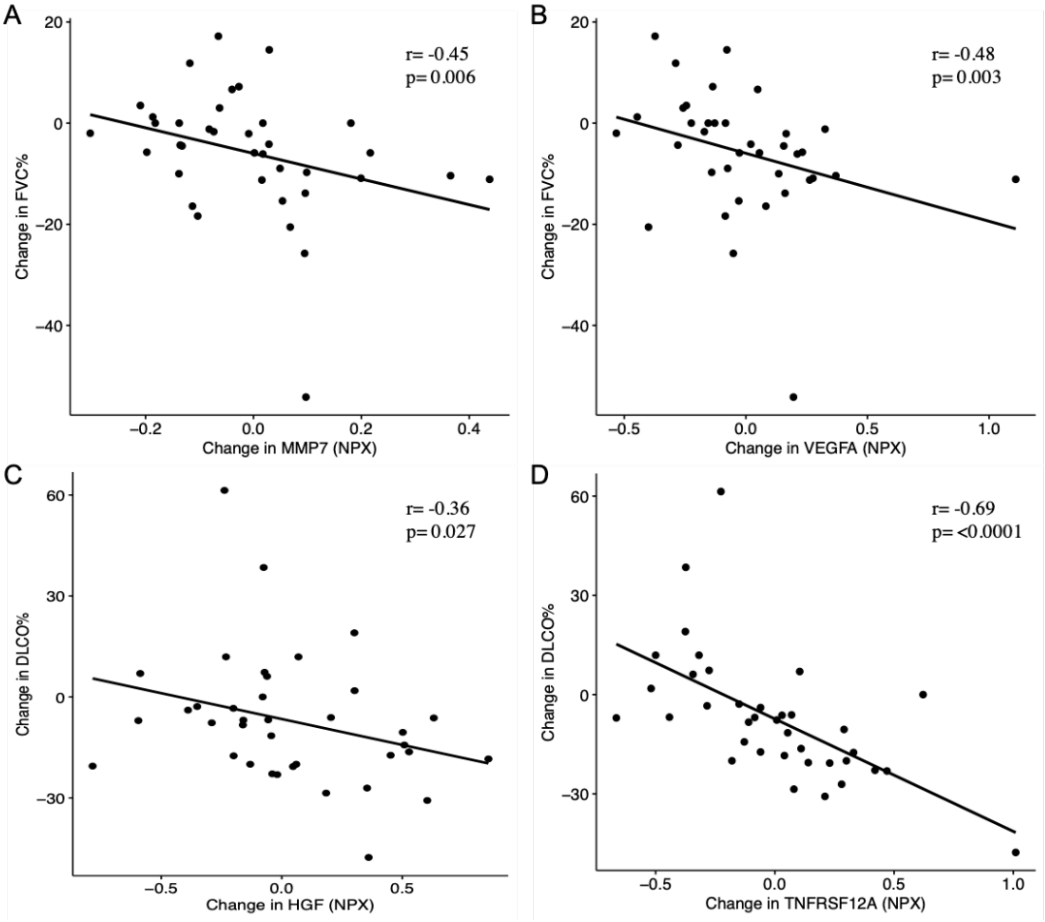

Figure 5A-D. Correlations between increasing concentrations in proteins MMP7(A), VEGFA(B), HGF(C) and TNFRSF12A and declining FVC% (A and B) and DLCO% (C and D)

Patients who had progressed (n=20) at follow up had higher concentration of interleukin 6 (IL-6, mean difference 0.45, 95% CI of difference 0.03–0.88, p = 0.04), NOS3 (0.51, 95% CI 0.02–1.01, p = 0.03), MMP7 (0.14, 95% CI 0.02–0.25, p = 0.03) and CASP-8 (0.36, 95% CI −0.01–0.73, p = 0.03) in the follow up sample compared to stable patients (n=17). No differences in protein concentrations were observed between the groups in the baseline sample. Comparison of changes in protein concentrations by progression status suggested that progressive patients had increasing concentrations of NOS3, HGF, VEGFA, MMP7 and TNFRSF12A (Table 4).

Table 4. Differences in changes of protein concentrations stratified by progression status

Protein	Progressive patients (mean difference) (95%CI) (n=20)	Stable patients (mean difference) (95%CI) (n=17)	p-value
NOS3	0.44 (0.002-0.89)	-0.04 (-0.21-0.12)	0.013
HGF	0.14 (-0.04-0.33)	-0.1 (-0.25 -0.05)	0.036
VEGFA	0.08 (-0.07-0.23)	-0.12 (-0.24-(-0.0001)	0.033
MMP7	0.04 (-0.03-0.12)	-0.06 (-0.13-0.005)	0.031
TNFRSF12A	0.15 (0.007-0.30)	-0.19 (-0.35-(-0.04))	0.0008

Kaplan-Meier and multivariate Cox analysis of progression over a 36 months time period demonstrated that patients with elevations in MMP7 and TNFRSF12A progressed faster (log rank p=0.0008, HR 63.0, 95% CI 4.36-917.7, p=0.002 and log rank p=0.019, HR 3.33, 95% CI 1.24-8.92, p=0.02, respectively).

An explorative analysis of the potential effects of antifibrotic treatment on protein concentrations showed that patients who were untreated at baseline and initiated treatment during follow up (n=13) had a statistically significant change in 30 proteins as opposed to untreated (n=12) and consistently treated (n=12) patients, where changes in one and 8 proteins were observed, respectively. Finally, decrease in concentrations of remodeling associated proteins EGF, FGF2, vascular endothelial growth factor receptor 2 (VEGFR-2) and proteins related to inflammation tumor necrosis factor superfamily 14 (TNFSF14) and interleukin 6 (IL-6) were seen among patients who initiated treatment (Table 7, paper III).

Discussion of paper III

We performed a proteomic investigation of the interplay between fibroblasts and ECM in tissue derived from healthy individuals and patients with IPF. We demonstrated that ECM from IPF lung altered the response and activation of fibroblasts compared to ECM from healthy lung. Fibroblasts are important players in the production of ECM. Therefore, knowledge on fibroblasts' behavior to the altered structural environment and how it drives pro-fibrotic and inflammatory cellular responses is important in order to elucidate disease mechanisms and develop new treatment targets. Previous studies of fibroblasts' response to IPF ECM have revealed activation of mechanosignalling pathways that propagate the rigidity of the ECM through amplification of pro-fibrotic mechanisms [48, 194, 195]. New experimental models that recapitulate the environment and the processes seen in fibrotic tissue are needed. Validating generated results in peripheral blood as we did in this study, strengthens the potential of the model and knowledge of specific proteins as disease relevant biomarkers.

By utilizing PEA technology, we were able to explore a broad set of proteins associated with remodeling, inflammation and chemotaxis, and provide a picture of the complexity and the ongoing processes and pathways involved in IPF biology. Leveraging the serum samples with clinical data, enabled us to link potential biomarkers to established measures of disease severity and progression. For instance, our results expand the literature on the diagnostic and prognostic utility of MMP7, one of the most described biomarkers in IPF to date [166, 196–199]. The elevated levels both in the *ex vivo* model and in patients' serum in addition to its associations with disease severity and progression confirm MMP7 as one of the more

interesting mediators in this area. Furthermore, the study shed light on other proteins such as TNFRSF12A, which, in the context of IPF, have been insufficiently studied, but found to play a role in other diseases with a fibrotic component [200–202].

Our results also highlight the upregulation and activity of inflammatory pathways. Despite several reports on inflammatory markers in peripheral blood, lung tissue and BAL in IPF [50, 173], the role of inflammation and its importance in the pathogenesis of IPF is controversial. This is motivated by several negative results in clinical trials of anti-inflammatory drugs [27, 28, 203]. Nonetheless, the results presented herein and in previous studies raises important questions into what extent inflammation is a driver of disease and progression and if there are targets and subgroups of patients that may benefit from anti-inflammatory treatment. Prospective studies on larger cohorts are therefore needed in the future.

The number of samples both in the *ex-vivo* model and the clinical part is a limitation which affected the statistical power which is important to have in mind when interpreting the results. The lack of a validation cohort of IPF patients is a limitation and is required to confirm or discard the generalizability of our results. Treatment with antifibrotics among a third (32%) of patients already at study start, may have resulted in an underestimation of protein concentrations. Other factors such as comorbidities or other pharmacological treatments were not considered in the study and may have had an impact. The age difference between IPF patients and controls were adjusted accordingly. Overall, the project shows the capabilities of the registry and the biobank and presents interesting pathways for future investigations in IPF.

5.4 IV. RHEUMATOID ARTHRITIS RELATED ANTIBODIES IN IDIOPATHIC PULMONARY FIBROSIS

A hallmark of RA is the serological presence of anti-citrullinated protein autoantibodies (ACPA). These autoantibodies target proteins who have been posttranslationally modified in which the amino acid arginine is converted into citrulline. ACPA bind to various citrullinated proteins including tenascin, fibrinogen, filaggrin, vimentin and histone [204–210]. Additionally, reactivity against other posttranslational modifications aside from citrullination such as carbamylation and acetylation by anti-modified protein autoantibodies (AMPA) have been described in RA [128, 211, 212].

We explored a spectrum of anti-modified protein autoantibody (AMPA) reactivities against posttranslational modifications associated with RA in serum from patients with IPF, healthy controls (HC) and patients with RA.

Altogether, 44% of IPF patients, 27% of healthy controls (HC) and 79% of RA patients, tested positive for autoantibodies against the modified peptides. More IPF patients had autoantibodies compared to HC (p=0.005). We identified 8 autoantibodies who differed between IPF and HC with regards to concentration and frequency. These included autoantibodies to the tenascin peptides $Cit_{(2033)}$-$TNC_{2025-2040}$; $Cit_{(2197)}$-$TNC_{2177-2200}$; $Cit_{(2198)}$-$TNC_{2177-2200}$, the fibrinogen peptides $Fib\beta_{36-52}$; $Cit_{(38,42)}$-$Fib\alpha_{36-50}$; $Cit_{(72)}$-$Fib\beta_{60-74}$ and the filaggrin peptides Acet-$Fil_{307-324}$, Carb-$Fil_{307-324}$ (Figure 6A-H). Autoantibodies targeting the other modified peptides tested, i.e. histone, LL37 and vimentin were not different between IPF vs HC (Supplementary Table 1, paper IV). In contrast to autoantibodies and reactivity seen in the RA cohort, IPF patients had both a lower frequency of autoantibody positive individuals and lower concentrations. Further, there were no reactivity against autoantigens typical for RA such as vimentin (Supplementary Table 1, paper IV). Presence of autoantibodies in the incident IPF sub-cohort (n=67) was found in 37% (n=25).

We then evaluated ACPA fine specificities, i.e. the number of reactivities in every patient in respective group. We were able to show that there were more individuals with >5 reactivities in IPF patients than HC (12% vs 3%, p=0.003), whereas the presence of high number of reactivities in RA patients were seen in 51% (p<0.0001 vs HC and IPF). We also compared results with tests of anti-CCP performed in the clinic. Forty-one percent (n=31) of patients with negative anti-CCP tests (n=75) showed presence of autoantibodies against at least one of the modified peptides. Similar results were found in the incident cohort where a third (33%, n =16) of the negative anti-CCP test (n=49) showed presence of autoantibodies.

Evaluation whether patients with IPF made up distinct clinical phenotypes based of their reactivity status showed no differences in terms of demographics, lung function, disease severity and comorbidities (Table 2, paper IV). Results remained non-significant after stratification by number of reactivities (Supplementary Table 3, paper IV).

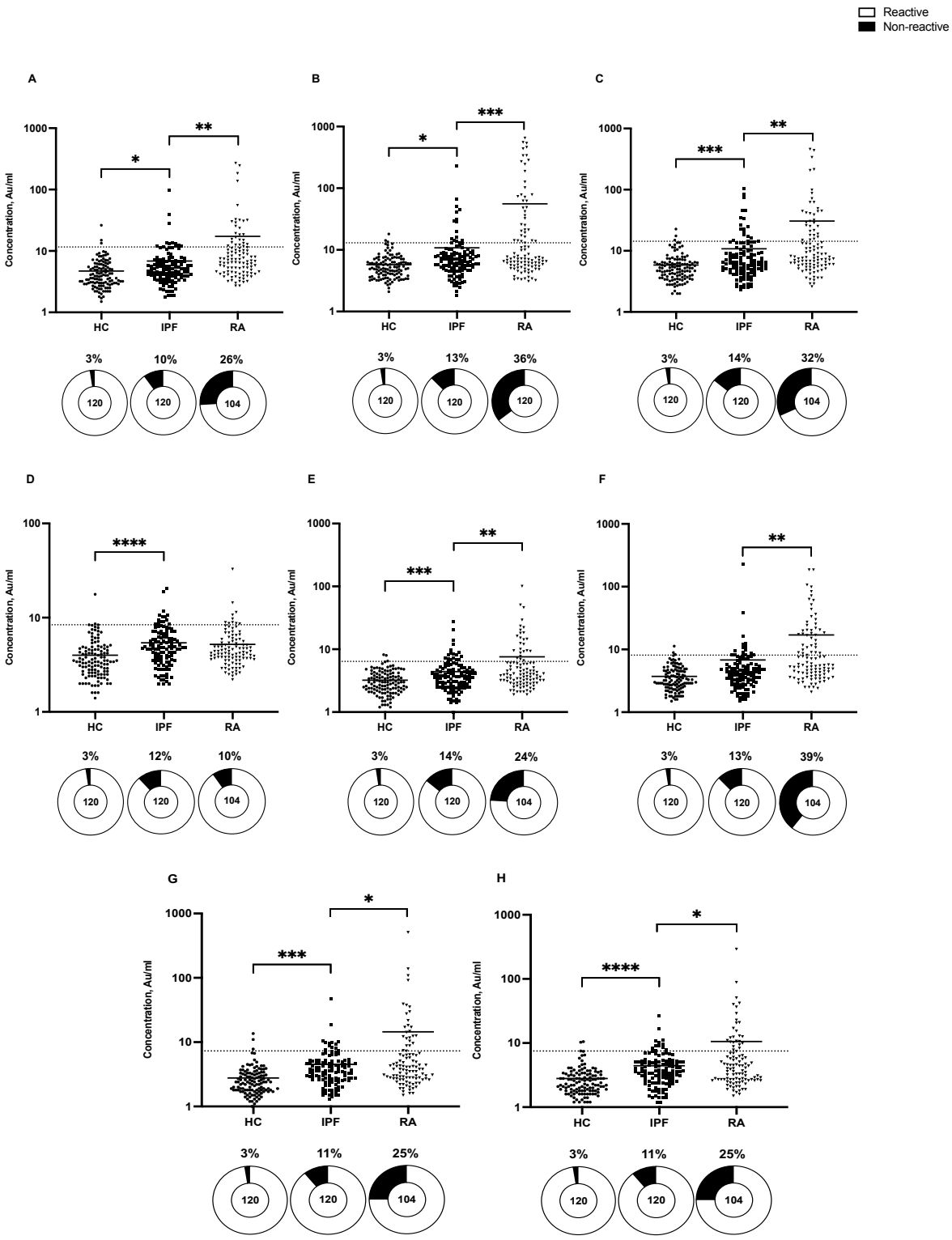

Figure 6A-H. Concentration and frequency of reactivity of autoantibodies against RA associated peptides found significantly increased in serum from IPF patients compared to healthy controls (HC). The horizontal dotted line indicate the cutoff for positivity towards each respective peptide. Pie chart represent the percentage of positivity in each cohort, with cohort size displayed in the middle. **A:** $Cit_{(2033)}$-$TNC_{2025-2040}$**, B:** $Cit_{(2197)}$-$TNC_{2177-2200}$**, C:** $Cit_{(2198)}$-$TNC_{2177-2200}$**, D:** $Fib\beta_{36-52}$**, E:** $Cit_{(38,42)}$-$Fib\alpha_{36-50}$**, F:** $Cit_{(72)}$-$Fib\beta_{60-74}$**, G:** Acet-$Fil_{307-324}$**, H:** Carb-Fil307-324; *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, **** $p < 0.0005$

No differences in survival stratified by the presence or absence of autoantibodies or by number of reactivities was observed in the full IPF-cohort (log-rank p=0.13 and log-rank p=0.20, respectively) (Figure 7A-B). Additionally, no differences were seen in time to disease progression, with regards to presence of autoantibodies or number of reactivities (Figure 3A-B, paper IV) (log rank p=0.019 and log rank p=0.33, respectively). Meanwhile, incident IPF patients, with presence of autoantibodies survived longer compared to incident patients with no autoantibodies (log-rank p=0.009) (Figure 7C). Further, when patients were grouped by number of reactivities the results were similar with longer survival in patients with >5 reactivities (log-rank p=0.034). Also, there was a trend towards longer survival also in patients with 1-5 reactivities (log-rank p=0.062) (Figure 7D). Although analysis of time to progression rendered statistically non-significant results, similar trends of beneficial prognosis in patients with presence of autoantibodies were observed (Figure 5A-B, paper IV).

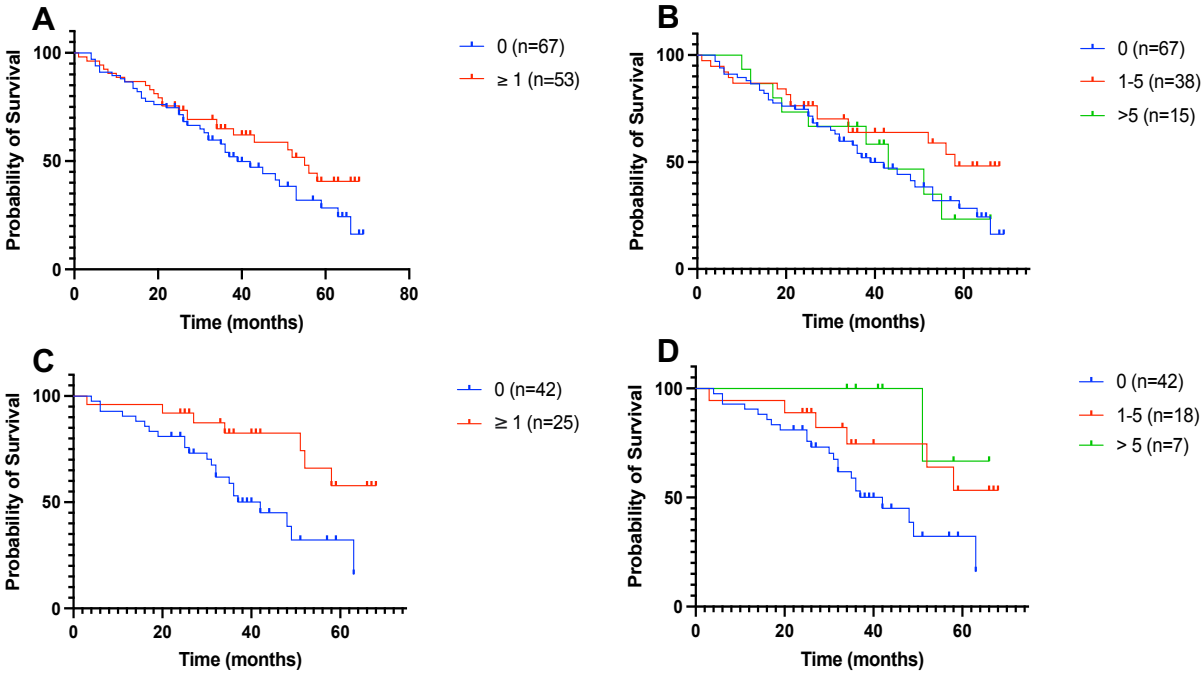

Figure 7A-D. Survival time estimates in the full cohort (A and B) and in the incident (time between diagnosis and serum collection <15 months) sub-cohort (C and D) of IPF patients. Time estimates are calculated from point of serum collection and stratified by presence or absence of antibodies (A and C) and number of reactivities (B and D) against RA-associated peptides in patients with IPF.

Discussion of paper IV

In this study we show how a substantial proportion of patients with IPF display autoantibody reactivity against posttranslational modified peptides that are known to be specific for RA. The prevalence and concentration of autoantibodies targeting specific epitopes of the peptides tenascin, fibrinogen and filaggrin was higher than in sex- and smoking matched healthy controls. In addition, almost 10% of patients with IPF presented with multiple reactivities (>5 reactivities) as opposed to 3% in HC. As expected, RA-patients demonstrated a comprehensive autoantibody reactivity against citrullinated peptides with elevated concentrations compared to IPF patients. Finally, explorative analyses in a cohort of incident patients suggested that presence and number of circulating autoantibodies may be associated with longer survival.

The post-translational modifications and the detection of ACPAs and AMPAs play important roles in the development and identification of RA [213]. ACPAs are prevalent in patients with RA-ILD and their presence and number of specificities have shown associations with ILD severity [104, 132, 133, 141]. The wide array of modified peptides tested in our study such as tenascin, may not be captured with the anti-CCP test and may thus explain both the substantially higher prevalence of ACPA reactivity reported (44%) and the presence of autoantibodies in anti-CCP negative patients. In light of the similarities observed between IPF and RA-ILD and the significance of reactivity against these posttranslational modifications in RA, our results raise questions on if the lung damage observed in these patients are in fact caused by AMPA, but different from the processes that we know of from RA? This due to the differences seen in reactivity pattern in the RA group. Perhaps this is a different entity of IPF? Further studies are certainly needed to address these questions. A limitation with our study is that we do not know if patients with presence of autoantibodies developed a connective tissue disease later on. Another limitation is the age difference between IPF and HC, as age may influence reactivity. Nonetheless, this study is to our knowledge the first to explore simultaneous ACPA and AMPA reactivity in IPF.

Collectively, our study shows how pathways associated with the adaptive immune system and autoimmunity are active in a proportion of IPF patients with potential prognostic implications. Future large prospective studies are needed to identify genetic, environmental and other factors resulting in the generation of ACPA and AMPA and their prognostic importance in IPF.

6 CONCLUSIONS

In this thesis, I have aimed, with the help of registry and biological data, to contribute to the complex and expanding puzzle of IPF. We have investigated clinical aspects of IPF, how it presents and develops and explored biological pathways through multiplex methods which may extend our knowledge on IPF's pathogenesis and heterogeneity.

From a methodological perspective, the studies highlight the possibilities and challenges when working with real-life data from registries. Knowledge of areas of improvement and opportunities is essential in order to place the registry in a valuable position to explore and address important research questions.

The main conclusions and the take-home-messages from this thesis are:

- There is a gender imbalance in lung function and in comorbidities, with males presenting with lower lung function and a higher burden of cardiovascular diseases and coronary heart disease in particular
- IPF patients enrolled in the Swedish IPF-registry have similar characteristics to patients enrolled in other IPF-registries with regards to demographics, clinical characteristics, comorbidities and survival
- We reinforce the literature on the importance of predictors of IPF progression such as GAP, CPI and measures of lung function, and demonstrate the agreement for mild impairment using GAP stage 1 was good to fair with other proposed criteria
- Patients treated with antifibrotics live longer
- We identified three clusters of IPF-patients distinguished by disease severity and cardiovascular diseases with differences in survival
- The pathological remodeling and architecture of the lung-ECM seen in IPF affects fibroblasts' behaviour by stimulating the release of mediators associated with remodeling, inflammation and chemotaxis such as MMP7 and CXCL13
- We confirmed protein signatures found elevated in a novel *ex-vivo* model in serum from patients with IPF, thereby validating the model as a promising pre-clinical tool to study the cell-ECM crosstalk in IPF
- Patients with IPF show elevated levels of multiple proteins associated with remodeling, inflammation and chemotaxis such as MMP7, MMP12, CXCL13, HGF, IL-6 and MCP-3
- Elevated levels of, among others, MMP7 and TNFRSF12A were associated with lung function decline and progression
- Compared to healthy controls, we found presence of AMPAs in a significant proportion of IPF patients, suggesting a potential role of the adaptive immune system and autoimmunity in a subgroup of patients with potential effects on mortality
- The pattern of autoreactivity against posttranslational modification of proteins in IPF patients was different from RA-patients, with fewer patients that had autoantibodies, less citrulline specific reactivity and lower concentrations of autoantibodies

7 POINTS OF PERSPECTIVE

These studies provide a glimpse of the opportunities and challenges when conducting research on registry data. Registries can help fill in the gaps from clinical trials and through the collection of clinical variables and biological samples at multiple time points, we have a unique opportunity to generate substantial quantity of data across several dimensions.

Registry-based research may be most useful when all data are complete. However, inherent to the nature of registries is missing data, which limits statistical power, analytical capabilities and the interpretation of results. Building a multicenter registry and biobank infrastructure within the framework of real-life routine care takes time and requires considerable resources. A plan to complete missing data will be executed in a near future in parallel to the regular data registrations. Furthermore, with Sweden in the forefront of registry-based data sources, linkage of registries could potentially be used to fill missing gaps in the registry. With several registries in Europe and the rest of the world collecting data and biological samples on IPF but also other ILDs, there is a big opportunity to increase power and capabilities of approaching genotypes, disease phenotypes and evaluate treatments. Collaboration is crucial to maximize the value of registries to the benefit of patients.

Gender differences in patients with IPF has gained more attention in recent years with studies demonstrating its implications in diagnosis, treatment patterns and outcomes. Attention to the role of these differences in ILD research will result in more accurate diagnostic and outcome assessments. Our cross-sectional study on gender differences in paper I, the cluster analysis in paper II and the investigations of serum samples performed in paper III and IV would be interesting to combine and advance in this regard, investigating potential gender differences and phenotypes across clinical and biological dimensions.

The results from the project exploring autoantibodies in patients with IPF raises interesting questions on the role of autoantigens in IPF pathology. With findings of autoantibodies in peripheral blood from serum, a natural extension would be to explore the generation of autoantibodies locally within the lung through analysis of BAL from patients with IPF. This in order to find out if a certain subset of patients with IPF have a humoral dysregulation were the fibrogenesis is antibody-mediated, which ultimately might mean the possibility of managing patients with targeted therapies available today.

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