Insights in Diagnostic and Prognostic Biomarkers in Interstitial Lung Disease

Sofia Moll

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Sofia Ana Moll

Thesis University of Utrecht

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Sofia Ana Moll

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Promotor:
Prof. dr. J.C. Grutters

Copromotoren:
Dr. A.D.M. Vorselaars
Dr. A.C.M. Platteel

Content of thesis

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General introduction and outline of the thesis

Abbreviation list

6MWT = Six minute walking test
6MWD = Six minute walking distance
AIP = Acute interstitial pneumonia
ANA = Antinuclear antibody
ATS = American Thoracic Society
ASS = Antisynthetase syndrome
BAL = Bronchoalveolar lavage
CA 15-3 = Cancer antigen 15-3
CCL18 = CC-chemokine ligand 18
COP = Cryptogenic organizing pneumonia
CTD = Connective tissue disease
DIP = Desquamative interstitial pneumonia
DLCO = Diffusing capacity of the lung for carbon monoxide
DM = Dermatomyositis
EGPA = Eosinophilic granulomatosis with polyangiitis
ERS = European Respiratory Society
FVC = Forced vital capacity
Hep-2 = Human Epithelial cell line-2
HP = Hypersensitivity pneumonitis
HRCT = High-resolution computed tomography
IIP = Idiopathic interstitial pneumonia
ILD = Interstitial lung disease
IPAF = Interstitial pneumonia with autoimmune features
IPF = Idiopathic pulmonary fibrosis
KL-6 = Krebs von den Lungen
LIP = Lymphoid interstitial pneumonia
MAA = Myositis associated antibody
MSA = Myositis specific antibody
NSIP = Non-specific interstitial pneumonia
OP = Organizing pneumonia
PFT = Pulmonary function test
PM = Polymyositis
PPFE = Pleuroparenchymal fibroelastosis
RA = Rheumatoid arthritis
RB-ILD = Respiratory bronchiolitis interstitial lung disease
sIL-2R = Soluble interleukin-2 receptor
SLE = Systemic lupus erythematosus
SP-A = Surfactant protein A

SP-D = Surfactant protein D

Ssc = Systemic sclerosis

UIP = Usual interstitial pneumonia

An introduction to diagnostic and prognostic biomarkers in interstitial lung disease.

Definition and aetiology

Interstitial lung diseases (ILDs) are a diverse group of rare, heterogeneous, diffuse parenchymal lung diseases, characterized by inflammation and/or fibrosis of the pulmonary interstitium (1–4). Fibrotic ILD is the result of fibroblast proliferation and extracellular matrix deposition of the lung parenchyma (5).

As described by the international consensus statements of The American Thoracic Society/European Respiratory Society (ATS/ERS), ILDs are classified based on underlying aetiology (6). Major categories include ILD secondary to connective tissue diseases (CTDs; i.e. systemic sclerosis (Ssc), antisynthetase syndrome (ASS), rheumatoid arthritis (RA)), ILD related to environmental exposures (i.e. hypersensitivity pneumonitis (HP), drug induced, pneumoconiosis), granulomatous disease (i.e. sarcoidosis) and idiopathic interstitial pneumonias (IIP) (7). The ARS/ERS has revised the classification of the latter group in 2013 into major, rare and unclassifiable forms of IIP. Idiopathic pulmonary fibrosis (IPF) is the most common form of IIP. Other types of IIP include non-specific interstitial pneumonia (NSIP), respiratory bronchiolitis interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), lymphoid interstitial pneumonia (LIP), pleuroparenchymal fibroelastosis (PPFE) and unclassifiable IIP (7).

In the past, patients were frequently misclassified as having a form of IIP such as IPF, whilst associations with known aetiologies such as CTDs or environmental exposures could have been identified. To date, more than 200 subtypes of parenchymal lung diseases have been described (8). However, despite ongoing updates of international guidelines based on scientific- and practice based evidence, it remains challenging for clinicians to distinguish one ILD subtype from another as patients may present with similar clinical characteristics (9). Decision making in therapeutic regiments and predicting course of disease depends on the underlying ILD subtype. Therefore, an accurate classification of ILD is crucial (7). Currently, standardized guidelines on diagnosis, treatment and prognosis in ILD are based on clinical, radiological, cellular and histological characteristics. Possibly, blood biomarkers could contribute to a more accurate diagnostic work-up, monitoring of treatment response and early signaling of progressive disease in ILD.

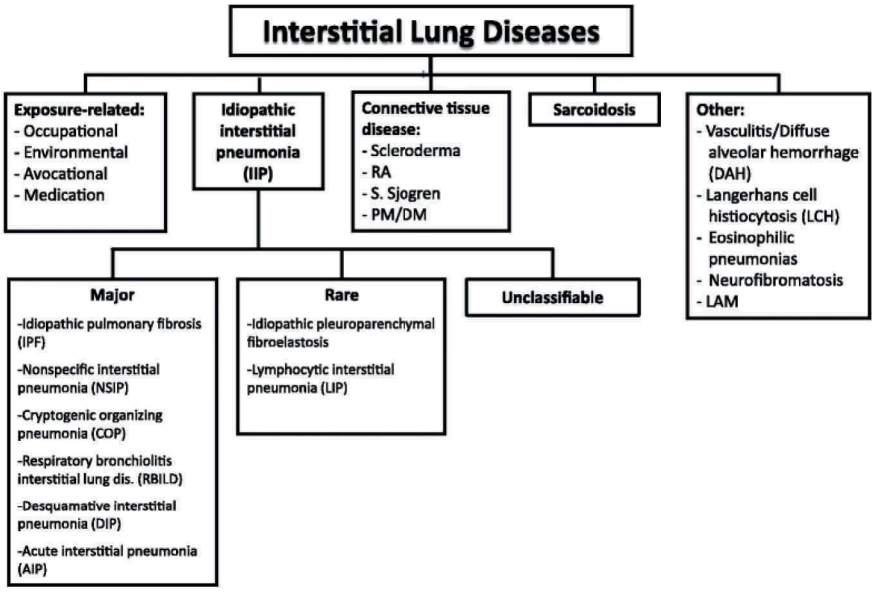

Figure 1. Classification of interstitial lung diseases, based on underlying etiology (10). Reprinted with permission, according to Creative Commons Attribution License requirements.

Diagnostic work-up of pulmonary fibrosis

The current principles of the diagnostic work-up of pulmonary fibrosis focus on the recognition of clinical characteristics of ILD and its underlying aetiology. Scientific research and expert's opinions have contributed to the establishment of uniformed approaches and classifications of ILDs at an international level (7,11–13). Current official ATS/ERS guidelines recommend a standardized diagnostic approach of ILD by obtaining a detailed patient history combined with serological, radiological and histopathological characteristics which are discussed in a multidisciplinary discussion between clinicians, experienced thoracic radiologists and pathologists to reach consensus on an ILD diagnosis (7,11–13). Clinical characteristics contributing to classification and diagnosis of ILD are discussed in the following paragraphs.

Clinical manifestations

Patients with pulmonary fibrosis can present with a wide variety of symptoms. A detailed history on clinical features, occupational, environmental and familial factors is essential, as certain factors are associated with particular ILDs. Dyspnoea and a non-productive cough are common clinical features in the majority of ILDs (14,15). A history of cigarette smoking is associated with an increased risk for smoking related IIPs, IPF and the development of pulmonary fibrosis in CTDs including RA, polymyositis (PM) and dermatomyositis (DM) (16–19). In addition, smoking is a risk factor for progressive ILD (20). Exposure to factors including birds, livestock, farming, wood and stone dust are associated with HP, IPF and pneumoconiosis (21,22). Particularly exposure to asbestos fibres should be explored, as it is a recognized

risk factor for the development of both asbestosis and IPF (23,24). Furthermore, it is necessary to obtain a detailed family history for ILD, as 2-20% of patients with IPF have a first-degree relative with an ILD (25). Moreover, shortened telomere length is a risk factor for development of human telomere disease, which is characterized by familial ILD and extra pulmonary manifestations including bone marrow abnormalities, liver disease, osteoporosis and premature aging (26,27). Radiation and drugs including amiodarone, antibiotics, rheumatology drugs and cancer drugs are recognized triggers of drug-/radiation-induced ILD (28,29).

The course of symptoms varies among ILDs. Patients with IPF generally present around the age of 60 years with increasing incidence with older age (15). Contrary, clinical features of CTD-ILD or exposure related ILD including HP can manifest at a much younger age (21,30). In addition, a female predisposition is seen in CTD-ILDs including DM and ASS (31–33), whereas patients with IIPs are predominately males (34–36).

Physical examination

Gas exchange abnormalities leading to hypoxemia are a hallmark feature of ILD and clinically detected by a low arterial oxygen saturation in rest and/or during exercise. In addition, functional limitations and a decreased exercise capacity in generally seen in ILD, as evaluated by the six minute walking test (6MWT)(37). A diagnosis of IPF should be considered in patients with digital clubbing and/or bibasilar inspiratory crackles, which is present in 25-50% of IPF, and symptoms that suggest a multisystem disease should be absent (38). Peripheral oedema may suggest right heart failure as a clinical feature of pulmonary hypertension secondary to ILD (39). Manifestations of autoimmune features including Raynaud phenomena, arthralgia, arthritis, myalgia and skin abnormalities are indicative for a CTD, but not all patients meet the established criteria of a CTD. Recently, this phenotype was designated as interstitial pneumonia with autoimmune features (IPAF) and added as a subgroup of IIP to standard ILD classifications (1). However, it remains challengeable to classify these patients, as pulmonary fibrosis may be the first or lone clinical manifestation of an associated CTD (4,9).

Pulmonary function test

Execution of pulmonary function tests (PFTs) is indicated in the diagnostic work-up of all ILD and include spirometry and diffusion capacity for carbon monoxide, carried out according to ATS/ERS recommendations (38). In general, a restrictive pattern is observed with impaired clinical parameters including forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO) (7,21,40). Approximately 20-30% of all diagnosed ILD show a progressive fibrosing ILD phenotype, which is defined as an absolute yearly deterioration in FVC and DLCO of respectively ≥5-10% and ≥10-15%, accompanied with a decrease in the six minute walking distance (6MWD)(41,42).

Radiology

The diagnostic approach to an ILD is strongly dependent on characteristics of radiological imaging, performed by high-resolution computed tomography (HRCT). Until recently, radiological ILD patterns were classified as a pattern of 'usual interstitial pneumonia' (UIP), 'possible UIP' or 'inconsistent

with UIP' (15). In 2018, the ATS/ERS statement on the diagnosis and treatment of IPF was updated and revised this classification into four patterns, including 'UIP', 'probable UIP', 'indeterminate for UIP' and 'alternative diagnosis' (38).

A UIP pattern is characterized by a sub pleural and basal distribution of honeycombing, peripheral traction bronchiectasis and traction bronchiolectasis (38). Features suggesting an alternative diagnosis are absent in a typical UIP pattern. A probable UIP or indeterminate UIP pattern is characterized by a sub pleural and basal distribution as well, but mild ground glass opacities or subtle reticulations may be present. Features suggestive of alternative ILD or other lung disease, include cysts (lymphangioleiomyomatosis), bronchiocentric fibrosis with mosaic attenuation (HP), pleural plaques (asbestosis), pleural thickening (CTD, drug-induced ILD) or nodules (pneumoconiosis, lung cancer) (38).

A radiological UIP pattern has 90-100% predictive value for a histological UIP. It is considered to be a hallmark for IPF and associated with impaired survival compared with non-UIP radiological patterns (38,43). Strikingly, up to 50% of IPF patients do not present with a typical radiological UIP pattern (44). Radiological patterns were reclassified in the updated official ATS/ERS statement to enhance decision making on additional investigations, such as bronchoalveolar lavage (BAL) and/or surgical lung biopsy. Consequently, an accurate ILD diagnosis could be reached without the necessity of invasive interventions (38). Patients presenting with the formerly known 'possible UIP' pattern who also had the presence of radiological peripheral traction bronchiectasis, showed a positive predictive value of 95% for a histological UIP (45). Therefore, this radiological phenotype was designated as 'probable UIP' and included to the standardized classification in the recent ATS/ERS update in order to avoid potentially unnecessary lung biopsies (38). Still, clinicians should be aware that a progressive fibrosing type of HP, RA-ILD or sarcoidosis may present with radiological characteristics of UIP as well (21,46). Moreover, a histological UIP pattern is revealed in 30% of lung biopsies of patients who present with radiological non-UIP features (47).

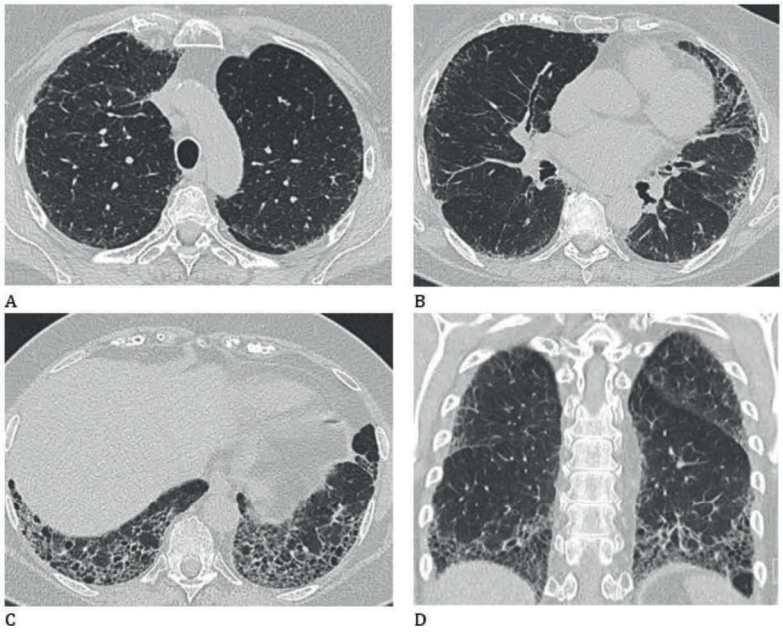

Figure 2. High-resolution computed tomography (HRCT) images from a patient demonstrating a pattern of usual interstitial pneumonia (UIP). (A-C). Transverse and (D) coronal CT section illustrating a sub pleural and basal distribution of honeycombing, peripheral traction bronchiectasis and traction bronchiolectasis. *Department of Radiology, St Antonius Hospital, Nieuwegein, The Netherlands*

BAL

Based on the clinical context and radiological findings on HRCT, a BAL can be performed for cellular analysis of BAL fluid (BALf) to exclude alternative diagnosis and reach an accurate ILD diagnosis (48). Lymphocytosis in BALf, defined as lymphocyte differential count ≥ 15%, is associated with pulmonary fibrosis including CTD-ILDs, COP and NSIP, whilst an extensive lymphocytosis (≥25%), including increased $CD4^+/CD8^+$ T-lymphocyte ratios, suggests a diagnosis of sarcoidosis or HP (21,48). Eosinophilia (≥1%) is indicative for eosinophilic pneumonias or drug-induced ILD but may suggest a non-ILD diagnosis as well including asthma, eosinophilic granulomatosis with polyangiitis (EGPA) or allergic bronchopulmonary aspergillosis (48). Neutrophilia (≥3%) can be demonstrated in patients with IPF, asbestosis or infectious diseases (48).

To date the prognostic value of cellular patterns in BALf is limited in ILD, since abnormal differential counts are indicative for a diagnosis, but not sensitive for predicting progression or mortality (48).

Histopathology

If an accurate ILD diagnosis cannot be defined based on clinical and radiological features and BAL, a histological lung biopsy is required for identification and classification of microscopic findings of ILD.

Histological patterns have been reclassified in the recent official ATS/ERS statement as well into a pattern of 'UIP', 'probable UIP', 'indeterminate for UIP' and 'alternative diagnosis' (38). A typical histological UIP pattern is characterized by a sub pleural and/or paraseptal distribution of patchy dense fibrosis and fibroblast foci, causing an architectural distortion of the lung which results in destructive scarring and honeycombing (38). Signs of inflammation, including interstitial infiltrates of lymphocytes and plasma cells, are usually mild in a typical UIP. A probable UIP shows histological features of a near-typical UIP without signs of alternative diagnoses, whereas an indeterminate UIP pattern has characteristics of both UIP and alternative diagnoses (38). Key features in histological lung biopsies suggesting alternative diagnoses include nonnecrotizing granulomas (sarcoidosis, HP), organizing pneumonia with fibrosis (COP), asbestos bodies (pneumoconiosis) or intraalveolar fibrosis with elastosis (PPFE).

It has been demonstrated in various studies that ILD patients with non-UIP histological characteristics, including NSIP and OP, have better survival rates compared to histological proven UIP (49). However, there is a risk on histological misclassification in patients with histologic variability (49).

Biomarkers

Despite standardized classification guidelines based on features as described in the previous paragraphs, it remains challenging to distinguish one ILD from another to establish an accurate diagnosis, treatment and prognosis (9). Multiple prediction models on histological outcomes using demographical, clinical and radiological characteristics have been proposed. Besides the fact that these models are based on former ATS/ERS diagnostic criteria, no consensus was reached on a standardized prediction tool (44,45,49–52). Individual prognosis in ILD is still unpredictable, as disease courses range from a slow deterioration to rapid declines with acute exacerbations and death (53,54). Currently, monitoring of disease activity, therapy response and progression is predominately assessed by PFT change and HRCT scanning (42). However, PFT declines are not specific enough to predict individual prognosis. Small yet relevant PFT changes can occur within the intrinsic variability of the PFT. Furthermore, PFT and HRCT scanning are time-consuming and costly. Moreover, PFT and HRCT scanning may be invasive in patients with a severe condition, resulting in possibly inaccurate values (55–58).

There is a need for reliable, non-invasive and objective markers for diagnosis, treatment response and prognosis in ILD. Biological markers, also referred to as biomarkers, are defined as objectively measured characteristics which are an indicator for physiological processes, pathological processes or pharmacological responses to therapeutic interventions (59). Biomarkers can have valuable applications in detection and monitoring of disease and include:

1. Diagnosis
2. Staging of disease
3. Indicator of progression of disease
4. Prediction and monitoring of response to treatment

Blood biomarkers can act as ideal biomarkers for ILD, as they can be easily sampled and analyzed and are generalizable. A substantial number of studies have investigated the application of serological lung biomarkers for diagnosis, treatment response and prognosis in ILD (60). The current evidence and

clinical challenges of circulating lung biomarkers is discussed in the following paragraphs, categorized according to their biology and source of production.

Precipitins

Presence of serum IgG specific to inhaled protein antigens from fungi, including *Aspergillus* spp. and *Micropolyspora faeni*, and bird droppings including parrots, pigeons, hens, ducks and turkeys, also referred to as precipitins, differentiate HP from other ILDs and healthy controls (21,61–64). Serum sampling of precipitins is recommended to identify potential exposures of HP, as 61-97% of patients with clinical exposure to inhaled antigens demonstrate associated precipitins (21,62,63). They also have a therapeutic value, as treatment regiments in HP include avoidance of the recognized antigen. The prognostic role of precipitins remains unclear though (21,65). It is thought that the presence of specific IgG antibodies reflects the autoimmune response to a specific antigen, indicating that a sufficient level of exposure has led to the development of sensitization (61). However, both the diagnostic and therapeutic roles of specific IgG antibodies in HP management can be challenging, as evidence is lacking on established physiological intervals for IgG levels against potential causal antigens (61).

Cytokines/chemokines

Soluble interleukin-2 receptor (sIL-2R) is considered to be a reflection of granuloma formation and suggested as a biomarker in sarcoidosis for diagnosis and staging of disease severity (66,67). Activation of Th1 cells leads to the expression of IL-2 receptor on the cell surface and release of sIL-2R into the circulation. sIL-2R plays a role in the regulation, activation, proliferation and survival of different T-cell subsets (68,69). Between 30-100% of patients with sarcoidosis have elevated sIL-2R levels (66,67). Although sIL-2R can be increased in systemic inflammatory conditions characterized by T-cell activation, including infectious diseases and other granulomatous- and autoimmune disease as well, high sensitivity and specificity (respectively 88% and 85%) was found for sarcoidosis, highlighting its diagnostic value (68). However, elevated sIL-2R levels can be found in non-sarcoidosis ILD, including IPF and HP as well, even though higher sIL-2R levels differentiate sarcoidosis from non-sarcoidosis ILD and were predictive for a chronic phenotype (70,71). Consequently, sIL-2R levels could potentially be used for disease monitoring rather than diagnostic purposes (70).

CC-chemokine ligand 18 (CCL18) is a chemokine which is secreted by alveolar macrophages and alternatively activated by Th2 cells and occurs at high levels in lung tissue (72). CCL18 is associated with immune-mediated fibrotic processes in ILD, as it is thought that high CCL18 levels induce collagen overproduction by lung fibroblasts through signaling of Sp1 and basal Smad3 activity (72–74). CCL18 levels are high in IPF compared to healthy controls but do not differentiate between different ILD subtypes (75). Its prognostic role was demonstrated in Ssc-ILD, in which increased baseline serum CCL18 levels were predictive for progression of pulmonary disease (72,76). Furthermore, a clear association was demonstrated between elevated serum CCL18 levels with acute exacerbation and survival in IPF (77–79).

Lung proteins

The alveolar epithelium is composed of alveolar type I pneumocytes, which provide the thin surface of the alveolus, and alveolar type II pneumocytes, which show a characteristic morphology with lamellar bodies and apical microvilli (80). Alveolar epithelial pneumocytes contribute to the regulated production of pulmonary surfactant. In addition, they play a key role in lung development, epithelial repair and pulmonary host defense. Alveolar type II pneumocytes are capable of self-renewal and trans differentiation into alveolar type I pneumocytes in response to epithelial injury. As a result, lung homeostasis is maintained (80).

Repetitive fibroblast-myofibroblasts activation leads to epithelial cell injury of alveolar type II pneumocytes. Consequently, the permeability of the alveolar-capillary barrier is enhanced and production of lung proteins increases (81,82). In the past decades, these proteins have been thoroughly studied as promising biomarkers of alveolar epithelial cell injury in fibrotic lung diseases.

Surfactant protein A (SP-A) and D (SP-D) are lipoprotein complexes which contribute to surfactant homeostasis and pulmonary host defense (83,84). These proteins are secreted by alveolar type II pneumocytes and Clara cells. It is thought that destruction of alveolar capillaries lead to an increased permeability of the alveolar-capillary barrier and production of surfactant proteins in pulmonary fibrosis. Elevated serum SP-A and SP-D levels distinguish an IPF from other ILDs, lung cancer and healthy controls (75,85–87). Furthermore, increased SP-D values are predictive for pulmonary involvement in CTD-ILD including Ssc and PM/DM (60,88,89). Increased baseline values of SP-A and SP-A are both associated with progressive disease and mortality, enhancing its prognostic value (90–93).

Mucins are recognised as biomarkers, as they play a role in cell growth and tissue remodelling, compatible with the processes as seen in fibrotic ILD including IPF. An increase in mucin production reflects the process of tissue damage (94). Krebs von den Lungen antigen (KL-6) is an epitope of the heavily weight glycosylated mucin 1 protein and expressed by alveolar type II cells (95). High serum KL-6 levels are found in ILD compared to other lung diseases and healthy controls (96–100). Furthermore, increased KL-6 levels are predictive for pulmonary involvement in CTD-ILD, including Ssc-ILD, DM and PM (89,101). Its diagnostic value for differentiation between various ILDs is questionable though, as contradictory findings were reported (97,102,103). Increased baseline and serial serum KL-6 measurements are associated with an acute exacerbation and mortality in IPF, but its predictive value in other ILDs is unknown (96,98,104–107). Despite the large number of scientific research on KL-6 as a promising biomarker for ILD, it might not act as an ideal biomarker, as serum sampling is costly, not easy to analyse and not generally applicable.

Considered as an alternative biomarker for KL-6 is cancer antigen 15-3 (CA 15-3), also located at the mucin 1 protein. Serum CA 15-3 sampling is less costly and readily available compared to KL-6 (108–110). It is originally known as a tumour biomarker, but elevated serum CA 15-3 levels are found in IPF as well. Increased CA 15-3 levels distinguish patients with IPF from sarcoidosis, Ssc-ILD and healthy controls (109,111). Secretion of CA 15-3 is also associated with fibroblast activity, progression of pulmonary fibrosis and fibrotic characteristics on HRCT (111). The prognostic value of CA 15-3 has been demonstrated in fibrotic ILD, including HP, Ssc-ILD and RA-ILD, in which increased CA 15-3 levels at diagnosis were associated with progression of disease and PFT deterioration (108,111–120). Whether CA

15-3 secretion and serum CA 15-3 levels are influenced by course of disease or by treatment regiments, is a topic of this thesis.

Autoantibodies

Recognition of a CTD-ILD can be challenging, as pulmonary manifestations can precede the systemic onset or remain the only clinical feature of a related CTD (4,9). Autoantibodies are associated with autoimmune processes as seen in CTDs. Therefore, testing for circulating autoantibodies is recommended in pulmonary fibrosis suspected for an underlying CTD (9,43,121–127).

Detection of antinuclear antibodies (ANA), which is preferably detected by an indirect immunofluorescence assay on antigens of Human Epithelial cell line-2 (Hep-2) cells and subsequent confirmation specific by ELISA or ELIA, is the most adequate screening method for relevant autoantibodies in CTDs (122). High positive predictive values (range 88-100%) for CTDs were demonstrated in ANA-positive patients and included Ssc, DM, PM, Sjögren's syndrome, systemic lupus erythematosus (SLE) and mixed connective tissue diseases (128). Approximately half of the patients with an unknown ILD show ANA positivity at screening during the diagnostic work-up, with increasing probability for development of clinical CTD features in ANA positive subjects compared to ANA negative subjects (12,129,130). Nevertheless, screening should be repeated during follow-up as an ANA-test might turn positive with change of clinical course in ILD (131).

The ANA fluorescence pattern is associated with nuclear and cytoplasmatic autoantibodies, including myositis antibodies (9,122,126). Myositis specific antibodies (MSA) and myositis associated antibodies (MAA) are related to idiopathic interstitial myopathies, but are also demonstrated in CTD-ILDs (3,125,132–137). Screening for t-RNA synthase antibodies, which are cytoplasmatic antibodies, during the diagnostic work-up is highly recommended, as they are strongly associated with pulmonary fibrosis in ASS, DM and PM (9,33,138–140). Antibodies specific for Jo-1 are a strong predictor for an ILD and present in 30-50% of DM patients with pulmonary fibrosis (9,33). Furthermore, antibodies specific for PL-7 and PL-12 are seen in 60-77% of patients with ASS-ILD (123,138,139). Myositis antibodies also have a prognostic value, as presence of both t-RNA synthase antibodies and anti-Ro52/SSA, which is a nuclear antibody, is characterized by a chronic and severe ILD phenotype (9,141).

Interestingly, reactivity against ANA and myositis antibodies also occurs in ILDs without clinical features or future development of a CTD, including HP and IIPs (123,133,135,142,143). The clinical relevance of autoantibodies in these ILD phenotypes in unclear. Consequently, decision making for diagnosis and treatment in clinical practice can be challenging for antibody positive ILD without established CTD (9). It is unknown whether circulating autoantibodies play a role in the fibrotic processes of ILD and if so, whether they have a pathogenic role or act as a bystander of disease. Possibly, certain antibodies could be more associated with ILD features such as fibrosis than other characteristics of CTD.

Aim of thesis

The use of blood biomarkers as objective, non-invasive indicators for physiological processes, pathological processes and pharmacological responses to therapeutic interventions is attractive and promising for the management of ILD. To date, routine use of blood biomarkers for ILD, including lung proteins and autoantibodies, is not recommended in clinical practice despite the established evidence. It is crucial to investigate the diagnostic, therapeutic and predictive values of blood biomarkers more thoroughly before implementation in standard ILD care. Potentially, blood biomarkers could improve phenotyping of ILD and contribute to the avoidance of invasive diagnostic interventions, including a BAL or surgical biopsy. Furthermore, they could be applied for disease monitoring and for early signaling of progressive disease. As a result, a more accurate diagnostic work-up, monitoring of treatment response and predicting course of disease would be achieved.

The aim of this thesis was to obtain novel insights on diagnostic and prognostic blood biomarkers in pulmonary fibrosis. An overview of novel, potential diagnostic and predictive blood biomarkers in fibrotic ILDs will be outlined in this thesis.

Outline of the thesis

In **chapter two**, the predictive value of serum CA 15-3 as a biomarker for PFT outcome and mortality will be evaluated in patients with non-fibrotic and fibrotic HP during immunosuppressive therapy.

Chapter three of this thesis describes the potential of serum CA15-3 as a predictive biomarker for response to anti-fibrotic treatment and mortality in patients with IPF.

In **chapter four**, the influence of genetic variation in the *CCL18* gene on CCL18 expression and serum CCL18 levels will be evaluated in IPF patients. In addition, the relation between CCL18 expression and serum levels with mortality will be assessed in these patients.

In **chapter five**, prevalence and clinical associations are presented of novel myositis autoantibodies, including antibodies specific for Ks, Ha, Zoα, and cN1A, in a large cohort of CTD-ILDs and other ILD including IIP. Antibody prevalence and associations in ILD will be compared to healthy controls to evaluate their potential as diagnostic biomarkers.

Finally, **chapter six** describes the prevalence and clinical associations of myositis antibodies of the conventional myositis line-blot, including antisynthetase antibodies, in a large cohort CTD-ILD and other ILD including IIP. In addition, the potential of antibodies specific for Mi-2β as a diagnostic biomarker for fibrotic ILD will be separately evaluated by analyses of BALf and histological lung biopsies.

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2

Change in serum biomarker CA 15-3 as an early predictor of response to treatment and survival in hypersensitivity pneumonitis

Sofia A. Moll
Ivo A. Wiertz
Adriane D.M. Vorselaars
Henk J.T. Ruven
Coline H.M. van Moorsel
Jan C. Grutters

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Abbreviation list

AUC = Area under the curve
CA 15-3 = Cancer antigen 15-3
DLCO = Diffusing capacity of the lung for carbon monoxide
ERS = European Respiratory Society
FVC = Forced vital capacity
HP = Hypersensitivity pneumonitis
HRCT = High-resolution computed tomography
ILD = Interstitial lung disease
IPF = Idiopathic pulmonary fibrosis
KL-6 = Krebs von den Lungen
PFT = Pulmonary function test
ROC = Receiving operating characteristic

Abstract

Background: Hypersensitivity pneumonitis (HP) is an interstitial lung disease with a heterogeneous course of disease and treatment response. Cancer antigen 15.3 (CA 15-3), part of mucin-1, is believed to reflect epithelial cell injury and lung permeability and could be a potential biomarker for treatment response in HP.

Object: To assess the value of CA 15-3 as a predictive biomarker in non-fibrotic and fibrotic HP during immunosuppressive therapy.

Methods: Serum levels of CA 15-3 and pulmonary function tests (PFTs) were retrospectively retrieved from 48 HP patients treated with prednisone or cyclophosphamide at initiation of therapy (baseline), after three and six months. Pearson's correlation coefficient was computed to assess correlations between change in serum levels and PFT. Survival was evaluated using Kaplan-Meier curves.

Results: After six months of immunosuppressive therapy CA 15-3 levels decreased significantly compared to baseline ($p=0.001$). Change in CA 15-3 after six months correlated with FVC change ($r=-0.469$; $p=0.001$). Correlations with FVC change were observed in prednisone-treated HP ($r=-0.514$; $p=0.005$) and fibrotic HP ($r=-0.417$; $p=0.007$). Three-month CA 15-3 change correlated with six-month FVC change ($r=-0.599$; $p<0.001$). CA 15-3 declines of at least 7.9% after six months were associated with increased survival compared to minor CA 15-3 changes (HR 0.34; $p=0.020$).

Conclusion: Serum CA 15-3 correlates with PFT during six months of immunosuppressive therapy in HP. Interestingly, early CA 15-3 changes could predict future PFT. Furthermore, a decrease in CA 15-3 is related to longer survival. Therefore, serum CA 15-3 is a promising biomarker for implementation in HP care.

Introduction

Hypersensitivity pneumonitis (HP) is an interstitial lung disease (ILD) caused by sensitization to a repeatedly inhaled antigen (1,2). In reaction to the antigen, a hypersensitivity response is provoked in pneumocytes of bronchioles and alveoli, leading to granulomatous inflammation, epithelial cell injury and increased lung permeability (1,3). The disease can manifest as a non-fibrotic HP, most commonly characterized by inflammation and a fever like clinical presentation, or as fibrotic HP characterized by pulmonary fibrosis and progressive loss of vital lung capacity as the result of repetitive cell injury and increased lung permeability (2,4). Although therapeutic evidence is scarce, avoidance of antigen and immunosuppressants are frequently prescribed (2,4,5).

Progressive HP is characterized by decline in pulmonary function tests (PFTs), in particular forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO) (2,5). Monitoring of disease activity, progression of disease and therapy response is mainly determined by longitudinal PFT (2) and high-resolution computed tomography (HRCT). Nonetheless, course of disease is variable and hard to predict. Small but relevant PFT changes can be within the intrinsic variability of the PFT. Moreover, PFT measurement and HRCT scanning are costly and may be invasive in HP patients with a severe condition (2,6). These factors necessitate the search for reliable, non-invasive predictors.

Possibly, disease activity and PFT course could be monitored accurately in HP by blood biomarkers like serum pneumoproteins (7). It is suggested that these pneumoproteins reflect epithelial cell injury, pulmonary production and lung permeability (8). Serum blood sampling is minimal invasive and biomarker measurement is easily accessible for most laboratories. A suggested pneumoprotein is Krebs von den Lungen antigen (KL-6), an epitope of the heavily weight glycosylated mucin 1 protein (9). It is expressed by alveolar type II cells in bronchi, bronchioles and alveoli (7,9) and increased levels of serum KL-6 are found in HP (10-13). However, KL-6 measurement is costly and not available for routine measurement on a large scale. Also located at mucin 1 is the epitope cancer antigen 15-3 (CA 15-3). In contrast to KL-6, serum CA 15-3 measurement is widely implemented in cancer care for treatment response and therefore available for routine analysis on a large scale. We previously showed that in fibrotic ILD, CA 15-3 can be used as an alternative biomarker for KL-6 (14). Elevated CA 15-3 levels are believed to reflect fibrotic formation and immunologic disease activity in various ILDs, including HP. Serum CA 15-3 levels are associated with PFT in fibrotic ILD like idiopathic pulmonary fibrosis (IPF) (15). However, it is unknown whether concentrations of circulating CA 15-3 reflect PFT change or treatment response with corticosteroids or other treatment regimens (14). To date, clinical value of CA 15-3 during follow up of HP patients under treatment is unknown. Change in CA 15-3 levels might be an early notice of therapy response and PFT change in HP patients.

This study retrospectively evaluated serum CA 15-3 as a predictive biomarker for HP treatment. We hypothesized that serum CA 15-3 levels decrease as pulmonary function improves during immunosuppressive therapy. Furthermore, we explored if (early change) in CA 15-3 levels would predict PFT change over time and survival.

Methods

A retrospective cohort study was conducted at the St Antonius ILD Centre of Excellence Nieuwegein, a tertiary ILD centre in the Netherlands. Medical records were retrieved of HP patients visiting the out-patient clinic between 2009 and 2016. To assess serum CA 15-3 as a biomarker for immunosuppressive therapy response, solely patients treated with corticosteroids or cyclophosphamide were included. The study was approved by the St Antonius institutional review board (registration number R05-08A and W14.056).

Patients were diagnosed as HP according to diagnostic recommendations of Salisbury et al in a multidisciplinary discussion with an ILD pulmonologist, experienced thoracic radiologist, and if needed a pathologist (2). Patients diagnosed as "HP likely" or "HP possible" were included. Lymphocytic alveolitis was classified as a bronchoalveolar lavage (BAL) cell count of >20% lymphocytes (smokers) or >30% lymphocytes (non-smokers). Patients without fibrotic high resolution computed tomography features were classified as non-fibrotic HP, whereas patients with fibrosis were designated as fibrotic HP.

Patients treated with immunosuppressive therapy were seen at the outpatient clinic every three months according to a standard protocol. Serum CA 15-3 assay was measured on an immunochemistry analyser (Cobas e601, Roche Diagnostics Ltd, Rotkreuz, Switzerland). Values above 30 kU/l were considered as elevated. The change of CA 15-3 between baseline and after three and six months was expressed in percentage change from baseline.

PFTs were routinely performed at baseline and six months after treatment initiation. Spirometry and diffusion capacity for carbon monoxide were executed at our hospital by a Jaeger Master Screen PFT (CareFusion Ltd, Houten, The Netherlands) and carried out according to European Respiratory Society (ERS) recommendations (16). The changes of FVC % predicted and DLCO % predicted are expressed in percentage change from baseline.

Solely HP patients treated with either corticosteroids or cyclophosphamide therapy and with at least two serum CA 15-3 samples and PFTs at set time points were included.

Corticosteroid treatment started at 0.5 mg/kg/day prednisolone and tapered to 0.15mg/kg/day within six months, unless otherwise decided for clinical reasons. Patients treated solely with corticosteroids were classified as the prednisone group. Patients treated with cyclophosphamide received corticosteroids as first line therapy before cyclophosphamide and were classified as the cyclophosphamide group. Treatment consisted of a four-week schedule during six months with six intravenous pulses dosed at 15mg/kg. All patients receiving at least four cyclophosphamide doses were included. Potential side effects were monitored with a monthly blood count and minimized by treatment with Mesna (sodium 2-mercaptoethane sulfonate, 200 mg) and instructions concerning high fluid intake and regular bladder voiding.

Statistical analysis

Baseline characteristics were expressed as mean and standard deviation or numbers and percentages, depending on the data. Statistical significance of the categorical characteristics between

baseline and follow up were compared with the Wilcoxon signed ranks test. Pearson's correlation coefficient (r) was computed between the variables at baseline and follow up measurement. Furthermore, three month biomarker values were correlated with six month PFT. The optimal cut-off value of CA 15-3 change was determined using receiver operating characteristic (ROC) curves. Survival rates were evaluated using Cox regression analysis and Kaplan-Meier survival curves.
A p-value less than 0.05 was considered statistically significant. The statistical analysis was performed with software IBM SPSS 24.0. Graphs were drafted in Graph Pad Prism 6.0.

Results

Medical records were retrieved of 105 HP patients that visited our outpatient clinic at the ILD department. Forty-eight HP patients were treated with corticosteroids or cyclophosphamide with available serum CA 15-3 at set time points and were included in this study. Seventeen patients were not treated with immunosuppressive therapy due to clinical improvement after eradication of antigen exposure and therefore excluded. Forty patients were excluded because follow up biomarker and PFT were measured on different set points. Table 1 summarizes the clinical characteristics at time of therapy initiation. Patients had elevated baseline CA 15-3 levels (mean 109.4; SD 95.6) with impaired baseline FVC (mean 70.1; SD 17.4) and DLCO (mean 40.1; SD 11.9). CA 15-3 levels declined noticeably after three months (mean 99.8; SD 89.2) and after six months (mean 78.9; SD 53.9)) of therapy (p=0.001. Table 2). Expressed as change in percentage, a negative CA 15-3 change was observed after three months (-11.1%) and six months (-16.1%) (p=0.004; Table 3, Figure 1a). Declines of CA 15-3 were observed in particular in prednisone treated HP (-21.7%; p=0.005, Figure 1b) and in non-fibrotic HP (-29.6%; p=0.123, Figure 1c).

Table 1. Characteristics of HP patients at time of start of therapy (n = 48)

Parameters	**Subjects**				
		Treatment		**Phenotype HP**	
	All	**Cyclophosphamide**	**Prednisone**	**Non-fibrotic HP**	**Fibrotic HP**
N - no (%)	48 (100.0)	20 (41.7)	28 (58.3)	8 (16.7)	40 (83.3)
Age (years)	62.3 (10.7)	64.4 (10.1)	60.8 (11.1)	58.3 (15.4)	63.1 (9.6)
Sex (M) - no (%)	16 (33.3)	4 (20.0)	12 (42.9)	4 (50.0)	11 (28.2)
Smoking					
Non-smoker - no(%)	22 (45.8)	9 (45.0)	14 (50.0)	3 (37.5)	20 (51.3)
Current/ex-smoker -	26 (54.2)	11 (55.0)	14 (50.0)	4 (62.5)	19 (48.7)
no(%)	12.6 (15.8)	14.6 (17.4)	9.6 (13.2)	12.0 (22.0)	12.7 (15.3)
Pack years - no (SD)					
Lung biomarker					
CA 15-3 (kU/l) – mean	109.4 (95.6)	114.2 (71.9)	106.0	86.8 (65.1)	114.0
(SD)			(110.6)		(100.6)
Pulmonary function					
FVC (%, pred) – mean	70.1 (17.4)	62.7 (18.0)	73.5 (24.5)	74.5 (28.8)	69.2 (14.5)
(SD)	40.1 (11.9)*	32.7 (7.1)	44.8 (12.0)	51.9 (14.9)	37.9 (10.0)
DLCO (%, pred) – mean					
(SD)					

CA 15-3 = cancer antigen 15.3; FVC = forced vital capacity; DLCO = diffusing capacity of the lung for carbon monoxide; *n = 44.

Although not significant, positive FVC changes (+1.9%) and DLCO changes (+3.0%) were observed after six months in the total cohort of patients (Table 3, Figure 1a). Positive PFT changes were most distinct in prednisone-treated HP (FVC: +6.8%; DLCO: +9.6 %; Figure 1b) and non-fibrotic HP (FVC: +19.6%; DLCO: +20.9%). However, negative PFT changes were observed in cyclophosphamide treated patients (FVC:-5.0%; p=0.004. DLCO:-9.3%, p=0.009, Figure 1c) and fibrotic HP (FVC: -1.6%; DLCO: -0.8%).

Table 2. Biomarker and PFT levels at baseline, 3 months and 6 months after treatment initiation in HP (n = 48)

Parameters	**Subjects**				
		Treatment		**Phenotype HP**	
	All	**Cyclophosphamide**	**Prednisone**	**Non-fibrotic HP**	**Fibrotic HP**
CA 15-3 (kU/l) (mean, SD)					
Baseline	109.4 (95.6)	114.2 (71.9)	106.4 (110.6)	86.8 (65.1)	114.0 (100.6)
3 months*	99.8 (89.2)	109.1 (55.3)	91.0 (113.6)	43.0 (31.2)	105.9 (91.5)
6 months	78.9 (53.9)	92.7 (44.1)	69.0 (58.7)	40.6 (23.9)	86.5 (55.2)
P-value**	0.001	0.082	0.002	0.058	0.005
FVC (%, pred) (mean, SD)					
Baseline	70.1 (17.4)	65.3 (14.7)	73.3 (18.9)	74.5 (28.8)	69.2(14.5)
6 months	71.7 (23.1)	62.7 (18.0)	78.6 (24.9)	85.6 (30.9)	69.0 (20.7)
P-value	0.903	0.083	0.255	0.263	0.605
DLCO (%, pred) (mean, SD)					
Baseline	40.1 (11.9)	32.7 (7.1)	44.8 (12.0)	51.9 (14.9)	37.9 (10.0)
6 months	41.8 (14.0)	30.8 (7.4)	48.1 (12.8)	55.6 (10.8)	38.9 (13.0)
P-value	0.835	0.009	0.096	0.735	0.957

CA 15-3 = cancer antigen 15.3, in kU/l; FVC = forced vital capacity; DLCO = diffusing capacity of the lung for carbon monoxide; $p < 0.05$ is considered statistically significant; *n = 31; **p = six month value compared to baseline

Figure 1. **A**. Change in percentage of CA 15-3 and PFT 6 months after start of therapy in all HP patients. **B**. Change in percentage of CA 15-3 and PFT 6 months after start of prednisone therapy. **C**. Change in percentage of CA 15-3 and PFT 6 months after start of cyclophosphamide therapy.

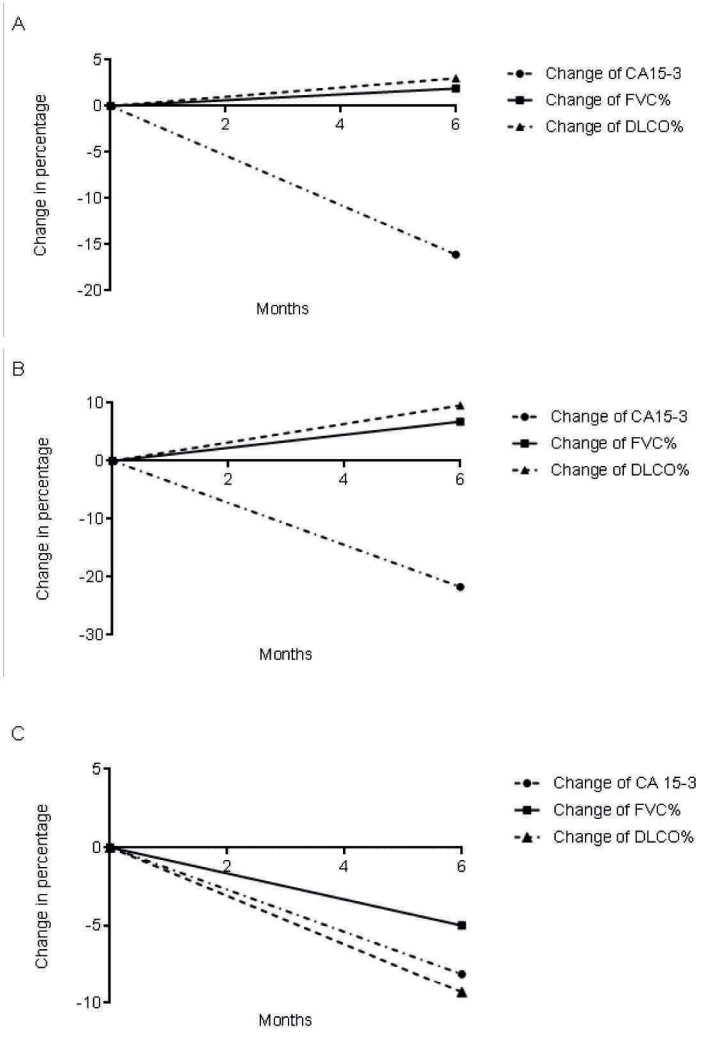


Table 3. Mean change in percentage of biomarker and PFT levels 6 months after treatment initiation in HP (n = 48)

Parameters	Subjects				
		Treatment		Phenotype HP	
	All	Cyclophosphamide	Prednisone	Non-fibrotic HP	Fibrotic HP
% - CA 15-3					
Three-month change, %*	-11.1 (28.9)	-3.6 (26.0)	-18.2 (30.3)	-23.4 (37.5)	-9.8 (28.3)
Six-month change, %	-16.1 (35.4)	-8.1 (32.2)	-21.7 (37.0)	-29.6 (39.6)	-13.4 (34.4)
P-value**	0.004	0.332	0.005	0.123	0.021
% - FVC					
Six-month change, %	+1.9 (22.1)	-5.0 (11.4)	+6.8 (26.5)	+19.6 (38.1)	-1.6 (15.9)
P-value	0.899	0.004	0.274	0.161	0.357
% - DLCO					
Six-month change, %	+3.0 (30.5)	-9.3 (11.1)	+9.6 (35.4)	+20.9(66.1)	-0.80 (15.0)
P-value	0.989	0.009	0.131	0.735	0.809

CA 15-3 = cancer antigen 15.3, in kU/l, expressed as change in percentage; FVC = forced vital capacity, in percentage of predicted, expressed as change in percentage; DLCO = diffusing capacity of the lung for carbon monoxide, in percentage of predicted, expressed as change in percentage; $p < 0.05$; *n = 31; **p = six month change compared to baseline; Parameters are expressed in mean and standard deviation

Correlations

Correlations between biomarker and PFT were evaluated at baseline and follow up measurements. Expressed as the percentage change from baseline in Table 4, moderate negative correlations were found between the six month CA 15-3 change with the six month FVC change ($r = -0,469$; $p = 0.00l$; see Figure 2a) and DLCO change ($r = -0.347$; $p = 0.028$), indicating that a negative change of CA 15-3 levels corresponds with a positive change of pulmonary function. Associations with FVC were most distinct in prednisone-treated HP ($r = -0.514$; $p = 0.005$) and fibrotic HP ($r = -0.417$; $p = 0.007$). In addition, associations between the three-month CA 15-3 change (n=31) and the six-month PFT change (n=48) were assessed, in order to evaluate if an early change of CA 15-3 could predict subsequent PFT change. CA 15-3 change at three months correlated strongly with the six-month FVC change ($r=-0.599$; $p<0.001$, see Figure 2b). Correlations with FVC were seen in particular in prednisone treated ($r=-0.612$; $p=0.012$) and fibrotic HP patients ($r=-0.551$; $p=0.002$). Overall, no significant correlations were demonstrated between baseline CA 15-3 levels with baseline PFT and only weak correlations were found between CA 15-3 levels and FVC at six months (data not shown).

Table 4. Pearson's correlation coefficients (r) between change in percentage of biomarker and change in percentage of pulmonary function parameters in HP patients (n = 48).

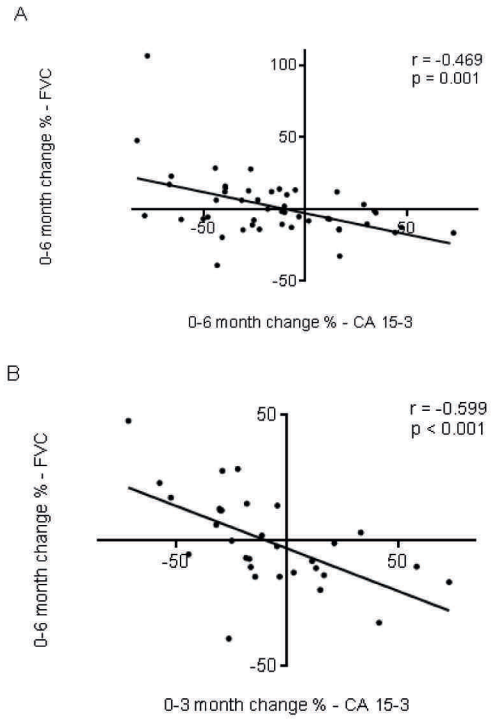
Subjects	Parameters			
	Correlations CA 15-3 –FVC			
	Three-month change CA 15-3, %* – Six-month change FVC, % (r)	P-value	Six-month change CA 15-3, % – Six-month change FVC, % (r)	P-value
All	-0.599	<0.001	-0.469	0.001
Cyclophosphamide	-0.469	0.060	-0.255	0.278
Prednisone	-0.612	0.012	-0.514	0.005
Non-fibrotic HP	-0.880	0.315	-0.575	0.136
Fibrotic HP	-0.551	0.002	-0.417	0.007
	Correlations CA 15-3 –DLCO			
	Three-month change CA 15-3, %* – Six-month change DLCO, % (r)	P-value	Six-month change CA 15-3, % – Six-month change DLCO, % (r)	P-value
All	-0.284	0.159	-0.347	0,028
Cyclophosphamide	0.286	0.423	0.504	0,066
Prednisone	-0.375	0.152	-0.446	0,022
Non-fibrotic HP	-0.998	0.039	-0.560	0,248
Fibrotic HP	-0.179	0.415	-0.133	0,459

CA 15-3 = cancer antigen 15.3, measured at 3 and 6 months after baseline, expressed as change in percentage; FVC = forced vital capacity, in percentage of predicted, measured at 6 months after baseline, expressed as change in percentage; DLCO = diffusing capacity of the lung for carbon monoxide, in percentage of predicted, measured at 6 months after baseline, expressed as change in percentage. r = Pearson's correlation coefficient; $p < 0.05$. *n = 31

Survival

Overall median survival was 54 months (inter quartile range 29.0-60.0). Within the study period 19 out of 48 patients died (39.6%). ROC curves of CA 15-3 change were carried out to define the optimal cut off value for progression of PFT. Progression was defined as an absolute FVC decline of more than 5% after six months. The ROC curve of three-month CA 15-3 change demonstrated an area under the curve (AUC) of 0.838 (p=0.002). A negative change of 3.88% was determined as the value with the highest sum of specificity and sensitivity (1.645) for predicting progression of PFT. Patients were categorized as having extensive (change <-3.88%) or little (change between -3.88-0% or positive change) CA15-3 decline. Survival analysis showed a significant better survival in patients with extensive CA 15-3 declines compared to little declines (Hazard ratio 0.25; 95% CI 0.09 – 0.71; p = 0.009, Figure 3a).

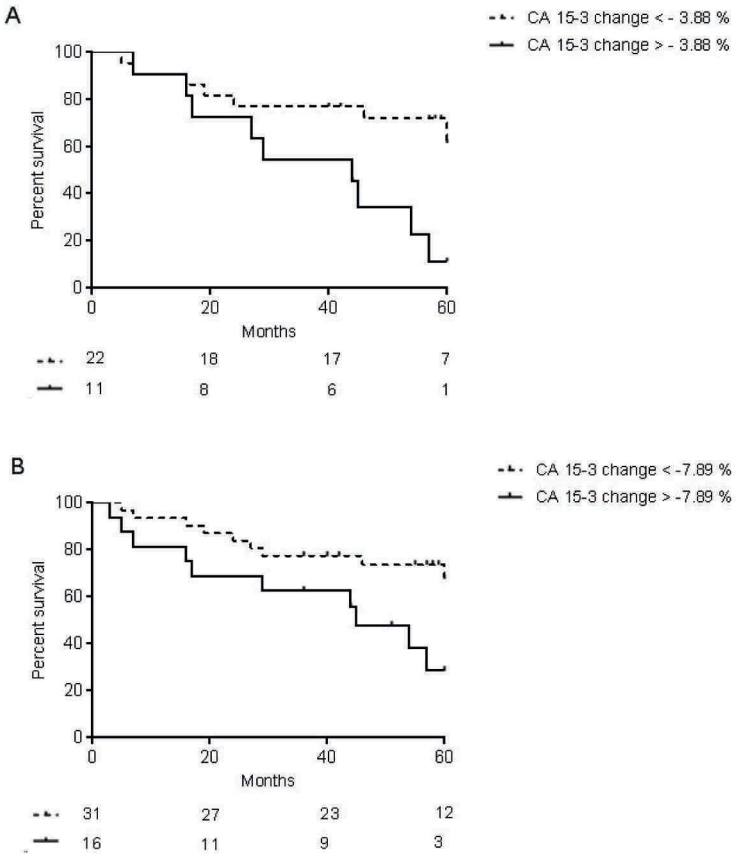
Figure 2. **A**. Pearson's correlation coefficients between0-6 months mean change in percentage of CA 15-3 and 0-6 months mean change in percentage of FVC in HP patients. **B**. Pearson's correlation coefficients between 0-3 months and 0-6 months mean change in percentage of CA 15-3 and mean change in percentage of FVC in HP patients.



The ROC curve of six-month CA 15-3 change demonstrated an AUC of 0.717 (p=0.014) with a negative change of 7.89% determined as cut off value for predicting PFT progression. Similarly, patients were categorized as having extensive (change <-7.89%) or little (change between -7.89-0% or positive change) CA15.3 decline, showing a survival in patients with extensive decline superior to little decline (Hazard ratio 0.34; 95% CI 0.14 – 0.84; p = 0.020, Figure 3b).

Figure 3. **A**. Kaplan Meier curve showing a significant better survival in patients with extensive CA 15-3 change after three months (change < - 3.88%) compared to little CA 15-3 change (change between - 3.88% and 0% or positive change) after three months (hazard ratio 0.25; 95% CI 0.09 – 0.71; p = 0.009, Figure 3A). The patient numbers at risk are represented.

B. Kaplan Meier curve showing a significant better survival in patients with extensive CA 15-3 change after six months (change < - 7.89%) compared to little CA 15-3 change (change between - 7.89% and 0% or positive change) after six months (hazard ratio 0.34; 95% CI 0.14 – 0.84; p = 0.020, Figure 3B). The patient numbers at risk are represented.



Discussion

We demonstrated that serum CA 15-3 has predictive value for lung function change and survival of HP patients. During treatment, CA 15-3 levels declined over time and correlated with positive FVC change, particularly in fibrotic HP and in prednisone treated patients. Interestingly, three-month CA 15-3 change was related to six-month FVC change, indicating that an early CA 15-3 change could predict future FVC change. Furthermore, CA 15-3 decrease was associated with a better survival compared to stable or increasing levels. Serum CA 15-3 therefore appears a potential predictive biomarker for use in clinical care of HP.

Elevated CA 15-3 and KL-6 levels, for which CA 15-3 can be used as an alternative biomarker, have previously been demonstrated in ILD including IPF and pulmonary sarcoidosis (10,11,13,17-24). In bird fancier's lung, KL-6 levels showed strong negative correlations with DLCO (14). A negative association between CA 15-3 and lung function (FVC) has been demonstrated in progressive IPF patients (15).

Immunosuppressive agents including corticosteroids and cyclophosphamide are regularly given as a treatment of HP (2,4,5,25,26). However, there is limited evidence of their effectiveness. Short-term benefit of prednisone has only been demonstrated in a small placebo-controlled study (27). Although HP is an ILD of frequent occurrence worldwide, biomarkers for treatment response have not yet been described in HP, in contrast to other ILDs (11,12,28-30). In ILD related to systemic sclerosis for example, change of ground-glass score, KL-6 and C-reactive protein were related to responsiveness of cyclophosphamide (31). In polymyositis and dermatomyositis related ILD, KL-6 was used as a biomarker for clinical response to immunosuppressive therapy (32). On the basis of our results we suggest further exploring to utility of CA 15-3 as predictive biomarker during treatment of HP, in particular for clinical decision making e.g. on the effectiveness of corticosteroids in individual cases.

Strongest correlations were found between the three month biomarker- and six months PFT changes. Although PFT did not significantly change during treatment, for the group as a whole, significant correlations were demonstrated with CA 15-3 change. It could be thought that in HP, damage or repair of lung tissue is reflected by an early, significant change of CA 15-3 levels. As a result, CA 15-3 levels would decline shortly after decreased disease activity and relate to little change of PFT which is observed later on. Of note, pulmonary function deteriorated in the cyclophosphamide treated patients, in contrast to prednisone treated HP. This result is possibly due to selection bias of progressive HP patients, as cyclophosphamide was solely administered as second line therapy after prednisone.

An interesting association between CA 15-3 and survival was demonstrated in our HP cohort. In IPF research, high baseline KL-6 levels and increasing levels during follow up were associated with poor survival compared to low or decreasing KL-6 levels (23,33,34). We observed a similar trend with CA 15-3 change on survival. It is hypothesized that elevated CA 15-3 levels reflect proliferation of type II alveolar pneumocytes, leading to wall damage. As a result, an active process of inflammation and fibrosis is induced, marked by pulmonary function deterioration (14). Our results suggest that descending CA 15-3

levels during therapy reflect restoration of parenchymal damage due to effective treatment, leading to improvement of PFT and longer survival rates.

This retrospective study has some limitations by selecting solely HP patients treated with immunosuppressive agents in an ILD referral centre with available follow up biomarker and PFT data. Selection bias of more severely impaired HP patients is possible, resulting in a reduced overall survival. These serum sampling and PFTs were all performed at the St Antonius Hospital, in order to have accurate follow up data with equal, standardized measurement points. A substantial number of HP patients are included in our study for analysis.

Serum CA 15-3 measurement could contribute to early decision making in therapy management of HP. As disease course is variable in HP, CA 15-3 measurement could improve predicting individual outcomes on disease course and survival. Serum CA 15-3 change could be more accurate to PFT change, as PFT changes are dependent of the intrinsic variability of the PFT (6).

A prospective evaluation in an HP replication cohort is needed to confirm CA 15-3 measurement as a biomarker of therapeutic effect of immunosuppressive and disease progression. It would be interesting to investigate CA 15-3 as a diagnostic biomarker and to evaluate associations between CA 15-3 with clinical symptoms like dyspnoea in HP. It should be noted that CA 15-3 levels can be increased by diseases other than ILD, such as breast cancer (35). Pneumoprotein CA 15-3, as part of mucin 1, is expressed in epithelial cells in lungs, breast and colon. Although not tumour specific, 60-80% of patients with early diagnose of breast metastases are characterized by high CA 15-3 levels (35,36).

In conclusion, we show that serum CA 15-3 could be useful as minimal invasive follow-up, prognostic biomarker for treatment-response during immunosuppressive therapy in HP. Furthermore, (early) changes of CA 15-3 could help predicting future PFT change upon therapy and survival.

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3

Serum biomarker CA 15-3 as predictor of response to anti-fibrotic treatment and survival in idiopathic pulmonary fibrosis

Sofia A. Moll
Ivo A. Wiertz
Adriane D.M. Vorselaars
Pieter Zanen
Henk J.T. Ruven
Coline H.M. van Moorsel
Jan C. Grutters

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Abbreviation list

ATS = American Thoracic Society
AUC = Area under the curve
CA 15-3 = Cancer antigen 15-3
CCL18 = CC-chemokine ligand 18
CEA = Carcinoembryonic antigen
DLco = Diffusing capacity of the lung for carbon monoxide
ERS = European Respiratory Society
FVC = Forced vital capacity
HP = Hypersensitivity pneumonitis
HRCT = High-resolution computed tomography
ILD = Interstitial lung disease
IPF = Idiopathic pulmonary fibrosis
IQR = Inter quartile range
KL-6 = Krebs von den Lungen
LDH = Lactate dehydrogenase
PFT = Pulmonary function test
RA = Rheumatoid arthritis
ROC = Receiver operating characteristic
Ssc = Systemic sclerosis
UIP = Usual interstitial pneumonia

Abstract

Background: Cancer antigen 15-3 (CA 15-3) is a baseline biomarker in idiopathic pulmonary fibrosis (IPF), but its value during follow-up is unknown.

Methods: Associations between serum CA 15-3 and pulmonary function tests (PFTs) during one-year follow-up were evaluated by a mixed model in 132 IPF treated with pirfenidone or nintedanib.

Results: Increased baseline (median 56 kU/l) and follow-up CA 15-3 levels were inversely associated with respectively FVC and DLco (estimates -5.21 and -4.69;$p<0.001$). Baseline and six-month CA 15-3 above 58.5 (HR 1.67;$p=0.031$) and 50.5 kU/l (HR 2.99;$p<0.001$) respectively showed impaired survival compared with lower levels.

Conclusion: CA 15-3 is associated with PFT during follow-up in IPF on anti-fibrotic treatment. Higher (follow-up) values are related with poor survival. Therefore, CA 15-3 is a promising follow-up biomarker in IPF.

Executive Summary

- Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease (ILD) of unknown cause with a heterogeneous prognosis.
- Blood biomarkers such as serum pneumoproteins have a predictive value in IPF as they reflect the process of remodelling and injury of the lungs.
- The epitope cancer antigen 15-3 (CA 15-3) is located at the mucin 1 protein, expressed at alveolar type II pneumocytes. Serum CA 15-3 has been thoroughly investigated as a prognostic blood biomarker in various fibrotic ILDs including rheumatoid arthritis-ILD, systemic sclerosis-ILD and hypersensivity pneumonitis.
- CA 15-3 is used a prognostic baseline biomarker in IPF, but its value during follow up is unknown.
- The current study evaluated the prognostic value of CA 15-3 during one-year follow up in IPF patients using anti-fibrotic therapy at the St Antonius Hospital in Nieuwegein, The Netherlands
- A repeated measures linear mixed model was used to analyse the associations between CA 15-3 and pulmonary function test (PFT) values over time. Survival was evaluated using Kaplan-Meier curves.
- A total of 132 IPF patients, predominately males (75.8%) with mean age 68.6 years (70 pirfenidone treated, 62 nintedanib treated) were included in the study. Mean follow-up duration was 47.0 months.
- Baseline CA 15-3 values were elevated in all IPF patients (median 56 kU/l).
- Increased baseline and follow-up CA 15-3 measurements were inversely associated with corresponding forced vital capacity (FVC; estimate -5.21; $p < 0.001$) and diffusing capacity of the lung for carbon monoxide (DLco; estimate -4.69 $p < 0.001$) values.
- IPF patients with baseline and six-month CA 15-3 levels above 58.5 (hazard ratio 1.67; $p = 0.031$) and 50.5 kU/l (hazard ratio 2.99; $p<0.001$) respectively were associated with an impaired survival compared to IPF patients with lower levels.
- No significant differences were observed between pirfenidone and nintedanib treated IPF patients in associations between CA 15-3 and PFT and in survival rates.
- In conclusion, serum CA 15-3 is associated with PFT course during one-year follow-up in IPF on anti-fibrotic treatment. Furthermore, higher baseline and follow-up CA 15-3 levels are related with poor survival.
- Serum CA 15-3 is a promising follow-up biomarker in IPF and could be implemented as a prognostic biomarker in IPF care.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive, fibrosing interstitial lung disease (ILD) of unknown cause (1). IPF patients are predominately male and the disease is characterized by progressive dyspnoea (2–4). IPF is the result of activated fibroblast-myofibroblasts after repetitive damage, which play an important role in tissue remodelling and injury of alveolar type II pneumocytes. As a result, pneumoproteins are secreted by type II pneumocytes (5,6).

The prognosis is extremely poor, with a median survival of 2-3 years after diagnosis, although this is very heterogeneous (7). Therefore, it is difficult to predict the exact prognosis (8). Currently, the six minute walking test is used to measure exercise tolerance as reflection of disease status in IPF (9). Furthermore, the GAP-index which is based on gender, age and pulmonary function tests (PFTs) is used for predictive purposes (10). More than 5% decline in forced vital capacity (FVC) and 15% decline in diffusing capacity of the lung for carbon monoxide (DLco) per year correlate with increased mortality in IPF (10–12). However, PFT declines are not specific enough to predict individual prognosis and PFTs may be invasive in patients with a severe condition (9,10,12,13).

Possibly, blood biomarkers such as serum pneumoproteins have a more accurately predictive value in IPF, as they reflect the process of remodelling and injury of the lungs (14). A well-studied serum biomarker is CC-chemokine ligand 18 (CCL18). It was demonstrated that increased baseline levels are associated with high mortality in IPF (15). Change in markers of the extracellular matrix were associated with disease progression and mortality in IPF as well (4,16). A suggested pneumoprotein is Krebs von den Lungen antigen (KL-6), an epitope of the mucin 1 protein which is expressed at alveolar type II pneumocytes (17). Mucins are recognised as biomarkers, as they play a role in cell growth and tissue remodelling, compatible with the processes as seen in IPF. (18) Increased baseline KL-6 predicted a higher risk on acute exacerbations and increased mortality in IPF at baseline and during follow up (17,19,20). KL-6 change was found to be an indicative of nintedanib response in IPF patients, as KL-6 levels declined during treatment and were associated with PFT (21). However, KL-6 analysis is expensive and not available on a large scale. The epitope cancer antigen 15-3 (CA 15-3), also located at mucin 1, is considered to be an alternative biomarker for KL-6 (14,22,23). It is a minimal invasive measurement available on a large scale and less expensive than the KL-6 ELISA kit (14). CA 15-3 was originally used as a tumour associated biomarker in neoplasms including lung carcinoma and breast cancer (24,25), but is also associated with fibroblast activity, progression of pulmonary fibrosis and fibrotic characteristics on high-resolution computed tomography (HRCT) scans (26).

The role of CA 15-3 as a prognostic biomarker has been confirmed in fibrotic ILDs including hypersensitivity pneumonitis (HP), systemic sclerosis (Ssc-ILD) and rheumatoid arthritis (RA-ILD)(22,26,35,27–34). Elevated baseline CA 15-3 levels were found in IPF and were higher compared to sarcoidosis, Ssc-ILD and healthy subjects (14,26). Furthermore, a negative correlation between serum CA

15-3 and FVC was found in progressive IPF patients referred for lung transplantation, whereas CA 15-3 was negatively associated with DLco in Ssc-ILD (29,36).

We previously demonstrated that (early) CA 15-3 change correlated inversely with (future) PFT change in hypersensitivity pneumonitis (32). However, CA 15-3 change over time has not been described in IPF. Furthermore, it is unknown whether CA 15-3 levels reflect PFT or treatment response of anti-fibrotic drugs, such as nintedanib and pirfenidone. These pharmaceuticals lead to a reduction of FVC decline in the majority of IPF patients (12,37). As fibrotic activity is reflected by CA 15-3 secretion by type II alveolar pneumocytes, it is possible that CA 15-3 reflects drug effectiveness as well.

This retrospective study investigated the prognostic value of serum CA 15-3 in IPF. We hypothesized that CA 15-3 during follow up was inversely associated with PFT in IPF patients using anti-fibrotic therapy. Furthermore, we evaluated if CA 15-3 predicted PFT outcome and survival.

Methods

A retrospective cohort study was performed at the St. Antonius Hospital Nieuwegein, a tertiary ILD centre in the Netherlands. Medical archives of patients diagnosed with IPF between February 2012 and February 2019 were examined. Patients were included if therapy with anti-fibrotic drugs, i.e. nintedanib or pirfenidone, was initiated within six months from date of diagnosis. Patients without anti-fibrotic treatment or initiation of therapy after six months were excluded. All patients gave written informed consent for retrospective study purposes. The study was approved by the St Antonius institutional review board (registration number R05-08A and W14.056).

Patients were diagnosed in a multidisciplinary discussion between a radiologist, ILD pulmonologist and if needed a pathologist, according to ATS/ERS/JRS/ALAT criteria for IPF: a pattern of usual interstitial pneumonia (UIP) found on HRCT and if available, in histological lung biopsy. Patients with a UIP secondary to known causes, including environmental exposures, collagen disease and drug induced lung fibrosis, were excluded.

IPF patients on anti-fibrotic treatment visited the outpatient clinic every three months according to a standard protocol. Serum CA 15-3 was routinely measured on an immunochemistry analyser (Cobas e601, Roche Diagnostics Ltd, Rotkreuz, Switzerland) according to the manufacturer's instructions. Samples were centrifuged and analysed within three hours after serum sampling. Values above 30 kU/l were considered as elevated. CA 15-3 levels measured at date of diagnosis (baseline) and during follow-up at three, six and twelve months were included for analysis.

PFTs were routinely performed at diagnosis and during therapy. Spirometry and diffusion capacity for carbon monoxide were executed at the St Antonius hospital by a Jaeger System (provided by CareFusion Ltd, Houten, The Netherlands) and carried out according to European Respiratory Society (ERS) recommendations (11). FVC percent predicted and DLco percent predicted measured at baseline and at three, six and twelve months of follow-up were included for analysis.

Patients with available biomarker and PFT measured at date of diagnosis and with at least one follow-up measurement were suitable for the study. Follow-up was censored after 72 months and ended if patients were referred to their own physician, if patients underwent a lung transplantation and in case of death. Patients were excluded from analysis if solely baseline measurements were available.

Other baseline variables evaluated in the study were age, sex, smoking status and GAP index. In addition, characteristics on HRCT and in histopathological lung biopsies were described. Characteristics were classified according to the most recent American Thoracic Society (ATS)/ERS recommendations as a pattern of UIP, probable UIP, indeterminate UIP or alternative diagnosis (38).

Statistical analysis

Baseline characteristics were expressed as mean and standard deviation for normally distributed data or as median and inter quartile range (IQR) for non-normally distributed data or as numbers and percentages. Independent sample T-test was executed to evaluate continuous data between the nintedanib and pirfenidone group, whereas Chi-square test was used to assess dichotomous data. A repeated measures linear mixed model was used to analyse the pulmonary function over time, as function of the observed CA 15-3 outcomes. In such an analysis for each patient, a regression equation is obtained depicting an intercept and the changes in PFT-outcomes between the fixed observation points. In this analysis the last observation serves as reference, while the intercept is the (PFT-) value at the start of the observation period (T=0). The correlation between successive observations needs to be taken into account: we used as starting point an autoregressive correlation structure and refined the analyses based on -2LL comparisons. The individual outcomes are summarised into cohort describing regression equations:

FVC (% of pred) = $\alpha + \beta_1$*CA 15-3 (ln) + β_2*time + β_3*treatment + β_4*time*treatment and

DLco (% of pred) = $\alpha + \beta_1$*CA 15-3 (ln) + β_2*time + β_3*treatment + β_4*time*treatment, where *time* reflects the observation points.

The last term in these equations is a time*treatment interaction, which tries to find a difference in time-trends between the two treatment groups. We ln-transformed CA-15-3 data to obtain a more suitable distribution.

Survival time was expressed in months from date of diagnosis. The optimal cut-off values of baseline and follow-up CA 15-3 levels were determined using receiver operating characteristic (ROC) curves on progression of pulmonary function. FVC progression was defined as an absolute FVC decline of at least 5% after one year, whereas DLco progression was defined as an absolute DLco decline of 15%. Survival plots were created using Kaplan Meier curves. A p-value less than 0.05 was considered as statistically significant. The statistical analysis was performed by software IBM SPSS 24.0. Graphs were drafted in Graph Pad Prism 8.3

Results

Baseline characteristics

A total of 132 patients diagnosed with IPF between 2012 and 2019, treated with nintedanib or pirfenidone within six months after diagnosis were identified. All patients had biomarker and PFT data available at diagnosis and at least on one follow-up time point. Table 1 summarizes the demographics of these patients at date of diagnosis. Subjects were predominately male (75.8%) with mean age 68.6 years (SD 7.8) and mean GAP index of 3.95 (SD 1.3). Overall, baseline serum CA 15-3 was increased (median 56.0 kU/l; IQR 34.0-89.8). FVC (% pred) was considerably high (79.5 SD 18.7) whereas low DLco (% pred) values were observed (40.9 SD 12.3). A radiological UIP pattern was predominately seen (63.6%), followed by probable UIP (31.8%) and indeterminate UIP (4.5%; table 1). Available lung biopsies of patients with a radiological probable UIP (n=13) and indeterminate UIP (n=6) all demonstrated a histological UIP pattern. No baseline differences were observed between pirfenidone and nintedanib treated patients (table 1). No statistical differences were found in median baseline CA 15-3 levels between patients with a radiological UIP, probable UIP and indeterminate UIP pattern (p = 0.508, data not shown), nor between patients with a histological UIP and probable UIP pattern (p = 0.901, data not shown).

Table 1. Characteristics of IPF patients at time of start of therapy

Parameters	**Subjects**			
		Treatment		
	All	**Pirfenidone**	**Nintedanib**	**P**
N - no (%)	132	70	62	
Age (years)	68.6 (7.8)	68.7 (8.0)	68.5 (7.8)	0.928
Sex (M) - no (%)	100 (75.8)	52 (74.3)	48 (77.4)	0.675
Current/ex-smoker – no (%)	102 (75.8)	57 (81.4)	48 (77.4)	0.226
GAP index – mean (SD)	3.95 (1.3)	3.96 (1.2)	3.95 (1.4)	0.394
Lung biomarker				
CA 15-3 (kU/l) – median (IQR)	56.0 (34.0-89.8)	55.5 (33.0-92.3)	58.0 (34.8-88.3)	0.659
Pulmonary function				
FVC (%, pred) – mean (SD)	79.5 (18.7)	78.0 (18.7)	81.3 (18.8)	0.304
DLco (%, pred) – mean (SD)	40.9 (12.3)	40.0 (11.4)	41.8 (13.3)	0.462
HRCT scan – no (%)				
UIP	84 (63.6%)	47 (67.1)	37 (59.7)	0.374
Probable UIP	42 (31.8%)	21 (30.0%)	21 (33.9%)	0.634
Indeterminate UIP	6 (4.5%)	2 (2.9%)	4 (6.5%)	0.415
Alternative diagnosis	-	-		
Histopathology – no (%)*				
UIP	27 (96.4%)	12 (92.3%)	15 (100%)	0.464
Probable UIP	1 (3.6%)	1 (7.7%)	-	0.464
Indeterminate UIP	-	-	-	-
Alternative diagnosis	-	-	-	-

GAP index = IPF severity index based on gender, age and pulmonary function test
CA 15-3 = cancer antigen 15-3; FVC = forced vital capacity, in percentage of predicted; DLco = diffusing capacity of the lung for carbon monoxide, in percentage of predicted
HRCT = high resolution computed tomography. Characteristics on HRCT and in histopathological lung biopsies were classified according to ATS/ERS 2018 recommendations. UIP = usual interstitial pneumonia
*n = 28
P < 0.05; difference in variables between pirfenidone and nintedanib group

CA 15-3 as marker for PFT

Mean follow-up duration was 47.0 months (SD 14.9). The results of the linear mixed model analysis to evaluate the effect of CA 15-3 on PFT outcome over time are listed in table 2. No significant differences in time trends were found between the pirfenidone and nintedanib group for FVC (p = 0.239) and DLco (p = 0.820) outcomes and we removed the time*treatment interaction from the analysis.
In the linear mixed model, the twelve-month measurement of PFT was set as reference point (0) of the effect of time on previous PFT outcomes. The estimates at baseline, 3 and 6 months were determined in comparison to the reference point. Overall, a significant effect of time on FVC (p = 0.030) and DLco (p < 0.001) values is evident, indicating that FVC and DLco values declined over time.

An overview of the regression coefficients are listed in Table 2. CA 15-3 (ln) measurements were inversely associated with the FVC (an estimated drop of 5.21 % predicted per unit increase in ln-CA 15-3; p < 0.001) and DLco (an estimated drop of 4.69 % predicted per unit increase in ln-CA 15-3; p < 0.001). For illustration, the course of pulmonary function and CA 15-3 measurements over time as calculated by the equation are shown for a pirfenidone and a nintedanib treated patient (Figure 1).

Table 2. Linear mixed model analysis of CA 15-3, FVC and DLco

Parameter	Estimate	SE [c]	P	95% CI
				FVC (% pred)
Intercept	100.69	6.00	< 0.001	88.89 – 112.48
Time			0.030	
- Baseline	2.22	0.85	0.011	0.51 – 3.88
- 3 months	2.23	0.80	0.007	0.62 – 3.82
- 6 months	1.34	0.74	0.074	-0.13 – 2.81
-12 months [a]	0	-	-	-
Treatment				
- Pirfenidone	-3.99	3.38	0.239	-10.67 – 2.68
- Nintedanib [b]	0	-	-	-
CA 15-3 (ln)	-5.21	1.37	< 0.001	-7.91 – -2.52
				DLco (% pred)
Intercept	54.21	4.42	<0.001	45.51 – 62.91
Time			<0.001	
- Baseline	5.21	0.73	<0.001	3.78 – 6.64
- 3 months	2.72	0.68	<0.001	1.38 – 4.06
- 6 months	2.24	0.57	<0.001	1.11 – 3.37
- 12 months [a]	0	-	-	-
Treatment				
- Pirfenidone	0.48	2.07	0.820	-3.62 – 4.57
- Nintedanib [b]	0	-	-	-
CA 15-3 (ln)	-4.69	1.05	<0.001	-6.75 – -2.63

CA 15-3 (ln) = cancer antigen 15-3, expressed as log transformed data; FVC = forced vital capacity, in percentage of predicted; DLco = diffusing capacity of the lung for carbon monoxide, in percentage of predicted
P < 0.05
[a] In the linear mixed model analysis, the 12 month pulmonary function measurement was set as reference point (0) for determination of the effect of time on previous pulmonary function outcomes. The estimates at baseline, 3 and 6 months were calculated in comparison to the reference point. Higher outcomes for both FVC and DLco were observed at baseline, 3 and 6 months compared to 12 months.
[b] In the linear mixed model analysis, nintedanib was set as reference point (0). The effect of pirfenidone treatment is thus calculated in comparison to nintedanib. A non-significant negative effect on pulmonary function outcome was observed compared to nintedanib treatment.
[c] Standard error

CA 15-3 as predictor of Survival

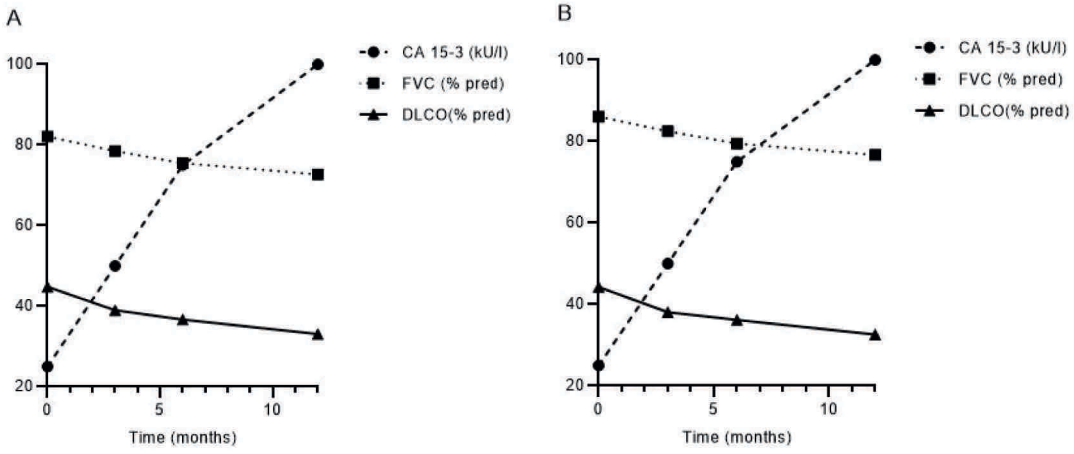
Within the study period, 74 out of 132 patients died (56.1%). Overall, median survival was 33 months (IQR 21.3-39.0). ROC curves were used to define cut-off CA 15-3 values to predict FVC progression and DLco progression. ROC curves of CA 15-3 levels and CA 15-3 change demonstrated no significant areas under the curve (AUCs) for FVC progression. Contrary, the AUC was significant for DLco progression

(0.650; p = 0.031), with baseline CA 15-3 level of 58.5 kU/l identified as the value with the highest sum of sensitivity and specificity (1.323). Patients were categorized as having low baseline CA 15-3 (CA 15-3 < 58.5) or high baseline CA 15-3 (CA 15.3 > 58.5) levels. Survival analysis demonstrated a significantly impaired survival of patients with high baseline CA 15-3 (median 29 months) compared to lower levels (median 50 months; hazard ratio 1.67; 95% CI 1.05-2.65; p = 0.031, Figure 2A). No significant differences were found in radiological and histological characteristics between patients with baseline CA 15-3 levels above 58.5 kU/l and those with lower levels (data not shown).

Figure 1

Patient A: The course of CA 15-3 (kU/l), FVC (% pred) and DLco (% pred) as illustrated by the linear mixed model analyses for a pirfenidone treated patient.

Patient B: The course of CA 15-3 (kU/l), FVC (% pred) and DLco (% pred) as illustrated by the linear mixed model analyses for a nintedanib treated patient.

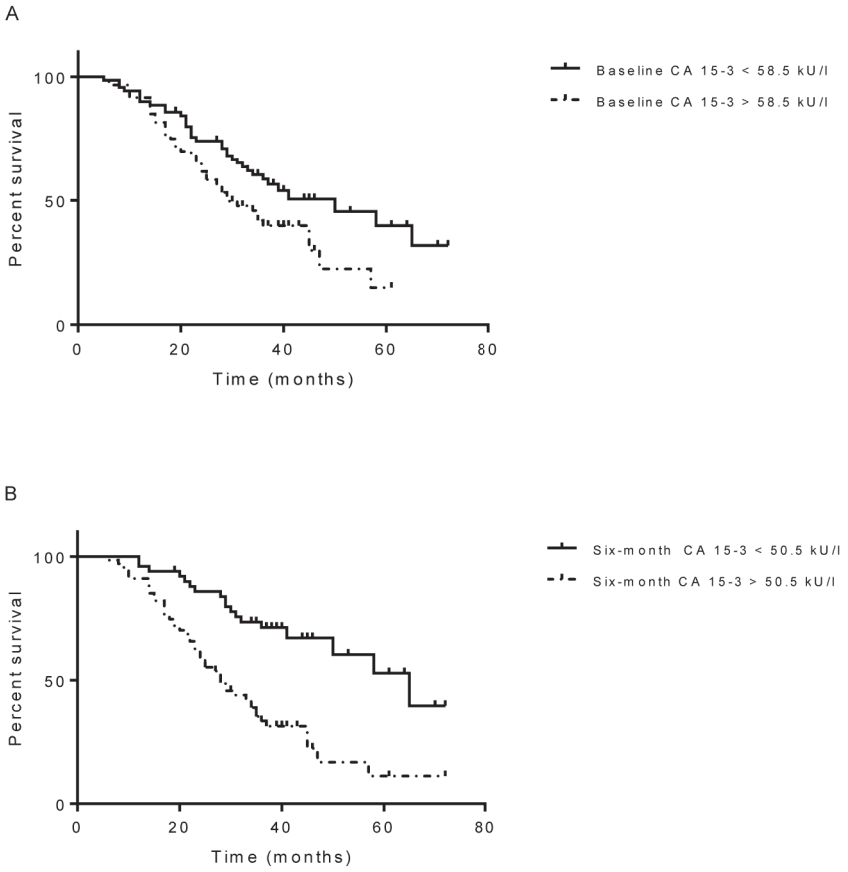


The ROC curve of the six-month CA 15.3 levels demonstrated an AUC of 0.694 (p = 0.006) with a value of 50.5 kU/l determined as cut off value for predicting DLco progression. Survival was significantly deteriorated in patients with six-month CA 15-3 levels above 50.5 kU/l (median 28.0 months) compared to those with lower levels (median 65 months; hazard ratio 2.99; 95% CI 1.74-5,13; p < 0.001, Figure 2B). Survival rates between pirfenidone and nintedanib treated IPF patients showed no significant differences (data not shown).

Figure 2

A. Kaplan Meier curve showing a significant impaired survival in patients with baseline CA 15-3 levels above 58.5 kU/l (median 29 months) compared to baseline CA 15-3 levels below 58.5 kU/l (median 50 months; hazard ratio 1.67; 95% CI 1.05-2.65; p = 0.031)

B. Kaplan Meier curve showing a significant impaired survival in patients with six-month CA 15-3 levels above 50.5 kU/l (median 28.0 months) compared to six-month CA 15-3 levels below 50.5 kU/l (median 65 months; hazard ratio 2.99; 95% CI 1.74-5,13; p < 0.001)



Discussion

In this study, the predictive value of serum CA 15-3 in IPF patients was evaluated. CA 15-3 was inversely associated with FVC (% pred) and DLco (% pred) during one-year follow-up in pirfenidone and nintedanib treated patients. Furthermore, increased baseline and six-month CA 15-3 levels predicted a significantly worse survival compared to lower levels. Therefore, serum CA 15-3 could be implemented as a prognostic biomarker in IPF.

Increased baseline CA 15-3 levels were found in IPF patients compared to healthy controls in previous research (26). In our study, CA 15-3 was strongly associated with pulmonary function during follow-up, in particular with DLco. Increased CA 15-3 levels and associations with severe lung involvement have been demonstrated in fibrotic ILDs including sarcoidosis, HP, Ssc-ILD and RA-ILD (22,26,29–35). Negative associations between baseline DLco and CA 15-3 were found in patients with sarcoidosis and Ssc-ILD (29,39). In contrast to our results, a weak positive association of CA 15-3 and FVC was found in IPF patients referred for lung transplantation, although selection bias of more progressive patients might have occurred in this study. (36). It was recently demonstrated that CA 15-3 is inversely associated with PFT in hypersensitivity pneumonitis (32). Our results add to these findings that CA 15-3 appears to be a predictive biomarker on baseline and during follow-up for PFT outcome in IPF as well,

Increased CA 15-3 levels above 50.8 and 50.5 kU/l at baseline and six months respectively were associated with poor survival compared to lower levels in our present study. Several IPF studies demonstrated decreased survival rates in patients with high baseline KL-6 compared to lower levels as well (19,40,41). Moreover, IPF patients with positive KL-6 change had a worsened survival and were associated with greater FVC declines compared to unchanged or negative KL-6 changes (40). Our study adds to previous findings that in addition to KL-6, increased CA 15-3 levels associate with decreased PFT outcome and survival during follow-up in IPF.

CA 15-3 has been associated with progression of pulmonary fibrosis and fibrotic characteristics on HRCT in IPF (26). In our study, we did not find differences in baseline CA 15-3 levels between IPF patients with a UIP, probable UIP or indeterminate UIP pattern on HRCT and/or in lung biopsies. Possibly, CA 15-3 levels do not specifically differentiate between the various interstitial findings on baseline, but could have a prognostic value for future radiological and/or histological progression. A similar phenomenon has been described in Ssc-ILD, in which patients with worsening of the interstitial HRCT score during follow-up had significant higher baseline CA 15-3 levels compared with patients with a stable interstitial HRCT score (29,30).

This retrospective cohort study has some limitations by selecting IPF patients in a tertiary ILD centre. Solely patients treated with anti-fibrotic therapy and with available follow-up CA 15-3 and PFT measurements were selected for analysis. Outcomes on PFT and survival might be impaired due to selection of more severe patients. Diagnosis and follow-up of patients was conducted by experienced ILD

pulmonologists and according to a standardized protocol. As a result, a large sample size was obtained with a considerable follow-up period and limited missing data.

According to ATS/ERS recommendations, the change in FVC is currently used as most important predictor in IPF (11). We are the first to demonstrate the predictive value of serial CA 15-3 levels on FVC, DLco and survival in a large cohort of IPF treated with anti-fibrotic therapy. We identified cut-off CA 15-3 values based on DLco progression by AUCs. The non-significant AUC for FVC progression in our study most probably originated in a lower percentual decline of the FVC (2.22%) compared to DLco (5.31%). In a follow-up study of nintedanib treated IPF patients, KL-6 was confirmed to predict DLco progression and indicative of therapy response (21). As associations between CA 15-3 and DLco were seen both in nintedanib and pirfenidone patients in our study, it could be hypothesized that CA 15-3 might reflect therapy response of anti-fibrotic treatment in IPF as well.

It is thought that DLco reflects the permeability of the alveolar surface (42). KL-6 was demonstrated to be a representative marker for increased alveolar-capillary permeability (43). Possibly, CA 15-3 is also more a reflection of alveolar-capillary permeability, and thus reflects DLco, due to lung injury than a reflection of fibroblast activity. As the condition in IPF patients declines in progressive disease, PFT may be invasive and even unreliable if maximal performance cannot be reached. Blood sampling is less invasive and more objective than PFT. Measurement of CA 15-3 could be used as an indicator of pulmonary function and treatment response of anti-fibrotic therapy in IPF.

For future research, it would be interesting to investigate if CA 15-3 has prognostic value for worsening of interstitial HRCT findings during follow-up, as seen in Ssc-ILD (29,30). Furthermore, evaluation of associations between CA 15-3 and dyspnoea severity is an interesting research topic, as KL-6 was found to be associated with dyspnoea severity, decreased physical activity and decreased walking distance in IPF (44,45). Moreover, it would be interesting to evaluate in future research whether CA 15-3 is associated with other biomarkers such as CCL18 and lactate dehydrogenase (LDH) or tumour associated biomarkers. Strong associations between CA 15-3 and tumour associated biomarkers carcinoembryonic antigen (CEA), CA 125 and CA 19-9 were observed in RA-ILD and idiopathic IP patients without underlying carcinoma (26,34,46–48). As mucin 1 upregulation is associated with smoking (49), it would be relevant for clinical interpretation of CA 15-3 to compare levels between smoking and non-smoking ILD patients. For clinical practice, it would be valuable to assess whether CA 15-3 levels predict IPF exacerbations, as elevated KL-6 levels were associated with a short follow up period before the onset of an acute exacerbation of IPF as well (20). In practice, preventive preparations for an exacerbation of IPF could then be taken into account when CA 15-3 is increasing (50).

Several trials have recently been published on nintedanib as treatment in systemic sclerosis related ILD and progressive interstitial lung diseases (51,52). It would be interesting to investigate if CA 15-3 is indicative for nintedanib response in other ILD related disease as well. In addition, it would be valuable to further assess CA 15-3 as biomarker for treatment response in a comparative study between non-treated and anti-fibrotic treated IPF.

We postulate that in the near future, the use of blood biomarkers will be implemented in standard ILD care including IPF for diagnostic, prognostic and early predictive purposes. Possibly, decision making on treatments regiments in ILD will be based on clinical parameters including the outcomes of these predictive blood biomarkers.

In conclusion, we demonstrated that serum CA 15-3 could be useful as minimal invasive, follow-up biomarker for treatment-response in IPF. Furthermore, CA 15-3 predicts PFT outcome and survival.

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4

Genetic variation in CCL18 gene influences CCL18 expression and correlates with survival in Idiopathic pulmonary fibrosis – Part A*

Sofia A. Moll

Ivo A. Wiertz

Benjamin Seelinger

Nicole P. Barlo

Joanne J. van der Vis

Nicoline M. Korthagen

Ger T. Rijkers

Henk J.T. Ruven

Jan. C. Grutters

Antje Prasse

Coline H.M. van Moorsel

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Abbreviation list

ATS = American Thoracic Society

AUC = Area under the Curve

CCL18 = CC-chemokine ligand 18

DLCO = Diffusing capacity of the lung for carbon monoxide

ERS = European Respiratory Society

FVC = Forced Vital Capacity

IIP = Idiopathic interstitial pneumonia

IPF = Idiopathic pulmonary fibrosis

IQR = Inter quartile range

LD = Linkage disequilibrium

PBMC = Peripheral blood mononuclear cells

ROC = Receiver operating curves

SNP = single nucleotide polymorphism

Ssc = Systemic sclerosis

Abstract

Background: IPF is a progressive fibrotic disease, characterized by fibroblast proliferation and extracellular matrix deposition. CC-chemokine ligand 18 (CCL18) upregulates the production of collagen by lung fibroblasts and is associated with mortality. This study was designed to evaluate the influence of single nucleotide polymorphisms (SNPs) in the *CCL18* gene on CCL18 expression and survival in IPF.

Methods: Serum CCL18 levels and four SNPs in the *CCL18* gene were analyzed in 77 Dutch IPF patients and 349 healthy controls (HCs). *CCL18* mRNA expression was analyzed in peripheral blood mononuclear cells (PBMCs) from 18 healthy subjects. Survival analysis was conducted dependent on CCL18-levels and -genotypes and validated in two German IPF cohorts (*Part B, not part of this thesis*).

Results: IPF patients demonstrated significantly higher CCL18 serum levels than healthy controls ($p<0.001$). Both in IPF patients and HCs, serum CCL18 levels were influenced by *rs2015086* C>T genotype, with highest CCL18-levels with presence of the C-allele. Constitutive *CCL18* mRNA-expression in PBMCs was significantly increased with the C-allele and correlated with serum CCL18-levels. In IPF, high serum levels correlated with decreased survival ($p=0.02$). Survival was worse with the CT-genotype compared to TT genotype ($p=0.01$).

Conclusion: Concluding, genetic variability in the *CCL18*-gene accounts for differences in *CCL18* mRNA-expression and serum-levels and influences survival in IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lung parenchyma, characterized by fibroblast proliferation and extracellular matrix deposition [1]. New therapeutic agents such as nintedanib and pirfenidone demonstrated a deceleration of disease progression [2,3], but the overall survival in IPF remains drastically impaired despite these agents [4]. There is substantial inter-individual difference in the clinical course of the disease, ranging from rapid decline to periods of relative stability for many years [1,5]. To predict the disease course, an increasing number of studies investigated the use of several biomarkers in IPF, including data from multicentric randomized trials [6–12].

CC-chemokine ligand 18 (CCL18) is the biomarker most consistently associated with outcomes in IPF and has been studied among several interstitial lung diseases [13–19]. A clear relationship has been demonstrated between elevated serum levels of CCL18 and clinical outcome in IPF patients including survival [16] and acute exacerbation rate [20], and has been confirmed in data from two randomized controlled trials [13]. Apart from IPF, elevated serum CCL18 levels reflected pulmonary fibrosis activity [21] and correlated with death or progression of pulmonary disease [15,17,22] in patients with systemic sclerosis (SSc) associated ILD.

CCL18 is predominantly expressed by alveolar macrophages and occurs at relatively high levels in lung tissue [23]. In response to CCL18, lung fibroblasts from healthy adults showed increased expression of collagen mRNA [24]. Furthermore, it was demonstrated that alveolar macrophages from patients with pulmonary fibrosis show an alternatively activated phenotype, which up-regulates the production of collagen by lung fibroblasts through the production of CCL18 [14]. As fibroblast contact and exposure to collagen increases spontaneous CCL18 production by alveolar macrophages, a positive feedback loop was suggested that maintains fibrosis.

The gene encoding CCL18 is small, positioned at the q arm of chromosome 17 and consist of 3 exons with a number of single nucleotide polymorphisms (SNPs), including the SNPs *rs2015086* and *rs712040*,present in the region. In macrophages of subjects with the A>G genotype of the *rs2015086* SNP, a threefold higher gene expression was found compared to those with the A/A genotype [25,26]. We hypothesized that genetic variation in the *CCL18* gene might be associated with increased CCL18 expression and may predispose to an unfavorable prognosis in subjects with IPF.

Experimental Section

Patients and clinical data

A retrospective cohort study was performed in an IPF cohort from the Netherlands. IPF patients diagnosed at St. Antonius Interstitial Lung disease Center Of Excellence, Nieuwegein, The Netherlands between 1998 and 2007 were assigned to the derivation cohort (Part A, presented herein). Diagnoses were reviewed and patients were included if the 2011 American Thoracic Society / European Respiratory Society (ATS/ERS) criteria were met [27]. Medical records were retrieved to determine survival status

and cause of death and baseline characteristics including age, sex, percentage of predicted forced vital capacity (%FVC) and diffusion capacity for carbon monoxide (%DLCO) were recorded. The findings were then validated in two independent prospectively-recruited German IPF cohorts (*Part B, not part of this thesis*). The study protocol was approved by the local Ethical Committee of the St. Antonius Hospital (registration number R05-08A) and all subjects gave written informed consent.

Blood sampling

Serum and blood for DNA extraction were collected at diagnosis. All serum samples of patients with the CC and CT genotype were analysed, and additionally a random sample from patients with TT were analysed. For details on DNA analyses, we refer to the supplement.

Serum CCL18 measurement by monoplex bead array

CCL18 levels were analyzed using a monoplex suspension bead array system in the derivation cohort. In the validation cohort an ELISA test was used to determine CCL18 levels, as described earlier in other articles [16]. For further details on CCL18 analysis, we refer to the supplement.

Single Nucleotide Polymorphism (SNP) genotyping and mRNA expression analysis

Patients were genotyped for multiple SNPs. Two SNPs with presumed functionality in the promoter region [26] were genotyped (*rs712040, rs2015086*). In addition, two haplotype tagging SNPs (*rs712042, rs712044*) were selected to cover genetic variability in the *CCL18* gene. The expression of *CCL18* mRNA in peripheral blood mononuclear cells (PBMCs) from 18 healthy donors was analyzed by quantitative RT-PCR amplification. See supplementary chapter for further details on genotyping and mRNA expression analysis.

Statistical analysis

Genotypes were tested for Hardy–Weinberg equilibrium using the website (*https://ihg.gsf.de/cgi-bin/hw/hwa1.pl*). Linkage disequilibrium (r2) was calculated using the computer program Haploview 4.1, Broad Instutitute at Massachusetts Institute of Technology abd Havard University, MA, USA [28]. Haplotypes were determined using Phase v2.1 (Department of Human Genetics, University of Chicago, IL, USA [29]. Data are presented as medians and inter quartile ranges (IQR). Statistical comparisons were made with the use of the Mann–Whitney-U test for two groups or Kruskal-Wallis for more than two groups. Receiver operating curves (ROC) were determined to define the optimal cut-off point for distinct CCL18 serum concentrations with the highest predictive accuracy for one year survival. In addition, the Kaplan Meier method was used for survival analyses.

For analysis of correlation, log-transformation was used to reach near normal distribution. The correlation between mRNA expression and serum levels was assessed using Pearson's correlation coefficients. Statistical analysis was performed using IBM SPSS statistics software for Windows (version 22.0; IBM, Armonk, NY, USA) and Graphpad PRISM 5 (GraphPad Software, La Jolla, CA, USA) and RStudio

version 1.2.5033 (RStudio Inc, Boston, MA, USA). Statistical significance was considered at a value of $p < 0.05$.

Results

Derivation cohort

A total of 77 IPF patients were included in this study, 58 male, 19 female, median age 61.4 years [IQR 54.1–71.6]). No significant differences were demonstrated in baseline characteristics between carriers of the different genotypes in the derivation cohort (Table 1). All patients were derived from a pre-anti-fibrotic treatment cohort.

CCL18 genotypes and allele carrier frequencies

DNA was present for 77 patients in the derivation cohort and for 349 healthy subjects (139 male (40%), 210 female (40%), median age 39.4 years, [IQR 28.3 – 49.1]). All controls and 71/77 IPF (93%) were of European descent. Five IPF patients (6%) were of North-American descent and one (1%) of North-African descent. Differences in ethnical background were not statistically different between healthy controls and IPF patients (p=0.184)

Genotypes and allele carrier frequencies in 77 IPF patients of the derivation cohort and 349 controls are summarized in Table 2. Healthy controls and IPF patients were in Hardy-Weinberg equilibrium for all polymorphisms. Comparison of the SNPs in the *CCL18* gene revealed no significant differences in allele frequencies between IPF patients and controls. The following SNPs, including the two SNPs with presumed functionality, showed strong linkage disequilibrium (LD): *rs712042*, *rs2015086* and *rs712040*; $76 < r2 < 0.90$ (Figure 1). Additionally, based on 4 SNPs, only 3 haplotypes were constructed with a frequency > 5%. Haplotype frequencies were not significantly different between IPF patients and healthy controls (data not shown). Although *rs712042* showed strongest LD, a presumably functional SNP was preferred for further evaluation. Therefore, the *rs2015068* polymorphism was selected for analysis.

Table 1. Baseline characteristics in IPF patients divided by *CCL18 rs2015086* genotype in the derivation cohort

Characteristics	**Derivation Cohort (n = 77)**			
***rs2015086* genotype**	**All (*n* = 77)**	**CT (*n* = 18)**	**TT (*n* = 59)**	***p***
Male, *n* (%)	58 (75)	11 (61)	47 (79)	0.200
Age, years (IQR)	61.4 (54-72)	61.3 (53-75)	62.8 (52-74)	0.900
Former or active smoker, *n* (%)	59 (76)	11 (61)	48 (81)	0.950
Baseline %FVC predicted, (IQR)	75.7 (62-87)	73.7 (52-88)	75.7 (63-89)	0.700
Baseline %DLCO predicted (IQR)	42.5 (33-56)	40.4 (32-64)	47 (31-60)	0.300

IQR (interquartile range); %FVC (Percent of predicted forced vital capacity); %DLCO (Percent of predicted diffusion capacity for carbon monoxide with single breath).

Table 2. Allele carrier and genotype frequencies in patients with IPF and healthy controls

Polymorphism	Allele and genotype	IPF (n = 77)	Healthy controls (n = 349)*
rs712040	C	16 (10%)	86 (12%)
	T	138 (90%)	612 (88%)
	CC	0 (0%)	7 (2%)
	CT	16 (21%)	72 (21%)
	TT	61 (79%)	270 (77%)
rs2015086*	C	18 (12%)	101 (15%)
	T	136 (88%)	593 (85%)
	CC	0 (0%)	10 (3%)
	CT	18 (23%)	81 (23%)
	TT	59 (77%)	256 (74%)
rs712042	A	136 (88%)	597 (86%)
	G	18 (12%)	101 (14%)
	AA	59 (77%)	258 (74%)
	AG	18 (23%)	81 (23%)
	GG	0 (0%)	10 (3%)
rs712044	A	97 (63%)	480 (69%)
	G	57 (37%)	218 (31%)
	AA	33 (43%)	172 (49%)
	AG	31 (40%)	136 (39%)
	GG	13 (17%)	41 (12%)

*healthy controls: n = 347 due to missing genotypes in 2 controls. No significant differences were observed.

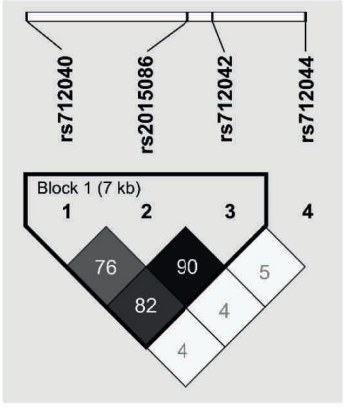

Figure 1. Linkage Disequilibrium (LD) map of 4 SNPs in the CCL18 gene: *rs712040* (location: CCL18 promotor), *rs2015086* (location: CCL18 promotor), *rs712042* (location: CCL18 intron), *rs712044* (location: CCL18 intron). The dark squares represent high r2 values and the triangle represents a haplotype block.

Serum CCL18 levels and CCL18 genotypes

Serum at time of diagnosis was available from 61/77 patients in the derivation cohort (79%). Characteristics of patients with available serum did not differ from the total group (see supplementary table 1). Serum levels were further measured in a selection of 204 healthy controls (86 male (42%), 118

female (58%), median age 40.1 years [IQR 29.5 – 51.0] who were enriched for the presence of the minor allele *rs2015086*. Characteristics of these healthy controls did not differ from the total group of controls (p ranging 0.768-0.999). Analysis of the derivation cohort showed that serum CCL18 levels were significantly higher in IPF patients (645 ng/ml [IQR 393 – 847]) compared with healthy controls (185 ng/ml [IQR 123-272]), $p < 0.0001$, (Figure 2A). Serum CCL18 levels (median 642 ng/ml [IQR 429-943] in eight patients who received low dose corticosteroids were not significantly different from IPF patients who did not receive immunosuppressive therapy (median 652 ng/ml; IQR 400-856; p = 0.880] at the time of sampling (data not shown).

In healthy controls, significant differences in CCL18 serum levels were observed between the carriers and non-carriers of the C-allele of the *rs2015086* polymorphism; TT 151 ng/ml (IQR 109-224), CT / CC 239 ng/ml (IQR 152-328), ($p < 0.0001$) (Figure 2B). No significant differences were found in CCL18 levels between the CT-group (241 ng/ml; IQR 156-327) and CC-group (212 ng/ml; IQR 137-483; p = 0.834).

Pronounced differences in CCL18 serum levels were observed between genotypes of the *rs2015086* polymorphism in IPF patients of the derivation cohort; TT 585 ng/ml (IQR 340 –793) and CT 817 ng/ml (IQR 681 – 1278), p = 0.002 (Figure 2C). No differences were present in the baseline characteristics between patients with CT and TT genotypes of *rs2015086* (Table 1).

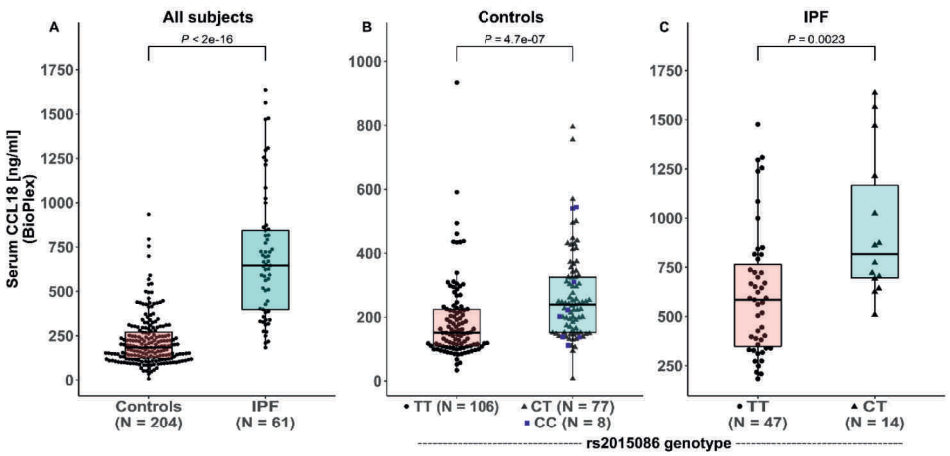

Figure 2. Serum CCL18 levels in healthy controls and patients with idiopathic pulmonary fibrosis (IPF) in the derivation cohort **(A)** and depending on *rs2015086* genotype in healthy controls **(B)** and IPF patients **(C)**.

CCL18 Genotypes and mRNA expression

The expression of *CCL18* mRNA in PBMCs was analyzed in 18 healthy controls. Six subjects had genotype CT for the *rs2015086* SNP and 12 subjects had TT. Subjects with the CT genotype had a 4-fold

higher gene expression ($3.0x10^{-5}$;[IQR $1.8x10^{-5}$ - $7.7x10^{-5}$]) than subjects with TT ($7.4x10^{-6}$ [IQR $1.1x10^{-6}$ - $1.8x10^{-5}$], $p = 0.007$), (Figure 3A). *CCL18* mRNA expression correlated significantly with serum CCL18 levels ($r = 0.73$, $p = 0.002$) (Figure 3B). Analysis of CCL18 mRNA expression for respectively the *rs712040, rs712042* and *rs712044* SNPs showed similar, but non-significant higher gene expression in the CT-genotypes compared with the TT-genotypes (data not shown). Weak to moderate, non-significant correlations between *CCL18* mRNA and serum CCL18 levels were found as well for respectively the *rs712040, rs712042* and *rs712044* SNPs (data not shown).

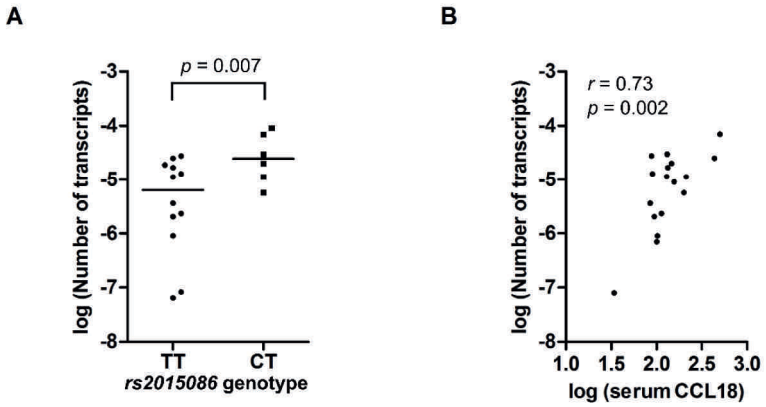

Figure 3. mRNA expression of *CCL18* in PBMCs from 18 healthy controls **(A)**, expressed as the number of *CCL18* transcripts per copy of β-actin according to *rs2015086* genotype. Scatterplot showing the correlation between serum CCL18 levels and the number of *CCL18* mRNA transcripts per copy of β-actin **(B)**. Values on the X and Y-axis represent log-transformed values.

Progression of disease and survival in IPF patients

Progression of disease was evaluated for IPF patients, based on FVC change and/or the clinical suspicion of an acute exacerbation. Of 77 patients, a total of 9 patients (13.2%) had a clinical suspicion of an acute exacerbation. An FVC decline of more than 10% after one year was considered as FVC progression. FVC values at one-year follow-up were availabe for 41 out of 77 IPF patients. Median FVC change after one year was -4.5% (IQR -9.4 – 2.3). Altoghether, 18 patients (44%) showed progression of disease. A trend towards higher baseline CCL18 levels (652 ng/ml; IQR 505-791) was observed in subjects with progression of disease compared with those without progression (592.8 ng/ml; IQR 328-853; p = 0.699). No significant differences were found for progression of disease between patients with CT and TT genotypes of rs2015086 (p = 0.342; data not shown). Frequencies of acute exacerbations did not differ between patients with the CT-genotype (14.3%) and TT-genotype of *rs2015086* (13.0%; p = 0.896).

Median survival in the derivation cohort was 35 months (95% CI 21.1 – 48.7). Within the study period 50 out of 77 IPF patients died (65%), and one patient was lost to follow-up. ROC analyses showed

that the highest Area under the Curve (AUC) was calculated for a serum CCL18 concentration of 500 ng/ml (AuC = 0.72). According to this cut-off level, patients were categorized as having high (serum CCL18 > 500 ng/ml) or low levels (serum CCL18 < 500 ng/ml). Median survival in the $CCL18^{low}$-group was 50.4 months (95% CI 31.9 – 68.9) and differed significantly from that of 27.6 months (95% CI 8.1 – 47.0) in the $CCL18^{high}$-group, ($p = 0.02$) (Figure 4A).

Survival was also analysed for dependency on CCL18 genotype. Patients with the *rs2015086* CT genotype showed a significantly worse survival (median 14.3 months (95% CI 0.0 – 35.9)) compared to the TT genotype (median 37.2 months (15.4 – 58.9), $p = 0.01$, Figure 4B). Patients were censored from the survival analysis if they were alive at end of follow-up ($n = 15$) or had received lung transplantation ($n = 11$). Censored patients were genotyped CT ($n = 4$) and TT ($n = 22$).

Survival rates were also analysed in three groups based on a combination of the serum CCL18 level and genotype: $CCL18^{low/TT}$ group: median survival 50.4 months (95% CI 25.4 – 75.4); $CCL18^{high/TT}$, median survival 37.2 months (95% CI 13.1 – 61.3) ; and $CCL18^{high/CT}$, median survival 14.3 months (95% CI 1.4 – 27.2), $p = 0.03$ (Figure 4C, calculated via the Log Rank Test using Kaplan Meier curves). There were no patients with low CCL18 and genotype CT.

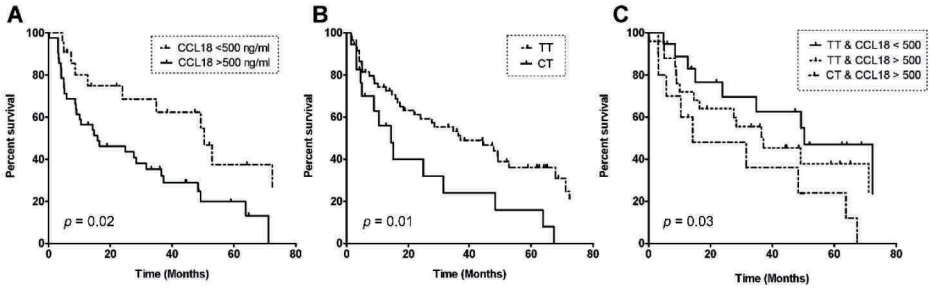

Figure 4. Kaplan-Meier curves for survival in patients with idiopathic pulmonary fibrosis depending on serum CCL18 level cut-off of 500 ng/ml **(A)**; *rs2015086*-genotype (B) and a combination of CCL18 cutoff and *rs2015086*-genotype **(C)**. P-values are calculated via the Log Rank Test using Kaplan Meier curves.

Discussion

CCL18 protein levels are known as a promising biomarker for IPF and this study showed that results are dependent on the *CCL18* genotype. The *rs2015086* CT polymorphism in the *CCL18* gene contributes to inter-individual differences in healthy controls, with individuals carrying the C allele having the highest CCL18 mRNA and protein expression. A similar genotypic effect on serum CCL18 levels was observed in patients with IPF, even though mean serum levels showed a 3.5 –fold increase compared to healthy controls. Both elevated serum CCL18 levels and CT genotypes were related to a significantly diminished long-term survival in IPF. Patients with the worst survival on the basis of

high serum CCL18 levels could be subdivided into intermediate and worse survival according to genotype.

Serum CCL18 concentrations reflect pulmonary fibrotic activity in patients with idiopathic interstitial pneumonias (IIPs) and Ssc with pulmonary involvement [15,21]. Prasse et al. demonstrated that increased serum CCL18 levels were associated with increased short-term (24 months follow-up) mortality in IPF patients [16]. In our study, we independently confirmed these results and added to this finding the predictive value of serum CCL18 for long-term survival. Further, we showed that serum CCL18 levels were genotype dependent. Subjects with the CT genotype display higher constitutive serum CCL18 levels. The CT-genotype of *rs2015086* caused a four-fold higher mRNA expression in PBMCs from healthy controls. The influence of genotypes on mRNA expression in IPF has not been investigated. Interestingly, Hägg et al. described that patients with carotic artery plaques and the CT genotype of *rs2015086* had a three-fold higher gene expression level in macrophages than subjects with the TT genotype [25]. This is in the same order of magnitude as our results and, with that, both the genotype-mRNA correlation and the protein-survival correlation have been demonstrated twice independently.

At presentation, patients with the *rs2015086* CT genotype did not show any significant differences in demographics or lung function parameters compared to patients with the TT genotype, as shown in table 1.We found an association between the *rs2015086* polymorphism and CCL18 serum levels in both controls and patients. Besides that, one may question whether higher constitutive CCL18 levels predispose to fibrotic disease. In order to investigate whether carriage of the C-allele predisposes to IPF we compared allele frequencies between cases and controls and found no significant differences, eventhough we had 80% power to detect an OR ≥2.1 under a dominant gene model. The absence of an association shows that carriage of the C-allele does not significantly predispose to IPF, however, due to limited sample size, small predisposing effects may still exist.

Alveolar macrophages are the main source of CCL18 in the lung and show an alternatively activated phenotype in IPF [14]. Fibroblast contact and exposure to collagen increases CCL18 production by alveolar macrophages and these macrophages up-regulate collagen production by lung fibroblasts via the production of CCL18. As such, we hypothesize that increase in CCL18 does not precede disease but occurs during disease and that the degree to which CCL18 increases is dependent on the presence of the C-allele.

In the search of a biomarker to predict prognosis in IPF, a great number of studies have focused on proteins in serum and BALF. This study is the first to show a genetic polymorphism correlating with serum biomarker levels and with disease course in IPF patients. Genotyping IPF patients for the *rs2015086* SNP in the CCL18 gene may therefore add substantial information to the interpretation of serum CCL18 levels with regard to the prediction of the disease course.

This study has some limitations related to the retrospective nature and relatively small sample size of the IPF cohort. Furthermore, the inclusion period started before 2011 and thereby, patients were selected before the era of new antifibrotic drugs like pirfenidone and nintedanib. We could not determine the potential negative effects of immunosuppressive therapy. The difference in age and sex between IPF and healthy controls may have influenced the results. However, with this approach we were able to evaluate a long follow-up period to determine the long term effect of the biomarker. To address these limitations, in Part B of this work we prospectively validated these findings in two independent German cohorts, one pre-antifibrotic and one with the majority of patients receiving anti-fibrotic therapy.

As serum CCL18 levels are increased in IPF and influence the disease course in IPF, it can be hypothesized that the *rs2015086* polymorphism may show similar effects in other fibrotic lung diseases. CCL18 expression is increased in patients with systemic sclerosis and in hypersensitivity pneumonitis [13,17,21,30]. Morbidity and mortality in these diseases are mainly caused by pulmonary fibrosis. Both diseases show a subset of patients who develop a phenotype in which progressive pulmonary fibrosis is the major cause of death. Further research is needed to investigate whether genetic variation in the CCL18 gene influences serum levels and disease course in systemic sclerosis and hypersensitivity pneumonitis.

Carriers of the CT genotype are at a disadvantage in terms of higher CCL18 levels and diminished prognosis. Interrupting the positive feedback loop by blocking CCL18 might be an interesting therapeutic intervention. IPF is a relentlessly progressive disease and therapy is not curative and in general does not stabilize disease [3,31,32]. As increased CCL18 levels stimulate fibroblasts to produce collagen, inhibiting CCL18 activity may directly inhibit fibrogenesis. Patients with the CT genotype may especially benefit from CCL18 blockade as they have the highest serum CCL18 levels.

In conclusion, we showed that genetic variability in the *CCL18* gene accounts for significant differences in *CCL18* mRNA expression and serum levels and showed to have a modifying role in the course of IPF. Our findings emphasize the value of serum CCL18 as a prognostic marker for IPF. Moreover, we confirmed in a replication cohort that future studies concerning CCL18 should take into account that mRNA and protein expression are influenced by genetic polymorphisms in the *CCL18* gene. The findings of this study are validated in part B (*not part of this thesis*).

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Supplement

Serum CCL18 analysis

Serum samples of IPF patients were collected at diagnosis. In addition, 100 serum samples of controls were selected for analysis including all controls arrying the minor C allele (n=26) supplemented with a random selection of controls with the rs2015086 TT genotype. Within two hours from blood withdrawal, blood samples were centrifuged for 10 minutes at 2200 rpm, serum was transferred to a new tube and stored at -20°C. Every two months stored samples were moved to –80°C until analysis. CCL18 levels in the derivation cohort were analyzed using a monoplex suspension bead array system. CCL18 antibodies (R&D systems, Minneapolis, MN, USA) were coupled to fluorescent carboxylated beads (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol (1). Data analysis was performed using the Bioplex 100 system and Bioplex Manager software version 4.1 (Bio-Rad Laboratories, CA, USA). The lower limit of detection was 0.9 pg/ml.

Genotyping of CCL18

In IPF patients, two SNPs with presumed functionality in the promoter region were genotyped (rs712040, rs2015086). In addition, two haplotype tagging SNPs (rs712042, rs712044) were selected to cover genetic variability in the CCL18 gene, using the Tagger program for the genomic region of CCL18 ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted using the pair wise tagging option, a minimum allele frequency setting of 10% and a high Illumina design score. The algorithm was set to select tags that would cover the Caucasian HapMap panel with an r2 of 0.8 or greater (2). DNA was extracted from whole blood samples and SNP typing was conducted using a custom Illumina goldengate bead SNP assay. The assay was performed in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, CA, USA).

RNA expression analysis

PMBCs from healthy donors were isolated from heparinized venous blood using Ficoll-Paque density gradient centrifugation and cryopreserved until further analysis.

Total RNA was isolated from PBMC using de RNeasy microkit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. 0.2 µg RNA was used for first-strand cDNA synthesis using the i-script cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). The obtained cDNA was diluted 1/10 with water of which 4 µl was used for amplification in a reaction volume of 20 µl. Primer sets were purchased from Sigma. The PCR was performed with the RT2 Real-TimeTM SYBR Green PCR master mix (SA-Biosciences, Frederick, USA) according to the manufacturer's protocol. Samples were amplified using a biorad MyiQ real time PCR detection system for 40 cycles (10 sec at 95°C, 20 sec at 55°C and 25 sec at 72°C.

The copy number of the CCL18 was normalized by the housekeeping gene β-actin, and is presented as the number of transcripts per 1 copy of β-actin (3).

Supplementary table 1: characteristics of all IPF patients compared with IPF patients with available serum CCL18

Characteristics			
***rs2015086* genotype**	**All IPF patients (*n* = 77)**	**IPF with serum CCL18 (*n* = 61)**	***p***
Male, *n* (%)	58 (75)	46 (75)	>0.999
Age, years (IQR)	61.4 (54-72)	61.9 (53-74)	0.796
Former or active smoker, *n* (%)	59 (76)	43 (77)	>0.999
Baseline %FVC predicted, (IQR)	75.7 (62-87)	73.0 (55-87)	>0.999
Baseline %DLCO predicted (IQR)	42.5 (33-56)	41.5 (33-54)	>0.999

IQR (interquartile range); %FVC (Percent of predicted forced vital capacity); %DLCO (Percent of predicted diffusion capacity for carbon monoxide with single breath); p-value calculated by Mann Whitney U test for continuous data and Chi-square test for dichotomous data.

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5

Prevalence of novel myositis autoantibodies in a large cohort of patients with interstitial lung disease

Sofia A. Moll

Mark G.J.P. Platenburg

Anouk C.M. Platteel

Adriane D.M. Vorselaars

Montse Janssen Bonàs

Claudia Roodenburg-Benschop

Bob Meek

Coline H.M. van Moorsel

Jan C. Grutters

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Abbreviations

ANA = Antinuclear antibody
ASS = Anti-synthetase syndrome
ATS = American Thoracic Society
cN1A = Cytosolic-5-nucleotidase-1A
COP = Cryptogenic organizing pneumonia
CPFE = Combined pulmonary fibrosis and emphysema
CTD = Connective tissue disease
DIP = Desquamative interstitial pneumonia
DM = Dermatomyositis
Dlco = Diffusing capacity of the lung for carbon monoxide
ERS = European Respiratory Society
FVC = Forced vital capacity
FEV1 = Forced expiratory volume in 1 second
Ha = tyrosyl-t-RNA synthetase
HP = Hypersensitivity pneumonitis
HRCT = High-resolution computed tomography
IBM = Inclusion body myositis
IIP = Idiopathic interstitial pneumonia
ILD = Interstitial lung disease
IMNM = Immune mediated necrotizing myopathy
IP = Interstitial pneumonia
IPAF = Interstitial pneumonia with autoimmune features
IPF = Idiopathic pulmonary fibrosis
Ks = Asparaginyl-t-RNA synthetase
MAA = Myositis associated antibody
MSA = Myositis specific antibody
NSIP = Non-specific interstitial pneumonia
PFT = Pulmonary function test
PM = Polymyositis
RA = Rheumatoid arthritis
Ssc = Systemic sclerosis
SLE = Systemic lupus erythematosus
UIP = Usual interstitial pneumonia
Zoα = Phenylanyl-t-RNA synthetase alpha

Abstract

Background: Connective tissue diseases (CTDs) are an important secondary cause of interstitial lung disease (ILD). If a CTD is suspected, clinicians are recommended to perform autoantibody testing, including myositis autoantibodies. In this study, prevalence and clinical associations of novel myositis autoantibodies in ILD are presented.

Methods: A total of 1194 patients with ILD and 116 healthy subjects were tested for antibodies specific for Ks, Ha, Zoα, and cN1A with a line-blot assay on serum available at time of diagnosis.

Results: Autoantibodies were demonstrated in 63 (5.3%) patients and 1 (0.9%) healthy control (p=0.035). Autoantibodies were found more frequently in females (p=0.042) and patients without a histological and/or radiological UIP (p=0.010) and a trend towards CTD-ILDs (8.4%) was seen compared with other ILD (4.9%;p=0.090). Prevalence of antibodies specific for Ks, Ha, Zoα and cN1A was respectively 1.3%, 2.0%, 1.4% and 0.9% in ILD. Anti-Ha and Anti-Ks were observed in males with unclassifiable IIP, HP and various CTD-ILDs, whereas anti-cN1A was seen in females with ASS, HP and IPF. Anti-Zoα was associated with CTD-ILD (OR 2.5; 95%CI 1.11-5.61; p=0.027).

Conclusion: In conclusion, a relatively high prevalence of previously unknown myositis autoantibodies was found in a large cohort of various ILDs. Our results contribute to the awareness that circulating autoantibodies can be found in ILDs with or without established CTD. Whether these antibodies have to be added to the standard set of autoantibodies analysed in conventional myositis blot assays for diagnostic purposes in clinical ILD care requires further study.

Introduction

Interstitial lung diseases (ILDs) are a heterogeneous group of diffuse parenchymal lung disorders, characterized by inflammation or fibrosis of the pulmonary interstitium. ILDs can be idiopathic or secondary to known causes including connective tissue diseases (CTDs) (1–5). It is challenging to distinguish CTD-ILD from other ILD as clinical, functional, radiological and pathological characteristics can be similar (6). Moreover, an interstitial pneumonia (IP) may be the first or single clinical manifestation of an underlying CTD (4,6). In general, outcomes on treatment response to immunosuppressive therapy and survival are better in CTD-ILD compared to the majority of ILDs without established CTD (5–10). Thus, discriminating these conditions in the diagnostic work-up is essential.

Serologic testing for autoantibodies by a myositis blot is recommended in pulmonary fibrosis suspected for an underlying CTD, which includes myositis specific antibodies (MSA) and myositis associated antibodies (MAA)(1,3,4,6,11–16). MSA and MAA are found in patients with idiopathic interstitial myopathies but also occur in patients with rheumatic diseases including CTD-ILDs (3–6,11–23). Moreover, myositis antibody positivity has been described in other ILD including hypersensitivity pneumonitis (HP) and idiopathic IPs (11,16,23–25). However, many suspected patients are negative for the standard set of autoantibodies. Here, we focus on relatively unknown antibodies, including antibodies specific for asparaginyl-t-RNA synthetase (anti-Ks), tyrosyl-t-RNA synthetase (anti-Ha), phenylanyl-t-RNA synthetase alpha (anti-Zoα) and cytosolic-5-nucleotidase-1A (anti-cN1A). Circulating antibodies specific for Ks, Ha and cN1A have been described in inclusion body myositis (IBM), systemic sclerosis (Ssc) and Sjögren's syndrome, whereas anti-Zo has been identified in anti-synthetase syndrome (ASS) with ILD (26–36). However, data is scarce on the prevalence and associations of these autoantibodies in CTD-ILD and other ILD.

The aim of this study was to evaluate the prevalence of antibodies to Ks, Ha, Zoα and cN1A in patients with CTD-ILD compared to various other ILDs and healthy controls, measured by a line blot assay. Clinical characteristics of ILD patients with autoantibody positivity are described.

Experimental Section

Patient selection

A retrospective cohort study was conducted at the St Antonius ILD Centre of Excellence Nieuwegein, a tertiary ILD referral center in the Netherlands. The majority of patients were diagnosed between 2000 and 2019 with an ILD with and without established CTD. Serum collected at date of diagnosis was evaluated for the presence of autoantibodies by a research myositis line-blot assay. Furthermore, serum of healthy, non-ILD blood donors were screened for autoantibodies and compared with ILD patients.

Diagnosis of ILD was assessed according to official recommendations of the American Thoracic Society/European Respiratory Society (ATS/ERS) in a multidisciplinary discussion with an ILD pulmonologist, experienced thoracic radiologist, and a pathologist, when required (37). All patients with pulmonary fibrosis were screened for an underlying CTD by the chest physician and referred to the rheumatologist for further diagnostic work-up if a CTD was suspected.

Patients were classified as having a diagnosis of CTD-ILD or ILD without established CTD (non-CTD-ILD). Patients were checked for any revisions of the ILD diagnosis during two years of follow-up, as an IP can occur two years before an associated CTD (3,6). CTD-ILDs included antisynthetase syndrome (ASS), Sjögren's syndrome, rheumatoid arthritis associated ILD (RA-ILD), systemic sclerosis (Ssc), dermatomyositis (DM), polymyositis (PM), immune mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM), overlap myositis, systemic lupus erythematosus (SLE), mixed CTD and other CTD-ILD. Non-CTD-ILDs included idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis (HP), unclassifiable idiopathic interstitial pneumonia (Unclassifiable IIP), non-specific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), pneumoconiosis, drug-induced IP and other ILD.

The baseline characteristics of patients with ILD tested for novel autoantibodies were described. This also included pulmonary function tests (PFTs), which were performed according to ERS recommendations. Furthermore, baseline characteristics on high-resolution computed tomography (HRCT) and in lung biopsies (when available) were described, and classified according to the most recent American Thoracic Society/European Respiratory Society recommendations as a pattern of usual interstitial pneumonia (UIP), probable UIP, indeterminate UIP or alternative diagnosis (38). Moreover, prevalence of patients meeting the non-serological criteria for interstitial pneumonia with autoimmune features (IPAF) was evaluated (39). In addition, presence of antinuclear antibodies (ANA) at baseline, PFT change after one year (expressed as an absolute delta positive or negative change) and survival outcomes were described.

The study was approved by the St Antonius institutional review board under protocol number 842002003 and patients provided written informed consent for research purposes.

Determination of antibodies

In the current study, we evaluated the prevalence of novel myositis antibodies as measured by a blot assay. Detection of antibodies by a line blot assay is concordant with the analysis by the gold standard, immunoprecipitation, and specific ELISAs (40). We therefore did not perform a method comparison between this line blot assay and other tests. Antibodies were detected in serum using a line blot assay (EUROLINE Myositis Research Profile, EUROIMMUN, Lübeck, Germany) in collaboration with Biognost. To date, this blot has been used for research purposes. Stability (stress test and real-time test including transport stability), reproducibility, interferences, serum/plasma-comparison and cross reactivity comply with CE standard for CE-certification of antibody testing against the concerned antibodies. This blot was run between 05-2019 and 07-2019 and identifies antibodies specific for asparaginyl-transfer-RNA synthethase (anti-Ks), tyrosyl transfer-RNA synthethase (anti-Ha), phenylanyl-transfer-RNA synthethase alpha (anti-Zoα) and specific for cytosolic-5-nucleotidase-1A (anti-cN1A). Analysis of the immunoblot strips was performed with the EUROLINEScan software (EUROIMMUN, Lübeck, Germany) according to manufacturer's recommendations as described for the EUROLINE Autoimmune Inflammatory Myopathies line blot assay. Staining strips were qualified as either negative (-), weakly positive (+) and positive (++), which corresponds with intensity levels of 0-10, 11-25 and >25 respectively. Antibody reactivity on a combined weak positive with positive intensity level and on a positive intensity level only was separately

evaluated. For further details, we refer to the methods and materials section of the study of Platteel et al (21).

Statistical analysis

Baseline characteristics were expressed as numbers and percentages or mean and standard deviation, depending on the type of data. Continuous and categorical variables were tested with a student's T-test/one-way ANOVA and Chi-square test/Fisher's exact test respectively. The statistical analysis was performed by software IBM SPSS 24.0. A p-value less than 0.05 was considered as statistically significant. Graphs were drafted in Graph Pad Prism 8.3.

Results

Baseline characteristics

A total of 131 patients with CTD-ILD and 1063 patients with non-CTD-ILD were included in this study (Table 1). Age and ANA positivity were statistically different between CTD-ILD and non-CTD-ILD patients. Further classification of baseline characteristics per ILD diagnosis can be found in the supplementary data (Suppl. Tables 1 and 2).

Table 1. Baseline characteristics of patients with ILD.

Subjects				
	All	**CTD-ILD[a]**	**Non-CTD-ILD[b]**	**P[g]**
N	1194	131	1063	
Age (y)	65.1 (11.2)	60.1 (11.4)	65.7 (11.0)	<0.001
Sex (m), %	773 (64.7)	73 (55.7)	700 (65.9)	0.022
History of smoking, %	801 (67.1)	69 (52.7)	732 (68.9)	0.073
Pulmonary function test[c]				
FVC (% pred)	80.6 (21.6)	80.4 (23.9)	80.6 (21.3)	0.940
FEV1 (% pred)	82.8 (21.2)	80.3 (22.9)	83.2 (21.0)	0.248
Dlco (% pred)	46.1 (15.8)	49.4 (17.2)	45.6 (15.7)	0.057
HRCT scan[d]				
UIP	345 (29.9)	23 (18.7)	322 (31.3)	0.005
Probable UIP	172 (14.9)	13 (10.6)	159 (15.4)	0.152
Indeterminate	233 (20.2)	37 (30.1)	196 (19.0)	0.004
Alternative	403 (35.0)	50 (40.7)	353 (34.3)	0.161
Histopathology[e]				
UIP	125 (34.8)	3 (9.1)	122 (37.4)	0.001
Probable UIP	15 (4.2)	2 (6.1)	13 (4.0)	0.573
Indeterminate	50 (13.9)	9 (27.3)	41 (12.6)	0.020
Alternative	169 (47.1)	19 (57.6)	150 (46.0)	0.205
ANA (%)[f]	138 (18.2)	31 (36.4)	107 (10.1)	<0.001

Data are expressed as mean and standard deviation or numbers and percentage within the diagnosis group. FVC = forced vital capacity, expressed in percentage of predicted; FEV1 = forced expiratory volume in 1 second, expressed in percentage of predicted; Dlco = Diffusing capacity of the lung for carbon monoxide; UIP = usual interstitial pneumonia; [a] CTD-ILD = connective tissue disease related interstitial lung disease; [b] non-CTD-ILD = ILD without established CTD; [c] n = 918; [d] n = 1153; [e] n = 359; [f] ANA = antinuclear antibody, expressed as % positive; n = 757; [g] $p < 0.05$, differences between CTD-ILD and non-CTD-ILD patients are calculated by a two-side unpaired T-test for continuous variables or Chi-Square test for dichotomous variables.

Prevalence of antibodies in ILD and healthy controls

Antibody prevalence of novel myositis autoantibodies was evaluated for all ILD, 116 healthy controls (Table 2) and per ILD diagnosis (Suppl. Tables 3 and 4). Regarding the antigens that stained 'positive', a total of 63 ILD patients (5.3%) demonstrated antibody reactivity, which was significantly higher compared to healthy controls (0.9%; p = 0.035; Table 2). Prevalence of antibody reactivity against myositis antibodies altogether on combined positive and weakly positive levels was also higher in ILD patients (10.0%) compared to healthy controls (2.6%; p = 0.009; Table 2). Anti-Ha was the most prevalent antibody found in ILD (2.0%), followed by anti-Zoα (1.4%) and anti-Ks (1.3%). In healthy controls, antibody reactivity at a positive level was observed in only one subject (0.9%; anti-cN1A). Prevalence of anti-Zoα reactivity on combined positive and weakly positive levels was significant higher in CTD-ILD compared to non-CTD-ILD (p = 0.047). Prevalence per antibody was not significantly different between all ILD patients and healthy subjects (Table 2), nor between the ILD subgroups (data not shown).

Table 2. Prevalence of novel myositis autoantibodies in patients with ILD.

Antibody	N (%)					
	All ILD	CTD-ILD[a]	Non-CTD-ILD[b]	P[c]	Healthy controls	P[d]
N	1194	131	1063		116	
Novel antibodies (p)	63 (5.3)	11 (8.4)	52 (4.9)	0.090	1 (0.9)	0.035
Novel antibodies (p+wp)	119 (10)	15 (11.5)	104 (9.8)	0.548	3 (2.6)	0.009
Ks (p)	15 (1.3)	3 (2.3)	12 (1.1)	0.222	-	0.388
Ks (p+wp)	24 (2.1)	3 (2.3)	21 (2.0)	0.741	-	0.262
Ha (p)	24 (2.0)	4 (3.1)	20 (1.9)	0.325	-	0.262
Ha (p+wp)	48 (4.0)	5 (3.8)	43 (4.0)	>0.999	1 (0.9)	0.119
Zoα (p)	17 (1.4)	4 (3.1)	13 (1.2)	0.106	-	0.390
Zoα (p+wp)	35 (2.9)	8 (6.1)	27 (2.5)	0.047	2 (1.7)	0.766
cN1A (p)	11 (0.9)	2 (1.5)	9 (0.8)	0.344	1 (0.9)	>0.999
cN1A (p+wp)	22 (1.8)	3 (2.3)	19 (1.5)	0.726	1 (0.9)	0.714

Data are expressed as numbers and percentage within the diagnosis group. (p) = positive level (wp) = weak positive level; a CTD-ILD = connective tissue disease related interstitial lung disease; b non-CTD-ILD = ILD without established CTD. c p< 0.05, considered significant; differences in frequencies between CTD-ILD and non-CTD-ILD patients, calculated by a Chi-Square or Fisher's exact test for dichotomous variables. d p< 0.05, considered significant; differences in frequencies between all ILD patients and healthy controls, calculated by a Chi-Square or Fisher's exact test for dichotomous variables.

Antibody positive ILD versus antibody negative ILD

Patients with antibody reactivity at intensity level 'positive' only were compared to patients without any antibody reactivity (Table 3). Patients with antibody reactivity on a 'weak positive' intensity level only were first excluded from this analysis (n = 56). Antibody positive subjects were more often females (47.6%) compared to antibody negative subjects (34.9%; p = 0.042). Furthermore, antibody positive ILD was less frequently characterized by a pattern of UIP in the biopsy (11.1%) compared to antibody negative ILD (35.8%; p= 0.032). Moreover, a trend towards absence of the UIP pattern on HRCT in antibody positive subjects was present (22.0%) compared to antibody negative subjects (30.9%; p= 0.158). Altogether, antibody positive subjects demonstrated less frequently a UIP pattern on either HRCT or in lung biopsies (15.9%) compared to antibody negative ILD as well (36.6%; p = 0.010, data not shown). No differences were found for age, ANA positivity or baseline PFT(Table 3). Additionally, a three-

way analysis comparing antibody positive ILD patients with antibody weak positive ILD and antibody negative ILD was performed (data not shown). Significantly more females (p = 0.049) and fewer patients with UIP patterns on either HRCT or in lung biopsies (p = 0.016) were also observed in the antibody positive ILD group compared to antibody weak positive ILD and antibody negative ILD.

Next, follow-up characteristics were evaluated for antibody positive ILD compared to antibody negative ILD. PFT change values were available in 36 antibody positive ILD and 678 antibody negative ILD. No significant differences were found in FVC change between antibody positive ILD (mean delta +2.0 % pred; SD 12.3) compared to antibody negative ILD (mean delta -1.1 % pred; SD 11.9; p = 0.145). Similarly, differences in DLCO change were not statistically significant as well between the antibody positive ILD group (mean delta -0.5 % pred; SD 9.6) compared to antibody negative ILD group (mean delta -1.7 % pred; SD 8.7; p = 0.481; data not shown). Survival analysis of the groups showed no differences between antibody positive ILD (mortality rate n = 26 (41.3%); median 38.6 months: IQR 22.9-70.4) and antibody negative ILD (mortality rate n = 529 (46.8%); median 31.6 months; IQR 18.6-56.0; p = 0.072).

Table 3. Characteristics of ILD patients with and without novel autoantibody reactivity.

	Novel autoantibody positive	**Novel autoantibody negative**	**p-value [d]**
N	63	1075	
Age (y)	64.6 (11.5)	65.1 (11.1)	0.713
Sex (m), %	33 (52.4)	699 (65.0)	0.042
History of smoking, %	39 (61.9)	722 (67.2)	0.557
Pulmonary function test			
FVC (% pred)	82.1 (20.8)	81.9 (34.6)	0.967
FEV1 (% pred)	84.6 (20.7)	84.4 (35.5)	0.981
Dlco (% pred)	47.4 (17.5)	46.0 (15.8)	0.607
HRCT scan [a]			
UIP	13 (22.0)	321 (30.9)	0.158
Probable UIP	9 (15.3)	154 (14.8)	0.928
Indeterminate UIP	15 (25.4)	202 (19.4)	0.262
Alternative	22 (37.3)	362 (34.8)	0.701
Histopathology [b]			
UIP	2 (11.1)	117 (35.8)	0.032
Probable UIP	1 (5.6)	14 (4.3)	0.560
Indeterminate UIP	3 (16.7)	44 (13.5)	0.722
Alternative	12 (66.7)	152 (46.5)	0.095
ANA (%)[c]	10 (24.4)	129 (17.9)	0.293

Data are expressed as mean and standard deviation or numbers and percentage per group. Included antibodies: anti-Ks, anti-Ha, anti-Zoα, anti-cN1A; on a positive level (antibody positive level; antibody reactivity on weakly positive level excluded) or negative level (antibody negative); FVC = forced vital capacity, expressed in percentage of predicted; FEV1 = forced expiratory volume in 1 second, expressed in percentage of predicted; Dlco = Diffusing capacity of the lung for carbon monoxide; UIP = usual interstitial pneumonia. [a] myositis antibody positive ILD: n = 59; myositis antibody negative ILD: n = 1039; [b] myositis antibody positive ILD: n = 18; myositis antibody negative ILD: n =327; [c] ANA = antinuclear antibody, expressed as % positive; myositis antibody positive ILD n = 41 ; myositis antibody negative ILD n = 716; [d] $p < 0.05$, differences between the groups calculated by a two-sided sample T-test for continuous variables or Chi-Square or Fisher's exact test for dichotomous variables.

CTD-ILD patients versus non-CTD-ILD patients

Overall, CTD-ILD patients were significantly younger compared with other ILDs ($p < 0.001$; Table 1). Furthermore, more females were observed in CTD-ILD (44.2%) compared with non-CTD-ILD (34.1%; $p = 0.022$). CTD-ILDs were frequently characterized by a radiological or histological pattern indeterminate for UIP, whereas a UIP pattern was more observed in non-CTD-ILDs, particularly in IPF (see Suppl. Tables 1 and 2). A trend towards higher prevalence of antibody reactivity was observed in CTD-ILD (8.4%) compared with other ILD (4.9%; $p = 0.090$). In both CTD-ILD and non-CTD-ILD, anti-Ha was the most prevalent antibody found (respectively 3.1% and 1.9%), followed by anti-Zoα and anti-Ks (Table 2). Patients with an IPF, unclassifiable IIP, HP and NSIP showed cN1A antibodies (range 0.3-1.5%). On the contrary, only two CTD-ILD patients (ASS) had anti-cN1A (Suppl. Tables 3 and 4). Reactivity against multiple antigens was rare and only occurred in Sjögren's syndrome (n = 1; anti-Ks and anti-Zoα), ASS (n =1; anti-cN1A and anti-Zoα) and unclassifiable IIP (n = 1; anti-Ha, anti-Ks and anti-Zoα). Patients with COP revealed no reactivity against the tested antigens. ILD patients who met the non-serological IPAF criteria (n = 11, all diagnosed as unclassifiable IIP) did not show antibody reactivity against the four tested antibodies on a positive or weakly positive level.

PFT change values were available in 89 CTD-ILD and 625 non-CTD-ILD patients. Non-CTD-ILD patients demonstrated significantly more FVC declines (mean delta -1.7 % pred; SD 11.7) compared to CTD-ILD patients (mean delta +4.1 % pred; SD 12; $p < 0.001$, data not shown). Similarly, DLCO declines were more observed in non-CTD-ILD patients (mean delta -2.2 % pred; SD 8.4) compared to CTD-ILD patients (mean delta +2.8 % pred; SD 10.1; $p < 0.001$). Survival was significantly better in CTD-ILD patients (mortality rate n = 42 (32.1%); median 54.0 months; IQR 33.8-84.1) compared to non-CTD-ILD patients (mortality rate n = 513 (48.3%); median 30.2 months; IQR 18.1-52.4; $p < 0.001$).

Characteristics per myositis antibody

Characteristics of anti-Ha positive ILD

Anti-Ha reactivity was observed in 24 patients who were classified as unclassifiable IIP (41.7%), HP (25.0%) or CTD-ILD (16.7%; Table 4, Figure 1). The CTD-ILDs consisted of ASS (n = 1), Ssc (n = 1) and Sjögren's syndrome (n = 2, Suppl. Table 3). Alternative patterns (other than UIP subcategories) were predominately seen on HRCT (34.8%) and in histopathological lung biopsies (75%, see Table 4; per ILD diagnosis see Suppl. Table 3 and 4).

Characteristics of anti-Ks positive ILD

Fifteen patients had anti-Ks antibodies, classifications including unclassifiable IIP (33.3%), HP (20.0%) and one patient with a desquamative interstitial pneumonia (DIP, 18.2%) (Figure 1). Anti-Ks positive CTD-ILDs (20.0%) consisted of patients with IBM (n = 1), RA-ILD (n = 1) and Sjögren's syndrome (n = 1; Suppl. Table 3). A variable palette of radiological patterns was observed, whereas available lung biopsies (n = 6) showed predominately alternative patterns (66.7%, see Table 4; per ILD diagnosis see Suppl. Table 3 and 4.

Table 4. Characteristics of ILD patients with positive novel antibody reactivity.

	Autoantibody				
	Ha	**Ks**	**Zoα**	**cN1A**	**p-value [d]**
N	24	15	17	11	
Age (y)	68.3 (11.3)	63.5 (12.0)	62.9 (12.9)	59.8 (7.3)	0.529
Sex (m), %	14 (58.3)	8 (53.3)	11 (64.7)	2 (18.2)	0.082
History of smoking, %	16 (66.7)	7 (46.7)	12 (75.0)	6 (54.5)	0.584
Pulmonary function test					
FVC (% pred)	83.3 (25.0)	81.0 (16.0)	77.6 (19.6)	76.5 (15.0)	0.984
FEV1 (% pred)	90.8 (26.5)	83.9 (15.6)	77.5 (15.8)	75.0 (14.6)	0.548
Dlco (% pred)	48.9 (16.0)	49.6 (17.6)	41.9 (13.4)	40.3 (16.8)	0.689
HRCT scan [a]					
UIP	5 (21.7%)	5 (35.7%)	2 (12.5%)	4 (40.0%)	0.048
Probable UIP	4 (17.4%)	3 (21.4%)	2 (12.5%)	-	0.278
Indeterminate UIP	6 (26.1%)	3 (14.3%)	5 (31.3%)	2 (20.0%)	0.652
Alternative	8 (34.8%)	4 (28.6%)	7 (43.8%)	4 (40.0%)	0.694
Histopathology [b]					
UIP	-	1 (16.7%)	-	1 (20.0%)	0.542
Probable UIP	-	-	-	1 (20.0%)	0.437
Indeterminate UIP	1 (25.0)	1 (16.7%)	-	1 (20.0%)	0.727
Alternative	3 (75.0)	4 (66.7%)	3 (100%)	2 (40.0%)	0.256
ANA (%)[c]	4 (23.5)	1 (10.0)	2 (16.7)	3 (50.0)	0.303

Data are expressed as mean and standard deviation or numbers and percentage per positive myositis antibody group; FVC = forced vital capacity, expressed in percentage of predicted; FEV1 = forced expiratory volume in 1 second, expressed in percentage of predicted; Dlco = Diffusing capacity of the lung for carbon monoxide; UIP = usual interstitial pneumonia. [a] Number of patients with available high resolution computed tomography (HRCT) scan: Ha (n = 23); Ks (n = 15); Zoα (n = 16); cN1A (n = 10); [b] Number of patients with available histopathological lung biopsies: Ha (n = 4); Ks (n = 6); Zoα (n = 3); cN1A (n = 5); [c] Number of patients with available antinuclear antibody (ANA): Ha (n = 17); Ks (n = 10); Zoα (n = 12); cN1A (n = 6) [d] $p < 0.05$, differences between the myositis antibodies calculated by a one way ANOVA for continuous variables or Chi-Square or Fisher's exact test for dichotomous variables.

Characteristics of anti-Zoα positive ILD

Seventeen patients had Zoα antibodies and were predominately male (64.7%) with a smoking history (75.0%; Table 4). Antibodies were seen in unclassifiable IIP (35.3%), HP (11.8%) and CTD-ILDs (23.6%), the latter groups consisting of ASS (n = 2), DM (n = 1) and Sjögren's syndrome (n = 1; Suppl. Table 3) patients. Zoα antibodies were found as well in DIP (n = 1), drug induced IP (flecainide-induced, n = 1) and combined pulmonary fibrosis and emphysema (CPFE, n = 1). Non-UIP radiological patterns were mostly observed. Available lung biopsies, all from non-CTD-ILDs, demonstrated a histological pattern alternative for UIP (Table 4; Suppl. Tables 3 and 4).

Characteristics of anti-cN1A positive ILD

Eleven subjects showed anti-cN1A reactivity and were predominately female (81.8%, Table 4). As illustrated in Figure 1, patients included HP (27.3%), IPF (18.2%) or CTD-ILD (18.2%, all with ASS). cN1A antibodies were found as well in smoking-related IP (SR-ILD) and respiratory bronchiolitis IP (RB-ILD). Almost half of anti-cN1A positive ILD subjects showed a radiological UIP pattern (40%), which was

significantly higher compared to the other antibody groups (p = 0.048). Concerning histological patterns, a variable palette of patterns was observed in these patients (Table 4 and Suppl. Tables 3 and 4).

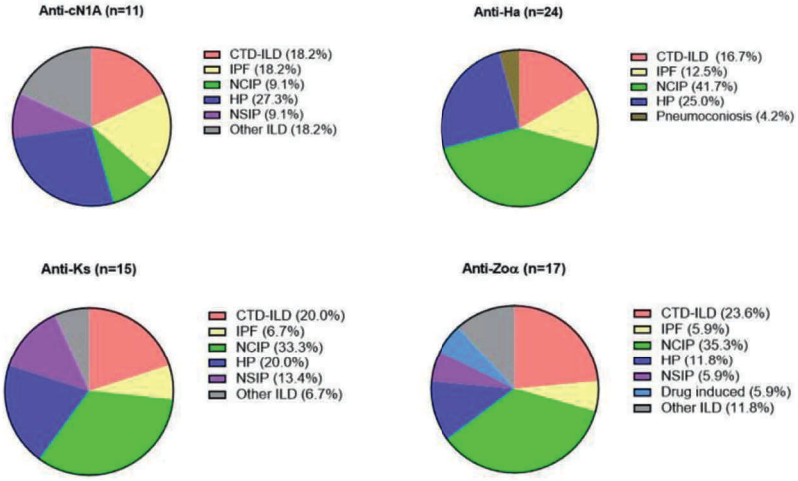

Figure 1. Prevalence of connective tissue disease related interstitial lung disease (CTD-ILD), idiopathic pulmonary fibrosis. (IPF), unclassifiable idiopathic interstitial pneumonia (unclassifiable IIP), hypersensitivity pneumonitis (HP), non-specific interstitial pneumonia; (NSIP), pneumoconiosis, drug induced interstitial pneumonia and other ILD, illustrated for each antibody with reactivity on a positive level.

Associations between antibodies and ILD

A logistic regression analysis was performed to evaluate associations between staining intensity levels and ILD classification. Antibody Zoα was found to be associated with CTD-ILD compared to other ILD when the "weakly positive" and "positive" groups were combined (OR 2.5; 95% CI 1.11-5.61; p = 0.027, Table 5). Further analysis demonstrated that the association was strongest for CTD-ILD compared to IPF within the group qualified as 'positive' (OR 9.6: p = 0.044, data not shown).

Table 5. Associations of novel myositis antibodies in ILD with and without established CTD.

Antibody	CTD-ILD (n = 131)			Non-CTD-ILD (n = 1063)					
	Number Neg	Number Weak pos	Number Pos	Number Neg	Number Weak pos	Number Pos	OR p OR wp OR wp+p[a]	95% CI[b]	p[c]
Ks	128	-	3	1042	9	12	2.04 ∞ 0.81	0.57-7.31 ∞ 0.34-3.95	0.276 ∞ 0.809
Ha	126	1	4	1020	23	20	1.62 0.35 0.94	0.55-4.81 0.05-2.63 0.37-2.42	0.386 0.309 0.900
Zoα	123	4	4	1036	14	13	2.59 2.41 2.50	0.83-8.07 0,78-7.43 1.11-5.61	0.100 0.127 0.027
cN1A	128	1	2	1044	10	9	1.81 0.82 1.29	0.39-8.48 0.10-6.42 0.38-4.41	0.450 0.847 0.687

CTD-ILD = connective tissue disease related interstitial lung disease; non-CTD-ILD = ILD without established CTD. a OR: odds ratio for positive level (OR p); odds ratio for weak positive level (OR wp); odds ratio for weak positive level + positive level (OR wp+p); b 95% confidence interval of odds ratio's; c Logistic regression analysis of CTD-ILD versus no established CTD-ILD (non-CTD-ILD) patients with positive, weak positive and negative antibody, with predicted probability for CTD-ILD.

Discussion

In this explorative study, we described the prevalence and clinical characteristics of a novel set of myositis related autoantibodies in a large cohort of patients with ILD. The pooled analysis showed that the prevalence of antibodies specific for Ha, Ks, Zoα and cN1A was significantly higher in ILD compared to healthy controls. Antibodies specific for Ha, Ks and Zoα were observed in unclassifiable IIP, HP and various CTD-ILDs, whereas cN1A antibodies were seen predominately in female subjects with ASS, HP and IPF. Furthermore, anti-Zoα was associated on a weakly positive and positive level with CTD-ILD compared to other ILD. In patients with circulating autoantibodies, radiological and/or histological non-UIP patterns on HRCT and/or in histological lung biopsies were predominately seen.

To date, little is known about the presence of these myositis antibodies in CTD-ILD and other ILD. Our study provides novel data on prevalence and clinical features of relatively unknown myositis antibodies measured by a line blot assay in a broad spectrum of ILD.

Antibodies specific for t-RNA synthetases have been thoroughly described in myopathies and CTD-ILDs, but were also identified in other ILDs including IPF (3–6, 11–25). Ha antibodies are seen in myopathies and in 40% of Ssc (28,35,36). We demonstrated novel data on presence of anti-Ha in a broad spectrum of ILD. Interestingly, most subjects had a preserved pulmonary function and included patients with an unclassifiable IIP, characterized by a radiological and/or histopathological pattern alternative for UIP. Possibly, these patients may have a phenotype that is characterized by a mild disease course.

Anti-Ks has been described in 0.3-7% of ILD patients (33,34). Interestingly, 70% had an IP without underlying CTD (33,34,41), which is agreement with 80% of anti-Ks positivity found our non-CTD-ILDs. Radiological and/or histological patterns of NSIP and OP (range 6-85.7%) have been described in ILD with anti-Ks (32,41–43) and other anti-t-RNA synthetases (11,16,23,25). These results are in congruence with the presence of non-UIP patterns in our study. Strikingly, COP patients demonstrated no reactivity against Ks, nor against any other antigen. We found more UIP patterns on HRCT (35.7%) but less in lung biopsies (16.7%) compared to respectively 5% and 80% found in small ILD studies (42,43). These results may be caused by the difference in study size and the absence of diagnostic lung biopsies in case of a typical radiological UIP.

A prevalence of 0.3% anti-Zo was found in ASS, of which 78% had an ILD (29,44). Our assay identified antibodies against the alpha unit of Zo (Zoα) in 1.4% of the ILD cases, including ASS. We demonstrated novel associations of anti-Zoα with CTD-ILD and idiopathic IPs. Radiological patterns of UIP (14%) and NSIP with OP (range 14-57%) have been described (29), which is in line with prevalence of UIP and non-UIP patterns in our study. Interestingly, 66% of anti-Zo positive ASS showed reactivity against anti-Ro52 (29). It is known that patients with both Ro52 and t-RNA synthase antibodies are characterized by chronic and severe ILD (6,19). Possibly, patients with combined Ro52 and Zoα antibodies show similar clinical outcomes.

Antibodies against cN1A were described in IBM, PM/DM, Ssc, SLE and Sjögren's syndrome (range 0-37%). However, associations with ILD have not been identified (26,27,30–32,43,45). We demonstrated novel data on presence of cN1A antibodies in ILDs including ASS, HP and IPF. Moreover, various radiological and histological patterns can be seen, including UIP. A predominance for female patients was observed, which is in agreement with a study with DM patients (27). Contrary, anti-cN1A positive patients with IBM, SLE and Sjögren's syndrome were mainly males (27,35,46).

This study was performed with patients whom were all diagnosed by a standardized multidisciplinary approach in a tertiary ILD center in the Netherlands. It is the first study to describe prevalence and clinical features of novel myositis antibodies in a large ILD cohort compared to healthy controls. This retrospective study has an important limitation, as a potential selection bias of more severely impaired patients with pulmonary fibrosis is possible due to the patient population in a referral center. However, we do not expect this to have any major impact on the distribution of autoantibodies. Furthermore, the line-blot used in this study has been used for research purposes only to date. Thus, validation for implementation is not complete yet. In time, the results of our study will contribute to final implementation of these antibodies for clinical use. A selection bias of patients who underwent surgical lung biopsies is possible, as subjects with a (probable) UIP pattern on HRCT might not undergo surgery for diagnostic purposes. Furthermore, prevalence of antibody reactivity was higher in ILD patients compared to healthy subjects, but statistical differences were not observed in prevalence per antibody. This result is probably due to the relatively low prevalence found per antibody.

The findings of this study raise the question why antibodies are present in idiopathic IP, including IPF. An IP can occur two years before an associated CTD (3,6), but antibodies are present in true idiopathic IP as well (11,16,23–25). In several studies, autoantibody producing plasma cells were identified in fibrotic lung tissue (47). Furthermore, T follicular helper cells, which induce the production of antigen-specific antibodies in germinal centres, where increased and activated in the peripheral blood of patients with IPF compared to healthy controls (48). Possibly, antibodies in idiopathic IP are randomly autoreactive and continuously generated at a certain stage of disease, without resulting in pathological autoimmunity as observed in CTD-ILDs. However, targets of these autoantibodies might actually do participate in the disease process culminating in pulmonary fibrosis i.e.. Although in general, treatment response for immunosuppressive drugs is better in CTD-ILDs compared with other ILDs (5–10), one can speculate whether specific treatment regiments should be reconsidered in antibody positive ILD without established CTD. Recently, the use of anti-fibrotic therapy has been successfully demonstrated in Ssc-IP and progressive fibrosing ILDs (49,50). It will be of interest to evaluate whether autoantibody positive idiopathic IP benefit from combining anti-fibrotic therapy with B cell targeted therapy, when compared with antibody negative idiopathic IP. Such a study will benefit from additional serological parameters to signal immune activation status to determine whether ILD progression and autoantibody detection is paralleled by an ongoing immune response (51,52). However, these studies may be difficult to realize because immunosuppressants can have a harmful effect in IPF in general (53,54).

In conclusion, our results contribute to the awareness that autoantibodies can be found in an IP without established CTD. Screening for antibodies on a regular basis could contribute to the identification of merely progressive fibrotic phenotype from those in which an ongoing autoimmune response which potentially feeds the fibrotic phenotype. A prospective cohort evaluation is needed to determine whether antibody positive idiopathic IP develop features of an associated CTD. Furthermore, it will be of interest to investigate associations between these novel antibodies with other myositis antibodies and treatment outcome.

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6

Prevalence and clinical associations of myositis autoantibodies in a large cohort of interstitial lung diseases

Sofia A. Moll

Mark G.J.P. Platenburg

Anouk C.M. Platteel

Adriane D.M. Vorselaars

Montse Janssen Bonàs

Raisa Kraaijvanger

Claudia Roodenburg-Benschop

Bob Meek

Coline H.M. van Moorsel

Jan C. Grutters

Abbreviations

ANA = Antinuclear antibody

ASS = Anti-synthetase syndrome

ATS = American Thoracic Society

BALf = Bronchoalveolar lavage fluid

CA 15-3 = Cancer antigen 15-3

CC16 = Clara cell secretory protein

CCL18 = CC chemokine ligand 18

COP = Cryptogenic organizing pneumonia

CPFE = Combined pulmonary fibrosis and emphysema

CTD = Connective tissue disease

DIP = Desquamative interstitial pneumonia

DM = Dermatomyositis

Dlco = Diffusing capacity of the lung for carbon monoxide

ERS = European Respiratory Society

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

HP = Hypersensitivity pneumonitis

HRCT = High-resolution computed tomography

IBM = Inclusion body myositis

IIP = Idiopathic interstitial pneumonia

ILD = Interstitial lung disease

IMNM = Immune mediated necrotizing myopathy

IP = Interstitial pneumonia

IPAF = Interstitial pneumonia with autoimmune features

IPF = Idiopathic pulmonary fibrosis

MAA = Myositis associated antibody

MSA = Myositis specific antibody

NSIP = Non-specific interstitial pneumonia

PFT = Pulmonary function test

PM = Polymyositis

RA = Rheumatoid arthritis

SLE = Systemic lupus erythematosus

SP-D = Surfactant protein D

Ssc = Systemic sclerosis

UIP = Usual interstitial pneumonia

YKL-40 = Chitinase-3-like protein 1

Abstract

Background: Serologic testing for autoantibodies is recommended in interstitial lung diseases (ILDs), as connective tissue diseases (CTDs) are an important secondary cause. Myositis antibodies are associated with CTD-ILD, but clinical associations with other ILDs are unclear. In this study, associations of myositis antibodies in various ILDs were evaluated.

Methods: 1463 ILD patients and 116 healthy subjects were screened for myositis antibodies with a line-blot assay on serum available at time of diagnosis. Additionally, bronchoalveolar lavage fluid (BALf) and histological lung biopsies were analysed.

Results: A total of 394 patients demonstrated reactivity to at least one antibody, including anti-Ro52 (36.0%), anti-Mi-2β (17.3%) and anti-Jo-1 (10.9%). Anti-Jo-1 (OR 6.4; p<0.100) and anti-Ro52 (OR 6.0; p<0.001) were associated with CTD-ILD. Interestingly, anti-Mi-2β was associated with idiopathic pulmonary fibrosis (IPF; OR 5.3; p=0.001) and hypersensitivity pneumonitis (HP; OR 5.9; p<0.001). Furthermore, anti-Mi-2β was strongly associated with a histological usual interstitial pneumonia (UIP) pattern (OR 6.5; p < 0.001). Anti-Mi-2β reactivity was identified in BALf and correlated with serum anti-Mi-2β (r=0.64; p=0.002).

Conclusion: In conclusion, novel associations of antibody Mi-2β with fibrotic ILD were found. Furthermore, serum anti-Mi-2β was associated with a histological UIP pattern and presence of anti-Mi-2β in BALf. We postulate that Mi-2β is associated with de-differentiation of alveolar epithelial cells, resulting in fibrotic ILD. Possibly, anti-Mi-2β could be implemented as a future diagnostic biomarker for fibrotic ILD, but further experimental research is imperative.

Introduction

Interstitial lung diseases (ILDs) are a group of heterogeneous, diffuse parenchymal lung diseases, characterized by inflammation and/or fibrosis of the pulmonary interstitium. ILDs can be idiopathic or secondary to known causes including environmental exposures, drugs or connective tissue disease (CTD) (1–4). In 15% of patients with ILD an underlying CTD is identified (3,5). Distinguishing CTD related ILD (CTD-ILD) from other ILD is challenging as clinical, functional, radiological and pathological characteristics could be similar (6). Moreover, an interstitial pneumonia (IP) may be the first or lone clinical manifestation of an associated CTD (4,6). In general, outcomes on treatment response to immunosuppressive therapy and survival are better in CTD-ILD compared to the majority of other ILDs (5–10). Thus, discriminating these conditions in the diagnostic work-up is essential.

Serologic testing for autoantibodies by a myositis blot is recommended in pulmonary fibrosis suspected for an underlying CTD and includes myositis specific antibodies (MSA) and myositis associated antibodies (MAA) (1,3,4,6,11–16). MSA and MAA are found in patients with idiopathic interstitial myopathies but also occur in patients with rheumatic diseases including CTD-ILDs (3–6,11–14, 15,16–22, 23). Presence of t-RNA synthase antibodies are strongly associated with ILD in antisynthetase syndrome (ASS), dermatomyositis (DM) and polymyositis (PM)(6,20,23–25). Furthermore, a combination of t-RNA synthase antibodies and anti-Ro52/SSA is characterized by chronic and severe ILD (6,19).

Myositis antibodies have also been identified in hypersensitivity pneumonitis (HP) and idiopathic IPs (11,16,26–28). Although it is known that an IP can precede future CTD (3,6), evidence is scarce on the clinical relevance of antibody positivity in patients meeting established criteria for other ILD. Therefore, it remains unclear how positive serologic testing in these ILDs should be interpret in clinical practice. Possibly, certain antibodies could be more associated with ILD features such as fibrosis than other characteristics of CTD.

To date, no major studies have compared myositis antibody positivity between CTD-ILD and other ILD. The aim of this study was to evaluate prevalence and clinical associations of myositis antibodies in these patients to enhance the diagnostic performance of serologic testing.

Methods

Patient selection

A retrospective cohort study was performed at the St Antonius ILD Centre of Excellence Nieuwegein, a tertiary ILD centre in the Netherlands. The majority of ILD patients were diagnosed between 2000 and 2019. Subjects who had been tested for myositis antibodies during diagnostic work-up were evaluated. In addition, ILD patients who were not screened for myositis antibodies were evaluated for the presence of antibodies in serum collected at date of diagnosis by a line-blot assay, run between 05-2019 and 07-2019. In addition, serum of healthy, non-ILD blood donors were screened for myositis antibodies as controls.

Diagnosis of ILD was assessed according to official American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations in a multidisciplinary discussion with an ILD

pulmonologist, experienced thoracic radiologist, and a pathologist, when required (29). All patients with pulmonary fibrosis were screened for an underlying CTD by the pulmonologist and referred to the rheumatologist for further diagnostic work-up if a CTD was suspected.

Patients were classified as having a CTD-ILD or ILD without established CTD (non-CTD-ILD). Patients were checked for any revisions of the ILD diagnosis during two years of follow-up, as an IP can precede an associated CTD (3,6). CTD-ILDs included antisynthetase syndrome (ASS), Sjogren's syndrome, rheumatoid arthritis associated ILD (RA-ILD), systemic sclerosis (Ssc), dermatomyositis (DM), polymyositis (PM), immune mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM), overlap myositis, systemic lupus erythematosus (SLE), mixed CTD and other CTD-ILD. Non-CTD-ILDs included idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis (HP), unclassifiable idiopathic interstitial pneumonia (Unclassifiable IIP), non-specific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP) and other ILD.

Patients with reactivity on a positive level against one or more antibodies were included for analysis. Only the first sample was included if more than one sample of a given patient was present. Subjects with solely weakly positive or negative antibody reactivity were excluded.

Baseline characteristics included pulmonary function tests, which were performed according to ATS/ERS recommendations (29). Serum pneumoproteins including cancer antigen 15-3 (CA 15-3), CC chemokine ligand 18 (CCL18), Clara cell secretory protein (CC16), chitinase-3-like protein 1 (YKL-40) and surfactant protein D (SP-D) were evaluated as well. Characteristics on high-resolution computed tomography (HRCT) scans and in lung biopsies (when available) were classified as a pattern of usual interstitial pneumonia (UIP), probable UIP, indeterminate UIP or alternative diagnosis according to recent ATS/ERS recommendations (29).

The study was approved by the St Antonius institutional review under protocol number 842002003 and patients provided written informed consent for research purposes.

Determination of antibodies

Antibodies were detected in serum using a line-blot assay (EUROLINE Autoimmune Inflammatory Myopathies, EUROIMMUN, Lübeck, Germany), in collaboration with Biognost. The assay identified MSA including antibodies against Jo-1, EJ, OJ, PL-7, PL-12, Mi-2α, Mi-2β, TIF1-γ, MDA5, NXP2 and SAE1 and MAA, including antibodies against Ku, PM/Scl-75, PM/Scl-100 and Ro52. Analysis of the immunoblot strips was performed with the EUROLINEScan software (EUROIMMUN, Lübeck, Germany) according to manufacturer's recommendations as described for the EUROLINE Autoimmune Inflammatory Myopathies line blot assay. Strips were scored as negative, weakly positive and positive, which corresponds with intensity levels of respectively 0-10, 11-25 and >25. Antibody reactivity on a positive and weakly positive intensity level was separately evaluated.

Statistical analysis

Baseline characteristics were expressed as numbers and percentages or mean and standard deviation. Continuous and categorical variables were tested with a student's T-test/one-way ANOVA and

Chi-Square test/Fisher's Exact test respectively. A binary logistic regression analysis was performed in CTD-ILD and non-CTD-ILD for testing antibody level and association, expressed as general odds ratios. The statistical analysis was performed by software IBM SPSS version 24.0. A p-value less than 0.05 was considered as statistically significant. Graphs were drafted in Graph Pad Prism 8.3.

Results

Baseline characteristics

A total of 1463 ILD patients were screened for myositis antibodies. One hundred CTD-ILD and 294 non-CTD-ILD patients were positive for at least one antibody and were included in this study. Baseline characteristics are listed in Table 1 and per ILD diagnosis in the supplementary data (Suppl. Tables 1 and 2). Significant differences between CTD-ILD and non-CTD-ILD were found for age (p<0.001), sex (p<0.001), smoking history (p=0.007) and serum CA 15-3 (p=0.003). Furthermore, radiological (p<0.001) and histopathological (p=0.002) UIP patterns were predominantly observed in non-CTD-ILDs (range 4.2-82.4%) including IPF.

Table 1	**Baseline characteristics of 394 patients with ILD**			
Subjects	**All**	**CTD-ILD**	**Non-CTD-ILD**	**P[e]**
N	394	100	294	
Age	63.6 (11.2)	59.1 (11.5)	65.1 (10.7)	< 0.001
Sex (m), %	242 (61.4)	46 (46.0)	196 (66.7)	< 0.001
History of smoking, %	272 (68.7)	56 (56.0)	212 (72.4)	0.007
Pulmonary function test[a]				
FVC	81.0 (20.1)	80.4 (23.2)	81.2 (19.0)	0.751
FEV1	83.3 (37.9)	86.3 (67.9)	82.3 (19.8)	0.571
DLCO	46.9 (16.2)	49.3 (15.0)	46.2 (16.5)	0.101
Pneumoprotein[b]				
CA 15-3	64.9 (64.2)	51.2 (46.8)	69.8 (68.9)	0.003
CC16	20.2 (26.1)	17.2 (46.8)	21.2 (15.1)	0.410
CCL18	174.2 (112.7)	164.7 (93.2)	177.3 (118.3)	0.331
SP-D	113.4 (194.1)	108.7 (175.1)	115.1 (200.8)	0.768
YKL-40	148.4 (133.7)	140.8 (122.0)	151.2 (137.8)	0.724
HRCT scan[c]				
UIP	96 (24.9)	11 (11.3)	85 (29.4)	< 0.001
Probable UIP	47 (12.2)	7 (7.2)	40 (13.8)	0.084
Indeterminate	75 (19.4)	24 (24.7)	51 (17.6)	0.126
Alternative	168 (43.5)	55 (56.7)	113 (39.1)	0.002
Histopathology[d]				
UIP	24 (20.5)	-	24 (27.0)	0.002
Probable UIP	12 (10.3)	5 (17.9)	7 (7.9)	0.129
Indeterminate	20 (17.1)	5 (17.9)	15 (16.9)	0.902
Alternative	61 (52.1)	18 (64.3)	43 (48.3)	0.140

Data are expressed as mean and standard deviation or numbers and percentage within the diagnosis group.
CTD = connective tissue disease; ILD = interstitial lung disease; non-CTD-ILD = ILD without established CTD; FVC = forced vital capacity; FEV1 = forced expiratory volume in 1 second; DLCO = diffusing capacity of the lung for carbon monoxide; CA 15-3 = cancer antigen 15-3; CCL18 = CC chemokine ligand 18; CC16 = Clara cell secretory protein; SP-D = surfactant protein D; YKL-40 = chitinase-3-like protein 1; UIP = usual interstitial pneumonia
[a] n = 383, data expressed mean and standard deviation in percentage of predicted
[b] n = 383, data expressed as mean and standard deviation in kU/l (CA 15-3) or ng/ml (CC16, CCL18, SP-D, YKL-40)
[c] n = 386, data expressed as numbers and percentage
[d] n = 117, data expressed as numbers and percentage
[e] p < 0.05, differences between CTD-ILD and non-CTD-ILD are calculated by a two-side unpaired T-test/One way Anova for continuous variables or Chi-Square test/Fisher's Exact test for dichotomous variables.

Frequencies of myositis antibodies in patients with ILD

Antibody prevalence was evaluated for all ILD, 116 healthy controls (Table 2) and per ILD diagnosis (Suppl. Tables 3 and 4). On a positive intensity level, the most prevalent antibodies in ILD were Ro52 (36.0%) and Mi-2β (17.3%), followed by Jo-1 (10.9%) and SRP (7.4%, Table 2). Antibody prevalence was significantly higher in ILD compared to controls except for antibodies EJ, MDA5, Mi-2α, NXP2 and OJ. Anti-Ro52 reactivity was high in ASS (83.9%) and Sjogren's syndrome (92.3%, Suppl. Table 3) but also observed in unclassifiable IIP, NSIP and COP (range 27.3-54.8%; Suppl. Table 4). Prevalence of antibodies Mi-2β, SRP and Ku was significantly higher in non-CTD-ILD compared to CTD-ILD (all p<0.05, see Table 2), in particular in IPF (respectively 26.5%, 11.8% and 8.8%) and unclassifiable IIP (respectively 17.9%, 14.2% and 7.5%; Suppl. Table 4). Mi-2β antibodies were observed in HP as well (26.4%).

Table 2	Frequency of myositis antibodies in ILD patients and healthy controls					
Antibody	**All ILD**	**CTD-ILD**	**Non-CTD-ILD**	**P[a]**	**Healthy controls**	**P[b]**
N	394	100	294		116	
MSA						
EJ	10 (2.5)	5 (5.0)	5 (1.7)	0.070	1 (0.9)	0.470
Jo-1	43 (10.9)	27 (27.0)	16 (5.4)	<0.001	-	<0.001
MDA5	8 (2.0)	2 (2.0)	6 (2.0)	0.980	-	0.208
Mi-2α	6 (1.5)	1 (1.0)	5 (1.7)	0.621	-	0.345
Mi-2β	68 (17.3)	7 (7.0)	61 (20.7)	0.002	1 (0.9)	<0.001
NXP2	7 (1.8)	4 (4.0)	3 (1.0)	0.051	-	0.359
OJ	7 (1.8)	1 (1.0)	6 (2.0)	0.496	-	0.359
PL-12	21 (5.3)	11 (11.0)	10 (3.4)	0.003	-	0.006
PL-7	24 (6.1)	5 (5.0)	19 (6.5)	0.597	-	0.006
SAE1	14 (3.6)	2 (2.0)	14 (4.8)	0.026	-	0.047
SRP	29 (7.4)	2 (2.0)	27 (9.2)	0.017	-	0.002
TIF1-γ	24 (6.1)	1 (1.0)	23 (7.8)	0.014	-	0.006
MAA						
Ku	20 (5.1)	1 (1.0)	19 (5.4)	0.032	-	0.011
PM/Scl 100	22 (8.4)	13 (13.0)	20 (6.5)	0.053	1 (0.9)	0.013
PM/Scl 75	46 (11.7)	13 (13.0)	33 (11.2)	0.633	-	<0.001
Ro52	142 (36.0)	67 (67.0)	75 (25.5)	<0.001	2 (1.7)	<0.001

Data are expressed as numbers and percentage of positive antibodies within each ILD diagnosis group. Weakly positive antibodies are excluded.
CTD = connective tissue disease; ILD = interstitial lung disease; non-CTD-ILD = ILD without established CTD
[a] p < 0.05, difference between CTD-ILD and other ILD patients calculated by Chi-Square test or Fisher's Exact test. [b] p < 0.05, difference between all ILD and healthy controls calculated by Chi-Square test or Fisher's Exact test

Associations between antibody level and ILD

Associations between antibody reactivity and ILD were evaluated (Table 3). At the positive intensity level, strong associations were found of antibodies Jo-1 (OR 6.4; p<0.001), PL-12 (OR 3.4; p=0.007) and Ro52 (OR 6.0; p<0.001) with CTD-ILD. Furthermore, odds ratios of less than one were found with antibodies Mi-2β (OR 0.3; p=0.002), SRP (OR 0.2; p=0.026) and Ku (OR 0.1; near-significant; p=0.058) with CTD-ILD. Expressed as a reversed odds ratio (1/OR), corresponding ratios were 2.7, 5.3 and 7.1 respectively, indicating odds in favour of non-CTD-ILD. A sub analysis of CTD-ILD compared to IPF showed strong associations of antibodies Mi-2β (OR 0.2; 1/OR 5.3; p=0.001), Ku (OR 0.1; 1/OR 10; p=0.034) and SRP (OR 0.1; 1/OR 7.7; p=0.013; Suppl. Table 5) with IPF. Similarly, antibodies Mi-2β (OR 3.2; p=0.015), Ku (OR 5.0 p=0.046) and TIF1-γ (OR 16.7; p=0.008, data not shown) were associated with unclassifiable IIP compared to CTD-ILD. Mi-2β antibody was associated with HP compared to CTD-ILD as well (OR 5.9; p<0.001, data not shown).

Table 3	Associations of myositis antibodies with ILD patients								
Antibody	**CTD-ILD (n = 100)**			**Non-CTD-ILD (n = 294)**					
	Number Neg	Number Weak pos	Number Pos	Number Neg	Number Weak pos	Number Pos	OR p OR wp[a]	95% CI[b]	p[c]
EJ	95	-	5	289	-	5	3.0	0.86-10.76	0.084
							-		
Jo-1	72	1	27	271	7	16	6.4	3.25-12.42	<0.001
							0.5	0.07-4.44	0.565
Ku	98	1	1	262	13	19	0.1	0.02-1.07	0.058
							0.2	0.03-1.59	0.130
MDA5	97	1	2	277	11	6	1.0	0.19-4.80	0.952
							0.3	0.03-2.04	0.199
Mi-2α	99	-	1	285	4	5	0.6	0.07-4.99	0.616
							-		
Mi-2β	91	2	7	211	22	61	0.3	0.12-0.60	0.002
							0.2	0.05-0.92	0.038
NXP2	95	1	4	287	4	3	4.0	0.89-18.3	0.071
							0.8	0.08-6.84	0.803
OJ	99	-	1	284	4	6	0.5	0.06-4.02	0.497
							-		
PL-12	89	-	11	278	6	10	3.4	1.41-8.36	0.007
							-		
PL-7	95	-	5	270	5	19	0.8	0.27-2.06	0.574
							-		
PM/Scl 100	87	-	13	260	14	20	1.9	0.93-4.07	0.078
							-		
PM/Scl 75	86	1	13	249	12	33	1.1	0.57-2.27	0.707
							0.2	0.03-1.88	0.175
Ro52	31	2	67	208	11	75	6.0	3.63-9.89	<0.001
							1.2	0.258-5.77	0.802
SAE1	98	2	-	271	9	14	-		
							0.6	0.13-2.89	0.538
SRP	90	8	2	232	35	27	0.2	0.04-0.82	0.026
							0.6	0.26-1.14	0.198
TIF1-γ	96	3	1	265	6	23	0.1	0.02-0.90	0.039
							1.4	0.34-5.63	0.653

CTD = connective tissue disease; ILD = interstitial lung disease; non-CTD-ILD = ILD without established CTD
[a] OR: odds ratio for positive level (OR p); odds ratio for weak positive level (OR wp).
[b] 95% confidence interval of odds ratio's
[c] Logistic regression analysis of CTD versus other patients with positive, weak positive and negative antibody, with predicted probability for CTD-ILD.

Associations between antibody level and radiological and histological characteristics

Next, associations of antibodies with radiological and histological patterns were evaluated. Anti-SAE1 was associated with a radiological UIP pattern (OR 3.2; p=0.036). Anti-Ro52 was associated with both radiological (OR 2.7; p<0.001, data not shown) and histological non-UIP patterns (OR 0.16; 1/OR 6.3; p=0.005; see Table 4). Interestingly, antibody Mi-2β was strongly associated with a histological UIP pattern (OR 6.5; p<0.001) but not significantly associated with a radiological UIP pattern (data not shown). However, evaluation of ILD patients classified per radiological pattern (from UIP to alternative diagnosis) showed that the association of anti-Mi-2β with histological UIP persisted within each radiological group (OR range 4.3-10, all p<0.05, data not shown), indicating that the association of serum Mi-2β antibodies with histological UIP was independent of the patients' corresponding radiological pattern. Other antibodies were not significantly associated with radiological patterns (data not shown).

Table 4	Associations of myositis antibodies with a histological UIP pattern and non-UIP pattern								
Antibody	**UIP (n = 24)**			**Non-UIP (n = 93)**					
	Number Neg	Number Weak pos	Number Pos	Number Neg	Number Weak pos	Number Pos	OR p OR wp[a]	95% CI[b]	p[c]
EJ	24	-	-	92	-	1	- -		
Jo-1	21	1	2	83	1	9	0.88 3.95	0.18-4.37 0.24-65.84	0.874 0.338
Ku	23	1	-	91	1	1	- 3.96	0.24-65.67	0.337
MDA5	23	1	-	91	1	1	- 3.96	0.24-65.67	0.337
Mi-2α	22	1	1	88	3	2	1.33 2.00	0.13-13.45 0.17-23.07	0.807 0.579
Mi-2β	10	3	11	77	3	13	6.52 7.70	2.31-18.41 1.36-43.46	<0.001 0.021
NXP2	24	-	-	92	-	1	- -		
OJ	23	1	-	92	-	1	- -		
PL-12	22	2	-	81	1	11	- 7.36	0.64-85.01	0.110
PL-7	21	1	2	86	2	5	1.64 2.05	0.30-9.04 0.18-23.67	0.571 0.566
PM/Scl 100	22	-	2	78	3	12	0.59 -	0.12-2.84	0.511
PM/Scl 75	18	2	4	76	2	15	1.13 4.22	0.33-3.80 0.56-32.03	0.848 0.164
Ro52	20	1	3	45	6	42	0.16 0.38	0.04-0.58 0.04-3.32	0.005 0.378
SAE1	22	-	2	90	1	2	4.09 -	0.55-30.67	0.171
SRP	15	7	2	78	11	4	2.60 3.31	0.44-15.50 1.11-9.91	0.294 0.033
TIF1-γ	19	3	2	87	1	5	1.83 13.74	0.33-10.16 1.35-139.36	0.489 0.027

UIP = usual interstitial pneumonia;
[a] OR: odds ratio for positive level (OR p); odds ratio for weakly positive level (OR wp).
[b] 95% confidence interval of odds ratio's
[c] Logistic regression analysis of histological UIP versus non UIP patients with positive, weakly positive and negative antibody, with predicted probability for UIP.

Antibody expression in bronchoalveolar lavage fluid (BALf) and histological lung biopsies

An analysis was performed in BALf and histological lung biopsies with regard to the association of antibody Mi-2β with histological UIP pattern. First, BALfs were retrieved of patients with serum Mi-2β antibodies on a positive intensity level and of a subset of patients without Mi-2β antibodies. BALf was tested for the presence of anti-Mi-2β by the line-blot assay (see Supplement for methods). To determine the extent of possible leakage of blood plasma products to the alveoli, an albumin BALf/serum ratio was calculated and used as an indicator for antibody leakage. Nine serum Mi-2β positive ILD and eleven serum Mi-2β negative ILD with available BALfs were included (Table 5). Of serum Mi-2β positive ILD, one patient (IPF) demonstrated Mi-2β reactivity on a positive level in BALf, one patient (HP) on a weakly positive level and three (n=2 HP and n=1; RA-ILD) on a borderline weakly positive level (intensity level 6-10).

Serum Mi-2β negative ILD patients did not show any Mi-2β reactivity in BALf. No differences were found in albumin BALf/serum ratios between serum Mi-2β positive and negative ILD patients (p=0.849). In addition, albumin BALf/serum ratios in serum Mi-2β positive ILD were not different as well between subjects with and without Mi-2β reactivity in BALf (p=0.568, data not shown). Mi-2β reactivity on a

combined (borderline) weakly positive and positive level in BALf was strongly associated with serum Mi-2β reactivity (r=0.64; p=0.002). Mi-2β reactivity on a positive level only in BALf was not significantly associated with serum Mi-2β reactivity (data not shown).

Table 5 Mi-2β measurement in bronchoalveolar lavage fluid in ILD patients

Patient	Diagnosis	Age (y)	Sex	HRCT scan	Histopathology	Serum Mi-2β	BALf Mi-2β	Albumin BALf/serum[a]
1	IPF	47	M	Probable UIP	-	Pos	Pos	1.29
2	HP	75	M	Alternative	-	Pos	Weak pos	1.27
3	HP	47	M	Alternative	Alternative	Pos	Borderline	0.22
4	HP	73	M	Alternative	-	Pos	Borderline	4.09
5	RA-ILD	54	M	Alternative	-	Pos	Borderline	1.67
6	HP	59	M	Alternative	-	Pos	Neg	1.49
7	HP	73	F	UIP	-	Pos	Neg	0.99
8	Unclassifiable IIP	72	F	Probable UIP	-	Pos	Neg	1.25
9	Unclassifiable IIP	50	F	UIP	Indeterminate UIP	Pos	Neg	1.29
10	IPF	74	M	UIP	-	Neg	Neg	1.44
11	COP	73	F	Alternative	Alternative	Neg	Neg	1.65
12	Sjogren's syndrome	75	M	Alternative	-	Neg	Neg	1.14
13	IPF	80	M	UIP	-	Neg	Neg	0.24
14	Sjogren's syndrome	73	F	Probable UIP	-	Neg	Neg	1.70
15	IPF	65	M	Probable UIP	UIP	Neg	Neg	1.07
16	IPF	76	M	UIP	-	Neg	Neg	0.87
17	ASS	62	M	Indeterminate UIP	Probable UIP	Neg	Neg	1.56
18	ASS	47	M	Alternative	-	Neg	Neg	4.05
19	Unclassifiable IIP	80	M	Alternative	-	Neg	Neg	0.56
20	HP	72	M	UIP	-	Neg	Neg	4.21

ILD = interstitial lung disease; IPF = idiopathic pulmonary fibrosis; HP = hypersensitivity pneumonitis; RA-ILD; rheumatoid arthritis associated interstitial lung disease; Unclassifiable IIP = unclassifiable idiopathic interstitial pneumonia; COP = cryptogenic organizing pneumonia; ASS: antisynthetase syndrome; HRCT = high resolution computed tomography; UIP = usual interstitial pneumonia; BALf = bronchoalveolar lavage fluid
Pos = antibody reactivity on a positive intensity level; Weak pos = antibody reactivity on a weakly positive intensity level; Borderline = antibody reactivity on a borderline weakly positive intensity level (6-10); neg = no antibody reactivity. [a] ratio of albumin level in BALf (mg/l) and serum (g/l).

Discussion

In this study, we evaluated prevalence, clinical characteristics and associations of myositis antibodies in a large cohort of ILD. Antibodies Jo-1 and Ro52 were strongly associated with CTD-ILD. Strikingly, we demonstrated stronger associations of anti-Mi-2β positivity with IPF, HP and unclassifiable IIP compared to CTD-ILD. Furthermore, Mi-2β antibody was strongly associated with a histological pattern of UIP. Interestingly, anti-Mi-2β reactivity was detected in BALf and correlated with serum Mi-2β reactivity in ILD. To date, the clinical value of positive myositis antibodies in other ILDs including idiopathic IP remains unclear. Possibly, testing of autoantibody Mi-2β in particular could be used as a diagnostic biomarker for fibrotic ILD in clinical practice.

Antibodies against t-RNA synthetases and Ro52 were demonstrated in various ILDs. Antibody Jo-1 is considered as a predictor for ILD and was observed in 27% of CTD-ILD, compared to 30-50% found in DM with ILD (6,20). Prevalence of MSA was high in non-CTD-ILD compared to studies with idiopathic IP, in which 6.6-24% of the subjects showed MSA including Jo-1, EJ, NXP-2, PL-7, TIF1-γ and SRP (11,16,26). Contrary, antibody PL-12 was infrequently observed in our IPF cohort (1.5%) compared to an IPF study

(5.3%)(27). Furthermore, patients with idiopathic IP and positive for antibodies EJ, PL-7 or PL-12 were radiologically and/or histologically characterized by a pattern of NSIP or UIP (16,11,28), which is in agreement with characteristics of IPF and unclassifiable IIP in our cohort. Antibody frequencies of PL-12 and PL-7 in ASS (both 9.7% respectively) were low compared to ASS-ILD studies (range 60-77%) (23,26,24). However, similarities in histological characteristics were observed in anti-PL-12 positive ILD (non-UIP) compared to findings in anti-PL-12 positive ASS (NSIP)(25). Furthermore, Ro52 antibodies were frequently observed in both our CTD-ILDs (67%) and in a study with CTD-ILDs including PM/DM and Sjogren's syndrome (60%)(30). Prevalence of antibody Ro52 in IPF (14.7%) was in congruence with previous IPF research (15.8%)(28).

Novel findings on associations of antibody Mi-2β with fibrotic ILDs were found, in particular IPF. In DM research, Mi-2β antibodies were demonstrated in 4-14% of the subjects and correlated with the presence of an IP (15,20). However, Mi-2β antibodies have not been described in patients with IPF, HP or unclassifiable IIP. Our study adds to previous research that serum Mi-2β antibodies are associated with ILDs without established CTD, including idiopathic IPs. In addition, we are the first to demonstrate anti-Mi-2β reactivity in BALf of ILD patients and its strong association with concurrent serum Mi-2β reactivity.

It is known that the Mi-2β antigen is part of the NuRD complex, which is regulated by the chromatin remodelling complex gene CDH4 (31). CDH4 is essential for specification of early B-cell lineage transcriptional program in lymphocytes (32). Inactivation of the CDH4/NuRD complex in mural models lead to extensive cardiac fibrosis due to de-differentiation of cardiac myocytes (33). In addition, high expression of Mi-2β was demonstrated in regenerating myofibers in mice. Moreover, Mi-2β expression was higher in muscle biopsies of DM patients, of which one had concurrent serum Mi-2β antibodies as well, compared to healthy controls (34,35). It could be hypothesized that the antibodies against Mi-2β are formed during the remodelling process following destruction of cells important for the structural integrity of alveoli, particularly alveolar type II pneumocytes, and release of the NuRD complex. This loss in structural integrity will be compensated by induction of fibrosis. Possibly, antibody Mi-2β could be used as a distinctive and diagnostic biomarker for pulmonary fibrosis with absence of extra thoracic features.

This study was performed with patients whom were all diagnosed by a standardized multidisciplinary discussion in a tertiary ILD centre in the Netherlands. It is the first study to describe prevalence and associations of myositis antibodies in a large cohort of patients with other ILD compared with CTD-ILD and healthy subjects. This retrospective study has some limitations, as selection bias of more severely impaired patients with pulmonary fibrosis is possible in a referral centre. Furthermore, overall prevalence of myositis antibodies in both CTD-ILD and other ILD might be overestimated, as only patients with at least one positive myositis antibody were included for analysis.

The findings of this research raise question why autoantibodies, are present in idiopathic IP, including IPF. It is acknowledged that IPF is the result of chronic activated fibroblast-myofibroblasts after repetitive damage, leading to tissue remodelling and injury of alveolar type II pneumocytes (36,37). The role of inflammation is controversial though, and sometimes described as an epiphenomenon or even co-

driver of disease (38). Increased numbers of auto-antibody producing plasma cells in human fibrotic lung tissue have been described in multiple studies, summarized in (38). Production of autoantibodies comes along with the increased expression of immunity- and inflammation-related genes (39), including many B cell related genes (39,40) and tertiary lymph nodes (38,41) during development of fibrosis. This supports a model in which autoreactive B cells are continuously primed and allowed to differentiate into plasma cells secreting autoantibodies. These may include long-lived autoreactive-plasma cells as well (38), with the ability to survive in the bone narrow and continuously secrete autoantibodies in absence of antigen stimuli. Furthermore, these plasma cells are resistant to immunosuppressive or B-cell depleting therapy (42). Thus, it could be hypothesized that certain autoantibodies, such as Mi-2β, are continuously produced in idiopathic IP and do not diminish after treatment with immunosuppressive therapy, whereas antibodies in active CTD-ILD would be more likely the result of short-lived autoreactive plasma cells which are sensitive for immunosuppressants. To investigate this hypothesis, a prospective study in CTD-ILD and non-CTD-ILD with multiple testing of Mi-2β antibodies before start and during therapy is imperative. Furthermore, a prospective cohort study is needed to confirm whether antibody positive ILD patients do not develop future auto immune features of an underlying CTD, as it is known that an IP can precede two years before clinical manifestations of an associated CTD (3,6).

For future research, it would be relevant to evaluate whether survival rates are less impaired in anti-Mi-2β positive ILD compared to antibody negative ILD. Moreover, it would be interesting to assess whether anti-fibrotic therapy reduces anti-Mi-2β signal during follow-up in ILD. In clinical practice, a novel approach of testing and interpretation of autoantibodies could be implemented. In Ssc related ILD, which is regularly treated by immunosuppressive therapy, the anti-fibrotic drug nintedanib slowed the progression of ILD (43)). Conversely, IPF patients with circulating autoantibodies could possibly be approached as a phenotype, which might be sensitive for the combination of both anti-fibrotic and immunosuppressive drugs.

In conclusion, we demonstrated associations of myositis antibodies including anti-Mi-2β in a large cohort of other ILDs compared to CTD-ILD. Possibly, Mi-2β antibody could be used as a diagnostic biomarker for fibrotic ILD in clinical practice.

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Supplement

Determination of antibodies in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALf) was screened for Mi-2β antibodies by a line-blot assay (EUROLINE Autoimmune Inflammatory Myopathies, EUROIMMUN, Lübeck, Germany), in collaboration with Biognost. Analysis of the immunoblot strips was performed with the EUROLINEScan software (EUROIMMUN, Lübeck, Germany) according to manufacturer's recommendations as described for the EUROLINE Autoimmune Inflammatory Myopathies line blot assay. All patients underwent bronchoalveolar lavages, prior to the date of diagnosis, for diagnostic purposes. Samples were stored at -80 °C until analysis.

After the start of run of the EUROLineScan, 1.5 ml of sample buffer was automatically pipetted on the strips, followed by an incubation time of 5 minutes. Next, 0.75 ml of the sample buffer was extracted from the scan and replaced by 0.75 ml supernatant BALf (dilution 1:1).

In addition, albumin levels in BALf (mg/l) and corresponding baseline serum (g/l) were retrieved. Next, albumin BALf/serum ratio was calculated as a marker for albumin leakage from the serum to the intrapulmonary space. This ratio was used as an indicator of leakage of other blood plasma products, including myositis antibodies, from the serum to the alveoli.

Supplementary table 1	Baseline characteristics of 100 CTD-ILD patients										
Subjects	**All CTD-ILD**	**ASS**	**Sjogren's syndrome**	**RA-ILD**	**Ssc**	**PM/DM**	**IBM**	**SLE**	**Mixed CTD-ILD**	**Other CTD-ILD[a]**	**P[f]**
N	100	31	26	11	10	6	1	1	10	4	
Age	59.1 (11.5)	58.7 (10.2)	57.2 (14.9)	63.0 (5.8)	59.5 (10.9)	59.0 (7.7)	69.0 (-)	68.0 (-)	57.5 (12.7)	62.3 (16.1)	0.879
Sex (m), %	46 (46.0)	14 (45.2)	5 (19.2)	8 (72.7)	7 (70.0)	4 (66.7)	1 (100.0)	1 (100.0)	3 (30.0)	3 (75.0)	0.009
History of smoking, %	56 (56.0)	17 (58.6)	12 (46.2)	9 (81.8)	6 (60.0)	3 (50.0)	1 (100.0)	1 (100.0)	6 (60.0)	1 (25.0)	0.401
Pulmonary function test[b]											
FVC	80.4 (23.2)	77.2 (22.1)	81.3 (24.6)	83.2 (23.4)	80.5 (17.4)	61.6 (27.0)	96.7 (-)	99.0 (-)	87.6 (27.3)	88.3 (23.9)	0.602
FEV1	86.3 (67.9)	77.2 (20.7)	80.4 (24.2)	141.8 (189.7)	83.8 (20.9)	61.4 (20.9)	97.6 (-)	81.0 (-)	83.1 (20.9)	81.1 (23.5)	0.361
DLCO	49.3 (15.0)	44.9 (14.5)	50.0 (13.7)	46.9 (8.8)	46.8 (14.4)	52.9 (24.2)	67.5 (-)	56.0 (-)	57.3 (16.3)	56.3 (22.9)	0.378
Pneumoprotein[c]											
CA 15-3	51.2 (46.8)	47.1 (40.3)	61.9 (60.5)	48.5 (35.3)	51.6 (32.9)	57.0 (30.2)	23.0 (-)	34.0 (-)	48.4 (66.7)	29.0 (29.7)	0.932
CC16	17.2 (46.8)	12.7 (7.6)	14.3 (7.9)	54.1 (129.5)	8.7 (3.4)	15.0 (8.6)	13.0 (-)	19.0 (-)	12.8 (10.0)	8.3 (1.7)	0.371
CCL18	164.7 (93.2)	180.9 (97.5)	141.2 (101.0)	175.5 (76.6)	212.5 (87.7)	132.2 (80.2)	-	217.0 (-)	157.9 (98.5)	108.5 (57.5)	0.459
SP-D	108.7 (175.1)	79.5 (110.3)	158.0 (251.2)	118.5 (134.5)	143.1 (241.8)	45.4 (46.7)	276.0 (-)	19.5 (-)	89.4 (125.0)	14.2 (12.6)	0.599
YKL-40	140.8 (122.0)	140.9 (113.4)	125.5 (107.6)	151.6 (79.2)	165.3 (137.4)	177.1 (251.7)	45.0 (-)	261.9 (-)	125.1 (145.0)	122.8 (68.3)	0.924
HRCT scan[d]											
UIP	11 (11.3)	3 (10.0)	1 (3.8)	5 (45.5)	1 (11.1)	1 (16.7)	-	-	-	-	0.088
Probable UIP	7 (7.2)	-	4 (15.4)	2 (18.2)	1 (11.1)	-	-	-	-	-	0.188
Indeterminate	24 (24.7)	8 (26.7)	8 (30.8)	-	2 (22.2)	-	1 (100.0)	1 (100.0)	3 (33.3)	1 (25.0)	0.040
Alternative	55 (56.7)	19 (63.3)	13 (50.0)	4 (36.4)	5 (55.6)	5 (83.3)	-	-	6 (66.7)	3 (75.0)	0.334
Histopathology[e]											
UIP	-	-	-	-	-	-	-	-	-	-	-
Probable UIP	5 (17.9)	2 (33.3)	2 (16.7)	-	-	-	-	-	1 (25.0)	-	0.505
Indeterminate	5 (17.9)	1 (16.7)	3 (25.0)	-	-	-	-	-	-	1 (33.3)	0.470
Alternative	18 (64.3)	3 (50.0)	7 (58.3)	-	3 (100.0)	-	-	-	3 (75.0)	2 (66.7)	0.468

Data are expressed as mean and standard deviation or numbers and percentage within the diagnosis group.
CTD-ILD = connective tissue disease related interstitial lung disease; ASS: antisynthetase syndrome; RA-ILD; rheumatoid arthritis associated interstitial lung disease; Ssc; systemic sclerosis; PM/DM; polymyositis/dermatomyositis; IBM; inclusion body myositis; SLE; systemic lupus erythematosus.
[a] Other CTD-ILD: IgG4 related disease (n = 3), Bechterew's disease (n = 1)
FVC = forced vital capacity; FEV1 = forced expiratory volume in 1 second; DLCO = diffusing capacity of the lung for carbon monoxide; CA 15-3 = cancer antigen 15-3; CCL18 = CC chemokine ligand 18; CC16 = Clara cell secretory protein; SP-D = surfactant protein D; YKL-40 = chitinase-3-like protein 1; HRCT = high resolution computed tomography; UIP = usual interstitial pneumonia
[b] n = 97, data expressed mean and standard deviation in percentage of predicted
[c] n = 99, data expressed as mean and standard deviation in kU/l (CA 15-3) or ng/ml (CC16, CCL18, SP-D, YKL-40)
[d] n = 97, data expressed as numbers and percentage
[e] n = 28, data expressed as numbers and percentage
[f] $p < 0.05$, differences between the CTD-ILD subgroups are calculated by a one way ANOVA for continuous variables (post-hoc Bonferroni test) or Chi-Square test/ Fisher's Exact Test for dichotomous variables

Supplementary table 2 Baseline characteristics of 294 ILD patients without established CTD (non-CTD-ILD)								
Subjects	**All Non-CTD-ILD**	**IPF**	**Unclassifiable IIP**	**HP**	**NSIP**	**COP**	**Other ILD[a]**	**P[f]**
N	294	68	106	53	31	11	25	
Age	65.1 (10.7)	67.4 (9.9)	66.3 (10.1)	64.0 (8.5)	62.0 (9.6)	67.0 (14.4)	59.2 (15.4)	0.006
Sex (m), %	196 (66.7)	55 (80.9)	69 (65.1)	34 (64.2)	17 (54.8)	4 (36.4)	17 (68.0)	0.022
History of smoking, %	212 (72.4)	58 (85.3)	76 (71.1)	35 (66.0)	17 (54.8)	4 (36.4)	22 (88.0)	0.001
Pulmonary function test[b]								
FVC	81.2 (19.0)	80.1 (15.6)	78.7 (19.3)	80.7 (20.0)	84.2 (23.3)	97.0 (17.7)	85.3 (15.4)	0.042
FEV1	82.3 (19.8)	84.1 (15.6)	81.0 (18.9)	78.8 (22.6)	83.4 (27.9)	90.6 (19.1)	85.3 (16.5)	0.392
DLCO	46.2 (16.5)	41.6 (13.4)	45.0 (15.9)	46.6 (19.3)	44.6 (11.1)	70.3 (14.0)	52.8 (16.2)	<0.001
Pneumoprotein[c]								
CA 15-3	69.8 (68.9)	78.3 (68.8)	66.2 (40.7)	95.1 (120.2)	57.2 (40.5)	27.3 (13.6)	41.7 (24.3)	0.005
CC16	21.2 (15.1)	25.3 (14.8)	22.0 (15.1)	22.6 (17.9)	15.8 (11.4)	9.8 (5.3)	13.9 (10.5)	0.001
CCL18	177.3 (118.3)	162.1 (69.1)	165.0 (81.2)	194.4 (175.2)	230.4 (180.2)	110.3 (67.5)	209.0 (141.6)	0.033
SP-D	115.1 (200.8)	107.0 (123.5)	94.0 (141.8)	208.9 (373.3)	125.0 (166.3)	24.7 (28.3)	63.0 (94.5)	0.010
YKL-40	151.2 (137.8)	164.8 (169.1)	157.3 (140.1)	122.1 (78.5)	121.9 (105.9)	140.9 (71.3)	183.8 (169.0)	0.350
HRCT scan[d]								
UIP	85 (29.4)	56 (82.4)	19 (17.9)	9 (18.0)	-	-	1 (4.2)	<0.001
Probable UIP	40 (13.8)	6 (8.8)	28 (26.4)	2 (4.0)	2 (6.7)	-	2 (8.3)	<0.001
Indeterminate	51 (17.6)	6 (8.8)	21 (19.8)	8 (16.0)	8 (26.7)	2 (18.2)	6 (25.0)	0.217
Alternative	113 (39.1)	-	38 (35.8)	31 (62.0)	20 (66.7)	9 (81.8)	15 (62.5)	<0.001
Histopathology[e]								
UIP	7 (7.9)	15 (100)	8 (25.8)	1 (4.8)	-	-	-	<0.001
Probable UIP	15 (16.9)	-	6 (19.4)	1 (4.8)	-	-	-	0.062
Indeterminate	43 (48.3)	-	4 (12.9)	4 (19.0)	4 (44.4)	1 (16.7)	2 (28.6)	0.067
Alternative	294	-	13 (41.9)	15 (71.4)	5 (55.6)	5 (83.3)	5 (71.4)	<0.001

Data are expressed as mean and standard deviation or numbers and percentage within the diagnosis group.
ILD = interstitial lung disease; non-CTD-ILD = ILD without established CTD; IPF = idiopathic pulmonary fibrosis; HP = hypersensitivity pneumonitis; Unclassifiable IIP = unclassifiable idiopathic interstitial pneumonia; NSIP = non-specific interstitial pneumonia; COP = cryptogenic organizing pneumonia; FVC = forced vital capacity; FEV1 = forced expiratory volume in 1 second; DLCO = diffusing capacity of the lung for carbon monoxide; CA 15-3 = cancer antigen 15-3; CCL18 = CC chemokine ligand 18; CC16 = Clara cell secretory protein; SP-D = surfactant protein D; YKL-40 = chitinase-3-like protein 1; HRCT = high resolution computed tomography; UIP = usual interstitial pneumonia
[a] Other ILD: desquamative interstitial pneumonia (n = 5), drug induced (n = 5), interstitial pneumonia with autoimmune features (n = 6), pneumoconiosis (n = 4), respiratory bronchiolitis interstitial pneumonia (n = 3) , sarcoidosis (n = 2).
[b] n = 282, data expressed mean and standard deviation in percentage of predicted
[c] n = 275, data expressed as mean and standard deviation in kU/l (CA 15-3) or ng/ml (CC16, CCL18, SP-D, YKL-40)
[d] n = 289, data expressed as numbers and percentage
[e] n = 89, data expressed as numbers and percentage
[f] $p < 0.05$, differences between other ILD subgroups are calculated by a one way ANOVA for continuous variables (post-hoc Bonferroni test) or Chi-Square test/Fisher's Exact Test for dichotomous variables

Supplementary table 3			Frequency of myositis antibodies in CTD-ILD patients								
Antibody	**N (%)**										
	CTD-ILD	**ASS**	**Sjogren's syndrome**	**RA-ILD**	**Ssc**	**PM/DM**	**IBM**	**SLE**	**Mixed CTD-ILD**	**Other CTD-ILD** [a]	**P**[b]
N	100	31	26	11	10	6	1	1	10	4	
MSA											
EJ	5 (5.0)	1 (3.2)	1 (3.8)	-	-	1 (16.7)	-	-	1 (10.0)	-	0.233
Jo-1	27 (27.0)	21 (67.7)	-	2 (18.2)	-	1 (16.7)	-	-	3 (30.0)	-	<0.001
MDA5	2 (2.0)	-	-	-	-	2 (33.3)	-	-	-	-	0.577
Mi-2α	1 (1.0)	-	-	-	-	-	-	-	1 (10.0)	-	0.789
Mi-2β	7 (7.0)	1 (3.2)	1 (3.8)	3 (2,3)	-	1 (16.7)	-	-	-	1 (25.0)	0.224
NXP2	4 (4.0)	-	1 (3.8)	-	-	-	1 (100.0)	1 (100.0)	1 (10.0)	-	0.183
OJ	1 (1.0)	-	-	-	-	-	-	-	1 (10.0)	-	0.789
PL-12	11 (11.0)	3 (9.7)	6 (23.1)	-	1 (10.0)	-	-	-	1 (10.0)	-	0.386
PL-7	5 (5.0)	3 (9.7)	2 (7.7)	-	-	-	-	-	-	-	0.660
SAE1	2 (2.0)	-	-	-	-	-	-	-	-	-	0.194
SRP	2 (2.0)	-	-	-	-	-	-	-	2 (20.0)	-	0.135
TIF1-γ	1 (1.0)	-	1 (3.8)	-	-	-	-	-	-	-	0.823
MAA											
Ku	1 (1.0)	-	-	1 (9.1)	-	-	-	-	-	-	0.969
PM/Scl 100	13 (13.0)	1 (3.2)	-	2 (18.2)	4 (40.0)	1 (16.7)	-	1 (100.0)	3 (30.0)	1 (25.0)	0.004
PM/Scl 75	13 (13.0)	2 (6.5)	-	-	6 (60.0)	1 (16.7)	-	-	3 (30.0)	1 (25.0)	0.023
Ro52	67 (67.0)	26 (83.9)	24 (92.3)	5 (45.5)	4 (40.0)	3 (50.0)	-	-	4 (40.0)	1 (25.0)	0.004

Data are expressed as numbers and percentage of positive antibodies within each ILD diagnosis group. Weak positive antibodies are excluded.
CTD = connective tissue disease; ILD = interstitial lung disease; ASS: antisynthetase syndrome; RA-ILD; rheumatoid arthritis associated interstitial lung disease; Ssc; systemic sclerosis; PM/DM; polymyositis/dermatomyositis; IBM; inclusion body myositis; SLE; systemic lupus erythematosus; MSA = myositis specific antibodies; MAA = myositis associated antibodies
[a] Other CTD-ILD: IgG4 related disease (n = 3), Bechterew's disease (n = 1)
[b] $p < 0.05$, difference between CTD-ILD patients calculated by Chi-Square test/ Fisher's Exact Test.

Supplementary table 4	Frequency of myositis antibodies in patients with non-CTD-ILD							
Antibody	**N (%)**							
	All non-CTD-ILD	**IPF**	**Unclassifiable IIP**	**HP**	**NSIP**	**COP**	**Other ILD[a]**	**P[b]**
N	294	68	106	53	31	11	25	
MSA								
EJ	5 (1.7)	4 (5.9)	1 (0.9)	-	-	-	-	0.089
Jo-1	16 (5.4)	6 (8.8)	3 (2.8)	4 (7.5)	-	2 (18.2)	1 (4.0)	0.277
MDA5	6 (2.0)	-	4 (3.8)	1 (1.9)	1 (3.2)	-	-	0.251
Mi-2α	5 (1.7)	1 (1.5)	4 (3.8)	-	-	-	-	0.138
Mi-2β	61 (20.7)	18 (26.5)	19 (17.9)	14 (26.4)	3 (9.7)	4 (36.4)	-	0.012
NXP2	3 (1.0)	1 (1.5)	1 (0.9)	1 (1.9)	-	-	-	0.604
OJ	6 (2.0)	1 (1.5)	1 (0.9)	2 (3.8)	-	1 (9.1)	1 (4.0)	0.525
PL-12	10 (3.4)	1 (1.5)	2 (1.9)	3 (5.7)	3 (9.7)	-	1 (4.0)	0.450
PL-7	19 (6.5)	5 (7.4)	5 (4.7)	6 (11.3)	3 (9.7)	-	-	0.177
SAE1	14 (4.8)	8 (11.8)	3 (2.8)	3 (5.7)	-	-	-	0.039
SRP	27 (9.2)	8 (11.8)	9 (8.5)	1 (1.9)	4 (12.9)	1 (9.1)	4 (16.0)	0.304
TIF1-γ	23 (7.8)	1 (1.5)	15 (14.2)	4 (7.5)	1 (3.2)	2 (18.2)	-	0.039
MAA								
Ku	19 (5.4)	6 (8.8)	8 (7.5)	-	2 (6.5)	-	3 (4.0)	0.116
PM/Scl 100	20 (6.5)	5 (7.4)	8 (7.5)	6 (11.3)	-	-	1 (4.0)	0.139
PM/Scl 75	33 (11.2)	7 (10.3)	9 (8.2)	7 (13.2)	3 (9.7)	1 (9.1)	6 (24.0)	0.531
Ro52	75 (25.5)	10 (14.7)	31 (29.2)	8 (15.1)	17 (54.8)	3 (27.3)	6 (24.0)	<0.001

Data are expressed as numbers and percentage of positive antibodies within each ILD diagnosis group. Weak positive antibodies are excluded. CTD = connective tissue disease; ILD = interstitial lung disease; non-CTD-ILD = ILD without established CTD; IPF = idiopathic pulmonary fibrosis; HP = hypersensitivity pneumonitis; Unclassifiable IIP = unclassifiable idiopathic interstitial pneumonia; NSIP = non-specific interstitial pneumonia; COP = cryptogenic organizing pneumonia; MSA = myositis specific antibodies; MAA = myositis associated antibodies

[a] Other ILD: desquamative interstitial pneumonia (n = 5), drug induced (n = 5), interstitial pneumonia with autoimmune features (n = 6), pneumoconiosis (n = 4), respiratory bronchiolitis interstitial pneumonia (n = 3) , sarcoidosis (n = 2).

[b] $p < 0.05$, differences between other ILD patients calculated by Chi-Square test/ Fisher's Exact Test.

Supplementary table 5	Associations of myositis antibodies with CTD-ILD and IPF patients								
Antibody	**CTD-ILD (n = 100)**			**IPF (n = 68)**					
	Number Neg	Number Weak pos	Number Pos	Number Neg	Number Weak pos	Number Pos	OR p OR wp[a]	95% CI[b]	p[c]
EJ	95	-	5	64	-	4	0.84	0.22-3.26	0.803
Jo-1	72	1	27	60	2	6	3.75	1.45-9.68	0.006
							0.42	0.04-4.71	0.479
Ku	98	1	1	58	4	6	0.10	0.01-0.84	0.034
							0.15	0.02-1.36	0.091
MDA5	97	1	2	63	5	-	-		
							0.13	0.02-1.14	0.065
Mi-2α	99	-	1	65	2	1	0.66	0.04-10.68	0.768
							-		
Mi-2β	91	2	7	45	5	18	0.19	0.08-0.49	0.001
							0.20	0.04-1.06	0.058
NXP2	95	1	4	64	3	1	2.70	0.29-24.67	0.380
							0.23	0.02-2.21	0.200
OJ	99	-	1	65	2	1	0.66	0.04-10.7	0.768
							-		
PL-12	89	-	11	66	1	1	8.16	1.03-64.76	0.047
							-		
PL-7	95	-	5	63	-	5	0.66	0.18-2.39	0.529
							-		
PM/Scl 100	87	-	13	62	1	5	1.85	0.63-5.47	0.264
							-		
PM/Scl 75	86	1	13	59	2	7	1.27	0.48-3.38	0.627
							0.39	0.03-3.87	0.387
Ro52	31	2	67	55	3	10	11.89	5.36-26.38	<0.001
							1.18	0.19-7.47	0.858
SAE1	98	2	-	58	2	8	-		
							0.59	0.08-4.32	0.605
SRP	90	8	2	48	12	8	0.13	0.03-0.65	0.013
							0.36	0.14-0.93	0.035
TIF1-γ	96	3	1	65	2	1	0.68	0.04-11.0	0.784
							1.02	0.17-6.25	0.987

CTD = connective tissue disease; IPF = idiopathic pulmonary fibrosis

[a] OR: odds ratio for positive level (OR p); odds ratio for weakly positive level (OR wp).

[b] 95% confidence interval of odds ratio's

[c] Logistic regression analysis of CTD-ILD versus IPF patients with positive, weakly positive and negative antibody, with predicted probability for CTD-ILD.

7

Summary and general discussion

Abbreviation list

BALf = Bronchoalveolar lavage fluid
CA 15-3 = Cancer antigen 15-3
CCL18 = CC-chemokine ligand 18
CTD = Connective tissue disease
DLCO = Diffusing capacity of the lung for carbon monoxide
HP = Hypersensitivity pneumonitis
HRCT = High-resolution computed tomography
IFNγ = interferon γ
IIP = Idiopathic interstitial pneumonia
ILD = Interstitial lung disease
IPAF = Interstitial pneumonia with autoimmune features
IPF = Idiopathic pulmonary fibrosis
KL-6 = Krebs von den Lungen
PBMCs = peripheral blood mononuclear cells
PFT = Pulmonary function test
SNP = single nucleotide polymorphism
UIP = Usual interstitial pneumonia

Summary

In this thesis, the potential of serological lung biomarkers was evaluated for diagnosis, treatment response and prognosis in patients with various fibrosing interstitial lung diseases (ILDs).

In **chapter two**, serum cancer antigen 15-3 (CA 15-3) was assessed as a biomarker for treatment response and prognosis in 48 patients with fibrotic and non-fibrotic hypersensitivity pneumonitis (HP) treated with immunosuppressive agents, namely prednisone or cyclophosphamide. The study showed that CA 15-3 levels decreased during follow-up in both the prednisone-treated and cyclophosphamide-treated HP cohorts. Further analysis revealed that CA 15-3 levels were inversely associated with pulmonary function test (PFT) outcomes, particularly in patients with a fibrosing form of HP. One of the most interesting findings was that early CA 15-3 decreases could predict future PFT improvement. Furthermore, HP patients who demonstrated extensive declines in CA 15-3 levels during their treatment had a longer survival compared to those with stable or increasing serum levels. These results suggest that serial CA 15-3 measurement can be useful as a predictive biomarker for treatment response and prognosis in both fibrotic and non-fibrotic HP patients.

As the value of CA 15-3 as a follow-up biomarker in idiopathic pulmonary fibrosis (IPF) is unknown, a similar analysis was performed in a large cohort of 132 IPF patients treated with anti-fibrotic drugs and described in **chapter three**. Evaluation of serial CA 15-3 measurements during one year treatment with pirfenidone or nintedanib demonstrated that serum CA 15-3 levels were inversely associated with PFT outcomes in both treatment groups. Furthermore, increased serum levels above 58.5 kU/l and 50.5 kU/l prior to start of therapy and during follow-up respectively were predictive for poor survival rates. These predictive findings were independent of the type of anti-fibrotic drug that was administered to IPF patients. This study shows that serum CA 15-3 can be used as a biomarker for treatment response and prognosis in IPF as well. In addition, serial measurements of CA 15-3 might enable more accurate i.e. earlier recognition of poor survival in IPF patients.

In **chapter four**, the influence of genetic variations in the *CC-chemokine ligand 18* (*CCL18)* gene on serum CCL18 levels and mortality was evaluated in 77 IPF patients and 349 healthy controls. Analysis showed that increased serum CCL18 levels were found in IPF patients. In both IPF and healthy controls, serum CCL18 levels were influenced by the same single nucleotide polymorphism (SNP) (*rs2015086* C>T genotype) in the *CCL18* gene, with the highest levels for those carrying the C-allele. The SNP demonstrated differences in *CCL18* mRNA expression as well in peripheral blood mononuclear cells (PBMCs) from healthy controls. Furthermore, mortality was highest in IPF patients with both the C-allele and high serum CCL18 levels compared to other genotypes and/or lower serum levels. The results show that genetic variability in the *CCL18*-gene accounts for differences in *CCL18* mRNA-expression and serum CCL18 levels and influences survival outcomes in IPF, emphasizing its value as a prognostic biomarker in patients with IPF.

Chapters five and **six** describe prevalence and associations of myositis antibodies in various ILDs, as the clinical relevance of these antibodies is unclear in ILDs without associated connective tissue disease (CTD), including idiopathic interstitial pneumonias (IIPs). In **chapter five**, four novel myositis antibodies were evaluated in 1194 patients with various fibrotic ILDs and included antibodies specific for Ks, Ha, Zoα, and cN1A. Patients were screened for presence of these antibodies using a line-blot assay on serum collected at time of diagnosis. Overall, a high prevalence of the concerning antibodies was found in ILDs compared to healthy controls. Antibodies were found in both CTD-ILD and other ILDs, including HP and IPF, but prevalence was highest in patients with radiological and/or histological characteristics other than usual interstitial pneumonia (UIP). Furthermore, presence of anti-Zoα was significantly more prevalent in CTD-ILDs compared to other ILDs, particularly IPF. The results contribute to the awareness that myositis antibodies can be present in ILD without established CTD during screening in the diagnostic work-up. Possibly, these antibodies should be added to the conventional myositis line-blot for diagnostic purposes.

Following the previous study, in **chapter six** prevalence and clinical associations of myositis antibodies of the conventional myositis line-blot in a large cohort of various ILDs was studied. Overall, myositis antibodies were present in both CTD-ILDs and other ILDs, measured in serum collected at time of diagnosis. Anti-Ro52 and several anti-synthetase antibodies, including anti-Jo-1, showed strong associations with CTD-ILDs. Interestingly, antibodies specific for Mi-2β were not primarily associated with CTD-ILDs, but highly associated with other fibrotic ILDs, including IPF and HP. Further analysis revealed a strong association between the presence of serum anti-Mi-2β and a histologically pattern of UIP. To better understand the clinical relevance of these findings, a subset of ILD patients was screened for presence of antibodies specific for Mi-2β in bronchoalveolar lavage fluid (BALf). For the first time we were able to detect anti-Mi-2β in BALf of ILD patients. Moreover, we demonstrated a clear association between presence of anti-Mi-2β in BALf and in serum. The results from this study also showed that autoantibodies can be present in ILD without established CTD. Moreover, a novel diagnostic biomarker for fibrotic ILD appears to be identified.

In conclusion, the various studies described in this thesis provide novel evidence on the potential of non-invasive, easily accessible and generalizable serological biomarkers for management of fibrotic ILD. It is possible that some of these markers will be implemented in standard ILD care for a more accurate diagnosis, monitoring of treatment response, and enhance decision making on therapeutic regiments and early prediction of progressive disease.

General discussion

Pathophysiology of blood biomarkers in ILD

As ILDs cover a whole spectrum of diffuse parenchymal lung diseases involving the pulmonary interstitium and present with overlapping clinical characteristics (1–4), it is crucial to identify biomarkers to improve diagnostic classification, monitoring and prediction of outcome of ILD patients (5). In the majority of fibrotic ILDs, epithelial injury and abnormal wound repair of alveolar type II pneumocytes have been recognized as key players in the pathogenesis of pulmonary fibrosis (6,7). It is thought that repetitive damage of the alveolar epithelium occurs in response to known or unknown stimuli, dependent on the underlying type of ILD. Consequently, fibroblast-myofibroblast activation is upregulated, resulting in increased permeability of the alveolar-capillary barrier and overproduction of lung proteins by alveolar type II pneumocytes (6,7).

The lung proteins that have been evaluated in this thesis, also referred to as blood biomarkers, all play a different role in the processes of fibrotic formation in patients with ILD.

Mucins

Mucins are high-molecular-weight glycoproteins, which are produced and secreted by alveolar type II cells, submucosal glands and serous cells. They are encode by MUC-genes, of which 16 have been identified in the lung (8). It is thought that mucins play a role in cell growth and tissue remodelling and reflect the process of tissue damage, fibroblast activity and progression of pulmonary fibrosis (8,9). A well-known mucin is MUC5B, a secreted gel-forming mucin which has been thoroughly investigated in IPF patients (8). Overproduction of MUC5B is associated with gas exchange abnormalities, impaired mucociliary clearance and pulmonary inflammation (8,10). Moreover, binding of transcriptional factors at the binding site of MUC5B promotor induces overexpression of MUC5B but also upregulation of cytokines in T cells during injury and fibrosis (8,11). Expression of MUC5B was found to be fourteen times higher in IPF compared to healthy controls and localized to IPF lesions, including honeycomb cysts (10). Various genetic variations in the *MUC5B*-gene, including MUC5B promotor variant *rs35705950*, are established risk factors for development of IPF (8).

In contrast to MUC5B, the exact role of the transmembrane mucin MUC1 has been less studied in pulmonary fibrosis. MUC1 is expressed at the basal level of epithelial cells, including alveolar type II cells (12). It was originally used as a tumour-associated molecule due to its overexpression and glycosylation in breast cancer, colon cancer and lung cancer (13,14). However, MUC1 overexpression has also been observed at hyperplastic alveolar type II cells and in fibrotic areas of the lung tissue in patients with pulmonary fibrosis, whereas it was not detectable in healthy controls, suggesting a role in fibrotic processes (15). Binding of interferon-γ (IFNγ) and fibrotic interleukin-6 (IL-6) at the single STAT binding site of MUC1 promotor induces overexpression of MUC1 protein, resulting in the release of the extracellular MUC1 domain (MUC-N/KL-6) (16). In vitro and in vivo studies demonstrated that Krebs von den Lungen (KL-6) upregulated the expression of collagen type I and III and induced myofibroblast activation, resulting in the proliferation and apoptosis of lung fibroblasts (17,18). Hence, serum KL-6 has

been proposed as a biomarker for pulmonary fibrotic activity. Elevated serum KL-6 levels were found in various patients with fibrotic ILDs and associated with acute exacerbation and mortality in patients with IPF (19–24). However, it is suggested that in response to MUC1 overexpression in fibrotic areas, not only MUC-N/KL-6 is released, but also the central protein core of MUC1 (MUC1/CA 15-3) (8). Elevated serum CA 15-3 levels are thought to reflect proliferation of type II alveolar pneumocytes, leading to wall damage. As a result, an active process of inflammation and fibrosis is induced, marked by pulmonary function deterioration (25).

Increased levels of serum CA 15-3 had already been demonstrated in patients with various fibrotic ILDs at diagnosis and associated with progression of disease and PFT deterioration (9,26,35,27–34), but its role as a reflection of disease activity during therapy remained unclear. Chapters two and three describe novel findings on the roles of CA 15-3 in patients with fibrotic lung disease. During immunosuppressive and anti-fibrotic therapy in respectively HP and IPF patients, descending serum CA 15-3 levels reflect the restoration of parenchymal damage due to effective treatment, resulting in improvement of PFT and survival rates, whereas increasing serum levels are predictive for clinical impairment and early mortality.

Chemokines

Chemotactic cytokines, also known as chemokines, play a key role in the positioning, trafficking and function of immune cells of the innate- and adaptive immune system, including leucocytes (36,37). During homeostasis, chemokines control the release of immune cells from the bone marrow, guide immune effector cells to sites of inflammation or infection and promote interactions between the innate and adaptive immune system (36,37). Over fifty chemokines have been identified, of which CCL18 is found at high levels in lung tissue and associated with immune-mediated fibrotic processes in ILD patients (37–40).

CCL18 is mainly secreted by alveolar macrophages, which are alternatively activated by Th2 cells in patients with pulmonary fibrosis (40). The release of CCL18 induces an overproduction of collagen by lung fibroblasts through Sp1 signaling and basal Smad3 activity (39,41,42). In response to fibroblast contact and collagen exposure, the production and release of CCL18 increases spontaneously, which suggests that CCL18 production maintains the process of fibrosis (40).

Previous studies have demonstrated that increased CCL18 levels did not distinguish between patients with different fibrosing lung diseases (43), but reflected pulmonary fibrotic activity and were an indicator of progression of disease and mortality, particularly in IPF patients (21,44–48). The results of chapter four demonstrate novel evidence on the role of genetic variability in the *CCL18*-gene, which influences the extent of *CCL18* mRNA-expression and eventually, the level of serum CCL18 that is released by alveolar macrophages in patients with IPF. Thus, differences in survival rates of IPF patients are associated with serum CCL18 levels, but more importantly, also by genetic variabilities in the *CCL18*-gene.

Autoantibodies

The role of inflammation has been recognized in the pathogenesis of CTD-ILDs and granulomatous ILD, but its role in IIPs remains controversial. Particularly for patients with IPF, there is an

ongoing discussion on the role of inflammation in the process of fibrosis. Sometimes, it is described as an epiphenomenon or even co-driver of fibrotic disease (49,50). For many years, IPF was thought to be a pulmonary disease which was primarily inflammatory driven, as histopathological evaluation of lung biopsies and BALf showed high levels of inflammatory cells including macrophages, neutrophils and fibroblasts (51,52). In the past decades, pathological studies have demonstrated that alveolar epithelial damage plays a key role in the fibrotic formation in IPF. In response to repetitive epithelial damage and impaired lung repair, cells of the alveolar epithelium produce mediators of fibroblast migration, activation and differentiation into myofibroblasts, resulting in increased alveolar-capillary barrier permeability and fibrosis (6,7). Although patients with CTD-ILDs and IIPs share several common biomarkers for disease activity and prognosis (53), little is known on the exact role of autoantibodies, including myositis antibodies, in IIP. It is unclear whether autoantibodies have a pathogenic role in the fibrotic processes in IIP or whether they act as a bystander of disease.

In small studies with fibrotic HP and IIPs, a considerable number of patients demonstrated myositis antibodies, including anti-Jo-1, anti-PL-7, anti-PL-12 and anti-Ro52. Antibody positive patients were more likely to show radiological and histopathological characteristics other than UIP patterns (54–58). Despite these evaluations, evidence was still lacking on the clinical relevance of myositis antibodies in ILDs without underlying CTD. The studies in chapters five and six are the first to describe associations of myositis antibodies in a large cohort of fibrotic ILD patients, including IIP. Surprisingly, one of the antibodies, anti-Mi-2β, was not associated with patients with CTD-ILDs, but with pulmonary fibrosis without related CTD, including HP and IPF. The associations found between presence of serum anti-Mi-2β and fibrotic characteristics in lung biopsies, corresponding with a UIP pattern, suggest a relation between the Mi-2β antigen and fibrotic processes. This hypothesis has previously been confirmed in cardiac myocytes, in which inactivation of the Mi-2β antigen lead to extensive cardiac fibrosis due to de-differentiation of cardiac myocytes (59).

The relation between anti-Mi-2β and UIP found in both CTD-ILDs and patients with other ILDs including IIP suggests that Mi-2β could serve as a biomarker for histological phenotyping of ILD. Possibly, Mi-2β antibodies are formed during the remodeling process, following destruction of cells important for the structural integrity of alveoli, particularly alveolar type II pneumocytes. Consequently, this loss in structural integrity will be compensated by induction of fibrosis.

The clinical meaning of presence of autoantibodies in IIP could be interpret in various ways. On the one hand, it is possible that those who were initially diagnosed as IIPs and showed presence of autoantibodies including Mi-2β in our studies, should in retrospect be reclassified as an immunological driven phenotype of ILD, including CTD-ILD. Anti-Mi-2β positive patients, who do not meet established criteria for CTD-ILD, could possibly act as an ILD phenotype, which might benefit from immunosuppressive drugs.

However,it is also possible that autoantibodies, including anti-Mi-2β, are autoreactive and continuously produced in pulmonary fibrosis in absence of a related CTD. Autoantibody producing plasma cells have been identified in human fibrotic lung tissue (60). During fibrogenesis, the production of autoantibodies is accompanied with the expression of inflammation- and immunity-related genes, including tertiary lymph nodes and B cell related genes (60–63). Thus, this espouses a theory in which

autoreactive B-cells are continuously primed and can differentiate into plasma cell secreting autoantibodies, including long-lived autoreactive-plasma cells, which are able to survive in the bone marrow and continuously secrete autoantibodies in absence of antigen stimuli (60). Furthermore, these plasma cells are not sensitive for immunosuppressive or B cell depleting therapy (64). Consequently, one could hypothesize that particular autoantibodies, such as anti-Mi-2β, are continuously produced in patients with IIP and do not diminish after immunosuppressive drugs, whereas autoantibodies in active CTD-ILDs are possibly more the result of short-lived autoreactive plasma cells, which are sensitive for immunosuppressants. Whether anti-Mi-2β positive ILD patients show an alternative prognosis compared to anti-Mi-2β negative ILD requires further study.

Implementation of blood biomarkers in personalized ILD care

Due to the heterogeneous nature of disease course in ILDs, it is still difficult to determine individual prognosis for a patient with various forms of pulmonary fibrosis. Routinely use of blood biomarkers as objective, non-invasive indicators for pathological processes and pharmacological responses to therapeutic interventions is promising for the management of ILD. It is crucial to perform scientific research on the possible values of serological biomarkers to enhance diagnostic criteria of ILD, propose novel therapeutic regiments, monitor course of disease more accurately and recognize early signs of progressive disease.

In general, personalized medicine is aimed on providing the best therapy for a maximal impact with minimal side effects for an individual (65). Potentially, determination of blood biomarkers could support, and ideally replace, clinical, radiological and histopathological findings to improve ILD phenotyping, evaluation of disease activity and therapy response of the individual patients. As a result, clinicians and patients are supported in their shared-decision making to decide what the best, appropriate therapy is for the concerning individual.

In clinical practice, blood biomarkers could act as additional, objective follow-up parameters for ILD patients with a severe condition, who have an increased risk for obtaining inaccurate PFT and/or high-resolution computed tomography (HRCT) values. But above all, blood biomarkers could possibly contribute to the avoidance of invasive diagnostic interventions for an individual, including a BAL and surgical lung biopsies, during the diagnostic work-up.

The possible applications of blood biomarkers evaluated in this manuscript for implementation in personalized ILD practise are proposed in the following paragraphs.

Early predictors of progressive ILD

The strong associations found in HP and IPF patients between increasing CA 15-3 levels and subsequent deterioration of diffusing capacity of the lung for carbon monoxide (DLCO) suggest that serum CA 15-3 could be an early biomarker for increased alveolar-capillary permeability in fibrotic lung disease. Monitoring of serial serum CA 15-3 levels could be implemented in ILD care as a cost-effective and easily derived follow-up biomarker for disease activity during treatment, early recognition and progression of disease in these patients. In addition, serum CA 15-3 could act as an important predictive marker for personal decision making on therapeutic regiments, as early fluctuations of CA 15-3 could contribute to

the determination of effectiveness of anti-fibrotic or immunosuppressive drugs. However, further research is imperative to validate the cut-off values found in our studies, before implementation in HP and IPF care.

In the search of a biomarker to predict prognosis for IPF patients, a great number of studies have focused on proteins in serum and BALF, including CCL18. We are the first to demonstrate that genetic polymorphisms influence serum biomarker levels and disease course in patients with IPF. Therefore, genotyping these patients for the *rs2015086* SNP in the CCL18 gene may add substantial information to the interpretation of serum CCL18 levels with regard to the prediction of the disease course and mortality in IPF.

Changing perspectives on immunological involvement in ILD

The associations found between myositis antibody Mi-2β and fibrotic ILD including IIP, shed a new light on the role of autoantibodies in the process of fibrotic formation in ILD. Possibly, antibody Mi-2β could be implemented in ILD care as a distinctive, novel diagnostic biomarker for pulmonary fibrosis with absence of extra thoracic features. In addition, antibody Mi-2β has a great potential to act as a non-invasive, diagnostic biomarker for fibrotic ILD, which is characterized by a histological UIP pattern. Thereby, autoantibody measurement could contribute to the avoidance of a surgical lung biopsy for histopathological phenotyping of ILD during the diagnostic work-up.

Although interstitial pneumonia with autoimmune features (IPAF) is acknowledged as an ILD phenotype and added to standard ILD classifications (1), it is still difficult to classify these patients, as pulmonary fibrosis may be the first or lone clinical manifestation of an associated CTD (4,5). Consequently, decision-making on treatment with immunosuppressive drugs or anti-fibrotic drugs remains challengeable in patients with pulmonary fibrosis who demonstrate autoantibodies, but not meet established criteria for IPAF or CTD-ILD. Recent results of the SENSCIS trials demonstrated that the anti-fibrotic drug nintedanib successfully slowed progression of disease in patients with Ssc-ILD, an ILD phenotype which has generally been treated with immunosuppressive drugs only (49,66,67). Conversely, is an interesting theory to evaluate whether IPF patients with presence of autoantibodies could be approached as a phenotype, which might benefit from combining anti-fibrotic therapy with B cell targeted therapy, when compared with autoantibody negative IPF patients. Such an analysis will benefit from additional serological parameters to signal immune activation status to determine whether ILD progression and autoantibody detection is paralleled by an ongoing immune response (68,69). As a result, different fibrotic ILD phenotypes could be defined based on presence of certain autoantibodies and contribute to development of personalized therapy schedules.

Future perspectives of blood biomarkers for ILD

ILDs are multifactorial diseases with complex underlying pathological processes, with clinically heterogeneous phenotypes and high inter-individual variability. In the multidisciplinary approach, clinicians strive for accurate and detailed phenotyping of ILD by discussing an individual's clinical, radiological and histopathological characteristics. However, it remains challengeable how to interpret and

incorporate a potential lung biomarker for daily, routine ILD management, as biomarkers have not been implemented in standard ILD care yet (70).

Concerning the investigated serological biomarkers of this thesis, we would propose personalized management schedules for the individual ILD patient. A patient suspected for pulmonary fibrosis should be screened routinely for presence of antibody Mi-2β for phenotyping a fibrotic ILD type, which is characterized by a histological UIP pattern. Thereby, invasive lung biopsies for additional histological classification for diagnosis and therapeutic decision-making could potentially be avoided in antibody positive individuals. Furthermore, we would propose to measure serum biomarker CA 15-3 in all patients classified as HP or IPF prior to start of therapy with respectively immunosuppressive and anti-fibrotic drugs. Serum CA 15-3 sampling should be repeated every three months to evaluate its fluctuations as a reflection of early treatment response and progressive disease. As this biomarker is widely available, serum could be sampled and analysed at nearby hospitals if one's ILD centre is localized at a considerable distance. The trends in CA 15-3 fluctuations during follow-up could support clinicians in evaluating therapy response and early progression of disease. As a result, this supports early decision-making on (dis)continuation of drugs with potential side effects months before an objective follow-up HRCT and/or PFT for disease activity can be obtained.

Future research should explore the function and role of serological biomarkers to enrich our knowledge of pathological processes in fibrotic ILD and contribute to improvement of treatment regiments. Sensitivity and specificity of serological biomarkers for pulmonary fibrosis should be evaluated in large, longitudinal studies with serial serum measurements. In addition, cut-off values of biomarkers should be further explored and validated to enhance (early) decision making on therapeutic regiments. Blood biomarkers as a reflection of histological ILD phenotyping and response to treatment could be further explored with the changing perspectives on the use of anti-fibrotic drugs for non-IPF disease and in the development of future, novel therapies in ILDs. It should be investigated whether ILD patients characterized by presence of certain biomarkers, i.e. antibody Mi-2β, would benefit from combined treatment schedules of anti-fibrotic and immunosuppressive drugs. Subsequent, it should be evaluated whether biomarkers, including CA 15-3, could reflect these combined treatment schedules. Moreover, research should focus on the development of other serological biomarkers and non-serological biomarkers for pulmonary fibrosis. In particular, research on proteomes in ILD is upcoming and recent results on circulating proteomes in IPF for identifying targets for future biomarker development are promising (71).

Ideally, multiparameter models based on large cohorts of ILD patients should be developed by incorporating biomarkers combined with relevant clinical, radiological and histopathological features. These models would be aimed on supporting personalized phenotyping of the individual ILD patient for clinical care. Based on these models, invasive investigations could potentially be avoided for decision-making on diagnosis and treatment.

The value of blood biomarkers as non-invasive and easily inferred parameters of pathological processes and therapeutic responses, which may precede pathological responses only later detected by the currently established clinical, radiological and histopathological parameters, is attractive for future ILD care. Dependent on the clinical ILD phenotype, follow-up HRCTs and/or PFTs could be replaced partly

by serial biomarker measurements for disease monitoring in future ILD care. Possibly, these management schedules are less invasive and cost-effective for ILD patients with a long follow-up of disease, although a detailed cost-effective analysis should be conducted to conform this hypothesis.

Conclusion

In conclusion, we found novel insights on the roles and applications of non-invasive, easily accessible and generalizable diagnostic and predictive serological lung biomarkers for fibrotic ILD management. Serum CA 15-3 was demonstrated to be a predictive follow-up biomarker for early treatment response and progressive disease in patients with HP and IPF and can contribute to earlier recognition of poor survival. Furthermore, we found that genetic variability in the *CCL18*-gene accounts for differences in serum CCL18 levels and influences survival outcomes in IPF, emphasizing its value as a prognostic biomarker in patients with IPF. Lastly, we identified antibody Mi-2β as a novel diagnostic biomarker for fibrotic ILD and could be used as a non-invasive biomarker for histological phenotyping. Potentially, these biomarkers can be implemented in standard ILD care for a more accurate diagnostic work-up, monitoring of treatment response, prediction of course of disease and early signaling of progressive disease for the individual patient.

The results of this manuscript contribute to knowledge on the clinical relevance of blood biomarkers and their value for personalized ILD care.

Future scientific research on blood biomarkers is imperative for further consolidation of the value in ILD practice.

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8

Nederlandse samenvatting

Inzichten in diagnostische en prognostische biomarkers in interstitiële longziekten

Introductie

Interstitiële longziekten (ILD's) zijn een diverse groep van ruim 200 zeldzame longaandoeningen, die gekenmerkt worden door littekenvorming (longfibrose) en/of ontsteking in de longen.

ILD's worden ingedeeld op basis van de onderliggende oorzaak. De belangrijkste categorieën zijn ILD's ten gevolge van bindweefselaandoeningen (connective tissue diseases (CTD), zoals reumatoïde artritis, sclerodermie, myositis), ILD's ten gevolge van granulomateuze (ophopingen van ontstekingsweefsel) ziekten (o.a. sarcoïdose), ILD's gerelateerd aan omgevingsblootstellingen (o.a. medicijnen, hypersensitiviteits pneumonitis (HP)) en als laatste de categorie idiopathische interstitiële pneumonieën (IIP's), waaronder idiopathische longfibrose (IPF) valt. Bij deze categorie is er geen duidelijke aanwijsbare oorzaak.

Voor het stellen van de diagnose, behandeling en voorspelling van het ziektebeloop (prognose), wordt er per patiënt met ILD gekeken naar een combinatie van kenmerken uit de voorgeschiedenis, symptomen en lichamelijk onderzoek, aangevuld door resultaten uit longfunctietesten, bloed analyse, radiologisch onderzoek en cel- of weefselonderzoek. Het blijft echter voor artsen een uitdaging om het ene type ILD van het andere te onderscheiden, omdat patiënten met verschillende ILD's dezelfde klinische eigenschappen kunnen vertonen. Een nauwkeurige classificatie van ILD is van groot belang, omdat behandelschema's en voorspelling van het ziekteverloop afhankelijk zijn van het onderliggende type ILD. Mogelijk kunnen biologische markers, ook wel biomarkers genaamd, zorgen voor een preciezere classificatie en daardoor bijdragen aan gepersonaliseerde zorg voor elke patiënt met ILD.

Biomarkers zijn meetbare stoffen die bepaalde processen in het lichaam weerspiegelen. Ze meten namelijk niet de ziekte of het ziekteproces zelf, maar functioneren als een substituut daarvan. In patiënten met longfibrose is aangetoond dat de concentratie van zulke stoffen in het bloed, waaronder longeiwitten en auto-antilichamen, verhoogd is ten opzichte van gezonde mensen. Ook zijn er tussen de type ILD's verschillen in concentraties in het bloed van zulke biomarkers. Omdat biomarkers nog niet standaard gebruikt worden in de zorg voor ILD-patiënten en in feite substituut metingen zijn die de ziekte(processen) weerspiegelen, is het belangrijk om de toegevoegde waarde van biomarkers voor de diagnose, behandeling en prognosestelling in ILD's met longfibrose grondiger te onderzoeken. Het gehalte van biomarker in het bloed zou kunnen bijdragen aan het nauwkeuriger classificeren van ILD. Hierdoor zouden ingrijpende diagnostische onderzoeken, zoals een bronchoscopie met lavage of chirurgische longbiopsie, potentieel vermeden kunnen worden. Bovendien zou het routinematig bepalen van biomarkers bij patiënten met ILD kunnen worden gebruikt als een objectieve, niet-ingrijpende surrogaat-test voor behandeleffect, activiteit van ziekte en zelfs kunnen helpen bij het vroegtijdig herkennen van achteruitgang van ziekte.

In dit proefschrift werden de inzichten beschreven van nieuwe, potentieel diagnostische en voorspellende bloed (serum) biomarkers, die onderzocht zijn in patiënten met verschillende fibrotische ILD's.

In **hoofdstuk twee** werd het eiwit cancer antigen 15-3 (CA 15-3) onderzocht als een serum biomarker voor effect van behandeling en prognose bij 48 patiënten met een fibrotische en niet-fibrotische vorm van hypersensitiviteits pneumonitis (HP). Deze patiënten werden behandeld met ontstekingsremmers, namelijk prednison of cyclofosfamide. De studie toonde aan dat serum CA 15-3 spiegels gedurende behandeling afnamen in zowel patiënten die behandeld werden met prednison als patiënten die behandeld werden met cyclofosfamide. Verdere analyse toonde aan dat CA 15-3-spiegels hoger waren bij patiënten met een verminderde longfunctie, vooral bij patiënten met een fibroserende vorm van HP. Een van de meest interessante bevindingen was dat een vroege daling van CA 15-3 spiegels voorspellend bleek voor betere longfunctie uitkomsten in de toekomst. Bovendien leefden HP patiënten met forse spiegel dalingen van serum CA 15-3 tijdens hun behandeling, langer vergeleken met patiënten met stabiele of stijgende serumspiegels. Deze resultaten suggereren dat het herhaaldelijk bepalen van serum CA 15-3 kan dienen als een voorspellende biomarker voor effect van behandeling en prognose bij HP patiënten met zowel fibrotische als niet-fibrotische vorm.

Omdat onbekend was wat de voorspellende waarde is van serum CA 15-3 als biomarker tijdens het ziektebeloop bij idiopathische longfibrose (IPF), werd een vergelijkbare analyse uitgevoerd in een grote studie met 132 IPF patiënten. Deze patiënten werden behandeld met anti-fibrotische geneesmiddelen, namelijk pirfenidon of nintedanib. De resultaten van de studie werden beschreven in **hoofdstuk drie**. Evaluatie van herhaaldelijke CA 15-3-metingen tijdens een behandeling van één jaar met het geneesmiddel pirfenidon of nintedanib toonde aan dat hoge serum-CA 15-3-spiegels gepaard gingen met een verminderde longfunctie in beide behandelingsgroepen. Bovendien hadden patiënten met verhoogde serumspiegels boven 58,5 kU/l (bij start van behandeling) en 50,5 kU/l (gedurende behandeling) een minder lange overleving. Deze voorspellende bevindingen waren onafhankelijk van het type anti-fibrotisch geneesmiddel dat IPF-patiënten gebruikten. Deze studie toont aan dat serum CA 15-3 ook kan worden gebruikt als een monitoring biomarker om het effect van behandeling en prognose bij IPF. Bovendien kunnen herhaaldelijke metingen van CA 15-3 vroegtijdiger een slechtere overleving bij IPF-patiënten herkennen.

Hoofdstuk vier legde zich toe op een andere biomarker, namelijk: CC-chemokine ligand 18 (CCL18). Bij 77 IPF patiënten en 349 gezonde mensen werd de invloed van veranderingen in erfelijk DNA materiaal, namelijk in het gen *CCL18,*, op de hoogte van CCL18 spiegels in het bloed en op overleving onderzocht. De studie liet verhoogde serum CCL18 spiegels bij IPF patiënten zien. In zowel IPF-patiënten als gezonde mensen werd de hoogte van serum CCL18 spiegels beïnvloed door dezelfde type genetische variant, namelijk single nucleotide polymorfisme (SNP) *rs2015086* C>T-genotype in het *CCL18*-gen. Patiënten met het C-allel hadden de hoogste serum CCL18 spiegels. Daarnaast toonde deze genetische variant ook verschillen in CCL18 mRNA expressie en in perifere mononucleaire bloedcellen (PBMC's) van gezonde mensen. Bovendien was de sterfte het hoogst bij IPF-patiënten met zowel het C-allel als verhoogde serum CCL18-spiegels in vergelijking met andere genotypen en/of lagere serumspiegels. De resultaten laten zien dat veranderingen in erfelijk materiaal in het *CCL18* gen zorgt voor verschillen in

CCL18 mRNA expressie, de hoogte van serum CCL18 spiegels en overleving bij IPF. Deze resultaten tonen aan dat serum CCL18 als een prognostische biomarker in patiënten met IPF kan worden gebruikt.

Vervolgens werd onderzocht in hoeverre speciale vormen van antilichamen (namelijk myositis antilichamen) als biomarkers bij verschillende typen ILD's kunnen dienen. Antilichamen zijn stoffen die door het afweersysteem worden gemaakt tegen antigenen, waardoor een afweerreactie wordt opgewekt. Antigenen zijn meestal lichaamsvreemde stoffen, zoals virussen of bacteriën. Antilichamen die gericht zijn tegen stoffen van het eigen lichaam, betreffen auto-antilichamen. **Hoofdstukken vijf** gaf inzicht in het voorkomen en associaties van bepaalde auto-antilichamen, namelijk myositis-antilichamen, in verschillende type ILD's. Het was al bekend dat myositis antilichamen geassocieerd zijn met ILD's gerelateerd aan bindweefselaandoeningen (CTD). Echter is onduidelijk wat de klinische betekenis is van aanwezigheid van myositis antilichamen in patiënten met andere vormen van. In dit hoofdstuk werden daarom vier nieuwe myositis-antilichamen geëvalueerd bij 1194 patiënten met verschillende fibrotische ILD's, namelijk antilichamen specifiek voor Ks, Ha, Zoα en cN1A. Patiënten werden gescreend op de aanwezigheid van deze antilichamen met behulp van een line-blot-test op serum op het moment van diagnosestelling. Deze antilichamen bleken vaker bij patiënten met ILD voor te komen in vergelijking met gezonde mensen. Antilichamen werden gevonden in zowel patiënten met CTD als andere ILD subtypes, waaronder HP en IPF. Het voorkomen was het hoogst bij patiënten met radiologische en/of weefselkenmerken die niet overeenkwamen met een zogenaamd patroon van 'usual interstitial pneumonia' (UIP). Bovendien kwam anti-Zoα vaker voor bij patiënten met CTD vergeleken met andere ILD subtypes, met name IPF. De resultaten dragen bij aan de kennis dat myositis antilichamen aanwezig kunnen zijn in ILD zonder vastgestelde CTD wanneer patiënten hierop gescreend worden in het diagnostisch traject. Mogelijk moeten deze antilichamen voor diagnostische doeleinden worden toegevoegd aan de reguliere myositis-line-blot.

Als vervolg op de vorige studie omschreven in hoofdstuk vijf onderzocht **hoofdstuk zes** het voorkomen en de associaties van myositis-antilichamen van de reguliere myositis line-blot in een groot aantal patiënten met verschillende type ILD's. In zowel patiënten met CTD als patiënten met andere ILD subtypes werden myositis antilichamen aangetoond, gemeten in serum dat afgenomen was op het moment van diagnosestelling. Het voorkomen van anti-Ro52 en verschillende anti-synthetase-antilichamen, waaronder anti-Jo-1, was sterk geassocieerd met CTD. Interessant was dat het voorkomen van antilichamen die specifiek zijn voor Mi-2β (anti- Mi-2β) niet zozeer geassocieerd waren met CTD, maar juist veel voorkwamen in andere fibrotische ILD subtypes, waaronder IPF en HP. Verder onderzoek onthulde een sterke relatie tussen de aanwezigheid van serum anti-Mi-2β en weefselkenmerken die overeenkwamen met een UIP-patroon. Om de klinische betekenis van deze bevindingen beter te begrijpen, werd een subgroep van ILD patiënten gescreend op de aanwezigheid van antilichamen die specifiek zijn voor Mi-2β in vloeistof verkregen bij spoeling van de longblaasjes (bronchoalveolaire lavagevloeistof (BALf)). Voor het eerst werd anti-Mi-2β vastgesteld in BALf van ILD patiënten. Bovendien hebben we een duidelijk verband aangetoond tussen de aanwezigheid van anti-Mi-2β in BALF en in serum. De resultaten van deze studie toonden ook aan dat auto-antilichamen in ILD aanwezig kunnen zijn

zonder vastgestelde CTD. Een potentieel nieuwe diagnostische biomarker voor fibrotische ILD lijkt te zijn geïdentificeerd.

Conclusie

In dit proefschrift zijn de diagnostische en prognostische waarden van een aantal bloed (serum) biomarkers beschreven bij patiënten met fibrotische ILD's. De meest belangrijke, nieuwe inzichten laten zich als volgt samenvatten. Het herhaaldelijk meten van serum CA 15-3 bleek een voorspellende biomarker te zijn voor het vroegtijdig herkennen van effect op behandeling, achteruitgang van ziekte en kans op overlijden bij patiënten met HP en IPF. Daarnaast werd vastgesteld dat genetische variaties in het *CCL18* gen de prognose beïnvloeden, aangezien deze variaties verschillen gaven in de hoogte van serum CCL18 spiegels en overlevingsduur in IPF. Ten slotte werd antilichaam Mi-2β geïdentificeerd als een nieuwe, potentiële diagnostische biomarker voor fibrotische ILD, waarbij dit auto-antilichaam zou kunnen worden gebruikt als een weerspiegeling van het type longfibrose op basis van weefselkarakteristieken.

De resultaten van dit proefschrift dragen bij aan de kennis over de klinische betekenis van serum biomarkers en hebben betekenis in het licht van gepersonaliseerde ILD zorg. Serum biomarkers weerspiegelen namelijk het ziekteproces en het effect van behandeling. Normaliter uiten deze zich mogelijk pas veel later in lichamelijke klachten. Ook zijn ze meestal pas later zichtbaar of aantoonbaar met behulp van beeldvormende- en weefselonderzoek, welke duur en invasief zijn. Daarom zijn biomarkers aantrekkelijk voor toekomstige ILD-zorg. Om serum biomarkers daadwerkelijk in de dagelijkse praktijk te gebruiken, is toekomstig wetenschappelijk onderzoek verder noodzakelijk. Idealiter zouden multiparametermodellen op basis van grote onderzoeksgroepen met ILD patiënten moeten worden ontwikkeld door biomarkers te combineren met relevante klinische, beeldvormende en weefselspecifieke kenmerken. Het inzetten van serum biomarkers draagt hierdoor bij aan gepersonaliseerde zorg, waarbij het type ILD van de patiënt nog beter gekarakteriseerd kan worden, met als doel om een zo goed mogelijke behandeling en prognose vast te stellen per persoon. Hiermee kan per individu afgewogen worden of bepaalde invasieve onderzoeken potentieel vermeden kunnen worden voor het stellen van de diagnose, behandeling en ziekte monitoring.

Concluderend zou het invoeren van serum biomarkers in de dagelijkse praktijk kunnen bijdragen aan een nauwkeuriger diagnostisch onderzoek, monitoring van effect van behandeling, voorspelling van het ziekteverloop en vroegtijdige signalering van achteruitgang ziekte voor de individuele patiënt met ILD.

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List of publications

List of publications

1. van Royen, F. S., **Moll, S. A.**, van Laar, J. M., van Montfrans, J. M., de Jong, P. A., & Mohamed Hoesein, F. A. A. (2019, March 1). Automated CT quantification methods for the assessment of interstitial lung disease in collagen vascular diseases: A systematic review. *European Journal of Radiology*, Vol. 112, pp. 200–206. https://doi.org/10.1016/j.ejrad.2019.01.024

2. **Moll, S. A.**, Wiertz, I. A., Vorselaars, A. D. M., Ruven, H. J. T., van Moorsel, C. H. M., & Grutters, J. C. (2020). Change in Serum Biomarker CA 15-3 as an Early Predictor of Response to Treatment and Survival in Hypersensitivity Pneumonitis. *Lung*, *198*(2), 385–393. https://doi.org/10.1007/s00408-020-00330-9

3. **Moll, S. A.**, Wiertz, I. A., Vorselaars, A. D. M., Zanen, P., Ruven, H. J. T., Van Moorsel, C. H. M., & Grutters, J. C. (2020). Serum biomarker CA 15-3 as predictor of response to antifibrotic treatment and survival in idiopathic pulmonary fibrosis. *Biomarkers in Medicine*, *14*(11), 997–1007. https://doi.org/10.2217/bmm-2020-0165

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5. **Moll, S. A.**, Platenburg, M. G. J. P., Platteel, A. C. M., Vorselaars, A. D. M., Janssen Bonàs, M., Roodenburg-Benschop, C., … Grutters, J. C. (2020). Prevalence of Novel Myositis Autoantibodies in a Large Cohort of Patients with Interstitial Lung Disease. *Journal of Clinical Medicine*, *9*(9), 2944. https://doi.org/10.3390/jcm9092944

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Curriculum vitae

Sofia Ana Moll was born on May 27th 1991 in Voorburg. After finishing A-levels (gymnasium) at St Maartenscollege Voorburg in 2009, she studied one year of courses in Pedagogical Sciences at the Utrecht University, followed by six months of courses in Health Sciences at the VU University. Subsequently, she was accepted at the international University College Utrecht to study a list of courses indicated as the Pre-Medical track. She started her study medicine in September 2011 at the Utrecht University where she received her medical degree in April 2018. During her studies, she followed a public health internship at the Palliative Care Support Trust at Queen Elizabeth Central Hospital in Blantyre, Malawi. Furthermore, she performed research on biomarkers in pulmonary fibrosis at the Interstitial Lung Diseases Centre of Excellence at the St Antonius Hospital in Nieuwegein, followed by a clinical internship in pulmonology in the same hospital. Fascinated by pulmonary fibrosis, she continued working at the St Antonius Hospital after her graduation and started her PhD project on biomarkers in interstitial lung diseases under the supervision of prof. dr. J.C. Grutters, while also working as a clinical physician of pulmonary medicine. Since January 2021, she has been doing her specialist training in pulmonary medicine at the St Antonius Hospital (head dr. F.M.N.H. Schramel).