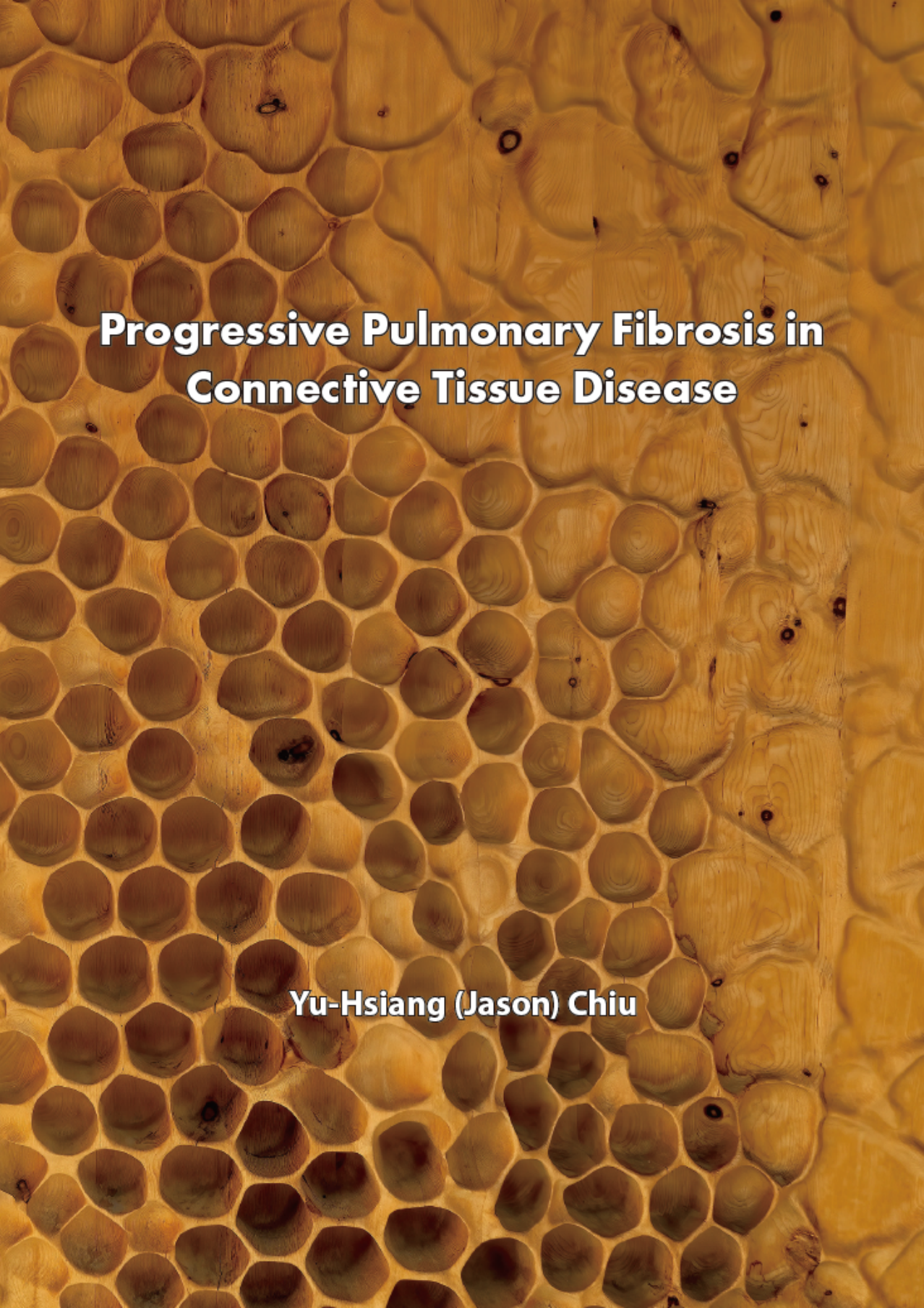
Progressive Pulmonary Fibrosis in Connective Tissue Disease
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(met een samenvatting in het Nederlands)

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Catalogue

Chapter 1
General introduction and thesis outline

1

Connective tissue diseases-associated interstitial lung disease

Connective tissue diseases (CTDs) are a group of systemic autoimmune diseases characterised by inflammation and tissue destruction, of which some develop fibrosis thereafter, including skin, internal organs and joints. (Figure 1) CTD can present heterogeneously, but pulmonary involvement, including airway disease and interstitial lung disease (ILD), can occur in all classifiable CTD [1]. The prevalence of ILD is approximately 5% in rheumatoid arthritis (RA) [2], 40–65% in systemic sclerosis (SSc) [3, 4], 30% in idiopathic inflammatory myopathies (IIM, depending on the subtype and up to 86% in anti-synthetase syndrome) [5-7], 4–13% in systemic lupus erythematosus (SLE) [8, 9], 2.7–36% in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) [10-12], 27% in primary Sjögren's syndrome (pSS; up to 65–90% if screening on non-symptomatic patients [13-15]) and 35–67% in mixed connective tissue disease (MCTD) [16, 17]. CTD-associated ILD (CTD-ILD) is one of the leading causes of morbidity and mortality in CTD patients and impairs patients' quality of life substantially [18].

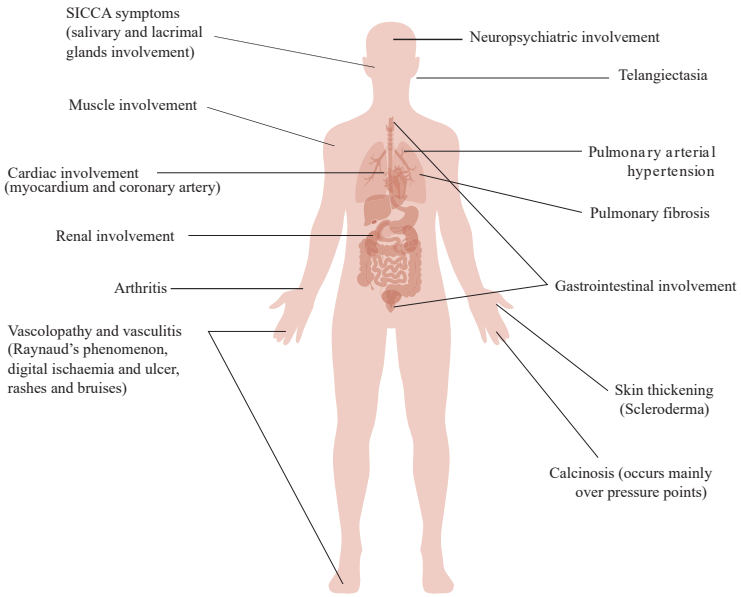

Figure 1. The main disease feature is joint involvement in rheumatoid arthritis, salivary and lacrimal glands in Sjögren's syndrome, muscles in idiopathic inflammatory myopathies and skin in systemic sclerosis. Kidney, central nervous system and blood vessels are commonly affected by systemic lupus erythematosus and anti-neutrophil cytoplasmic antibodies-associated vasculitis. Pulmonary involvement can occur in all connective tissue diseases. Modified from J. Spierings with permission (https://doi.org/10.33540/168).

Pulmonary involvement can occur at any stage of the disease, even before the onset of a CTD-specific manifestation [19, 20]. Initially, patients often have heterogenic nonspecific symptoms, and a timely diagnosis is challenging in CTD-ILD. The most common ILD associated symptoms are dyspnoea, cough and fatigue [21]. Some patients might be asymptomatic, especially pSS patients [13]. Patient report outcome (PRO) tools can be used to assess impact of ILD symptoms on several domains. Respiratory symptoms can be reflected on health-related quality of life evaluation, including Short-form 36 (SF-36), Health Assessment Questionnaire disability index (HAQ-DI) and multi-dimensional HAQ (MDHAQ) and functional class assessment [22-25]. Focusing on breathlessness, Saint George's Respiratory Questionnaire (SGRQ, with a modified version developed for IPF, SGRQ-I), University of California San Diego Shortness of Breath Questionnaire (UCSD-SOBQ), Dyspnoea-12 (D-12), King's Brief Interstitial Lung Disease health status questionnaire (K-BILD), Functional Assessment of Chronic Illness Therapy (FACIT)-Dyspnoea, Mahler Baseline Dyspnoea Index (BDI) and Transition Dyspnoea Index (TDI) have been validated in ILD [26-34].

Extrapulmonary involvement can mimic ILD presentation or influence pulmonary symptoms, i.e., skin tightness, muscle weakness, enthesitis and arthritis at thoracic cage, fatigue, cardiac involvement and pulmonary arterial hypertension. On pulmonary auscultation, bibasilar Velcro-like crackles can be heard in 60–79% of ILD patients [35]. For high risk patients, further evaluation is recommended [25, 36-38]. Nonetheless, more research is needed to optimize risk stratification and guidance on which patients needs additional diagnostic assessments for ILD.

The most informative evaluation modalities are pulmonary function tests (PFT) and high-resolution computed tomography (HRCT). PFT can show reduced lung volume, usually measured by forced vital capacity (FVC), and reduced diffusing capacity, measured by diffusing capacity of carbon monoxide (DLCO). However, the diagnosis of CTD-ILD is mainly based on pulmonary imaging. The gold standard of pulmonary imaging for diagnosing ILD is HRCT [37]. Heterogeneous radiologic patterns can be seen in HRCT, which are associated with prognosis [39].

The American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Asociación Latinoamericana de Tórax (ATS/ERS/JRS/ALAT) defined the diagnostic criteria for these radiological ILD patterns. Since 2018, ATS/ERS/JRS/ALAT categorised HRCT features into usual interstitial pneumonia (UIP) pattern, probable UIP pattern, indeterminate for UIP pattern and alternative diagnosis, which includes patterns such as nonspecific interstitial pneumonia (NSIP), lymphocytic interstitial pneumonia (LIP) and organising pneumonia (OP). The typical HRCT feature of UIP is honeycombing and traction bronchiectasis (Figure 2A); irregular thickening of interlobular septa and reticulation can also be found in UIP [37]. LIP is categorised by variably sized thin-

walled cysts and in most cases also ground-glass opacity (GGO) (Figure 2 B) [36]. The typical radiological NSIP features is bilateral GGO and fine reticulation with mostly sub-pleural sparing, leading in some cases to traction bronchiectasis [40]. NSIP feature can be heterogeneous and can be sub-classified into fibrotic (Figure 2C), cellular (Figure 2D) and mixed NSIP patterns. OP typically shows peripheral or peribronchial patchy consolidations on HRCT (Figure 2E) [40]. More than one feature may be observed on HRCT in a single patient; therefore, the predominant HRCT features can also be concisely classified as fibrotic or inflammatory [41-44]. Features such as reticulation, traction bronchiectasis and honeycombing are fibrotic, whereas ground-glass opacity and consolidation are inflammatory.

In CTD-ILD, NSIP or the combination of NSIP/OP are the most prevalent HRCT patterns, while there is disease variation depending on the type of CTD [7]; UIP is more prevalent in patients with RA, while LIP is mainly seen in patients with pSS (NSIP is the most prevalent pattern in pSS) [42, 45]. Several studies have shown that CTD patients with UIP, mostly in RA, have a poor prognosis, like IPF patients [46-51]. However, how HRCT patterns contribute to prognostication in CTD-ILD remains controversial because of conflicting data [45, 52-55]. Moreover, HRCT assessment relies on experts, and there is considerable interobserver variation on HRCT patterns [56, 57]. Patterns as honeycombing, tree-in-bud nodules and cystic lesions can reach higher agreement, whereas intralobular lines had poor interobserver agreement [57]. Interestingly, the extent of fibrosis and disease trajectory are powerful prognostic determinants in CTD-ILD [36, 58]. An artificial intelligence-assisted quantitative analysis could help to better track disease progression and treatment response in the future [59]. However, more studies need to be performed before we can implement this modality in clinical practice confidently. Furthermore, multidisciplinary meetings remain needed to discuss ILD diagnosis and personalised, tailor-made treatment strategy [36, 37, 60].

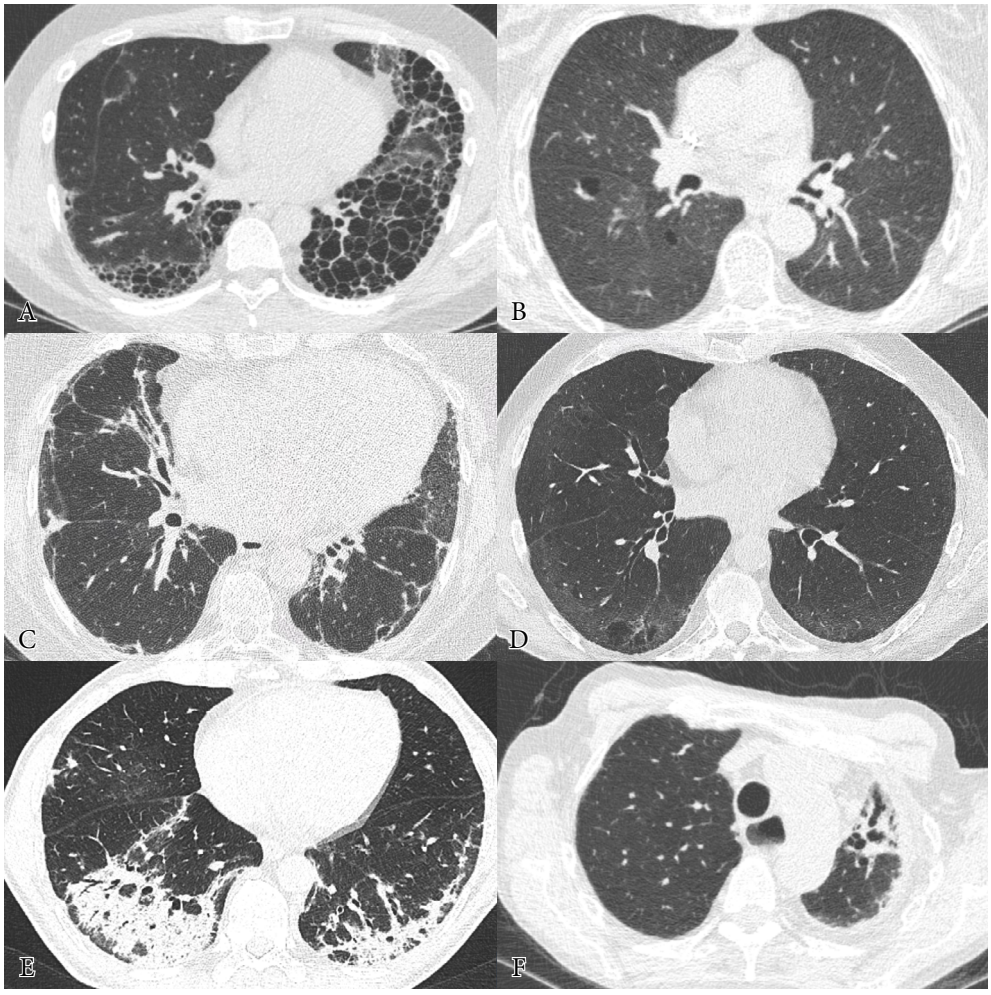

Figure 2. HRCT patterns in usual interstitial pneumonia (UIP, A), lymphocytic interstitial pneumonia (LIP, B), fibrotic nonspecific interstitial pneumonia (fNSIP, C), cellular NSIP (D), organising pneumonia (OP) and Pleuroparenchymal fibroelastosis (PPFE, F). The images were from our patients reported in Chapter 2.

Pathophysiology of CTD-ILD

The pathophysiological causality between CTD and ILD is still not fully understood. The general concept for CTD-ILD is that excessive extracellular matrix accumulates in lung, which is induced by the underlying inflammation, repetitive tissue repair and associated pathophysiology, including autoimmunity, vasculopathy, oxidative stress, cellular senescence and epithelial/endothelial to mesenchymal transition (Figure 3) [61].

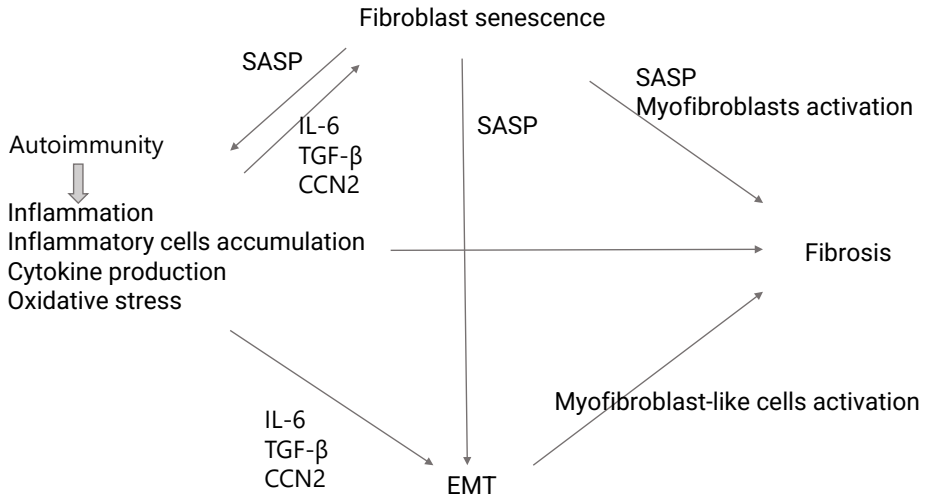

Figure 3. Pathophysiological relation between autoimmunity and fibrosis. Abbreviation: SASP, senescence-associated secretory phenotype; EMT, endothelial/epithelial to mesenchymal transition; CCN2, Cellular Communication Network Factor 2, also known as connective tissue growth factor (CTGF).

The pathogenesis of CTD-ILD is complex and multi-factorial. Fibrosis associated genes, including CCN2 (cellular communication network factor 2, also known as connective tissue growth factor), TGFB (transforming growth factor beta), FBN1 (fibrillin 1), SPARC (secreted protein acid rich in cysteine), KCNA5 (potassium voltage-gated channel subfamily A member 5), uPAR (urokinase-type plasminogen activator receptor), FLI1 (friend leukaemia integration 1), FRA2 (Fos-related antigen 2) and major histocompatibility complex haplotype, abnormalities have been observed in SSc patients and validated inducing a fibrotic phenotype in animal models [62-64]. Several inducible mechanisms have been also utilised in animal models. Bleomycin, monocrotaline, reactive oxygen species and angiotensin II stimulate inflammation, endothelial cells and fibroblasts activation [62, 64, 65]. In a pulmonary arterial hypertension model, induced by vascular endothelial growth factor receptor antagonist (semaxinib) and toll like receptor 7/8 agonist (resiquimod), pulmonary inflammation and fibrosis were also observed [66]. Immunisation of autoantigen observed in clinical patients, including DNA topoisomerase I, Jo-1 and melanoma differentiation-associated protein 5 (MDA5), can also induce murine ILD, while a second-hit stimulation is mostly needed [62, 64, 67-69].

Biomarkers

Due to the complexity and heterogeneity of CTD-ILD, it would be valuable to identify biomarkers to improve diagnosis, monitor disease activity and predict outcomes.

Biomarkers, including analytes (cells, protein, lipid, glycan, micro RNA, extracellular vesicles, etc.), anatomic features and physiological characteristics, indicate biological/pathogenic processes and responses to treatment or disease course [70]. Ideal biomarkers should be non-invasive and accurate. In addition, composite biomarkers may improve clinical relevance and merit further investigation [71]. Blood-based biomarkers associated with pneumocyte and extracellular matrix remodelling, inflammation, oxidative stress, circulating cells and micro-ribonucleic acid have emerged and been investigated in CTD-ILD [71, 72].

Serum non-inflammatory markers have shown an overarching predictive value in identification, severity estimation and prognostication of ILD in CTD patients [71]. For example, selectin-E, endothelin 1, vascular endothelial growth factor (VEGF), intercellular adhesion molecule 1 (ICAM-1 or CD54) and vascular cell adhesion molecule 1 (VCAM-1 or CD106) are vascular markers associated with vasculopathy and endothelial activation. Connective tissue remodelling markers include metalloproteinase family, CCN2, YKL-40 (chitinase-3-like-1) and galectin family that can reflect activity of tissue remodelling in fibrosis. Pulmonary epithelium markers, including Krebs von den Lungen 6 (KL-6) and surfactant protein A and D, indicate turnover and remodelling of pneumocytes. Moreover, other mucin glycoproteins such as CA125 and CA153 have shown correlation with KL-6 [73]. Furthermore, inflammatory markers like cytokines and chemokines may unravel underlying CTD pathogenic pathways and are associated with disease severity and prognosis [71].

Prognosis and progressive pulmonary fibrosis

The disease course of CTD-ILD is heterogeneous. Some patients have subclinical or minimal disease, and the disease course may be chronic but indolent [13, 74]. In contrast, some patients have severe illness which is difficult to control [75, 76]. Furthermore, a part of the CTD-ILD patients develops progressive pulmonary fibrosis (PPF) with increased mortality risk [37, 51]. Therefore, identifying patients at risk for progressive severe disease or PPF is crucial in patient management. Baseline demographic and pulmonary function appear to be overarching prognostic factors in patients with ILD. A clinical prediction model using gender, age and pulmonary function (GAP index) has been developed for predicting mortality in patients with IPF [77, 78]. The GAP index is also applicable in RA-ILD [79]. Moreover, the performance of prognostication improved by combining GAP index and Charlson comorbidity index in patients with CTD-ILD, IPF and other ILD [80]. In addition, a prediction model with smoking history, age and DLCO (SADL model) has been developed for predicting mortality in SSc-ILD [81]. However, despite the baseline risk factors, patients' condition can change during the disease course; a rapid progression in pulmonary function and HRCT, identified as PPF, can occur at any time during the

disease trajectory. The criteria for PPF differ between studies, which complicates study comparison and clinical implication [82, 83]. Table 1 lists the PPF criteria in randomised controlled trials (RCT) and guidelines.

Table 1. Classification criteria for progressive pulmonary fibrosis in randomised controlled trials and guidelines

Trial and time frame	Criteria definition
INBUILD criteria: any of the criteria within two years [51]	≥10% relative decline in FVC
	≥5% and <10 % relative decline in FVC with progressive fibrosis on HRCT or worsening of respiratory symptoms
	Deterioration of both HRCT fibrosis and respiratory symptoms
RELIEF criteria: 6–24 months [84]	≥5% absolute decline in FVC per year
OMERACT 2015 criteria: any of the criteria within one year [1]	≥10% relative decline in FVC
	≥5% and <10 % relative decline in FVC and ≥ 15% relative decline in DLCO
ATS/ERS/JRS/ALAT 2022 criteria: at least two of the criteria within one year [37]	Worsening of respiratory symptoms
	Progression of fibrosis on HRCT: a. Increased extent or severity of traction bronchiectasis and bronchiolectasis b. New ground-glass opacity with traction bronchiectasis c. New fine reticulation d. Increased extent or increased coarseness of reticular abnormality e. New or increased honeycombing f. Increased lobar volume loss
	Lung function deterioration: ≥5% absolute decline in FVC and/or ≥10% absolute decline in DLCO

Abbreviation: FVC, percent predicted value of forced vital capacity; DLCO, percent predicted value of diffusing capacity of carbon monoxide; HRCT, high resolution computed tomography.

Treatments

The general approach to managing patients with CTD-ILD is to control the underlying inflammation, in which immunosuppressive therapy is the cornerstone. The choice of immunosuppressants, including glucocorticoids, conventional disease modifying anti-rheumatic drugs (cDMARD), biologic DMARDs (bDMARDs), targeted synthetic DMARDs (tsDMARDs) and hematopoietic stem cell transplantation (HSCT), depends on the underlying CTD. Intravenous immunoglobulin (IVIG) and plasmapheresis have been utilised in refractory cases, especially in IIM-ILD and lupus pneumonitis [25]. In severe cases with non-reversable pulmonary damage, lung transplantation can be an option of salvage therapy.

Glucocorticoids

Glucocorticoids are a cornerstone of immunosuppressants originated from the endogenous hormone, cortisone, with not only rapid onset anti-inflammatory effect

but also side effects owning to multi-targets [85, 86]. A combination of glucocorticoids and cDMARD has been investigated and revealed some benefits as initial treatment in SSc and IIM. In induction therapy for SSc-ILD, a combination of pulse cyclophosphamide and high dose glucocorticoids (1 mg/kg/day prednisolone for 4 weeks followed by tapering gradually) showed more GGO reduction on HRCT, lung function and symptoms improvement than a combination of pulse cyclophosphamide and low dose glucocorticoids (< 10 mg/day) [87]. A combination of glucocorticoids and cyclosporin revealed better lung function and HRCT improvement and survival than glucocorticoids alone in patients with IIM-ILD [88, 89]. However, the risk and benefit of long-term glucocorticoid use remain elusive in CTD-ILD.

cDMARDs

Most of the robust evidence comes from studies in SSc. The scleroderma lung study I (SLS I) showed oral cyclophosphamide improved patients' pulmonary function and skin thickness in the first year and continued to do so for several months after stopping therapy [90]; however, the effects faded away after two years [91]. Maintenance therapy with oral azathioprine may be an option after discontinuing cyclophosphamide [92]. The SLS II study further indicated that mycophenolate mofetil (MMF) is as effective as cyclophosphamide, with better tolerability and less toxicity [93].

Calcineurin inhibitors (CNI), including cyclosporin, voclosporin and tacrolimus, and m-Tor inhibitors, including sirolimus, are T-cell targeted. In observational studies, a combination of tacrolimus and conventional therapy in IIM-ILD and undifferentiated connective tissue disease (UCTD) associated ILD revealed pulmonary stabilisation and glucocorticoid dose reduction [94, 95]. Tacrolimus also reported comparable effect but a favourable safety profile to MMF in progressive SSc-ILD [96].

Some immunosuppressants have been suspected pulmonary toxicity and concerned for aggravating CTD-ILD [97]. However, the causative relationship between immunosuppressants and CTD-ILD is difficult to established. The underlying CTDs that are treated with immunosuppressants are also associated with ILD. Methotrexate (MTX) is an anchor drug for RA and revealed more evidence for this concern. Recent cohorts and meta-analysis revealed more evidence that MTX use is not associated with onset or progression of ILD in RA patients and may even be beneficial [98, 99].

Biologic and targeted synthetic DMARDs

The reported RCT on b/tsDMARDs was performed in SSc. Tocilizumab stabilised FVC decline and fibrosis on HRCT in patients with SSc but did not show a significant effect on skin thickness [100-102]. Compared with cyclophosphamide, rituximab treated SSc-ILD patients had more FVC and skin improvements [103]. In observational studies, abatacept

showed efficacy and safety in treating patients with RA-ILD [104]. Janus kinase inhibitors (JAKi) interferes with multiple cytokine pathways and may be beneficial in treating CTD-ILD. Add-on tofacitinib with triple therapy (glucocorticoid, cyclophosphamide and CNI) in patients with anti-MDA5^{+} IIM-ILD and rapid progression (reduced PaO2 >10 mmHg or newly-emerging GGO on HRCT within 4 weeks) reduced HRCT progression and serum ferritin levels [105]. There are ongoing RCTs studying efficacy and safety of JAKi in SSc-ILD and RA-ILD (NCT05177471, NCT05246293, NCT04311567).

HSCT

In patients with diffuse cutaneous SSc (dcSSc) patients, three RCTs (ASSIST trial, ASTIS trial and SCOT trial) revealed that autologous HSCT achieved long-term benefits in skin thickness reduction, pulmonary stabilisation and event free survival, however, with an increased risk of upfront treatment related mortality [106-108]. Therefore, selecting patients at high risk of serious disease complications but with preserved cardiac and pulmonary function is crucial to minimise treatment-related complications. In addition, whether early dcSSc patients benefit from autologous HSCT is being investigated in our ongoing RCT (UPSIDE trial) [109].

Antifibrotic therapy

In case of PPF in CTD-ILD patients, add-on of antifibrotic therapy is recommended. The two antifibrotic oral-available small molecules, nintedanib and pirfenidone, have shown benefits in selected patients with CTD-ILD. In 2019, the INBUILD trial repurposed nintedanib for non-IPF ILD, including CTD-ILD, with PPF and showed an FVC decline of -80.8 ml/year in the nintedanib group and -187 ml/year FVC decline in the placebo group [51]. The post-hoc analysis of subgroups revealed that nintedanib reduced annual FVC decline 125.7 ml/year (95%CI 22.5–228.8) in UIP CTD-ILD and 37.5 ml/year (95%CI -108.2–183.2) in non-UIP CTD-ILD [110]. Moreover, in SSc patients without selection of PPF (SENSCIS trial), the FVC decline was -52.4 ml/year in the nintedanib group and -93.3 ml/year in the placebo group [111]. Pirfenidone was studied in RCTs in non-IPF ILD with PPF, RA-ILD and SSc-ILD. Although the three major pirfenidone trials (RELIEF trial, TRAILD1 and SLS III) were unfortunately early terminated due to slow recruitment, pirfenidone also attenuated FVC decline compared to placebo and upfront combination therapy of pirfenidone with MMF had more rapid improvement in FVC than MMF alone [84, 112].

Lung transplant

Lung transplantation can be a lifesaving procedure in severe CTD-ILD. Due to prevailing organ shortage, the recipient candidates should be carefully selected [113]. Patients' recovery and long-term survival associated factors, including disease severity, nutritional

status, degree of frailty, comorbidities (especially multiorgan involvement in CTD), psychosocial circumstances, and health-related behaviours, should be fully evaluated. Early referral of end-stage and/or refractory CTD-ILD patients is recommended as extrapulmonary manifestations may require special consideration [113, 114].

Thesis aims and outline

CTD-ILD is a heterogeneous disease, and a better understanding of prognostic factors, including clinical characteristics and biomarkers, is vital to optimise managing patients with CTD-ILD. This thesis addresses biomarkers and clinical risk factors for PPF and mortality in patients with CTD-ILD and further explores the potential pathophysiology in SSc, the most fibrosis-predominant CTD.

Chapters 2 and **3** describe clinical prognostic factors in CTD-ILD and the validation of PPF criteria. **Chapters 4** and **5** illustrate the identification and clinical application of biomarkers in CTD-ILD. **Chapter 6** summarises the role of autologous HSCT in SSc-ILD, the most notorious CTD in its fibrotic feature. **Chapter 7** presents a bedside-to-bench approach to identify potential pathophysiological mechanisms in fibrosis using skin biopsies from SSc patients. **Chapter 8** summarises the findings of this thesis and discusses the clinical implication and direction of future research.

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Chapter 2

Predictors for progressive fibrosis in patients with connective tissue disease associated interstitial lung diseases

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Abstract

Background

Connective tissue disease associated interstitial lung disease (CTD-ILD) is associated with decreased quality of life and high mortality risk. Outcome and treatment response is unpredictable. This study aimed to identify clinical predictors for CTD-ILD with poor outcome.

Methods

We performed a retrospective single centre cohort study in outpatients with CTD-ILD seen between 2004 and 2018. Clinical and biochemical data, pulmonary function tests (PFT) and high-resolution computed tomography (HRCT) results were analysed. Overall survival and progressive fibrosing ILD (PF-ILD, defined as a significant deterioration of PFT or HRCT) after two years of follow-up were assessed.

Results

In total, 150 patients with CTD-ILD were included. Thirty (20%) deaths occurred during a median follow-up of 40 months (IQR 27.3–60.8), which were attributed to pulmonary infection in six (4%), respiratory failure due to PF-ILD in ten (7%) and due to other causes in fourteen patients. PF-ILD occurred in 76 (50.7%) patients and was associated with poor overall survival (adjusted HR 5.73, 95%CI 1.17–28.11). Age, smoking, C-reactive protein, and steroid-use were independently associated with increased mortality risk as well. Furthermore, patients with diabetes mellitus (adjusted OR 4.52, 95%CI 1.10–18.51), steroid-use (adjusted OR 2.26, 95%CI 1.04–4.93), and a fibrotic HRCT pattern at baseline (adjusted OR 3.11, 95%CI 1.15–8.38) had a higher risk of PF-ILD.

Conclusion

PF-ILD is associated with increased mortality in patients with CTD-ILD. Patients with a fibrotic HRCT pattern at baseline, diabetes mellitus and steroid-use have a higher risk of developing PF-ILD.

Introduction

Interstitial lung disease (ILD) in patients with connective tissue disease (CTD) is a heterogeneous disease which negatively impacts quality of life and is associated with increased mortality [1, 2]. Systemic sclerosis (SSc), rheumatoid arthritis (RA), idiopathic inflammatory myopathy (IIM), primary Sjögren syndrome (PSS), systemic lupus erythematosus (SLE), and mixed CTD (MCTD) are CTDs with distinctive clinical features, yet ILD can occur in each condition. A subset of patients develops progressive fibrosis (PF-ILD), which is characterised by a rapid deterioration in symptoms, decline in lung function and/or progressive fibrosis on high-resolution computed tomography (HRCT) [3-5].

The management of CTD-ILD has improved in the last decades. Multidisciplinary collaboration and routine screening with HRCT and pulmonary function test (PFT) are now widely accepted and implemented [2, 6, 7]. Furthermore, the therapeutic armamentarium has expanded and includes immunosuppressants and more recently, antifibrotic therapy [3, 8, 9]. Moreover, autologous stem cell transplantation can be effective in carefully selected patients with SSc associated ILD [10-12]. Despite the advances in management and treatment, some patients with CTD-ILD still develop PF-ILD. To optimize management of CTD-ILD, predictors for progressive fibrosis early in the disease course are needed. This study aimed to identify clinical predictors for CTD-ILD with poor outcome.

Methods

Design

A retrospective cohort study was performed at the University Medical Centre Utrecht, a tertiary referral hospital for CTDs.

Patients

All patients with an established diagnosis of CTD-ILD who were treated at the outpatient clinics of the Department of Rheumatology and Clinical Immunology and the Department of Pulmonology between 2004 and 2018 with a minimal follow-up of one year were included. All patients met the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for a CTD or fulfilled the proposed criteria for interstitial pneumonia with autoimmune features (IPAF), or ILD with undifferentiated connective tissue disease (UCTD) [13-18]. This study was conducted in accordance with the amended Declaration of Helsinki [19]. The institutional medical ethics committee of UMC Utrecht approved the study (study number 19/148).

Data collection

Demographical, clinical, radiological and pulmonary function data were retrieved from medical records. Data included age, sex, obesity (body mass index ≥ 35), disease duration, and smoking (pack-years). Treatment history and current therapies were registered.

Laboratory results

The following laboratory results were collected from medical records: erythrocyte sedimentation rate, C-reactive protein (CRP), creatine kinase, creatinine, rheumatoid factor, anti-CCP antibody, antineutrophil cytoplasmatic autoantibodies, antinuclear antibody and specific antinuclear antibodies, myositis-specific antibodies, carbohydrate antigen 15.3 (CA15.3) and soluble IL-2 receptor (sIL-2R).

Pulmonary function tests

PFT was performed in standard spirometry according to the American Thoracic Society (ATS) / European Respiratory Society (ERS) recommendations [20, 21]. The diffusing capacity for carbon monoxide (DLCO) was measured using the single-breath method (Masterlab, Jaeger, Wurzburg, Germany). Values were expressed as percentage of predicted. Data at baseline, six months, one year, two years, and last follow up were recorded. A significant and clinically relevant change in PFT was defined as ≥ 10% change in forced vital capacity (FVC) or ≥ 15% change in DLCO within 2 years [22]. A change in FVC of < 10% or DLCO of < 15% within 2 years was defined as stable.

Pulmonary Imaging

HRCTs were evaluated at baseline, one year, and two years follow-up. HRCT patterns were classified according to the classification for idiopathic interstitial pneumonia [23], listing them as consistent with usual interstitial pneumonia (UIP), probable UIP or alternative diagnosis. The consistent UIP and probable UIP were summarised as UIP. The alternative diagnosis category was then classified as non-specific interstitial pneumonia (NSIP), lymphocytic interstitial pneumonia (LIP), desquamative interstitial pneumonia (DIP), nodular lymphocytic hyperplasia (NLH), organising pneumonia (OP) or pleuroparenchymal fibro-elastosis (PPFE). The NSIP pattern was categorised as fibrotic, cellular, or mixed [24]. The predominant HRCT patterns were categorized into fibrotic patterns (UIP, fibrotic NSIP, and PPFE) or inflammatory patterns (cellular and mixed NSIP, LIP, DIP, NLH, and OP) [25, 26]. The percentage of lung involvement and changes of both inflammation and fibrosis on HRCT over time were evaluated independently by two radiologists, which were classified as progression, stable, or regression. Two experienced chest radiologists independently reviewed the HRCTs with blinding to clinical information or pathology diagnosis. In case of discrepancies, a third expert (pulmonologist) was consulted to reach consensus.

Progressive fibrosing interstitial lung disease

PF-ILD was defined when the following occurred: ≥ 10% decline in FVC, ≥ 15% decline in DLCO, and/or progressive fibrotic changes on HRCT (either in the fibrotic or inflammatory predominant group at baseline) within 2 years. The term PF-ILD has recently been popularised and refers to a subset of patients who develop rapid pulmonary function decline with subsequently high mortality rates [27]. However, different criteria for PF-ILD are used in literature. The INBUILD trial (nintedanib) set a two-year period for FVC decline of more than 10%, FVC decline between 5–10% and deterioration of respiratory symptoms, or deterioration of respiratory symptoms and progressive fibrotic changes on HRCT [4, 8]. Meanwhile, the pirfenidone trial used a six months period for a decline in FVC of more than 5% or a significant symptomatic worsening [3], and the RELIEF trial used a period of at least six months with a maximum of 24 months for a decline in FVC of more than 5% [28]. The ILD guideline from the British Thoracic Society in collaboration with the Thoracic Society of Australia and New Zealand and the Irish Thoracic Society also suggested that a decline ≥10% in FVC or ≥15% in DLCO in the first 6–12 months is a mortality risk factor [22]; therefore, we combined these criteria in our study and defined PF-ILD as ≥ 10% decline in FVC, ≥ 15% decline in DLCO, or progressive fibrotic changes on HRCT within two years. Baseline HRCTs were classified as predominant inflammatory or predominant fibrotic pattern. HRCTs may contain both inflammatory and fibrotic components and change in inflammation and fibrosis was evaluated separately during follow-up. Only the progression of fibrosis on HRCT was included in our PF-ILD criteria. Patients with a predominantly inflammatory pattern on HRCT could, however, also show progression of their fibrotic component.

Statistical analysis

Descriptive statistics were used to describe patient characteristics. Survival was analysed using Kaplan-Meier survival analysis, and the difference between groups was examined in a log-rank test. The hazard ratios (HR) of clinical characteristics for death were calculated using Cox regression analysis. Correlations between biomarkers and the progression of PFT and HRCT were examined by logistic regression. Factors with a univariate p-value less than 0.2 were included in the multivariable regression [29]. Normality of the data was assessed with the Shapiro-Wilk test. Categorical variables were presented in frequencies, and the differences between groups were estimated with Fisher's exact test. The difference of continuous variables between groups was determined using the Mann-Whitney U or unpaired T-test test as appropriate. The interobserver agreement in the HRCT scores was tested using Cohen's kappa. The correlation between the variation of serum markers and PFT over-time was evaluated with Spearman's Rho. Missing data were excluded from the individual analysis. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using R 3.5.1.

Results

Patient characteristics

A total of 150 patients with CTD-ILD were identified and included (Table 1). The median follow-up duration was 40 months (IQR 27.3–60.8). In 117 (78%) patients, CTD was diagnosed before ILD onset. In these patients, median disease duration of CTD was fourteen months (IQR 2–73). CTDs included 53 (35%) SSc, 29 (19%) IIM, 24 (16%) RA, 19 (13%) PSS, sixteen (11%) UCTD, five (3%) SLE, and four (3%) MCTD. Immunosuppressants or immunomodulatory treatment for CTD and/or ILD was used in 128 (85%) patients (Table 1). In 72 (48%) patients, two or more immunosuppressants/immunomodulators were combined.

Table 1. Baseline patient characteristics

Characteristic	Patients
Female, n(%)	95 (63.3)
Age (years), median (IQR)	57 (48–68)
Disease Duration of CTD (months), median (IQR)	14 (2–73)
Systemic sclerosis, n(%)	53(35.3)
Sjögren's syndrome, n(%)	19(12.7)
Myositis, n(%)	29(19.3)
Rheumatoid arthritis, n(%)	24(16)
SLE, n(%)	5(3.3)
MCTD, n(%)	4(2,7)
UCTD, n(%)	16(10.7)
Comorbidities, n(%)	
Coronary artery disease	18 (12.0)
Congestive heart failure	15 (10)
Pulmonary hypertension	17 (11.3)
Diabetes mellitus	15 (10.0)
Cerebrovascular event	5 (3.3)
Obesity (BMI ≥ 35)	11 (7.3)
Smoking status, n(%)	
Current	9 (6.0)
Former	69 (46)
Never	71(47.3)

(Continued)

Characteristic	Patients
Immunosuppressants, n(%)	
azathioprine	12 (8)
mycophenolate mofetil	48 (32)
cyclophosphamide	1 (0.7)
rituximab	20 (13.3)
methotrexate	16 (10.7)
cyclosporine	5 (3.3)
tacrolimus	1 (0.7)
leflunomide	4 (2.7)
adalimumab	3 (2)
etanercept	1 (0.7)
infliximab	1 (0.7)
belimumab	1 (0.7)
tocilizumab	1 (0.7)
prednisolone	78 (52)
Immunomodulatory treatment, n(%)	
hydroxychloroquine	22 (14.7)
IVIG	2 (1.3)
HSCT	3 (2)
Fibrotic CT patterns, n(%)	
UIP	12 (8)
Fibrotic NSIP	18 (12)
PPFE	1 (0.7)
OP/UIP	1 (0.7)
LIP/UIP	1 (0.7)
Inflammatory CT patterns, n(%)	
Cellular NSIP	55 (36.7)
Mix NSIP	36 (24)
OP	6 (4)
LIP	6 (4)
NSIP/OP	12 (8)
LIP/NSIP	2 (1.3)
Percentage of lung involvement, median (IQR)	17.5 (10.0–27.5)
Baseline FVC, median (IQR)	80.0 (65.0–94.6)
Baseline DLCO, median (IQR)	54.5 (43.3–66.0)

Abbreviations: CTD, connective tissue disease; MCTD, mixed connective tissue disease; SLE, systemic lupus erythematosus; UCTD, undifferentiated connective tissue disease; IVIG, intravenous immunoglobulin; HSCT, hematopoietic stem-cell transplantation; TNFi, tumor necrosis factor alfa inhibitor; UIP, usual interstitial pneumonia; NSIP, non-specific interstitial pneumonia; PPFE, pleuroparenchymal fibro-elastosis; OP, organising pneumonia; LIP, lymphocytic interstitial pneumonia; FVC, percentage of predicted forced vital capacity; DLCO, percentage of predicted single-breath diffusing capacity for carbon monoxide.

Radiology and pulmonary function test

At baseline, 33 (22%) patients had a predominant fibrotic pattern and 117 (78%) patients a predominant inflammatory pattern (Table 1). Of patients with predominant fibrotic

patterns, eleven (33.3%) had SSc, seven (21.2%) RA, six (18.2%) PSS, five (15.2%) IIM, three (9.1%) UCTD, and one (3.0%) SLE. Of patients with predominant inflammatory patterns, 42 (35.9%) had SSc, 24 (20.5%) IIM, 17 (14.5%) RA, thirteen (11.1%) UCTD, thirteen (11.1%) PSS, four (3.4%) MCTD, and four (3.4%) SLE. No follow-up HRCT was done in 29 patients at one year and in 73 patients at two years of follow-up. Fibrosis on HRCT had progressed in 27 (22%) after one year of follow-up and six more patients progressed at two years. Inflammatory features on HRCT progressed in 32 (26%) patients, stabilised in 49 (41%), and improved in 40 (33%) after one year of follow-up; inflammatory features progressed in fourteen (18%), stabilized in 46 (60%), and improved in 17 (22%) patients at two years follow-up (Figure 1). In 35 (47%) patients who did not develop PF-ILD, no HRCT was available at two years, yet PFT showed no significant decline at last follow-up. Three (4%) patients without PF-ILD did not follow-up HRCT at two years and PFT after two years. The kappa value for HRCT patterns was 0.4 among the radiologists. In addition, the kappa value for the progression of inflammation and fibrosis on HRCT was 0.6 and 0.5, respectively.

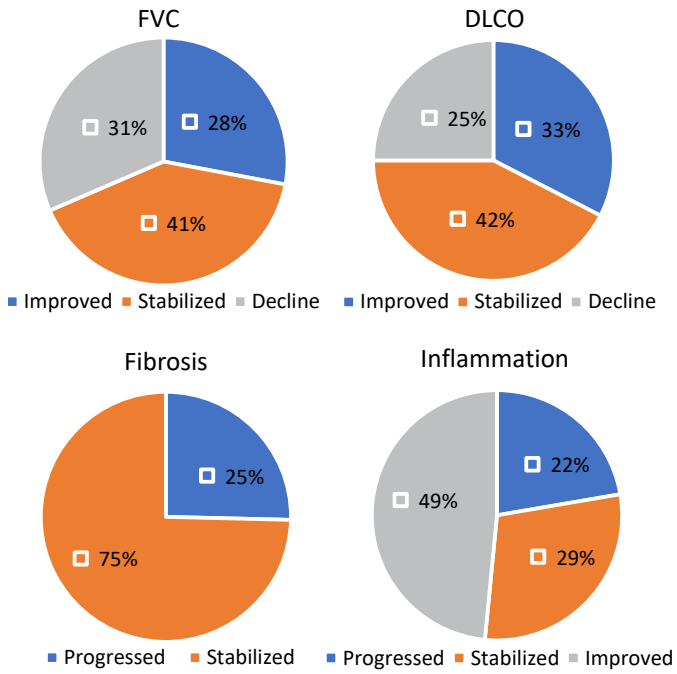

Figure 1. The upper two pie charts illustrate the follow-up pulmonary function test. Fibrosis and inflammation on high-resolution computed tomography at one year and two years follow-up are combined in the lower two pie charts.

Median baseline FVC was 80.0% (IQR 65.0–94.6%) and DLCO 54.5 % (IQR 43.3–66.0%). Baseline FVC and DLCO did not differ between patients with inflammatory and fibrotic HRCT patterns (p=0.051 and 0.629, respectively). After two years of follow-up, FVC improved in 40 (28%), stabilized in 58 (41%), and declined in 45 (31%) patients. DLCO improved in 43 (33%), stabilized in 56 (42%), and declined in 33 (25%) patients after two years (Figure 1). There was no follow-up of FVC in seven patients, and DLCO in 18 patients. PF-ILD occurred in 76 (50.7%) patients. The prevalence of PF-ILD did not differ between CTD, with fifteen (63%) RA, nine (56%) UCTD, 27 (51%) SSc, two (50%) MCTD, thirteen (45%) IIM, eight (42%) PSS, and two (40%) SLE. PF-ILD occurred in 51 (44%) patients with predominant inflammatory HRCT pattern at baseline and 25 (76%) patients with predominant fibrotic HRCT pattern at baseline. A trend of FVC improvement was seen in both the PF-ILD and non-PF-ILD group at six months and one year of follow-up. After two years of follow-up, deterioration of both FVC and DLCO was seen in the PF-ILD group. (Figure 2).

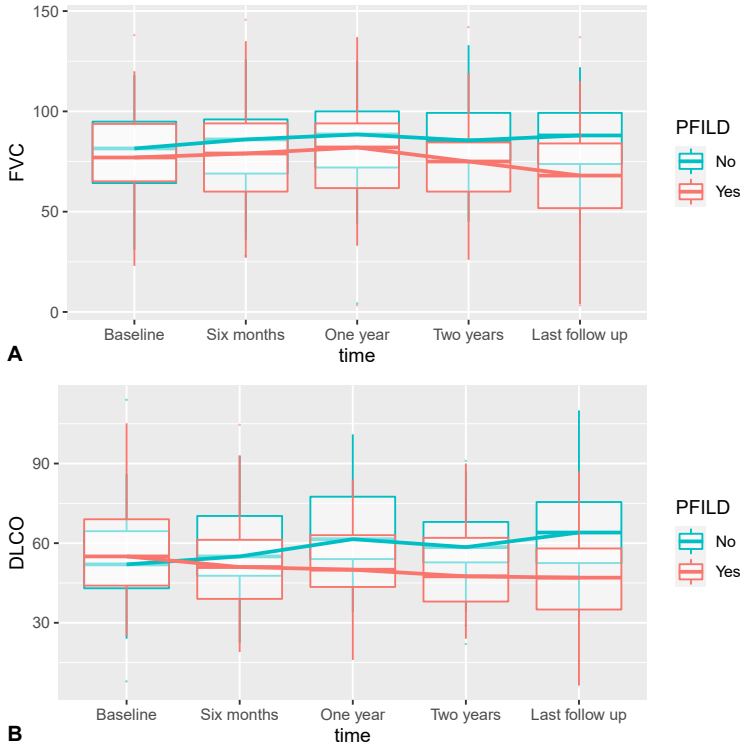

Figure 2. Pulmonary function change over time between patients fulfilled progressive fibrosing interstitial lung diseases (PF-ILD) or not. FVC, percentage of predicted forced vital capacity; DLCO, percentage of predicted single-breath diffusing capacity for carbon monoxide.

Clinical features and the risk of progression

Correlations between clinical factors and change in lung function, HRCT, and occurrence of PF-ILD were examined. Changes in CA15.3 were negatively correlated with change in FVC (Rho -0.308, p= 0.037). Change in CRP was negatively correlated with change in FVC (Rho -0.302, p= 0.006) and DLCO (Rho -0.268, p= 0.019). Patients with inflammatory HRCT patterns at baseline had a lower risk of FVC decline than patients with a fibrotic pattern (adjusted OR 0.24, 95%CI 0.09–0.64). Patients with congestive heart failure had an increased risk of DLCO deterioration at two years follow-up (adjusted OR 27.41, 95%CI 1.79–419.2). Patients with diabetes mellitus (adjusted OR 4.52, 95%CI 1.10–18.51), steroid-use (adjusted OR 2.26, 95%CI 1.04–4.93), and fibrotic HRCT pattern at baseline (adjusted OR 3.11, 95%CI 1.15–8.38) had a higher risk of developing PF-ILD; conversely, patients with obesity (adjusted OR 0.16, 95%CI 0.03–0.85) and positive anti-dsDNA (adjusted OR 0.16, 95%CI 0.03–0.78) revealed a lower risk of developing PF-ILD (Table 2).

Table 2. Predictors of PF-ILD with multivariable adjustment in logistic regression

Risk factors	Crud OR (95%CI)	P	Adjusted OR (95%CI)	p
Coronary artery disease	2.13 (0.78–6.41)	0.155	1.09 (0.31–3.81)	0.898
Congestive heart failure	2.96 (0.96–11.11)	0.075	2.10 (0.50–8.78)	0.308
Pulmonary hypertension	2.59 (0.91–8.51)	0.089	1.47 (0.41–5.25)	0.552
Diabetes mellitus	2.96 (0.96–11.11)	0.075	4.52 (1.10–18.51)	0.036
Obesity	0.34 (0.07–1.23)	0.121	0.16 (0.03–0.85)	0.031
rituximab-use	0.48 (0.17–1.24)	0.138	0.39 (0.12–1.26)	0.115
steroid-use	2.01 (1.06–3.89)	0.035	2.26 (1.04–4.93)	0.040
Fibrotic HRCT pattern	4.04 (1.75–10.27)	0.002	3.11 (1.15–8.38)	0.025
Anti-Ro	0.61 (0.31–1.18)	0.144	0.61 (0.28–1.33)	0.217
Anti-CCP	2.56 (0.96–7.61)	0.070	1.45 (0.46–4.58)	0.522
Anti-dsDNA	0.30 (0.06–1.04)	0.078	0.16 (0.03–0.78)	0.024
Anti-Scl-PM	0.40 (0.10–1.30)	0.144	0.64 (0.16–2.48)	0.517

OR, odds ratio; 95%CI, 95% confidence interval; HRCT, high-resolution computed tomography.

Survival analysis

During follow-up, 30 (20%) patients died. Eighteen patients died due to respiratory failure caused by PF-ILD (n=10, 7%), pulmonary infection (n=6, 4%), pulmonary hypertension (n=1, 0.7%), and pulmonary flare of lupus (n=1, 0.7%). After two years of follow-up, patients with improved FVC and DLCO demonstrated better survival; PF-ILD was associated with poor prognosis (Figure 3). There was no significant difference between the inflammatory HRCT at baseline with PF-ILD and the fibrotic HRCT at baseline with PF-ILD, HR 0.908 (95% CI 0.40–2.05, p= 0.817). The difference in mortality risk between patients with PF-ILD, baseline predominant inflammatory HRCT patterns and no progression of fibrosis on follow-up HRCT (n = 26) and patients with PF-ILD

and progression of fibrosis on follow-up HRCT (n = 31) was also insignificant, HR 1.26 (95% CI 0.51–3.11, p= 0.617). A UIP pattern at baseline showed an insignificant increased mortality risk, HR 1.227 (95% CI 0.45–3.34, p=0.689). The risk of FVC decline, DLCO decline or progressive fibrosis on HRCT were all insignificant too (Table S1). Age (adjusted HR 1.08, 95%CI 1.02–1.14, p= 0.009), smoking (adjusted HR 7.01, 95%CI 1.99–24.68, p= 0.002), steroid-use (adjusted HR 5.11, 95%CI 1.01–25.92, p= 0.049), CRP (adjusted HR 1.01, 95%CI 1.00–1.02, p= 0.022), and PF-ILD (adjusted HR 5.73, 95%CI 1.17–28.11, p= 0.031) were associated with increased mortality risk (Table 3). No dose-effect of smoking on mortality risk was observed. There was a dose-response effect of steroid use with a 10-year mortality rate of 2.4% (n=41) for low dose (≤7.5 mg/day), 13.9% (n=36) for medium dose (>7.5 and ≤30 mg/day) and 100% (n=1) for high dose (>30 mg/day). Baseline FVC and DLCO were not correlated with steroid use (p value 0.051 and 0.181, respectively).

Table 3. Predictors of mortality with multivariable adjustment in Cox regression

Risk factor	Crud HR (95%CI)	P-value	Adjusted HR (95%CI)	P-value
Age	1.11 (1.06–1.15)	<0.001	1.08 (1.02–1.14)	0.009
Smoking	1.64 (0.79–3.43)	0.187	7.01 (1.99–24.68)	0.002
Congestive heart failure	1.86 (0.75–4.58)	0.179	0.57 (0.16–2.05)	0.386
MMF-use	0.55 (0.23–1.35)	0.195	0.94 (0.24–3.63)	0.928
Steroid-use	4.37 (1.67–11.45)	0.003	5.11 (1.01–25.92)	0.049
CRP	1.01 (1.00–1.02)	0.028	1.01 (1.00–1.02)	0.022
PF-ILD	3.31 (1.24–8.82)	0.017	5.73 (1.17–28.11)	0.031
Anti-centromere	3.34 (1.14–9.79)	0.028	3.74 (0.88–15.94)	0.074
Anti-Ro	2.98 (1.42–6.23)	0.004	2.79 (0.77–10.12)	0.119
AMA	12.14 (2.69–54.89)	0.001	13.43 (0.64–282.81)	0.095

MMF, mycophenolate mofetil; HR, hazard ratio; 95%CI, 95% confidence interval; CRP, c-reactive protein; PF-ILD, progressive fibrosing interstitial lung diseases; AMA, Anti-mitochondrial antibody.

Discussion

PF-ILD describes a high-risk population in patients with ILD and manifests as deterioration in pulmonary symptoms, imaging and function. We identified that having a fibrotic HRCT pattern at baseline, diabetes mellitus, and steroid use are clinical predictors for PF-ILD. In our study, approximately half of patients with CTD-ILD developed PF-ILD. Furthermore, ageing, smoking, high CRP at baseline, steroid-use and PF-ILD were associated with increased mortality in patients with CTD-ILD.

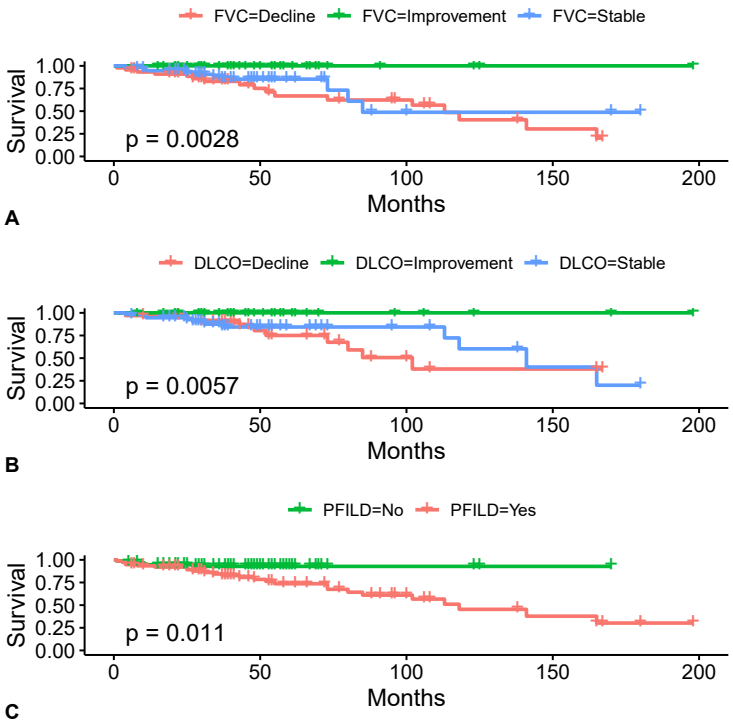

Figure 3. The Kaplan-Meier plots for survival analysis among predicted forced vital capacity (FVC), predicted single-breath diffusing capacity for carbon monoxide (DLCO), and progressive fibrosing interstitial lung diseases (PFILD).

Our results are in line with previous SSc-ILD and non-IPF (idiopathic pulmonary fibrosis) ILD trials in which PF-ILD patients were identified as a high-risk group for poor outcome [3, 4, 30-32]. The early SSc-ILD trial also found that a rapid pulmonary function decline predicts mortality [33, 34].

Other factors associated with increased mortality in our study were age, smoking, steroid-use, and CRP. Smoking is a known risk factor for mortality in many diseases and has also shown increased mortality in previous CTD studies [35]. The higher risk of PF-ILD on mortality in patients with steroids is partly because higher steroid dose was used in the more severely ill patients. However, we did not find a difference in baseline lung function and administration of steroids. In vitro steroids have mixed effects on fibroblast function and contradictory profibrotic and anti-fibrotic effect of steroids on fibroblasts are reported [36, 37]. There are some clinical studies that evaluated the impact of steroids on CTD-ILD. Although the post-hoc analysis from INBUILD trial showed a non-significant influence of steroid use on the effects of nintedanib, steroid revealed an impact in placebo group. The mean rate of annual FVC decline in the placebo group was

206.4 ml/year in steroid users (n = 184) and 165.8 ml/year in non-steroid users (n=147) [38]. Furthermore, development of irreversible organ damage was associated with steroid treatment independent of underlying CTD activity in patients with SLE [39]. These data suggest that steroid treatment could be detrimental in CTD-ILD. Also, steroids increased the risk of death and hospitalization in patients with IPF [40]. Therefore, it is essential that clinicians are aware of the risks associated with steroid use and minimise long-term steroid administration in patients with CTD-ILD, i.e. by combining steroids with other disease-modifying antirheumatic drugs at the onset of disease and thereafter proactively reduce steroid dosage.

Other immunosuppressants did not show a significant effect on mortality or progression to PF-ILD in our study. We did observe a trend towards improved survival in patients treated with mycophenolate mofetil (MMF), but possibly because of small sample size (48 patients) this did not reach significance. Benefits from immunosuppressants were reported in previous clinical studies, which observed preservation of pulmonary function in patients with SSc-ILD treated with MMF or cyclophosphamide [41], and benefits of tocilizumab, rituximab, and abatacept were seen in patients with ILD and SSc, RA, and IIM [6]. MMF is a key anchor drug for SSc-ILD [31], because of its' efficacy and safety profile and optional use combined with antifibrotic agents. MMF inhibits inosine monophosphate dehydrogenase for the de novo synthesis of guanosine purines, which majority suppress lymphocytes. Interestingly, MMF might also reduce pulmonary function decline in patients with IPF, suggesting potential anti-fibrotic effects [42]. The effects of the antifibrotic drug nintedanib have been evaluated in clinical trials and is a valuable addition to immunosuppressants. In the CTD-ILD subgroup analysis of INBUILD trial, the difference of annual FVC reduction rate between nintedanib and placebo was 104 ml/year (95%CI 21.1–186.9) [43]. Nintedanib may therefore reduce pulmonary function decline in CTD patients with PF-ILD. However, patients receiving immunosuppressants, including azathioprine, cyclosporine, tacrolimus, rituximab, cyclophosphamide, mycophenolate mofetil or oral corticosteroids (>20 mg/day) at baseline were excluded from INBUILD trial. Consequently, the CTD-ILD patients in the INBUILD trial are different from our real-world cohort and studies are needed to determine the optimal timing of nintedanib and effects of combination therapy with immunosuppressants.

Another interesting finding was that patients with obesity and positive anti-dsDNA had a lower risk of developing PF-ILD. Although excess soft tissue in the abdominal cavity and chest is associated with reduction in total lung capacity and FVC [44], the role of obesity in PF-ILD has not been reported.

In our study, we also searched for serum biomarkers to predict lung function decline. We found CRP and CA15.3 were correlated with pulmonary function decline; for every units

increased in CRP at baseline, the mortality hazard increased with one percent. CRP is a general biomarker which reflects the systemic inflammation and underline disease activity. The background autoimmunity not only plays a crucial role in pulmonary fibrosis but also contributes to systemic organ dysfunction. Control of CTD activity should always be part of pivotal therapy in patients with CTD-ILD. CA15.3, which is a mucin glycoprotein, has been used as a tumour marker for breast cancer. Another glycoprotein Krebs von den Lungen 6 (KL-6) has shown a high correlation with CA15.3 [45]. KL-6 is highly expressed on regenerated type II pneumocytes, and the serum KL-6 level has shown a diagnostic, prognostic, and monitoring value for progressive fibrosis in IPF and CTD-ILD [46-49]. Since KL-6 testing is not available worldwide, monitoring CA15.3 and CRP may be the best choice for clinicians in treating patients with CTD-ILD.

There are limitations in this cohort study. Firstly, it would have been interesting to include disease-specific activity scores in the analyses. However, comparison of disease activity between groups would have been difficult because the scores are disease-specific, and the groups would have been too small for comparison within groups. In addition, there might be confounding in the prediction model, therefore we adjusted for multiple variables in the regression analysis to minimise the risk of confounding. Also, in patients without HRCT at two years follow-up, we could have missed progression of fibrosis. Furthermore, the Cohen's kappa value for the different ILD patterns between the two pulmonary radiologists was not high in our study; a relatively low Cohen's kappa value was also seen in other ILD studies [50]. Clinical symptoms were not systematically scored in our cohort. A prospective study design combining physician global scores and patient report outcomes may provide more evidence on the risk stratification of clinical symptoms. Moreover, immunosuppressants/modulators used at inclusion were administered to treat the underlying CTD activity. In case of mild ILD without other major organ involvement, patients did not receive immunosuppressants. The median baseline HRCT involvement was 17.5% (Table 1) and 87 patients (58%) showed less than 20% lung involvement. Therefore, not all patients in our cohort were treated for ILD.

In conclusion, our study shows that a fibrotic HRCT pattern at baseline, diabetes mellitus, and steroid treatment increases the risk of developing PF-ILD, whereas positive anti-dsDNA and obesity revealed a lower risk of developing PF-ILD in patients with CTD-ILD. In addition, we found that PF-ILD, age, smoking, steroid use, and CRP are associated with increased mortality risk. These findings have important implications for clinical monitoring and confirm the central place of routine PFT and HRCT in follow-up in order to detect PF-ILD. Since antifibrotic treatment can be used in addition to intensive immunosuppressive treatment nowadays, it is important to closely monitor patients with CTD-ILD for PF-ILD. Furthermore, education on smoking cessation and minimising steroid use are also crucial in managing CTD-ILD.

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Supplementary

Table S1. P value of univariate risk factor analysis

Predictor	Mortality	FVC decline	DLCO Decline	Fibrosis progression	Inflammation progression	PF-ILD
Age	<0.001	NS	NS	NS	NS	NS
Disease Duration	NS	NS	NS	NS	NS	NS
Smoking	NS	NS	NS	NS	NS	NS
Coronary artery disease	NS	NS	NS	NS	NS	NS
Congestive heart failure	NS	NS	0.010	NS	NS	NS
Pulmonary hypertension	NS	NS	0.017	NS	NS	NS
Diabetes mellitus	NS	NS	NS	NS	NS	NS
Cerebrovascular event	NS	NS	NS	NS	NS	NS
Obesity	NS	NS	NS	NS	NS	NS
Azathioprine	NS	NS	NS	NS	NS	NS
MMF	NS	NS	NS	NS	NS	NS
Rituximab	NS	NS	NS	NS	NS	NS
steroid	0.003	NS	NS	NS	NS	0.035
Methotrexate	NS	NS	NS	NS	NS	NS
Hydroxychloroquine	NS	NS	NS	NS	NS	NS
UIP pattern HRCT	NS	NS	NS	NS	NS	NS
Fibrotic pattern HRCT	NS	0.008	NS	NS	NS	0.002
CA15.3	NS	NS	NS	NS	NS	NS
sIL2R	NS	NS	0.034	NS	NS	NS
ESR	NS	NS	NS	NS	NS	NS
CRP	0.028	NS	NS	NS	NS	NS
Creatine kinase	NS	NS	NS	NS	NS	NS
FVC	NS	NS	NS	NS	NS	NS
DLCO	NS	NS	0.022	NS	NS	NS
Anti-Jo-1	NS	NS	NS	NS	NS	NS
Anti-centromere	0.028	NS	NS	NS	NS	NS
Anti-Ro	0.004	NS	NS	NS	NS	NS
Anti-La	NS	NS	NS	NS	NS	NS
Anti-CCP	NS	NS	NS	NS	NS	NS
Anti-dsDNA	NS	NS	NS	NS	NS	NS
Anti-Scl-PM	NS	NS	NS	NS	NS	NS
Anti-SCL70	NS	NS	NS	NS	NS	NS
Rheumatoid factor	NS	NS	NS	NS	NS	NS
Anti-nucleosomes	NS	NS	NS	NS	NS	NS
Anti-histones	NS	NS	NS	NS	NS	NS
Anti-MDA5	NS	NS	NS	NS	NS	NS
Anti-Ro52	NS	NS	NS	NS	NS	NS
Anti-Sm	NS	NS	NS	NS	NS	NS
AMA	0.001	NS	NS	NS	NS	NS
Anti-RNP	NS	NS	NS	NS	NS	NS
Anti-PCNA	NS	NS	NS	NS	NS	NS

Mortality was analysed in Cox regression and other outcomes were analyzed in logistic regression. NS,

nonsignificant; FVC, forced vital capacity; DLCO, single-breath diffusing capacity for carbon monoxide; PF-ILD, progressive fibrosing interstitial lung disease; MMF, mycophenolate mofetil; HRCT, high-resolution computed tomography. sIL2R, soluble IL-2 receptor; ESR, erythrocyte sedimentation rate; CRP, c-reactive protein; AMA, Anti-mitochondrial antibody; Anti-RNP, anti-ribonucleoprotein; Anti-PCNA, antibodies to the proliferating cell nuclear antigen.

Table S2. Immunomodulatory therapy in each connective tissue disease

Disease	AZA	MMF	CYC	Rituximab	IVIG	HSCT	MTX	HCQ	TNFi	prednisolone	Others
Systemic sclerosis	0	24	0	2	1	3	7	1	0	16	0
Sjögren's syndrome	0	3	0	1	0	0	2	6	1	12	0
Myositis	7	5	1	11	1	0	2	2	0	24	6
RA	2	4	0	5	0	0	4	6	3	14	7
SLE	1	2	0	0	0	0	0	3	0	4	1
MCTD	0	2	0	0	0	0	0	1	1	3	0
UCTD	2	8	0	1	0	0	1	3	0	5	1

AZA, azathioprine; MMF, mycophenolate mofetil; CYC, cyclophosphamide; IVIG, intravenous immunoglobulin; HSCT, hematopoietic stem-cell transplantation; MTX, methotrexate; HCQ, Hydroxychloroquine; TNFi, tumor necrosis factor alfa inhibitor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; UCTD, undifferentiated connective tissue disease.

Chapter 3

Prognostication of progressive pulmonary fibrosis in connective tissue disease-associated interstitial lung diseases: A cohort study

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Abstract

Background

Connective tissue diseases-associated interstitial lung disease (CTD-ILD) is a heterogeneous condition that impairs quality of life and is associated with premature death. Progressive pulmonary fibrosis (PPF) has been identified as an important risk factor for poor prognosis. However, different criteria for PPF are used in clinical studies, which may complicate comparison between trials and translation of study findings into clinical practice.

Methods

This is a retrospective single center study in patients with CTD-ILD. The prognostic relevance of PPF definitions, including INBUILD, ATS/ERS/JRS/ALAT 2022, and simplified progressive fibrosing (simplified PF) criteria, were examined in this cohort and validated in the other reported Dutch CTD-ILD cohort.

Results

A total of 230 patients with CTD-ILD were included and the median follow-up period was six (3—9) years. Mortality risk was independently associated with age (adjusted HR 1.07, $p < 0.001$), smoking history (adjusted HR 1.90, $p = 0.045$), extent of fibrosis on HRCT at baseline (adjusted HR 1.05, $p = 0.018$) and baseline DLCO (adjusted HR 0.97, $p = 0.013$). Patients with regular pulmonary function tests in the first two years (adjusted HR 0.42, $p = 0.002$) had a better survival. The prognostic relevance for survival was similar between the three PPF criteria in the two cohorts.

Conclusion

Higher age, smoking, increased extent of fibrosis and low baseline DLCO were associated with poor prognosis, while regular pulmonary function evaluation was associated with better survival. The INBUILD, ATS/ERS/JRS/ALAT 2022, and simplified PF criteria revealed similar prognostication.

Introduction

Connective tissue diseases (CTD) are characterized by dysregulation of the immune system resulting in inflammation and subsequent tissue damage followed by fibrosis. In CTDs with lung involvement, inflammation and/or fibrosis of pulmonary parenchyma leads to deterioration of lung function, cough and shortness of breath. Interstitial lung disease (ILD) occurs in approximately 15% of CTD patients, depending on the type of CTD, and is associated with high mortality and decreased quality of life [1].

The disease course of CTD-associated ILD (CTD-ILD) is heterogeneous. Therefore, clinical characteristics and risk factors for poor prognosis are crucial in managing patients with CTD-ILD. In previous studies, several biomarkers, fibrotic high-resolution computed tomography (HRCT) at baseline, senior age, smoking, steroid use and progressive pulmonary fibrosis have been identified as predictors of poor prognosis in CTD-ILD [2-4].

Particularly, rapid deterioration of respiratory symptoms, lung function and progressive fibrosis on HRCT are referred to as progressive fibrosing interstitial lung diseases or progressive pulmonary fibrosis (PPF) [3, 5-7]. Identification of patients with PPF is crucial for clinical practice, as these patients have a poor prognosis and may benefit from antifibrotic drugs similar to patients with idiopathic pulmonary fibrosis (IPF) in randomized controlled trials [8, 9]; however, the definition of PPF criteria differ between studies. Furthermore, the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Asociación Latinoamericana de Tórax (ATS/ERS/JRS/ALAT) defined scientific societies-approved criteria in the 2022 guideline [7]. The variety in criteria complicates study comparison and clinical implication. In this study, we aimed to explore the prognostic relevance of the different PPF criteria in patients with CTD-ILD.

Methods

Study population

This is a single center retrospective cohort study performed at the ILD Center of Excellence, St. Antonius Hospital, Nieuwegein, the Netherlands. Patients diagnosed with CTD-ILD or interstitial pneumonia with autoimmune features between 2005 and 2021 were included when at least a baseline HRCT was available [10-12]. Baseline was defined as the time of ILD diagnosis. All patients were discussed in multidisciplinary team meetings. Clinical characteristics, laboratory results and pulmonary function tests (baseline, six months, one year and two years) were retrieved from the electronic medical records. This study was approved by the Medical Research Ethics Committees United (MEC-U, number R05-08A) and all patients provided written informed consent.

Pulmonary imaging

HRCT results were collected at baseline, one and two years. Baseline HRCT patterns were classified according to the classification for idiopathic interstitial pneumonia [13, 14], listing as consistent with usual interstitial pneumonia (UIP), probable UIP, alternative diagnosis or indeterminate for UIP. Probable and consistent with UIP were summarized as UIP. The alternative diagnosis was then classified as non-specific interstitial pneumonia (NSIP, including fibrotic, cellular, or mixed [15]), lymphocytic interstitial pneumonia (LIP), organizing pneumonia (OP), desquamative interstitial pneumonia, nodular lymphocytic hyperplasia, pleuroparenchymal fibro-elastosis and acute interstitial pneumonitis (AIP). The predominant HRCT features were categorized into fibrotic, including features as reticulation and honeycombing, or inflammatory, including ground-glass opacity and consolidation [3, 16-18]. The changes in fibrosis and inflammation over time were classified as progression, stable, or regression. Extent of fibrosis on HRCT was evaluated at all time points. HRCTs were evaluated by two experienced thoracic radiologists who were blinded to clinical information and pathology diagnosis.

Criteria for progression

The INBUILD criteria included patients with ≥10% relative decline in percentage of predicted forced vital capacity (FVC), ≥5% and <10 % relative decline in FVC with progressive fibrosis on HRCT or worsening of respiratory symptoms, or deterioration of both HRCT fibrosis and respiratory symptoms within two years despite standard (anti-inflammatory) treatment [8]. The ATS/ERS/JRS/ALAT 2022 criteria were met with at least two of the following criteria; worsening of respiratory symptoms, fibrotic progression on HRCT and lung function deterioration (≥5% absolute decline in FVC and/or ≥10% absolute decline in percentage of predicted hemoglobin adjusted diffusing capacity of the lung for carbon monoxide (DLCO)) occurring within one year and without alternative explanation [7]. The simplified progressive fibrosing (simplified PF) criteria were met with any of the following: ≥10% relative decline in FVC, ≥15% relative decline in DLCO, or progression of fibrosis on HRCT within two years (Table S1) [3, 6]. The prognostic relevance for mortality over time was evaluated for the INBUILD criteria, the ATS/ERS/JRS/ALAT 2022 criteria, and simplified PF criteria. The prognostic relevance of the three PPF criteria was then validated in a previously published Dutch CTD-ILD cohort at University Medical Center Utrecht (UMCU) [3].

Statistical analysis

Categorical variables were presented in frequencies, and the difference between groups was examined in Fisher's exact test. The distribution of the data was assessed in histograms. The continuous variables were presented in medians (interquartile range, IQR), and the difference

between groups was determined using the Wilcoxon rank sum test. The hazard ratios (HR) for mortality risks were calculated using Cox regression, and variables with a p-value < 0.1 were included in a multivariable analysis with age, gender, smoking, comorbidities and underlying CTD. The prognostic relevance for mortality and the PPF criteria was examined in the time-dependent receiver operator characteristic (ROC) model and visualized in area under curve (AUC) over time. Risk factors for PPF were examined in logistic regression. Missing data were omitted from each regression analysis. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using R 4.0.3.

Results

Patient characteristics

A total of 230 patients were included in this cohort, of which 122 (53%) were female. The median age was 63 (IQR 54—69) years. The median follow-up period was 6 (3—9) years. The underlying CTD diagnosis included rheumatoid arthritis (RA) in 77 patients (33%), idiopathic inflammatory myopathies (IIM) in 38 (17%), primary Sjögren's syndrome (pSS) in 33 (14%), undifferentiated connective tissue disease (UCTD) in 32 (14%), systemic sclerosis (SSc) in 24 (10%), mixed connective tissue disease (MCTD) in eight (3%), systemic lupus erythematosus (SLE) in eight (3%), overlap syndrome in six (3%), spondyloarthropathy in three (1%) and antineutrophil cytoplasmic antibody-associated vasculitis in one (0.4%). Patients with RA, including RA overlap syndromes, were older (median 65 (IQR 62—73) years) than non-RA patients (median 60 (50—67) years, p < 0.001). A total of 133 (58%) patients were past smokers, and 13 (6%) patients were current smokers. The median tobacco exposure was 18 (10—30) pack-years at baseline. In 104 (45%) patients, the diagnosis of CTD and ILD occurred within six months of one another. ILD was diagnosed in 100 (43%) patients with pre-existing CTD for more than six months, and the median CTD duration at ILD diagnosis was six (IQR 2—13) years. Twenty-six (11%) patients were diagnosed with CTD more than six months after ILD diagnosis. Antinuclear antibodies were positive in 106 (46%) patients. Other autoantibodies were detected, including rheumatoid factor in 71 (31%) patients, anti-SSA in 70 (30%), anti-citrullinated peptide antibodies in 61 (27%), and anti-Jo-1 in 25 (11%). The median body mass index was 27 (IQR 24—30). The median Charlson's comorbidity index was 3 (IQR 2—4), including 32 (14%) coronary artery disease, 23 (10%) diabetes mellitus, 14 (6%) chronic obstructive pulmonary disease, 12 (5%) cerebrovascular accident, 11 (5%) heart failure, eight (3%) pulmonary arterial hypertension, six (3%) peripheral vascular disease (PVD) and three (1%) chronic kidney disease. The most commonly used immunomodulators at baseline were corticosteroids in 165 (72%) patients, methotrexate in 64 (28%), azathioprine in 55 (24%), mycophenolate mofetil in 47 (20%), and hydroxychloroquine in 45 (20%). Four patients were on antifibrotics at baseline (nintedanib (n=2) and pirfenidone (n=2)). (Table 1)

Table 1. Patient characteristics

Baseline characteristics	Patients
Age, median (IQR)	63 (54—69)
Gender (Female), n (%)	122 (53)
BMI, median (IQR)	27 (24—30)
Immunomodulators, n (%)	
Corticosteroids	165 (72)
Steroid dose (mg/day), median (IQR)	15 (5—30)
Azathioprine	55 (24)
Mycophenolate mofetil	47 (20)
Methotrexate	64 (28)
Leflunomide	12 (5)
Hydroxychloroquine	45 (20)
Cyclophosphamide	34 (15)
Sulfasalazine	12 (5)
Rituximab	22 (10)
Tumour necrosis factor inhibitors	29 (13)
Abatacept	3 (1)
Tocilizumab	3 (1)
Tofacitinib	1 (0.4)
Anti-fibrotics, n (%)	
Nintedanib	2 (1)
Pirfenidon	2 (1)
Charlson's comorbidity index, median (IQR)	3 (2—4)
Autoantibodies, n (%)	
Antinuclear antibody	106 (46)
Rheumatoid factor	71 (31)
Anti-citrullinated peptide antibodies	61 (27)
Anti-dsDNA	5 (2)
Anti-SSA	70 (30)
Anti-SSB	16 (7)
Anti-U1-RNP	12 (5)
Anti-SM	5 (2)
Anti-SCL-70	12 (5)
Anti-RNA polymerase III	1 (0.4)
Anti-centromere	8 (3)
Anti-PM-SCL	8 (3)
Anti-Jo-1	25 (11)
Anti-PL12	7 (3)
Anti-Th/To	3 (1)
Anti-Ku	3 (1)
Anti-Ej	2 (1)
Anti-Oj	1 (0.4)
Anti-SAE	2 (1)
Anti-MDA5	1 (0.4)
Anti-TIF1γ	1 (0.4)
Anti-Mi2α	1 (0.4)

(Continued)

Baseline characteristics	Patients
Anti-Mi2β	2 (1)
Anti-MPO	1 (0.4)
Anti-PR3	1 (0.4)
Anti-Cardiolipin IgG	1 (0.4)
Anti-Cardiolipin IgM	1 (0.4)
Anti-β2-glycoprotein IgG	2 (1)
Negative for autoantibodies, n (%)	21 (9)

Abbreviation: IQR, interquartile range; BMI, body mass index.

Radiology and pulmonary function progression

Various HRCT patterns were observed at baseline: UIP in 63 (27%, of whom 35 patients had RA) patients, fibrotic NSIP in 21 (9%), cellular NSIP in 25 (11%), mixed NSIP in 79 (34%), OP in 34 (15%), LIP in four (2%), AIP in one (0.4%), two (1%) combined OP and mixed NSIP, and one (0.4%) indeterminate. UIP patterns were observed in 35 (45 %) RA patients, including RA overlap syndrome, and 28 (18%) in other CTD, $p < 0.001$. HRCT features were predominantly fibrotic in 117 (51%) patients and predominantly inflammatory in 113 (49%). The predominantly fibrotic HRCT consisted of 63 (100%) UIP, 31 (39%) mixed NSIP, 21 (100%) fibrotic NSIP, one LIP, and one indeterminate pattern. Patients with fibrotic HRCT patterns were older compared to patients with inflammatory patterns (respectively, 65 (IQR 60—74) and 59 (IQR 49—65) years old, $p < 0.001$). Low extent of fibrosis (< 20% [19]) on baseline HRCT occurred in 214 (93%) patients; in the predominant fibrosis group, 102 (87%) patients had low extent of fibrosis on HRCT at baseline. In patients with predominantly inflammatory patterns, 38 out of 68 patients (56%) had less inflammation at one year and 26 out of 47 patients (55%) at two years. HRCTs were unavailable in 95 patients at one year (50 in the predominantly fibrotic and 45 in the predominantly inflammatory group), and 144 patients at two years of follow-up (78 in the predominantly fibrotic and 66 in the predominantly inflammatory group).

In the first two years, 112 (49%) patients had regular pulmonary function tests at six months, one year and two years. The serial change in pulmonary function was shown in Figure 1. A relative decline ≥10% in FVC was seen in 22 (10%) patients at six months, 22 (10%) at one year, 32 (14%) at two years and 39 (17%) at the last follow-up. A relative decline ≥15% in DLCO was observed in 20 (9%) patients at six months, 28 (12%) at one year, 40 (17%) at two years and 40 (17%) at the last follow-up (Figure S1).

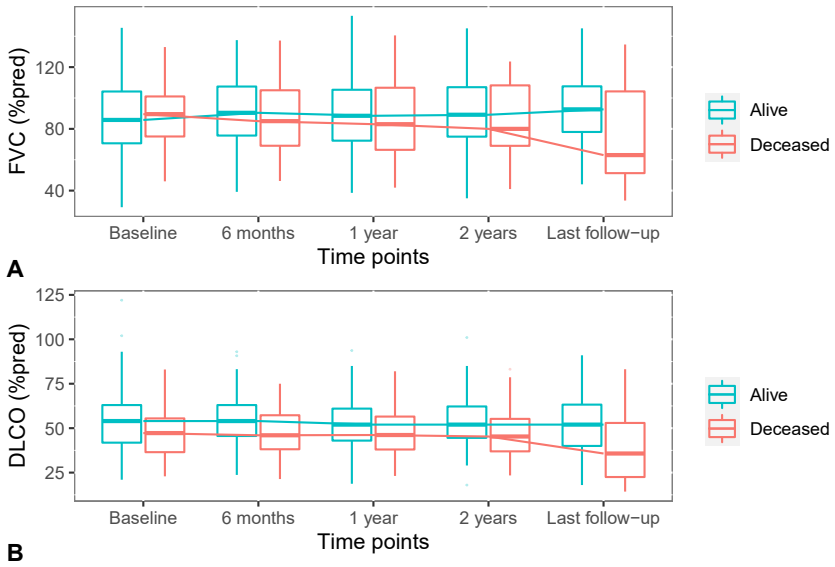

Figure 1. Serial change in pulmonary function test including percentage of predicted forced vital capacity (FVC) and hemoglobin adjusted diffusing capacity of the lung for carbon monoxide (DLCO).

PPF in the first two years was observed in 61 (27%) patients meeting INBUILD criteria, 53 (23%) meeting ATS/ERS/JRS/ALAT criteria, 136 (59%) meeting simplified PF criteria and 125 (54%) when using simplified PF criteria with a threshold for HRCT ≥ 5% increase in the extent of fibrosis. The prevalence of PPF in each CTD was shown in Table S2. Diagnosis of SSc, azathioprine use, PVD, regular follow-up pulmonary function, NSIP pattern and ANA positivity were revealed as predictors for more than two PPF criteria in univariable analysis; TNF inhibitor use was associated with reduced PPF risk. After multivariate adjustment, PVD and NSIP pattern remained significant as predictors for more than two PPF criteria (Table S3). In RA patients, baseline HRCT with fibrotic NSIP pattern was associated with PPF meeting ATS/ERS/JRS/ALAT criteria (OR 6.04, p = 0.012) and INBUILD criteria (OR 7.60, p = 0.004). For other CTDs, no risk factors could be identified for more than two PPF criteria.

Survival analysis

During follow-up, 68 (30%) patients died. The cause of death was ILD related in 17 (25%) patients, malignancy in nine (13%), COVID-19 in five (7%), other pulmonary infection in four (6%), heart failure in two (3%) and combined ILD and heart failure in four (6%), thrombosis in one (1%) and unknown in 26 (38%). Survival was independently associated with age (adjusted HR 1.07, p < 0.001), smoking history (adjusted HR 1.90, p = 0.045), and extent of fibrosis on HRCT at baseline (adjusted HR 1.05, p = 0.018). Higher

baseline DLCO (adjusted HR 0.97, p = 0.013) and regular pulmonary function tests in the first two years (adjusted HR 0.42, p = 0.002) were associated with better survival. (Table 2). In subgroup analysis, the association between UIP patterns and mortality was insignificant in RA patients (HR 1.3, p = 0.448) but significant in patients with other CTDs (adjusted HR 2.27, p = 0.030).

Table 2. Prognostic factors for survival

Risk	HR	P-value	Adjusted HR	P-value
Male	2.19	0.002 *	1.63	0.093
Age	1.08	< 0.001 *	1.07	< 0.001 *
Charlson's score	1.47	< 0.001 *		
PAH	3.16	0.028 *	2.29	0.118
Smoking history	2.46	0.002 *	1.90	0.045 *
RA	2.32	< 0.001 *	1.18	0.555
CTD duration	1.04	0.005 *	1.02	0.232
TNFi	1.89	0.040 *	1.71	0.142
Hospitalized infection	1.82	0.017 *	1.25	0.405
Extent of fibrosis	1.03	0.019 *	1.05	0.018 *
DLCO	0.98	0.030 *	0.97	0.013 *
Regular PFT	0.399	< 0.001 *	0.42	0.002 *
UIP	2.71	< 0.001 *	1.61	0.077
mNSIP	0.52	0.021 *	0.60	0.078
OP	0.44	0.038 *	0.53	0.129
NSIP	0.581	0.027 *	0.78	0.327
Fibrotic patterns	2.56	< 0.001 *	1.64	0.094

Abbreviation: PAH, Pulmonary arterial hypertension; RA, rheumatoid arthritis; CTD, connective tissue disease; TNFi, Tumor necrosis factor inhibitors; DLCO, percentage of predicted hemoglobin adjusted diffusing capacity of the lung for carbon monoxide; PFT, pulmonary function test; UIP, usual interstitial pneumonia; mNSIP, mixed non-specific interstitial pneumonia; OP, organizing pneumonia. * p < 0.05.

None of the PPF criteria (in the first two years) achieved significant relation with mortality in Cox regression. The prognostic relevance did not differ between simplified PF criteria, INBUILD and ATS/ERS/JRS/ALAT criteria; the prognostic value improved in simplified PF criteria with defining HRCT progression with a ≥5% increase in fibrosis. The prognostic relevance of the PPF criteria with mortality risk over time in both cohorts is shown in Figure 2; The prognostic value of PPF criteria increased during the first 3 years and achieved a plateau thereafter in both cohorts.

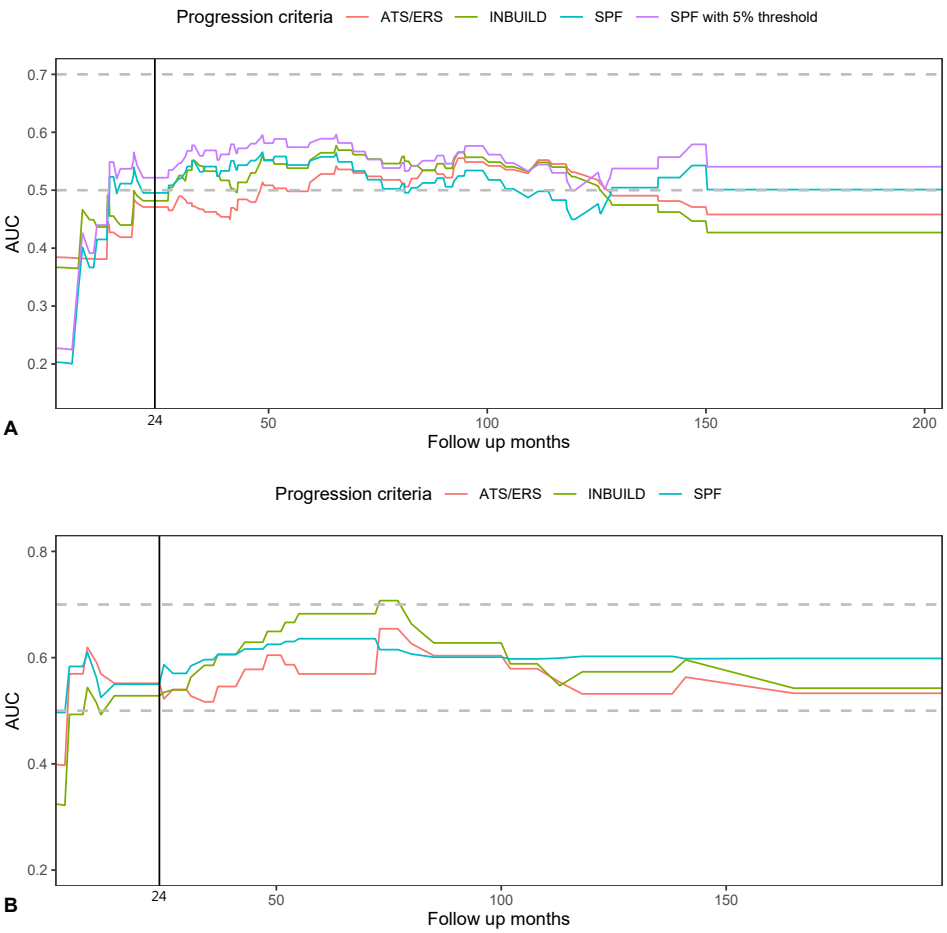

Figure 2. The prognostic relevance to mortality and progressive pulmonary fibrosis (PPF) is shown in this time dependent receiver operator characteristic (ROC) model. The figure demonstrates the area under the ROC curve (AUC) over the follow-up period in this cohort (A) and the validation cohort (B). The vertical line indicates the timepoint of 24 months when PPF was identified. A higher AUC reflects a better correlation of the criteria with prognosis. The PPF criteria, including ATS/ERS/JRS/ALAT criteria (ATS/ERS), INBUILD criteria (INBUILD), and the simplified progressive fibrosing criteria (SPF), did not substantially outcompete each other. The prognostic value in AUC improved in SPF with defining HRCT progression with a ≥5% increase in fibrosis (SPF with 5% threshold) in the present cohort (A).

Discussion

This study explored the characteristics of patients with early CTD-ILD and their prognostic correlation with PPF. Increased age, smoking, and increased extent of fibrosis were associated with higher mortality risk, while higher baseline DLCO and regular

pulmonary function tests were associated with reduced mortality risk. The prognostic relevance with mortality did not differ between simplified PF criteria, INBUILD and ATS/ERS/JRS/ALAT 2022 criteria.

The risk factors associated with mortality in this cohort are in line with identified risk factors in previous studies. Age and smoking are overarching risk factors across diseases [20]. Patients with early diagnosis and subsequently low extent of fibrosis on HRCT and better DLCO, have a larger window of opportunity to initiate treatment in order to decrease the risk of progression. In addition, a large proportion of patients in this study had low extent of fibrosis at baseline, in contrast to previous studies, including the INBUILD trial and the validation cohort, in which more patients had high extent of fibrosis [3, 8]. The correlation between mortality and PPF was also more prominent in patients with extensive lung fibrosis than in those with limited lung fibrosis in another SSc-ILD cohort [6].

In several studies, UIP pattern was observed more often in RA patients and was associated with mortality and DLCO decline [21, 22]. In our study, RA patients were older and had UIP patterns more frequently than patients with other CTDs. However, this was not significantly associated with mortality. We did find an association with UIP pattern and mortality in the non-RA group. Similarly, in a recent RA-ILD study, UIP pattern was not associated with mortality or FVC decline at 2 years [23]. A possible explanation is that treatment strategies in RA have improved tremendously in the last decades, whereas disease control in other underlying CTD diseases has proven more challenging. Moreover, not only UIP pattern was associated with predominant fibrosis; also, fibrotic NSIP and some other patterns could be linked to predominant fibrosis and were associated with increased risk for PPF. This finding is in line with the results of the validation cohort; predominantly fibrotic HRCT patterns revealed an increased risk for PPF [3, 18]. Patients with predominantly inflammatory HRCT may respond better to anti-inflammatory treatment than those with predominantly fibrotic HRCT and therefore reduce the risk of PPF.

There may be a different risk profile of PPF in each CTD, while baseline severity, including lung function and HRCT, seems to be an overarching risk. In the European Scleroderma Trials and Research (EUSTAR) database, a large registry of SSc patients in Europe, male gender, higher modified Rodnan skin score and reflux/dysphagia symptoms were associated with FVC decline over 5 years in patients with SSc-ILD [24]. In patients with RA-ILD, low baseline FVC/DLCO, UIP pattern, and steroid-use (> 10 mg/day) were associated with progressive lung function decline [25]. A positive serum anti-MDA5 is associated with rapid progression in IIM patients, but distinct clinical course was observed in subgroups [26, 27].

In recent years, PPF has received attention in trials increasingly, especially after the randomized trials with antifibrotic treatment. The natural history of PPF in ILD, including CTD-ILD, appears to be comparable with idiopathic pulmonary fibrosis (IPF) [28]. Nevertheless, definitions of PPF vary across studies. The ATS/ERS/JRS/ALAT 2022 criteria were the first consensus of scientific societies but were based on data from IPF [7]. As emphasized in the ATS/ERS/JRS/ALAT 2022 guideline, PPF should be utilized in prognostication instead of diagnosis. We examined the prognostic correlation of these PPF criteria in the time-dependent ROC model. The prognostic correlation with mortality was similar between the three PPF criteria and achieved a plateau after three years in this cohort (predominant CTD in RA) and the validation cohort (predominant CTD in SSc); the AUC in time-dependent ROC model was higher in the validation cohort than this cohort.

The strength of this study is that we validated the prognostication with two real world CTD-ILD data. The prognostic relevance was visualized in time dependent ROC model. Most patients were diagnosed early with low extent of fibrosis at baseline. However, the proportion of missing data was relatively high and can be regarded as limitation of this study (Figure S1). As the St. Antonius Hospital is an ILD referral center, patients are often evaluated once for expert opinion after which follow-up will take place at local hospitals, which could largely explain the missing data at follow-up. In addition, patient reported respiratory symptoms were not systematically scored in the medical records, therefore we did not include this parameter in our analysis. In the validation cohort, 23 (15%) patients reported symptom progression from dyspnea on exertion to dyspnea at rest or oxygen requirement in the first two years. Because of the missing data at follow-up, the proportion of patients with PPF may be underestimated. Nonetheless, regular pulmonary function test in the first two years was associated with a significant preferable prognosis. A second limitation is that the reading of HRCT, which relies on experienced radiologists, may be variation in interobserver agreement, and radiological progression of most of the criteria is descriptive [3, 7-9, 29, 30]. An artificial intelligence-aided quantitative HRCT evaluation could improve accurate detection of changes, although these techniques are not universally available yet [31, 32]. Since CTD-ILD is a heterogenous manifestation, further research in biomarkers and artificial intelligence-aided HRCT analysis could support tailored clinical decision making.

In conclusion, we identified risk factors for mortality and examined prognostication of PPF in CTD-ILD patients. CTD-ILD is a rather heterogenous disease and the current PPF criteria may not be applicable universally. Disease control of the underlying CTD, multidisciplinary evaluation and systematic assessment of respiratory symptoms, pulmonary function, and HRCT are instrumental to identify high-risk patients and tailor treatment strategies [33]. Further research is needed to explore optimal use of PPF criteria in managing patients with CTD-ILD.

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Supplementary

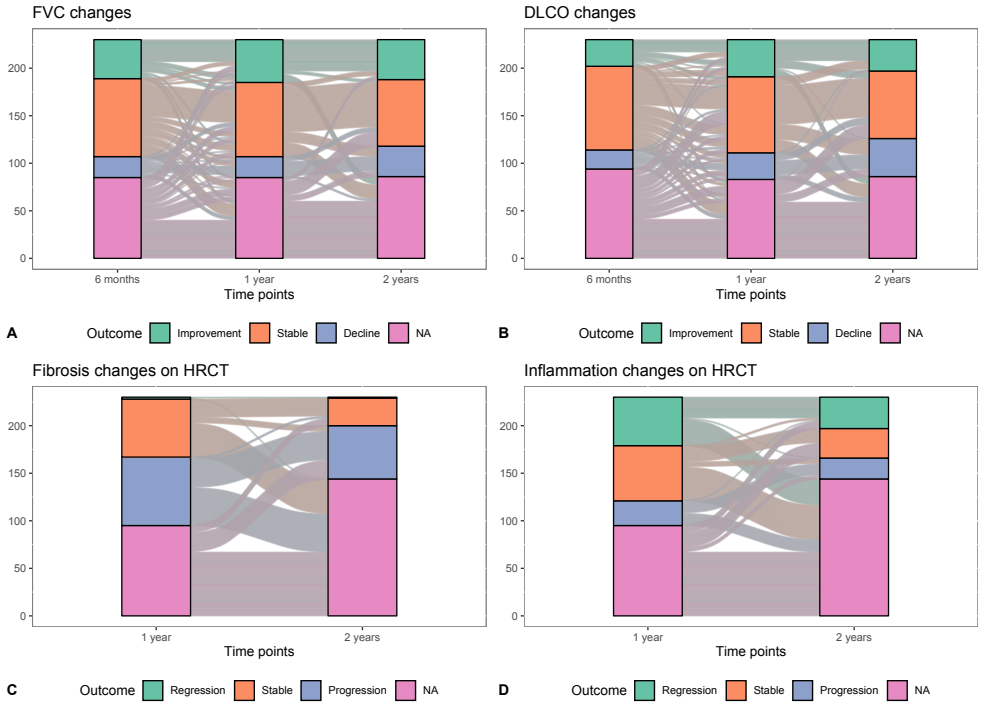

Figure S1. The alluvial plot shows the progress in forced vital capacity (FVC), diffusing capacity of the lung for carbon monoxide (DLCO), high-resolution computed tomography (HRCT) inflammation and HRCT fibrosis. Pulmonary function ≥ 10% in FVC and ≥ 15% relative change were defined as decline or improvement. NA, not available.

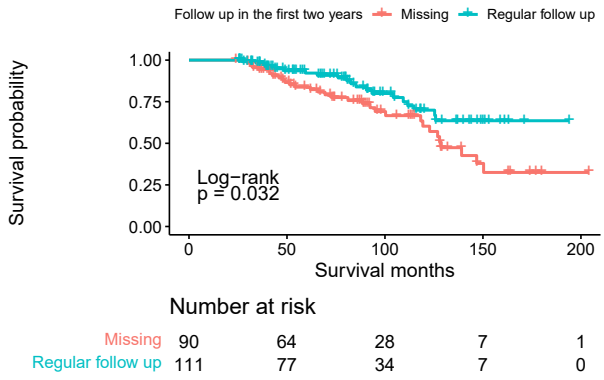

Figure S2. The Kaplan-Meier curve after the first two years when progressive pulmonary fibrosis was identified in patients with connective tissue disease- associated interstitial lung disease. The patients with missing follow up in the first two years had higher mortality risks in the disease course thereafter. (unpublished)

Table S1. Criteria for progressive pulmonary fibrosis

Criteria names	Criteria definition
INBUILD criteria: any of the criteria within two years	≥10% relative decline in FVC
	≥5% and <10 % relative decline in FVC with progressive fibrosis on HRCT or worsening of respiratory symptoms
	Deterioration of both HRCT fibrosis and respiratory symptoms
ATS/ERS/JRS/ALAT 2022 criteria: at least two of the criteria within one year	Worsening of respiratory symptoms
	Progression of fibrosis on HRCT: a. Increased extent or severity of traction bronchiectasis and bronchiolectasis b. New ground-glass opacity with traction bronchiectasis c. New fine reticulation d. Increased extent or increased coarseness of reticular abnormality e. New or increased honeycombing f. Increased lobar volume loss
	Lung function deterioration: ≥5% absolute decline in FVC and/or ≥10% absolute decline in DLCO
Simplified progressive fibrosing criteria: any of the criteria within two years	≥10% relative decline in FVC
	≥15% relative decline in DLCO
	Progression of fibrosis on HRCT

Table S2. The prevalence of progressive pulmonary fibrosis (PPF) by ATS/ERS/JRS/ALAT 2022 criteria (ATS/ERS), INBUILD criteria (INBUILD), and the simplified progressive fibrosing criteria (simplified PF) in each CTD

	RA, n = 77	IIM, n = 38	pSS, n = 33	UCTD, n = 32	SSc, n = 24	MCTD, n = 8	SLE, n = 8	Overlap, n = 6	SpA, n = 3	AAV, n = 1
ATS/ERS	13 (17%)	5 (13%)	9 (27%)	9 (28%)	10 (42%)	3 (38%)	2 (25%)	1 (17%)	1 (33%)	0
INBUILD	15 (19%)	7 (18%)	10 (30%)	8 (25%)	13 (50%)	3 (38%)	2 (25%)	3 (50%)	1 (33%)	0
Simplified PF	42 (55%)	24 (63%)	23 (70%)	19 (59%)	14 (58%)	5 (63%)	4 (50%)	3 (50%)	2 (67%)	0

Abbreviation: RA, rheumatoid arthritis; IIM, idiopathic inflammatory myopathies; pSS, primary Sjögren's syndrome; UCTD, undifferentiated connective tissue disease; SSc, systemic sclerosis; MCTD, mixed connective tissue disease; SLE, systemic lupus erythematosus; overlap, overlap syndrome; SpA, spondyloarthropathy; AAV, antineutrophil cytoplasmic antibody-associated vasculitis.

Table S3. Predictors for progressive pulmonary fibrosis

Variable	ATS/ERS		INBUILD		Simplified PF		Simplified PF + 5%	
	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
PVD	7.14 (1.35 –52.63)*	7.59 (1.29– 60.63)*	5.86 (1.11– 43.08)*	6.56 (1.17– 50.35)*				
DM	2.38 (0.94– 5.80)	2.59 (0.94– 6.94)	2.35 (0.95– 5.68)	2.41 (0.92– 6.12)				
BMI					0.96 (0.90– 1.01)	0.95 (0.90– 1.01)		
RA	0.55 (0.26– 1.07)	0.70 (0.31– 1.50)						
SSc	2.31 (0.99– 5.22)*	2.04 (0.82– 4.90)	3.61 (1.62– 8.11)*	3.67 (1.60– 8.46)*				
pSS							2.14 (0.99– 4.93)	1.85 (0.83– 4.37)
CTD duration					1.00 (0.99– 1.00)	1.00 (0.99– 1.00)	1.00 (0.99– 1.00)*	1.00 (1.00– 1.00)
Steroid use					1.76 (0.98– 3.15)	1.44 (0.78– 2.66)	1.73 (0.97– 3.10)	1.43 (0.77– 2.66)
Steroid dose					0.98 (0.96– 1.00)			
AZA					4.34 (1.59– 15.24)*	3.46 (1.23– 12.37)*	3.14 (1.28– 8.89)*	2.57 (1.01– 7.44)
TNFi					0.33 (0.13– 0.79)*	0.50 (0.17– 1.39)	0.26 (0.09– 0.66)*	0.41 (0.13– 1.18)
Regular PFT	3.15 (1.65– 6.21)*	2.78 (1.41– 5.64)*	2.33 (1.28– 4.32)*	2.12 (4.03– 0.019)			1.77 (1.05– 3.02)*	1.60 (0.93– 2.77)
FVC					0.99 (0.97– 1.00)*	0.99 (0.98– 1.01)		
UIP	0.46 (0.20– 0.98)	0.48 (0.19– 1.11)			0.52 (0.29– 0.93)	0.6 (0.32– 1.14)		
NSIP	2.81 (1.46– 5.71)*	2.50 (1.24– 5.29)*	1.79 (0.99– 3.34)	1.58 (0.83– 3.05)	2.06 (1.21– 3.54)*	2.5 (1.40– 4.54)*	1.76 (1.05– 3.00)*	1.79 (1.02– 3.17)*
ANA	1.91 (1.03– 3.61)*	1.43 (0.67– 3.09)	2.04 (1.13– 3.72)*	1.52 (0.79– 2.93)				
Anti-U1-RNP	3.64 (1.09– 12.14)*	3.62 (1.01– 13.02)*						

The table demonstrates the predictors for each progressive pulmonary fibrosis (PPF) criteria. The multivariate analysis was adjusted for variables with p value < 0.1 excluding FVC, DLCO, and HRCT patterns. Data with p value < 0.05 were marked with *. Abbreviation: ATS/ERS, PPF by 2022 ATS/ERS/JRS/ALAT guideline; INBUILD, PPF by inclusion criteria of INBUILD trial; Simplified PF, PPF by previous cohort; Simplified PF + 5%, the simplified PF criteria with a threshold for HRCT ≥ 5% increase in the extent of fibrosis; OR, odds ratio; aOR, adjusted odds ratio; PVD, peripheral vascular disease; DM, diabetes mellitus; BMI, body mass index; RA, rheumatoid arthritis; SSc, systemic sclerosis; pSS, primary Sjögren's syndrome; CTD, connective tissue diseases; PFT, pulmonary function test; UIP, usual interstitial pneumonia pattern, NSIP, non-specific interstitial pneumonia pattern; ANA, antinuclear antibody.

Chapter 4

KL-6, CXCL11 and CTGF are potential biomarkers in response to treatment in connective tissue disease-associated interstitial lung disease

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Abstract

Objective

To identify biomarkers that are associated with the response to treatment in connective tissue disease-associated interstitial lung disease (CTD-ILD).

Methods

An exploratory set of 38 biomarkers were measured in serum of patients with CTD-ILD at baseline and one year of follow-up. ILD patterns were classified according to the classification for idiopathic interstitial pneumonia and categorised into fibrotic or inflammatory. The predictivity of baseline biomarker, responsiveness of biomarkers to treatment, and correlations between the variation of biomarkers and pulmonary function test after one year of treatment were examined.

Results

Sixteen patients were included with a median age of 51 years old (IQR 45–62). Patients with inflammatory HRCT patterns showed less decline in forced vital capacity (FVC), 7.7% versus 33.3% in patients with fibrotic HRCT patterns. In patients with inflammatory HRCT patterns, CXCL11 reduced from a median 307.8 pg/ml to 253.8 pg/ml (p-value 0.011), CTGF from 48.5 pg/ml to 9.5 pg/ml (p-value 0.033) and KL-6 from 1221 U/ml to 543 U/ml (p-value 0.040). Additionally, an increase in levels of galectin-3 at one-year follow-up was associated with improved FVC (Rho 0.5, p = 0.048).

Conclusion

In this study, CXCL11, CTGF, and KL-6 reduction were associated with inflammatory HRCT patterns and better pulmonary outcome. In contrast to previous research in severe ILD, there was a positive correlation between changes of galectin-3 and FVC in our study. Further research in a larger group and focusing on combining biomarkers to predict outcome and prognosis is needed.

Introduction

Interstitial lung disease (ILD) consists of a heterogeneous group of parenchymal lung disorders with variable degrees of inflammation and fibrosis [1]. Connective tissue disease associated interstitial lung disease (CTD-ILD) is common and an important cause of morbidity and mortality [2].

In recent studies, several proteins have been identified, showing correlations with high resolution computed tomography (HRCT) characteristics and clinical outcomes [3-5]. Unfortunately, there are few accurate markers that can guide risk stratification and treatment decisions in daily practice. This study aimed to identify biomarkers in CTD-ILD correlating with radiographic characteristics and response to immunosuppressive therapy at one-year follow-up.

Methods

Patients

This is a retrospective cohort study. Patients with ILD and eligible for a specific CTD diagnosis or fulfilled the proposed criteria for interstitial pneumonia with autoimmune features were included from the Utrecht Infection and Immunology cohort Biobank Study with a minimum follow-up of one year and two longitudinal samples [6, 7]. The institutional medical ethics committee of the University Medical Centre Utrecht has approved the study (study number 19/148). Demographical, clinical, radiological and pulmonary function test (PFT) data were retrieved from medical records.

Serum biomarkers

Serum samples were collected at inclusion and after one year as per protocol of the Utrecht Infection and Immunity cohort [7]. Biomarkers involved in inflammatory and fibrotic pathways were measured in multiplex immunoassay (xMAP, Luminex Austin TX USA), including interleukin (IL)-1 receptor antagonist (IL-1RA), IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, tumour necrosis factor-alpha, interferon gamma, CD223, CCL2, CCL3, CCL4, CCL7, CCL8, CCL18, CCL19, CXCL9, CXCL10, CXCL11, CXCL14, osteoblast-specific factor 2, granulocyte-macrophage colony-stimulating factor, connective tissue growth factor (CTGF), matrix metalloproteinase (MMP)-1, MMP-7, soluble programmed death-1, P-selectin, YKL-40, serum amyloid A1, soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1 and galectin-3. Surfactant protein D was analyzed by the enzyme-linked immunosorbent assay (bio-techne®), and Krebs von den Lungen 6 (KL-6) was analyzed by chemiluminescence enzyme immunoassay (Lumipulse®).

Pulmonary function tests

PFT values were expressed as percentage of the predicted values. A PFT change less than 10% in predicted forced vital capacity (FVC) and less than 15% in single-breath diffusing capacity for carbon monoxide (DLCO) was defined as stable [8].

HRCT imaging

Two experienced chest radiologists independently and blindly reviewed the two time points HRCTs, at baseline and preferable at one year after treatment initiation. In case of discrepancies, a pulmonologist was consulted to reach consensus. The dominant ILD patterns were categorised into fibrotic (usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), and pleuroparenchymal fibro-elastosis (PPFE)) or inflammatory (lymphocytic interstitial pneumonia (LIP), cellular and mixed NSIP, and organising pneumonia (OP)).

Statistical analysis

Differences among groups were determined using the Wilcoxon rank sum test for continuous variables and the Fisher's exact test for categorical variables. The difference between biomarkers at baseline and after one year was examined with Wilcoxon signed rank test. Prediction using biomarkers for determining outcome of PFT and HRCT were examined by logistic regression. Risk factors and biomarkers with a univariate p value less than 0.2 were included in the multivariable logistic regression [9]. Correlations between the variation of biomarkers and PFT after one year of treatment were examined using Spearman's rank correlation coefficient. A p value <0.05 was considered statistically significant. All statistical analyses were performed using R 3.5.1.

Results

Patient characteristics

Serum samples of sixteen patients were available at baseline and after one year. The median follow-up duration was 36 months (IQR 30.5–48.8). The established CTD diagnoses, comorbidities, immunosuppressive therapies, and HRCT patterns were summarized in Table 1. In twelve (75%) patients, two or more immunosuppressants were combined. Regarding HRCT patterns, thirteen patients had an inflammatory and three a fibrotic pattern.

Table 1. Baseline demography

Characteristic	Patients, n (%)
Female	12 (75)
Age (years), median (IQR)	51 (45–62)
Disease duration of CTD (months), median (IQR)	6 (2–22)
CTD	
SSc	5 (31.3)
Sjogren's syndrome	1 (6.3)
Myositis	6 (37.5)
Rheumatoid arthritis	1 (6.3)
SLE	1 (6.3)
MCTD	1 (6.3)
UCTD	1 (6.3)
Comorbidities	
Coronary artery disease	2 (12.5)
Congestive heart failure	0
Pulmonary hypertension	0
Diabetes mellitus	0
Cerebrovascular event	1 (6.3)
Obesity (BMI ≥ 35)	0
Smoking status	
Current	0
Former	8 (50)
Never	8 (50)
Immunosuppressive treatment	
Azathioprine	2 (12.5)
Mycophenolate mofetil	7 (43.8)
Methotrexate	3 (18.8)
Hydroxychloroquine	6 (37.5)
Cyclosporin	1 (6.3)
Rituximab	1 (6.3)
HSCT	1 (6.3)
Steroid	11 (68.8)
Fibrotic HRCT patterns	
UIP	0
Fibrotic NSIP	2 (12.5)
PPFE	1 (6.3)
Inflammatory HRCT patterns	
Cellular NSIP	6 (37.5)
Mixed NSIP	4 (25)
NSIP/OP	2 (12.5)
LIP/NSIP	1 (6.3)

Abbreviations: IQR, interquartile range; BMI, body mass index; CTD, connective tissue disease; HRCT, high resolution computed tomography; HSCT, hematopoietic stem-cell transplantation; LIP, lymphocytic interstitial pneumonia; MCTD, mixed connective tissue disease; NSIP, non-specific interstitial pneumonia; OP, organising pneumonia; PPFE, pleuroparenchymal fibro-elastosis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease; UIP, usual interstitial pneumonia.

Clinical features and imaging

The FVC after one year of treatment improved in eight (50%) patients, stabilized in six (37.5%), and declined in two (12.5%). In patients with inflammatory HRCT patterns, there was one (7.7%) patient with FVC decline; furthermore, one (33.3%) patient with a fibrotic HRCT pattern had FVC decline. The DLCO improved in seven (53.8%) patients, stabilized in five (38.5%), and declined in one (7.7%) patient with inflammatory HRCT patterns. One patient with a baseline fibrotic HRCT pattern did not receive follow-up HRCT. Progression of fibrosis on HRCT was seen in three (20%) patients and stable fibrosis in 12 (80%) patients. Progression of inflammation on HRCT was also seen in three (20%) patients; six (40%) were stable, and six (40%) showed regression in inflammation.

Biomarkers and lung function

Biomarkers measured at baseline are visualized in a heatmap. (Figure 1) Patients with the same CTD seem to cluster together. Increase in levels of galectin-3 at one-year follow-up was associated with improved FVC (Rho 0.5, p = 0.048). (Figure 2D) Other biomarkers did not demonstrate a direct correlation with FVC or DLCO, and none were predictive for significant FVC or DLCO decline in logistic regression at follow-up. (Table S1)

Biomarkers and HRCT

Two of the biomarkers showed significant difference at baseline between patients with fibrotic and inflammatory HRCT patterns. MMP-1 was higher in patients with fibrotic than inflammatory HRCT patterns with a median 158,389.5 pg/ml (IQR 144,169.6–214,009.7) and 52,385.3 pg/ml (IQR 2.4×10^4–6.7×10^4), respectively (p= 0.004). Galectin-3 was lower in patients with fibrotic than inflammatory HRCT patterns with a median 75,281.2 pg/ml (IQR 46,455.2–82,916.8) and 100,175.8 pg/ml (IQR 89,519–114,321), respectively (p= 0.039). A decrease in CXCL11, KL-6, and CTGF was observed in patients with inflammatory HRCT patterns (n = 13), and these markers were associated with less HRCT progression and FVC decline. (Figure 2) CXCL11 reduced from a median 307.8 pg/ml (IQR 200.6–517.3) to 253.8 pg/ml (IQR 191.8–344.5, p-value 0.011), CTGF from 48.5 pg/ml (IQR 4.7–110.7) to 9.5 pg/ml (IQR 4.7–36.9, p-value 0.033) and KL-6 from 1221 U/ml (IQR 361–1769) to 543 U/ml (IQR 303–1419, p-value 0.040). None of the baseline serum biomarkers could predict progression of inflammation or fibrosis on HRCT in logistic regression. (Table S1)

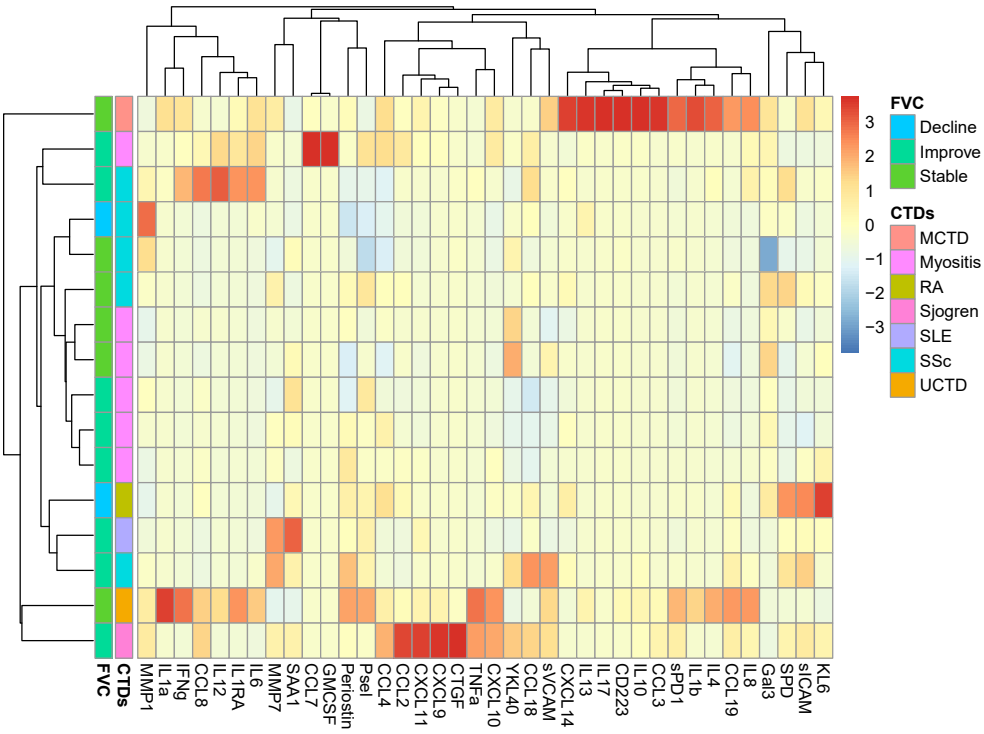

Figure 1. Baseline biomarkers heatmap.
Hierarchical clustering of biomarkers measured at baseline was visualized in the heatmap. The concentration of each biomarker was normalized and presented in colour, the closer to red the higher. MCTD, mixed connective tissue disease; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; UCTD, undifferentiated connective tissue disease. KL6, Krebs von den Lungen 6; sICAM, soluble intercellular adhesion molecule-1; SPD, surfactant protein D; Gal3, Galectin-3; sPD1, soluble programmed death-1; sVCAM, soluble vascular cell adhesion molecule-1; YKL40, also known as chitinase 3-like 1; TNFa, tumor necrosis factor alpha; CTGF, connective tissue growth factor; Psel, P-selectin; GMCSF, granulocyte-macrophage colony-stimulating factor; MMP, matrix metalloproteinase; SAA1, serum amyloid A1; IL1RA, interleukin (IL)-1 receptor antagonist; IFNg, interferon gamma.

Discussion

In this study, we investigated 38 biomarkers in relation to radiologic patterns of pulmonary inflammation and fibrosis and clinical response in patients with CTD-ILD. We found that levels of CXCL11, CTGF, and KL-6 decreased in patients with inflammatory HRCT patterns during treatment, which was associated with better pulmonary outcome compared to patients with fibrotic HRCT patterns. In addition, there was a positive correlation between changes in galectin-3 and FVC. While FVC improved after treatment, the level of galectin-3 increased.

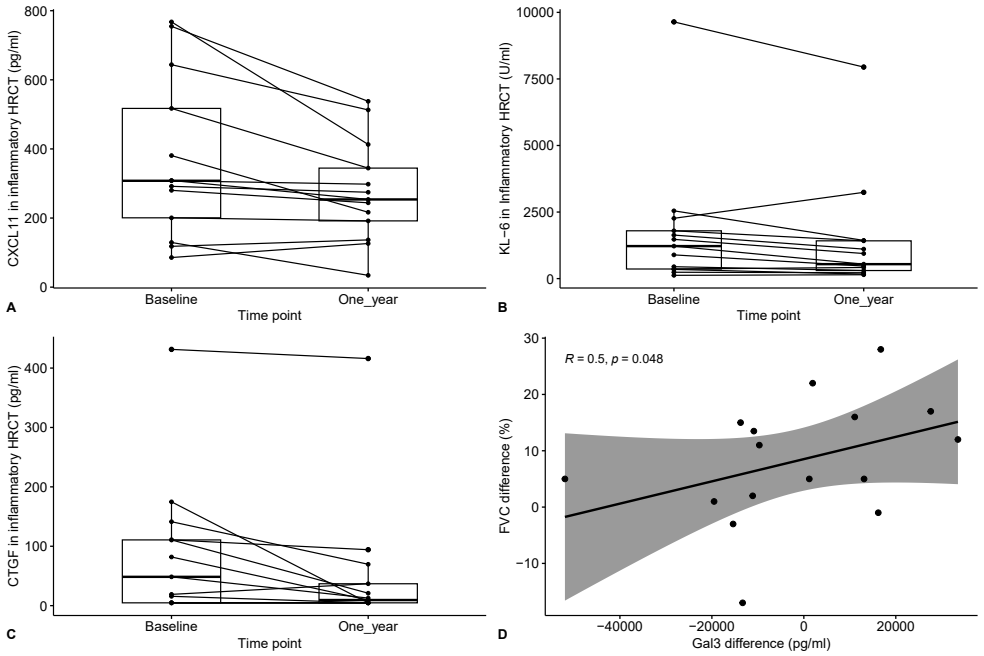

Figure 2. Decline in CXCL11 (A), Krebs von den Lungen 6 (KL-6) (B), and connective tissue growth factor (CTGF) (C) over one year of treatment was associated with inflammatory high resolution computed tomography (HRCT) pattern. (D) Correlation between changes of Galectin-3 (Gal3) and forced vital capacity (FVC) after one year of treatment. An increase of Galectin-3 reflected the improvement of lung function.

We observed that KL-6, a glycoprotein expressed on epithelial cells and regenerated type II pneumocytes [10], dropped in patients with inflammatory HRCT patterns during treatment with immunosuppressants. In previous studies, KL-6 was found to correlate with disease extent and increased mortality in CTD-ILD [4, 5, 10, 11]; our finding is in line with that sequential changes of KL-6 play a role in monitor ILD progression [11, 12].

New biomarkers, associated with clinical response, that were identified in our study are serum CTGF and CXCL11. CTGF is a profibrotic growth factor produced by fibroblasts in response to TGFβ and CXCL11 is a ligand of CXCR3, involved in pulmonary vascular remodelling and fibrosis [13, 14]. These two new biomarkers may therefore have additional value as predictive biomarkers in CTD-ILD and need to be evaluated in a larger population.

Another new finding in our study was the relation between increased serum galectin-3 and improvement in FVC during treatment. Galectins are involved in inflammation, cell migration, autophagy, and signalling. Serum concentrations of galectin-3 were found elevated in IPF and CTD-ILD patients in previous studies [15]. In contrast to the previous study that showed higher baseline galectin-3 level was associated with poor outcome,

our study suggested that raised serum galectin-3 during follow-up pointed to improved lung function. A possible explanation could be that galectin-3 plays different roles in patients with IPF and CTD-ILD.

Interestingly, the visualized biomarkers heatmap showed that the biomarker concentrations are determined less by ILD but more by underlying CTD manifestations. These provide a hint that while researchers exploit cytokines and chemokines as biomarkers, underlying inflammatory pathophysiology of CTD should be considered as effect modifiers.

Our study has some limitations. Firstly, the number of patients with CTD-ILD and available serum samples was low, and the number of patients with stable ILD was relatively large. Consequently, the comparison between patients with and without response and the baseline biomarkers predictivity were limited. Secondly, because of the retrospective character of our study, there were some missing data.

In conclusion, decrease in concentrations of CXCL11, CTGF, and KL-6 during treatment was associated with inflammatory HRCT patterns and better pulmonary outcome. In contrast to previous research in ILD, we found a positive correlation between changes in galectin-3 concentrations and FVC. Our findings need to be confirmed in a larger cohort and focusing on combining biomarkers to predict outcome. Also, further research into individual biomarker profiles and biomarker-based therapy is important to pave the way towards precision medicine in CTD-ILD.

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Supplementary

Table S1. P value of univariate risk factor analysis of baseline biomarkers

Baseline biomarkers	FVC decline	DLCO decline	Fibrosis progression on HRCT	Inflammation progression on HRCT
IL-1RA	0.99	0.45	0.99	0.85
IL-1α	0.99	0.78	0.99	0.78
IL-1β	0.71	0.99	0.65	0.45
IL-4	0.99	0.76	0.99	0.50
IL-6	0.56	0.29	0.60	0.50
IL-10	0.99	0.83	0.99	0.47
IL-12	0.99	0.31	0.99	0.67
IL-13	0.98	0.84	0.99	0.39
IL-17	0.99	0.99	0.99	0.99
TNFα	0.99	0.99	0.99	0.79
IFNγ	0.99	0.91	0.99	0.70
CD223	0.99	0.82	0.75	0.42
CCL2	0.74	0.49	0.46	0.44
CCL3	0.99	0.99	0.99	0.43
CCL4	0.92	0.28	0.54	0.84
CCL7	0.99	0.99	0.99	0.58
CCL8	0.59	0.88	0.49	0.39
CCL19	0.76	0.73	0.45	0.61
IL-8	0.99	0.95	0.99	0.36
CXCL9	0.64	0.94	0.46	0.39
CXCL10	0.50	0.53	0.38	0.75
CXCL11	0.45	0.99	0.21	0.37
CXCL14	0.76	0.47	0.86	0.29
OSF2	0.45	0.78	0.32	0.59
GMCSF	0.99	0.99	0.99	0.99
CTGF	0.74	0.65	0.79	0.83
MMP7	0.31	0.82	0.16	0.28
sPD1	0.64	0.76	0.54	0.42
P-selectin	0.48	0.31	0.61	0.17
YKL-40	0.71	0.75	0.61	0.20
CCL18	0.79	0.55	0.29	0.12
SAA1	0.67	0.68	0.42	0.71
sICAM	0.21	0.49	0.56	0.54
sVCAM	0.46	0.64	0.28	0.07
MMP1	0.19	0.69	0.43	0.84
Gal3	0.59	0.73	0.22	0.44
KL-6	0.15	0.41	0.22	0.76
SPD	0.15	0.57	0.75	0.79

IL-1RA, IL-1 receptor antagonist; TNFα, tumour necrosis factor α; IFNγ, interferon γ; OSF2, osteoblast-specific factor 2; GMCSF, granulocyte-macrophage colony-stimulating factor; CTGF, connective tissue growth factor; MMP, matrix metalloproteinase; sPD1, soluble programmed death-1; SAA1, serum amyloid A1; sICAM, soluble intercellular adhesion molecule-1; sVCAM, soluble vascular cell adhesion molecule-1; Gal3, galectin-3.

Chapter 5

KL-6 as a biomarker of interstitial lung disease development in patients with Sjögren syndrome: a retrospective case-control study

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Abstract

Objectives

Krebs von den Lungen-6 (KL-6) is expressed on regenerating type II pneumocytes and has been recognized as biomarkers in interstitial lung disease (ILD). We aim to identify the role of the serum KL-6 level in patients with newly diagnosed Sjögren syndrome (SS), as well as the correlation between the immunoassays.

Methods

Patients with newly diagnosed SS and receiving HRCT for clinical reason during follow-up were included. Baseline KL-6 level was measured via enzyme-linked immunosorbent assay (ELISA) and latex particle-enhanced turbidimetric immunoassay (LETIA).

Results

Of the 39 patients, 21 (53.85%) developed interstitial lung disease (ILD) by the conclusion of the follow-up period. The median time to diagnosis of ILD was 2.24 years (IQR 1.15–4.34) in the ILD group. The median serum KL-6 level, measured using ELISA, was 1232 U/ml (IQR 937–2242) and 764.5 U/ml (IQR 503.25–1035.75) in the ILD group and the non-ILD group, respectively ($p = 0.001$). The median LETIA for serum KL-6 was 329 U/ml (IQR 235–619) and 245 U/ml (IQR 215.25–291) in the ILD group and the non-ILD group, respectively ($p = 0.074$).

Conclusion

Serum KL-6 levels were higher in newly diagnosed SS patients with ILD diagnosis during follow-up. Thus, the serum KL-6 level can serve as a valuable biomarker to identify hidden ILD in patients with newly diagnosed SS patients. However, the immunoassay procedure may influence the efficacy of the prediction and its clinical association.

Introduction

Sjögren syndrome (SS) is an autoimmune disease presenting with exocrine gland inflammation and extra-glandular involvement [1]. Approximately one-fifth of primary SS (pSS) patients presented symptomatic pulmonary involvement with reduced quality of life and a fourfold increase in their mortality rate [2]. However, a high fraction of pSS patients without clinical symptoms could be identified abnormality on pulmonary high resolution computed tomography (HRCT) [3].

Krebs von den Lungen-6 (KL-6), also called episialin, was initially identified using a murine IgG1 monoclonal antibody in a BALB/c mouse, immunized with a human pulmonary adenocarcinoma cell line [4]. It is a membrane-associated glycoprotein, which was classified as cluster 9 mucin-1 (MUC1), that is expressed in epithelial cells, especially type II pneumocytes, respiratory bronchiolar epithelial cells, bronchial gland serous cells, fundic gland cells, ductal epithelial cells of mammary glands, pancreas and salivary glands [5, 6]. Furthermore, it is highly expressed in regenerating pneumocytes from patients with ILD and adenocarcinoma of the lung, pancreas, or breast; whereas in healthy lung tissue, type I pneumocytes, goblet cells, and mucous cells of the bronchial glands do not express KL-6 [7]. The concentration of KL-6 is highest in the epithelial lining fluid, followed by the serum and then the bronchoalveolar lavage fluid, which suggests a high permeability through the air-blood barrier [7]. KL-6 also functions as a chemotactic factor for human fibroblasts, and the high KL-6 concentration found in epithelial lining fluid may trigger intra-alveolar fibrosis [8].

Other biomarkers, including anti-Ro antibodies and complement 3 (C3), were reported to be associated with the development of ILD in a cohort of 315 Chinese pSS patients [9]. However, the serum KL-6 level is directly related to the process of recovery from pneumonitis and the following fibrosis. We aim to identify the role of the serum KL-6 level in patients with newly diagnosed SS, as well as the correlation between the latex particle-enhanced turbidimetric immunoassay (LETIA) with enzyme-linked immunosorbent assay (ELISA) and association with clinical phenotypes.

Material and Methods

Study subjects

In this retrospective case-control study, patients who were diagnosed with pSS between 2011 and 2018 fulfilled the American-European Consensus Group Criteria for Sjögren's Syndrome, and had an available baseline serum sample for KL-6 level evaluation, were included [1]. All patients received a chest high resolution computed tomography

(HRCT) for clinical reason in the follow-up period. Clinical information and laboratory results on inclusion were retrieved from electronic medical records. A diagnosis of ILD was established by the pulmonologist, and the HRCT patterns were recorded according to the classification of idiopathic interstitial pneumonia [10]. The results of pulmonary function testing and pulmonary arterial pressure detected by transthoracic echocardiography at the end of follow-up were recorded. An experienced pulmonologist reviewed all pulmonary imaging, including a chest X-ray and HRCT.

The KL-6 level at baseline was detected with ELISA (MBS2601395; MyBioSource, CA, USA) and LETIA (Nanopia), following the manufacturer's instructions. Immunologic assay on inclusion included antinuclear antibody (ANA), anti-Ro/La, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), complement 3 (C3) and complement 4 (C4). This study was approved by the Institutional Review Board of Tri-Service General Hospital and the National Defense Medical Center in Taiwan (1-107-05-137).

Statistical analysis

We used the Chi-square test to compare categorical variables. The continuous variables were tested using the Wilcoxon rank sum test or t test according to the Shapiro-Wilk normality test. Correlation between continuous variables was examined with Spearman's correlation. A p-value <0.05 was considered statistically significant. Biomarker-predicted values were estimated with logistic regression, receiver-operating characteristic (ROC) curve and the area under curve (AUC). We use Youden's J statistic to find an optimal cut-point. The effect size of independent two-sample comparison was examined in power analysis. Statistical analyses were conducted using the R software, version 3.5.2.

Results

Of the 39 patients with pSS, 21 (53.85%) had developed ILD by the end of the follow-up period, with a median of 2 years (IQR 4.47–0.91). The median age of the participants was 59 years (IQR 53–63). Women comprised 87.2% of the participants. The median time to diagnosis of ILD was 2.24 years (IQR 1.15–4.34) in the ILD group. There were coexisting malignancies in seven patients, including two patients with breast cancer and one patient with lung adenocarcinoma in the non-ILD group and one with breast cancer, one with lymphoplasmacytic lymphoma, one with pancreatic adenocarcinoma, and one with gastric mucosa-associated lymphoid tissue lymphoma in the ILD group. Five patients had non-productive cough lasting for at least 3 months at baseline, including two in the ILD group and three in the non-ILD group. Other baseline characteristics including European League Against Rheumatism Sjögren's syndrome disease activity index (ESSDAI), immunomodulators, and comorbidities did not show a difference between

the ILD and non-ILD groups (Table 1). Five patients received labial minor salivary gland biopsy with a focus score 1, three of whom were in the ILD group and two of whom were in the non-ILD group.

Table 1. Patient characteristics

Characteristics	Total	ILD (n = 21)	Non-ILD (n = 18)	p-value
Age (years)	57.41 (10.98)	59.76 (8.94)	54.67 (12.66)	0.220
Female, n (%)	34 (87.18%)	18 (85.71%)	16 (88.89%)	1
Follow-up period (years)	2 [0.91; 4.47]	2.24 [1.15; 4.34]	1.95 [0.74; 4.85]	0.806
ESSDAI	5 [2; 7]	4 [2; 7]	5 [3.25; 7]	0.327
Non-productive cough, n (%)	5 (7.80%)	2 (9.52%)	3 (16.67%)	0.853
Immunomodulators, n (%)				
Hydroxychloroquine	36 (92.31%)	20 (95.24%)	16 (88.89%)	0.889
Methotrexate	3 (7.69%)	1 (4.76%)	2 (11.11%)	0.889
Azathioprine	9 (23.08%)	3 (14.29%)	6 (33.33%)	0.305
Prednisolone	6 (15.38)	4 (19.05)	2 (11.11)	0.811
Smoking, n (%)	2 (5.13%)	1 (4.76%)	1 (5.56%)	1
Hypertension, n (%)	10 (25.64%)	5 (23.81%)	5 (27.78%)	1
Diabetes, n (%)	2 (5.13%)	1 (4.76%)	1 (5.56%)	1
Asthma, n (%)	3 (7.69%)	2 (9.52%)	1 (5.56%)	1
COPD, n (%)	2 (5.13%)	1 (4.76%)	1 (5.56%)	1
Coexisting malignancy, n (%)	7 (17.95%)	4 (19.05%)	3 (16.67%)	1

Data were presented as mean (standard deviation) or median [interquartiles] according to normality. ILD: interstitial lung disease; ESSDAI, the EULAR Sjögren's syndrome disease activity index; COPD: chronic obstructive pulmonary disease.

Baseline serum and clinical markers are summarized in Table 2, with missing data indicated. Two patients were not arranged pulmonary function test and echocardiogram was not available in 16 patients. The median serum KL-6 level, measured using ELISA, was 1232 U/ml (IQR 937–2242) and 764.5 U/ml (IQR 503.25–1035.75) in the ILD group and the non-ILD group, respectively (p = 0.001). (Figure 1) The median serum KL-6 in LETIA was 329 U/ml (IQR 235–619) and 245 U/ml (IQR 215.25–291) in the ILD group and the non-ILD group, respectively (p = 0.074). The effect size was 0.528 for ELISA KL-6 and 0.289 for LETIA KL-6. Serum KL-6 did not differ between patients with and without coexisting malignancy (p = 0.770 for ELISA and 0.985 for LETIA). Serum KL-6 level did not differ between patients with and without positive anti-Ro, including ELISA (p = 0.529) and the LETIA (p = 0.867). The correlation between ELISA and LETIA for serum KL-6 was 0.40 (p = 0.012). The correlation between ESSDAI and KL-6 was insignificant in ELISA (r = 0.01, p = 0.929) and low in LETIA (r = 0.32, p = 0.048).

Table 2. Baseline serum and clinical markers

Biomarkers	Total	ILD	Non-ILD	p-value
KL-6 (ELISA, U/ml)	997 [757; 1497]	1232 [937; 2242]	764.5 [503.25; 1035.75]	0.001
KL-6 (LETIA, U/ml)	258 [219.5; 435.5]	329 [235; 619]	245 [215.25; 291]	0.074
LDH (U/L)	185.67 (39.61) Missing: 18	188.42 (38.29)	182 (43.36)	0.729
ANA	320 [40; 640] Missing: 2	320 [40; 640]	160 [30; 1280]	0.565
ANA 1:80, n (%)	26 (70.27%)	15 (71.43%)	11 (68.75%)	1
Anti-Ro (U/ml)	240 [47; 240]	234 [21; 240]	240 [156.25; 240]	0.373
Anti-Ro positive, n (%)	34 (87.18%)	18 (85.71%)	16 (88.89%)	1
Anti-La (U/ml)	0.7 [0; 13.3]	0.4 [0; 1.9]	0.85 [0.03; 17.65]	0.408
Anti-La positive, n (%)	11 (28.21%)	5 (23.81%)	6 (33.33%)	0.763
Anti-dsDNA, n (%)	1 (2.56%)	0	1 (5.56%)	0.938
ACPA, n (%)	1 (2.56%)	0	1 (5.56%)	0.938
ATA, n (%)	1 (2.56%)	1 (5.88%)	0	1
Anti-B2GP, n (%)	1 (2.56%)	0	1 (5.56%)	0.938
AMA, n (%)	1 (2.56%)	1 (5.88%)	0	1
C3 (mg/dL)	100.97 (19.67) Missing: 4	99.9 (14.63)	101.98 (2.87)	0.757
Low C3, n (%)	4 (11.43%)	1 (5.88%)	3 (16.67%)	0.638
C4 (mg/dL)	21.97 (6.4) Missing: 4	22.26 (7.06)	21.68 (6.05)	0.796
Low C4, n (%)	8 (22.86%)	4 (23.53%)	4 (22.22%)	1

Data were presented as mean (standard deviation) or median [interquartile range] according to normality. KL-6: Krebs von den Lungen-6, ELISA: enzyme-linked immunosorbent assay, LETIA: latex particle-enhanced turbidimetric immunoassay, LDH: lactate dehydrogenase, ANA: antinuclear antibodies, ACPA: anti-citrullinated protein antibodies, ATA: anti-topoisomerase I antibodies (anti-Scl-70), Anti-B2GP: anti-beta 2-glycoprotein, and AMA: Antimitochondrial antibodies.

The AUC of the ROC curve was 0.810 and 0.669 on ELISA and LETIA of serum KL-6, respectively (Figure 2). The optimal cut-point was 922 U/ml and 402 U/ml for ELISA and LETIA of serum KL-6, respectively. The risk factors for ILD are summarized in Table 3. Serum KL-6 level higher than the cut-point revealed an increased risk of ILD, odds ratio 12 (95%CI 2.51, 57.48) in ELISA and 7.27 (95%CI 1.33, 39.86) in LETIA. The rest of the biomarkers did meet the significance level to incorporate into multivariable adjustment [11].

At the end of follow-up, the median percentage of predicted forced vital capacity (FVC) was 84.00 (IQR 74.30–88.00) and 86.00 (IQR 74.80–90.40) in the ILD and non-ILD groups, respectively (p-value 0.798); the median diffusing capacity for carbon monoxide (DLCO) was 72.55 (IQR 59.80–84.28) and 90.35 (IQR 83.15–101.50) in the ILD and non-ILD groups, respectively (p-value <0.001). The median pulmonary artery pressure measured by transthoracic echocardiography was 36.00 (IQR 28.50–40.50) mmHg in patients with ILD and 28.00 (IQR 27.00–36.00) in patients without ILD (p = 0.292).

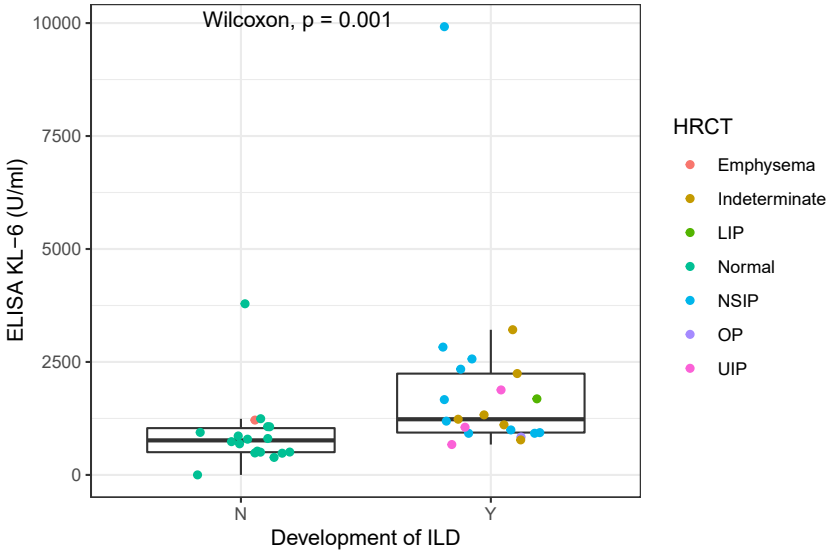

Figure 1. The KL-6 level, measured using enzyme-linked immunosorbent assay, in patients with and without interstitial lung disease (ILD) development, shows significantly higher values in patients with the development of ILD. Colors discriminated against different CT patterns. N and Y represent the non-ILD and ILD groups, respectively. LIP, lymphocytic interstitial pneumonitis; NSIP, nonspecific interstitial pneumonia; OP, organizing pneumonia; UIP, usual interstitial pneumonia; ILD not meeting above-mentioned patterns was recorded as indeterminate.

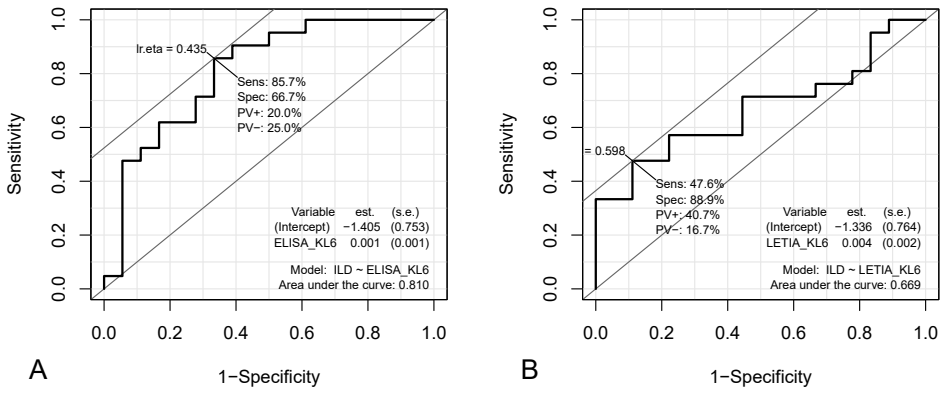

Figure 2. Receiver-operating characteristic curve analysis of two methodologies of serum KL-6. Investigation of the ability of KL-6 level to identify the development of ILD in patients with Sjögren syndrome revealed an area under the curve of 0.810 and 0.669 on enzyme-linked immunosorbent assay (ELISA, A) and latex particle-enhanced turbidimetric immunoassay (LETIA, B) of serum KL-6, respectively.

Table 3. Predictors for interstitial lung disease in patients with Sjögren syndrome

Biomarkers	OR (95%CI)	P
ELISA KL-6	12 (2.51,57.48)	< 0.001
LETIA KL-6	7.27 (1.33,39.86)	0.011
Old age	1.56 (0.32,7.7)	0.58
Smoking	0.85 (0.05,14.64)	0.911
Hypertension	0.81 (0.19,3.43)	0.777
Diabetes	0.85 (0.05,14.64)	0.911
Asthma	1.79 (0.15,21.54)	0.639
COPD	0.85 (0.05,14.64)	0.911
ESSDAI	0.96 (0.83,1.1)	0.539
ANA positive	1.56 (0.41,5.95)	0.518
Anti-Ro positive	0.75 (0.11,5.07)	0.767
Anti-La positive	0.62 (0.15,2.54)	0.51
Low C3	0.31 (0.03,3.34)	0.305
Low C4	1.08 (0.22,5.22)	0.927

The two methods of KL-6 were defined as positive with a greater than calculated cut-point by Youden's J statistic. Age greater than 65 was defined as old age. COPD: chronic obstructive pulmonary disease; ESSDAI, the EULAR Sjögren's syndrome disease activity index; ANA greater than 1:80 was defined as positive. ELISA: enzyme-linked immunosorbent assay, LETIA: latex particle-enhanced turbidimetric immunoassay, LDH: lactate dehydrogenase, ANA: antinuclear antibody.

Discussion

KL-6 is a mucin-like high-molecular-weight glycoprotein expressed in regenerating type II pneumocytes [12]. Serum KL-6, measured with various immunoassays, has shown diagnostic and prognostic value in ILD. Immunoassays with ELISA, LETIA, and chemiluminescent enzyme immunoassay (CLEIA) have been reported in studies. Its levels are highly associated with ILD activity in patients with radiation pneumonitis, idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), hypersensitivity pneumonitis, sarcoidosis, rheumatoid arthritis (RA), polymyositis, dermatomyositis, systemic sclerosis (SSc), and a combination of autoimmune diseases, which were primarily measured by ELISA [5, 13-22]. LETIA KL-6 has demonstrated higher levels in patients with CTD-ILD and provided an independent prognostic prediction for the mortality of patients with RA- associated ILD [23, 24]. Moreover, serial change in serum KL-6, measured in CLEIA, was associated with the improvement of lung function and HRCT in patients with IPAF [25, 26]. In a recent trial with newly diagnosed CTD patients, serum KL-6 was higher in CTD-LID patients than patients with non-ILD CTD, pneumonia, and healthy population; there was also a correlation between serum KL-6 and extent of HRCT involvement [22]. Serum KL-6 increased mortality hazard with a cutoff value greater than 800 U/mL in a Japanese cohort [27]. An increase in the levels of KL-6 in the serum might reflect an increase in the number of regenerating type II

pneumocytes, secondary to pulmonary damage [21]. Our data also suggested that serum KL-6 level is higher in pSS patients with insubstantial chest X-ray changes (non-radiographic) and developing ILD. Furthermore, KL-6 tended to induce chemotaxis, proliferation and inhibit apoptosis of human fibroblast [8, 28]. However, the predictivity of KL-6 may vary according to the immunoassay methodology. Serum KL-6 in both immunoassays was elevated in patients with pSS who developed ILD during follow-up, but the LETIA did not achieve significance. Other markers, such as autoimmunity markers (ANA, anti-Ro/La, C3, and C4), inflammation markers (ESR and CRP), and LDH, did not exhibit a significant difference [29-31]. Although LETIA is much more efficient than ELISA, only requiring approximately 10 minutes with an automated clinical chemistry analyzer versus 4 hours, respectively, the implication regarding clinical association needs to be carefully taken into consideration.

Pulmonary symptoms are present in 6–15% of the patients with pSS; among these, dyspnea and cough are the most common [29, 30]. Although most patients with pulmonary involvement are non-symptomatic, they present with functional decline [32]. There was a lower DLCO in patients with ILD. FEV1, FVC, FEV1/FVE, or pulmonary arterial pressure, upon transthoracic echocardiography, did not reveal a significant difference. The decline in DLCO was most likely the result of an ILD other than restrictive lung disease, obstructive lung disease, or pulmonary hypertension.

ILD may be highly misdiagnosed or delay-diagnosed in patients with pSS [3], although the progression of ILD is relatively less frequent in patients with pSS than other CTD [33]. The pSS patients who developed ILD exhibited increased morbidity and mortality, and required additional medical resources. Our data support that KL-6 is a valuable biomarker in identifying hidden ILD in patients with pSS.

This study was a retrospective case-control study with small sample size and possible selection bias. The effect size in power analysis was large enough for ELISA KL-6 but small for LETIA KL-6. The HRCT was performed at the end of follow-up, but not on inclusion. There might be hidden ILD patients on inclusion until they received HRCT. Except for KL-6 level, other serum biomarker levels were measured clinically with existing missing data. Moreover, although the serum KL-6 level was associated with ILD and adenocarcinoma in the literature review, the coexisting malignancy did not significantly affect the serum KL-6 level in both ELISA and LETIA.

Conclusion

Serum KL-6 levels were significantly higher in SS patients with developing ILD and might denote early pulmonary damage. Serum KL-6 level can serve as a valuable biomarker for identifying hidden ILD in patients with newly diagnosed SS, and regular screening may

be warranted. However, the methodology of immunoassay may influence the efficacy of the prediction and clinical association.

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Chapter 6

Autologous stem-cell transplantation in systemic sclerosis-associated interstitial lung disease: early action in selected patients rather than escalation therapy for all

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Abstract

Systemic sclerosis (SSc) is a rare rheumatic disease characterised by inflammation, vasculopathy and fibrosis of skin and internal organs. A common complication and a leading cause of death in SSc is interstitial lung disease (ILD). The current armamentarium of treatments in SSc-ILD mainly includes immunosuppressive therapies and has recently been expanded with anti-fibrotic agent nintedanib. Autologous stem cell transplantation (SCT) is increasingly used in progressive diffuse cutaneous SSc. This intensive treatment has been studied in three randomised trials and demonstrated to improve survival and quality of life. In the subsets of patients with SSc-ILD, SCT resulted in stabilisation and modest improvement of lung volumes and disease extent on high resolution computed tomography, but less impact was seen on diffusion capacity. Comparison of SCT outcomes with results from SSc-ILD trials is difficult though, as lung involvement per se was not an inclusion criterion in all SCT trials. Also, baseline characteristics differed between studies. The risk of severe treatment-related complications from SCT is still considerable and patients with extensive lung disease are particularly at risk of complications during transplantation. Therefore SCT should only be provided by experienced multidisciplinary teams in carefully selected patients. Future research needs to include comprehensive pulmonary evaluation and establish whether SCT early in the disease might prevent irreversible pulmonary damage and reduce treatment-related complications. Also, more insight in mechanisms of action of SCT in the lung and predictors for response will improve the use of this treatment in SSc-ILD. In this review the role of SCT in the treatment of SSc-ILD is summarised.

Introduction

Systemic sclerosis (SSc) is a rare connective tissue disease characterised by inflammation, vasculopathy and fibrosis of skin and internal organs [1]. The clinical presentation of SSc is heterogenous and manifestations range from limited skin thickening to generalised skin involvement with severe internal organ damage. In the diffuse cutaneous disease subset (dcSSc) major organ involvement (heart, kidney and lungs) are common [2]. Notably, pulmonary complications such as interstitial lung disease (ILD) compromise quality of life and are the leading cause of death in SSc.

In the last years the understanding of pathogenic pathways has improved. Damage to alveolar epithelial and endothelial cells leading to inflammation are regarded as the first central events in SSc-ILD [3]. Ongoing damage and impaired healing of lung tissue together with aberrant innate and adaptive immune responses and myofibroblast function are believed to create a profibrotic milieu in the lung [4, 5]. Non-specific interstitial pneumonia is the most commonly observed radiological and histological pattern in SSc-ILD [6]. Other patterns include usual interstitial pneumonia, organising pneumonia and diffuse alveolar damage.

Risk factors for development of SSc-ILD include dcSSc, shorter disease duration, male sex and older age at disease onset [7, 8]. Also the presence of anti-topoisomerase I antibodies has been identified as a predictor for SSc-ILD [9]. The clinical course of SSc-ILD is variable as some patients have stable disease while others develop extensive and progressive disease [10, 11]. Therefore, pulmonary function tests (PFTs) and chest high resolution computed tomography (HRCT) play a central role in detection and follow-up of SSc-ILD [12].

Current management options of SSc-ILD include immunosuppressive therapies and the recently approved anti-fibrotic agent nintedanib. In the case of refractory ILD, lung transplantation can be considered [13]. Treatment recommendations and algorithms published over the years generally place mycophenolate mofetil (MMF) as the preferred first-line therapy and cyclophosphamide (CYC) and rituximab as second and third line, respectively [14-16]. The place of autologous haematopoietic stem cell transplantation (SCT) in SSc-ILD has been a matter of debate. SCT has been shown to improve long-term event-free survival and overall survival in dcSSc patients, but the risk of treatment-related mortality restricts its use to a selection of patients. Notably, in the recently published European consensus statement on management of SSc-ILD, 80% of the expert panel agreed that SCT is a potential treatment in the case of rapid progressive and refractory lung disease [17]. In this review we summarise the evidence on the effects of SCT on SSc-ILD and discuss the potential role of SCT in the treatment of SSc-ILD.

Autologous stem cell transplantation

SCT is an intensive immunomodulating therapy that has been used in the treatment of autoimmune diseases for more than 25 years [18]. In the early years, SCT was mainly used to treat refractory cases with inflammatory arthritis [19]. However, after the introduction of effective and less toxic biologic and other targeted agents, the role of SCT in the treatment of rheumatoid arthritis and juvenile idiopathic arthritis has diminished [20, 21]. In contrast, SCT is still performed in patients with Crohn's disease, multiple sclerosis and SSc [21]. In addition, recent reports on experiences with SCT in systemic lupus erythematosus, Behcet's disease and vasculitis illustrate the need for this treatment in refractory cases of other rare autoimmune conditions [22, 23].

SCT is thought to reset the immune system through elimination of autoreactive immune cells and regeneration of a new, rebalanced immune system. The exact mechanisms driving this reset are, however, not completely known [24]. Autologous SCT consists of four steps (Figure 1). The first step includes mobilisation of haematopoietic stem cells using chemotherapy (mostly CYC) and growth factors [granulocyte colony-stimulating factor (G-CSF)] to stimulate migration of stem cells from bone marrow to the blood so they can be collected using leukapheresis. This step is followed by conditioning which aims to eradicate autoreactive immune cells. Regimens used for conditioning can be either myeloablative or non-myeloablative and vary from high-intensive to intermediate-intensive schemes. In autoimmune diseases non-myeloablative intermediate intensive regimens are most commonly used. The third step is the reinfusion of autologous stem cells. Ex vivo graft selection ($CD34^+$ selection) prior to reinfusion has been a matter of debate, although two studies recently demonstrated superiority of $CD34^+$ selection compared with reinfusion of unselected cells in remission rate [25, 26]. The reinfusion of stem cells shortens aplasia from conditioning and allows a naïve immune system to emerge.

An important issue in SCT is the treatment-related mortality attributed to medication used for mobilisation and conditioning which can lead to severe infections, haemorrhage or cardiopulmonary toxicity. Therefore selection of patients, close monitoring during treatment and an experienced multidisciplinary team are key to ensure optimal and safe treatment. Also, benefits and risks need to be discussed with the patient carefully in order to make a balanced decision about treatment [27].

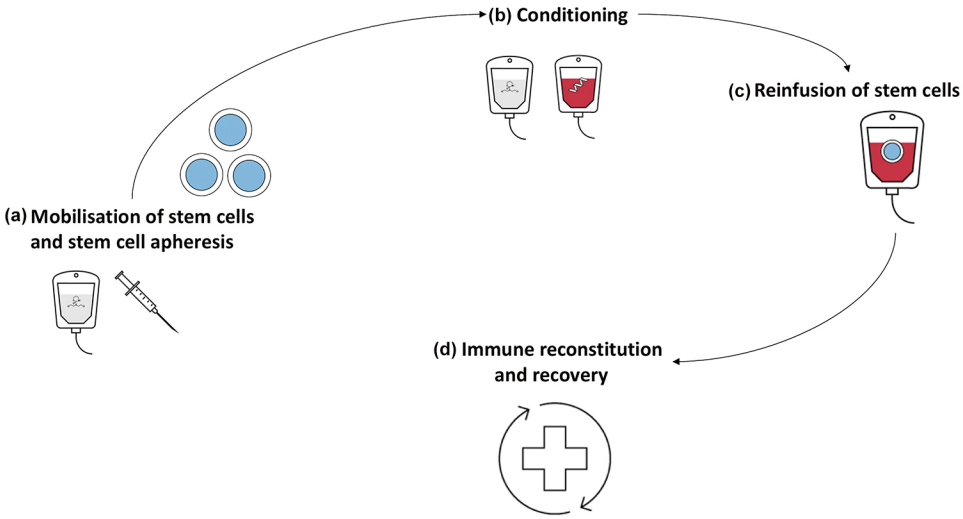

Figure 1. Autologous stem cell transplantation. (a) The first step in stem cell transplantation (SCT) is the mobilisation of stem cells from the bone marrow. This is most often done using chemotherapy, such as cyclophosphamide, to stimulate the production of stem cells in the bone marrow due to cytopenia. Granulocyte colony-stimulating factor is used to further facilitate the production and release of stem cells in the peripheral circulation. Subsequently, the stem cells are harvested using leukapheresis. (b) The next step is conditioning, which takes place approximately 4–6 weeks after mobilisation and leukapheresis. Myeloablative or highly immunosuppressive agents are administered, aiming to eliminate autoreactive B and T cells. Conditioning regimens in SCT for systemic sclerosis often include cyclophosphamide, anti-thymocyte globulin or total body irradiation. (c) Directly after completion of the conditioning scheme, stem cells are reinfused. Mostly graft manipulation is used (CD34+ selection), to improve efficacy of the treatment. (d) The last step involves supportive care during the aplastic phase, which normally takes 1 to 3 weeks until recovery. Full reconstitution of the immune system can take 6–9 months. Depending on the course of the treatment and condition of the patient pre-transplantation rehabilitation takes up several months.

Impact of SCT on SSc-ILD

The benefits of SCT in progressive dcSSc on survival have been demonstrated in three controlled trials [28-30]. In a meta-analysis a reduction of all-cause mortality compared with controls treated with CYC in progressive dcSSc [risk ratio (RR) 0.5 (95% confidence interval (CI), 0.33–0.75] was reported [31]. Quality of life and skin involvement were also significantly better in patients treated with SCT. Although not all SSc patients had lung involvement in these trials and hence pulmonary endpoints were used as the sole primary outcome, the impact on lung disease is reported as co-primary or secondary outcome in all published trials and cohorts. Lung function parameters forced vital capacity (FVC) and diffusing capacity of the lungs for carbon monoxide (DLco) are most often reported and changes observed in HRCTs are described in a couple of studies. Change in pulmonary symptoms, patient reported outcomes or functional scores related to lung disease have not been reported yet.

Impact of SCT on lung function

All three randomised controlled transplant trials in dcSSc report that SCT has beneficial effect on FVC but not significantly on DLco. The ASSIST study (American Scleroderma Stem Cell versus Immune Suppression Trial), which used >10% increase in FVC at 12 months as one of the two primary outcome measures, reported a significant improvement in FVC in the SCT group in 80% of patients (n = 8), while the mean FVC decreased in patients randomised to CYC (n = 9) one year post-transplantation [28]. The mean rate of change of FVC in the SCT group was 10% in two years. Change in DLco did not differ significantly between groups. Four patients in the ASSIST trial had limited cutaneous SSc (lcSSc) with ILD. In the two patients treated with SCT lung function improved, whereas the two patients treated with cyclophosphamide pulse therapy experienced deterioration of pulmonary function.

The ASTIS trial (Autologous Stem Cell Transplantation International Scleroderma) observed a mean change in FVC of +6.3% at two years in the patients treated with SCT (n = 79) compared with −2.8% in the control arm (n = 77) (p = 0.004). A decrease of −4.7% in DLco in SCT-treated patients compared with −4.1% in the control group (p = 0.84) at 2 years of follow-up was seen [29]. The SCOT trial (Scleroderma: Cyclophosphamide Or Transplantation) reported beneficial effects of SCT (n = 36) compared with cyclophosphamide (n = 39) on FVC but not on DLco [30]. Fewer patients in the SCT group had a decrease of ≥10% of FVC (n = 4) and more patients had improvement of FVC > 10% (n = 13), compared with the control group (n = 8 and n = 7, respectively) in the intention to treat analysis at 54 months. The majority of patients included in the ASTIS trial had mild ILD compared with patients in SCOT; this is reflected in the lung function results at baseline and should be taken into account when comparing these three trials.

Large observational studies reported similar findings for FVC and showed a modest positive effect on DLco as well. A retrospective analysis of transplant SSc patients in the Netherlands (N = 92, median follow-up time 4.6 years, 96% dcSSc, median disease duration 1.5 years, 36% had ILD) showed a median increase of FVC of +10% at 5 year follow-up and median increase of DLco from +6%. The Brazilian SCT cohort study (N = 70, median age 35.9, 57% female, 96% dcSSc, median disease duration at SCT was 2 years) reported stabilisation of both FVC and DLco after SCT [32]. In patients with progressive ILD with decline in FVC or DLco > 10% in 6 months before SCT (n = 51), improvement of both FVC and DLco after treatment was observed at 5 year follow-up.

An analysis of the cohort of the European bone marrow transplant organisation (EBMT) (N = 80, per cent dcSSc not reported, follow-up time 2 years) reported an increase in FVC of +7% at 2 year follow-up (p < 0.001) [26]. DLco stabilised [+0.2% at 2 years (p = 0.01)]. A previous analysis in this cohort (N = 57, median age 40 years, 70% female, 88% dcSSc, median disease duration at SCT 36 months) showed no significant change in FVC or DLco

during a follow-up period of 36 months, although serial long function parameters were available in only a small number of patients (n = 26 at 12 months, n = 18 at 24 months and n = 10 at 36 months) and 31% of patients had pulmonary arterial hypertension, which could influence lung function results too [33].

A study by Nash et al. (N = 34, median age 41 years, 76% female, all dcSSc, median disease duration 21 months) followed patients for a median of 4 years (range 1–6) and reported a mean change in FVC from baseline to final evaluation of +2.1% (p = 0.50) and DLco of −6.0% (p = 0.05) [34]. Also, an observational Italian study (N= 18, median age 41 years, 72% female, all dcSSc, median disease duration at SCT 24 months) showed stabilisation of DLco at 60-month follow-up [35]. (Table 1).

Disease extent on imaging

Extent of ILD is generally assessed using thoracic HRCT scans and changes after SCT are described in only a couple of studies, which also use different outcome measures. In the retrospective Dutch cohort available HRCTs at baseline and 5 year follow-up were evaluated using Goh scores [a visual scoring (in per cent) of extent of SSc-ILD at HRCT] [36]. Median Goh scores improved from 14% (7–34%, n = 39) at baseline to 8% (3–23%, n = 16) at 5 year follow-up [37]. Estimated mean improvement per year was −1.0 (95% CI −1.9 to 0.0). Another Dutch single-centre study evaluated HRCTs retrospectively at baseline and 1 year follow-up in 51 patients treated with either SCT (n = 20) or CYC (n = 31) [38]. A composite ILD score included assessment of total ILD extent, reticulations and ground glass opacities. Patients treated with SCT had clear improvement of ILD extent on HRCT at 1 year follow-up, and improved more (but not significantly) compared with the CYC-treated group [−5.1% of ILD score compared with −1.0% in the CYC group (p = 0.535) respectively]. Also, change in HRCT was weakly associated with change in PFT. Nash et al. evaluated HRCTs of 21 patients treated with SCT [34]. The six patients who survived after 1 year follow-up had fewer 'ground-glass' abnormalities compared with baseline; however, more interstitial fibrosis was present compared with baseline [28]. In the ASSIST trial the extent of lung disease decreased in patients treated with SCT at 2 year follow-up while this increased in controls [28].

Table 1. SCT studies and effect on ILD

Studies (N)	Regimen	Effect on lung function		Effect on HRCT
		FVC	DLco	
ASSIST N=10 (SCT) (70%*) N=9 (CYC) (89%*) Mean FU: 2.6 years Primary outcome: improvement at 12 months	- Mobilisation: CYC 2 g/m^2, G-CSF - Conditioning: CYC (200 mg/kg), rabbit ATG - CD34 selection: no - Comparator; CYC iv 6 months	Baseline (median): SCT: 62% (range 53–70) CYC: 67% (range 43–84) Median change in 1 years: SCT: +20% CYC: -9%	Baseline (median): SCT: 58% (range 29–82) CYC: 75% (range 29–111) Median change in 1 years: SCT: +9% CYC: -7%	Baseline (ILD on scan): SCT: 70% CYC: 89% Change at 2 years: Extent of ILD decreased after SCT but increased in controls
ASTIS N=79 (SCT) (86%*) N=77 (CYC) (86%*) Median FU: 5.8 years Primary outcome: EFS at 24 months	- Mobilisation: CYC 4 g/m^2, G-CSF - Conditioning: CYC (200 mg/kg), rabbit ATG - CD34 selection: yes - Comparator; CYC iv 6 months	Baseline (mean): SCT: 82% (SD 19) CYC: 81% (SD 18) Mean change in 2 years SCT: +6.3% (SD 18.3) CYC: -2.8 (SD 17.2)	Baseline (mean): SCT: 59% (SD 14) CYC: 58% (SD 14) Mean change in 2 years SCT: -4.7% (SD 13.7) CYC: -4.1 (SD 17.6)	Baseline (ILD on scan): SCT: 87% CYC: 80%
SCOT N=36 (SCT) (100%*) N=39 (CYC) (100%*) Mean FU: 54 months Primary outcome: GRCS at 54 months	- Mobilisation: G-CSF - Conditioning: CYC (120 mg/kg), equine ATG - TBI (800 cGy) - CD34 selection: yes - Comparator; CYC iv 6m	Baseline (mean): SCT: 74% (SD 15) CYC: 74% (SD 17) Change ITT group at 54 months: SCT N=13 improvement** N=10 no change N=4 worsening** CYC N=7 improvement N=6 no change N=8 worsening	Baseline (mean): SCT: 54% (SD 8) CYC: 53% (SD 8) Change ITT group at 54 months: SCT N=4 improvement*** N=19 no change N=13 worsening*** CYC N=5 improvement N=10 no change N=24 worsening	Baseline (ILD on scan): SCT: 100% CYC: 95% Change at 54 months: SCT - Decrease ILD scores - Stable fibrosis CYC - No change ILD score - Increase fibrosis
Nash et al. N=34 (79%*) Median follow-up 4 (range 1–6) years Primary outcome: improvement of mRSS and HAQ-DI	- Mobilisation: G-CSF - Conditioning: TBI (800cGY), CYC (120 mg/kg), and equine ATG (90 mg/kg) - CD34 selection: yes - Comparator; none	Baseline (median): 71 (range 27–103) Mean change in 4 years +2.1% (95% CI -5.2–9.3, (p=0.560)) +1.7 per yr (95% CI 0.4–3.0, p=0.010)	Baseline (median): 62 (range 40–83) Mean change in 4 years -2.3% (95% CI -9.9–4.9)(p=0.310)) +0.4 per year (95% CI 1.4–0.7, p=0.50)	Baseline HRCT (n=34): - Normal: 21% - Ground-glass: 35% - Fibrosis: 74% Change: 18%: ILD reactivation 18%: decreased ground-glass, increased fibrosis

(Continued)

Studies (N)	Regimen	Effect on lung function		Effect on HRCT
		FVC	DLco	
Bijnen et al. N=92 (36%*) Median follow-up: 5 years (IQR 2–12) Primary outcome: EFS	- Mobilisation: CYC 2–4 g/m^2, G-CSF - Conditioning: CYC (200 mg/kg), rabbit ATG - CD34 selection: yes - Comparator; none	Baseline (median, n=66): 84% (range 68–102%) Median at 5yrs (n=40) 94% (range 81–107) +2.5 (1.9–3.0) per year	Baseline (median, n=67): 55% (range 42–67%) Median at 5 years (n=38) 61% (range 53–73) +1.6 (1.0–2.2) per year	Median Goh scores Baseline (median, n=39) 14% (range 7–34%) At 5 years (median, n=16): 8% (range 3–23%) -1.0 (-1.9–0.0) per year
Henes et al. N=80 (86%*) Follow-up: 2 years Primary outcome: PFS at 2 years	- Mobilisation: CYC 1–4 g/m^2, G-CSF - Conditioning: CYC (50–240 mg/kg), rabbit ATG, thiotepa 10 mg/kg - CD34 selection: both - Comparator; none	Baseline (mean, n=37) 74% (SD 16.9) Mean at 1year: 80% (SD 17); Mean at 2 years: 81% (SD 19)	Baseline (mean, n=35) 60% (SD 19.3) Mean at 1year: 60% (SD 18); Mean at 2 years: 60% (SD 19)	-
Henrique-Neto et al. N=70 (84%*) Follow-up: 8 years Primary outcome: -	- Mobilisation: CYC 2 g/m^2, G-CSF - Conditioning: 200mg/kg CYC and 4.5mg/kg ATG - CD34 selection: yes - Comparator; none	Baseline (median, n=70): 70 (range 35–122) N=66 stabilisation Median at 5 years (N=51): 75% (range 48–110, p=0.020)	Baseline (median, n=70): 70 (range 48–125) N=66 stabilisation Change at 5 years (N=51): 76% (range 50–115, p=0.030)	-
Farge et al. N=57 (57%*) Follow-up: 36m	- Mobilisation: CYC 4 g/m^2, +/- G-CSF - Conditioning: CYC (150–200 mg/kg), other chemotherapy, rabbit ATG, TBI - CD34 selection: both - Comparator; none	Baseline (n=47): 57% had FVC<70% No significant change during 36 months of FU	Baseline (n=47): 64% had DLco <70% No significant change during 36 months of FU	
Del Papa et al. N=18 (67%*) Follow-up: 60m Primary outcome: -	- Mobilisation: CYC 4 g/m^2, G-CSF - Conditioning: CYC (200 mg/kg), rabbit ATG - CD34 selection: yes		Baseline (median): 68% (range 51–100) Median at 60m 62% (range 30–85)	-

* Percentage of patients with ILD.

**>10%

***>15%

ASSIST, American Scleroderma Stem Cell versus Immune Suppression Trial; ASTIS, Autologous Stem Cell

Transplantation International Scleroderma; ATG, anti-thymocyte globulin; CI, confidence interval; CYC, cyclophosphamide; DLco, diffusing capacity of the lungs for carbon monoxide; EFS, event free survival; FU, follow-up; FVC, forced vital capacity; G-CSF, granulocyte colony-stimulating factor; GRCS, global rank composite scores; ILD, interstitial lung disease; IQR, interquartile range; ITT, Intention-to-treat; iv, intravenous; mRSS, modified Rodnan skin score; PFS, progression free survival; SCOT, Scleroderma: Cyclophosphamide Or Transplantation; SCT, stem cell transplantation; SD, standard deviation; TBI, total body irradiation.

A German study used automated quantitative analysis on HRCTs of 26 patients (median age 41 years, 54% female, median disease duration 3.5 years) treated with SCT at 6 months and 2 years of follow-up [39]. Based on FVC at 6 months patients were classified as responders (n = 20) and non-responders (n = 6). In these 20 responders DLco also significantly improved and total lung volume increased, lung density and high attenuation values decreased significantly. Additionally, structural and architectural properties of involved lung tissue parenchyma on chest computed tomography were analysed in 23 patients [40]. Fibrotic features increased in non-responders (n = 5) at 6 and 12 months. In both responders (n = 18) and non-responders significant changes in these properties were observed at 6 months and at 12 months in responders only. A small French study (N = 9, median age 41 years, 67% female) qualitatively evaluated HRCTs at 6 up to 36 months and reported improvement on short term evaluation and stabilisation at the last follow-up scan [41]. In conclusion, although different measures and scores were used in the studies and follow-up time was relatively short, most reported either stabilisation or improvement of lung disease on HRCT in patients treated successfully with SCT. In a sub-analysis of the SCOT trial, HRCTs were quantitatively scored on fibrosis and ILD scores during 54 months of follow-up [42]. Patients treated with SCT showed decreased ILD scores and stable lung fibrosis compared with patients treated with CYC in the control arm.

Progressive ILD or relapse after SCT

Approximately 17% of patients with SSc relapsed post-SCT [20]. In the Brazilian cohort study, 17 (18%) patients developed disease reactivation, mostly ILD (n = 11, 12%), requiring immunosuppressive medication [32]. In the study by Nash et al. 29% (n = 6) experienced reactivation of lung disease after treatment [34]. No data on newly developed ILD after SCT is described in the literature.

Patient selection and pulmonary complications

A main concern in SCT is the risk of complications related to the treatment. Treatment-related mortality was considerably higher in dcSSc patients treated with SCT compared with control arms in the three randomised trials [RR 9.00 (95% CI, 1.57–51.69)] [31]. Patient selection for SCT therefore focuses on identifying patients at risk for SSc-related organ damage who are still in a fit state to undergo this intensive treatment without severe adverse events. This is reflected in the inclusion and exclusion criteria of trials

(Table 2). It can be argued that the effect of SCT in patients with severe and active ILD might be larger compared with patients treated with mild ILD and therefore this needs to be taken into account while comparing results of different trials. Also, early SCT in patients with limited pulmonary disease may show less impact on present ILD in patients, but could prevent development or progression of ILD, which is currently being investigated in the UPSIDE trial [43].

Severe pulmonary damage pre-treatment could place patients at risk of severe and even fatal treatment complications (an overview of pulmonary complications related to autologous SCT is provided in Table 3) [44]. In previous studies pulmonary complications were an important cause of death or organ failure after SCT. In the ASTIS trial, 15 (19%) severe pulmonary adverse events had occurred in the transplant group compared with six (7.8%) in the CYC arm [29]. Fatal events included pulmonary haemorrhage, pulmonary oedema, acute respiratory distress syndrome (ARDS) triggered by G-CSF and pulmonary infection. In the SCOT trial, most events of organ failure were lung related as well [30]. Five (13%) patients in the SCT arm died due to ARDS and pulmonary haemorrhage. In the recently published cohort studies fewer pulmonary complications were reported, which may be attributed to improved supportive care and increased awareness or possible underreporting. Thus, patient selection and collaboration with a multidisciplinary team including pulmonologists, infectious disease specialists and intensive care specialists is key to minimise risks for patients undergoing SCT.

Mechanism of action of SCT

Immune reconstitution following SCT and the working mechanism of SCT have been studied in dcSSc and other autoimmune diseases, that is, multiple sclerosis and Crohn's disease [21]. Autoreactive immune cells and immune memory cells are erased, followed by reconstruction with $CD34^+$ haematopoietic stem cells, which provide a chance to reshape by antigenic selection that may be different from the first triggering of diseases. Changes after SCT in both the innate immune system and the adaptive immune system have been described. In the SCOT trial normalisation of the interferon (IFN) signature, circulating neutrophils and NK cells was seen after treatment with SCT, but not in controls treated with CYC [45]. Also, the diminished IFN and neutrophil gene signatures were associated with improved FVC. Other studies investigating reconstitution of innate immune responses reported changes in serum cytokine profiles after SCT, that is, IL-2 and IL-8, suggesting a shift in Th balance [46-48].

Table 2. Inclusion and lung-related exclusion criteria used in clinical trials

Studies	Inclusion criteria	Lung related exclusion criteria
ASSIST	Age <60 years dcSSc Disease duration ≤4 years mRSS ≥15 + Internal organ involvement Lung: DLco <80% or FVC -10% within 12 months + HRCT abnormalities	TLC < 45% of predicted PAH
ASTIS trial	Age 18–65 years dcSSc Disease duration ≤4years mRSS >15 Internal organ involvement Lung: DLco and/or FVC ≤80% + HRCT abnormalities	DLco < 40% of predicted PAH
SCOT	Age 18–69 years dcSSc Disease duration ≤4 years mRSS ≥16 Internal organ involvement Lung: FVC <70% or DLco <70% + HRCT abnormalities	DLco < 40% of predicted FVC < 45% of predicted PAH
UPSIDE trial	Age 18–65 years dcSSc Disease duration ≤2 years AND: mRSS ≥15 OR: Internal organ involvement Lung: DLco and/or FVC ≤85% and HRCT abnormalities or relative change in FVC >-10% or DLco >-15% within 12 months	DLco < 40% of predicted PAH

ASSIST, American Scleroderma Stem Cell versus Immune Suppression Trial; ASTIS, Autologous Stem Cell Transplantation International Scleroderma; DLco, diffusing capacity of the lungs for carbon monoxide; ESR, estimated sedimentation rate; FVC, forced vital capacity; Hb, haemoglobin; HRCT, high resolution computed tomography; mRSS, modified Rodnan skin score; PAH, pulmonary arterial hypertension; SCOT, Scleroderma: Cyclophosphamide Or Transplantation; TLC, total lung capacity; UPSIDE, UPfront autologous hematopoietic Stem cell transplantation versus Immunosuppressive medication in early DiffusE cutaneous systemic sclerosis.

Table 3. Pulmonary complications related to autologous stem cell transplantation

Treatment phase	Complications
Mobilisation	Pulmonary oedema
	G-CSF related alveolitis
Conditioning	Pulmonary oedema
	Toxicity ATG or cyclophosphamide
	Radiation related lung damage
Post-SCT	Reconstitution
	Haemorrhage
	Infection (bacterial, viral, fungal)
	Viral reactivation (CMV, EBV)
	TRALI after transfusion

ATG, anti-thymocyte globulin; CMV, cytomegalovirus; EBV, Epstein-Barr virus; G-CSF, granulocyte colony-stimulating factor; SCT, stem cell transplantation; TRALI, transfusion-related acute lung injury.

The T cell receptor (TCR) repertoire showed up to 90% renewal two years after SCT [49]. Broadening of the TCR repertoire is reflected by the increase in number of TCR-rearrangement excision circles and represents thymic output [49]. Moreover, the Th1/Th2 ratio was found to increase after SCT at 1 month post-transplantation and reached a plateau after 6 months [47]. B cell composition also changes following SCT and a decrease of IL-6- and TGF-β1 producing B cells and an increase of $CD19^{+}CD24^{hi}CD38^{hi}$ B regulatory cells (Bregs) were observed after treatment in 22 patients [50]. Interestingly, the number of $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs at baseline was also associated with post-SCT remission. In another study (N = 17) decline in both naïve and memory B cells was seen until one year post-transplantation and lower peripheral B cell levels were associated with infectious complications [51].

Although more insight has been gained in the reconstitution of circulation immune cells, it is still unclear how SCT induces immunological changes in peripheral tissue, including the lungs. As illustrated by the varied clinical response reported, improvement is much more prominent in skin compared with lungs or the gastrointestinal tract; SCT may impact pathogenic processes in every organ differently [34]. Although there is limited evidence on predictors for pulmonary outcome after SCT, several biomarkers are correlated with clinical response in studies investigating other treatments for SSc-ILD. For instance, decrease in serum level of Krebs von den Lungen 6 and surfactant protein D are associated with improvement of FVC after SCT [46], and changes in bronchoalveolar lavage proteins have shown to predict treatment response [52].

Implications for further research

With three randomised controlled clinical trials completed and countries sharing their experiences with SCT with cohort studies, understanding of the effects of SCT on organ complications such as ILD has grown. Currently the UPSIDE trial is ongoing and investigates upfront SCT in early disease compared with other immunosuppressive therapy and the impact on ILD, using lung function and imaging (automated quantitative HRCT analysis and positron emission tomography) scans to assess changes in the lung after treatment [43]. Also the use of post-transplant MMF in order to prevent (pulmonary) relapse is currently under investigation (NCT01413100). Still, no studies have been done investigating the impact of SCT compared with immunosuppressive medication in the long-term using lung involvement as a primary outcome measure. Future research focused on lung involvement is therefore needed. Additionally, studies are required to investigate refined treatment strategies with similar or better effects but lower toxicity making SCT suitable for patients with more extensive disease who are currently excluded for this treatment. Also the impact of SCT in patients with lcSSc-ILD has yet to be established as only very few cases with lcSSc-ILD treated with SCT were included in the studies and outcomes in this subset are described only in the ASSIST trial. Furthermore, as mentioned above, not much is known about the impact of SCT on lung-related patient reported outcomes. Mechanistic studies investigating changes in the lungs during and after SCT could improve understanding of the different effect of the treatment on ILD compared with skin fibrosis and might help to identify biomarkers predicting response to SCT or immunosuppressive treatment in early onset.

Discussion

In this review we summarised the results of SCT on SSc-ILD. Autologous SCT showed a modest but clinically relevant improvement of lung volumes and disease extent on imaging; however, no consistent effect on DLco has been reported. This small effect on DLco may be explained by coexisting pulmonary vascular disease which is less affected by SCT [53]. Moreover, other factors can influence FVC, such as myositis or other chest problems, or affect DLco, including anaemia, intrapulmonary or intracardiac shunts and cardiac disease [54]. That DLco results can be affected by cardiac involvement was also shown in a retrospective analysis of 90 SSc patients treated with SCT [55]. In this study DLco did not improve significantly after treatment in the whole group, but only in patients with normal cardiac tests (echo and electrocardiogram) at baseline. Thorough pre-transplant screening in microvascular and cardiac disease is therefore essential not only for risk assessment during the treatment but also to anticipate response.

Robust evidence for the efficacy of SCT in SSc-ILD is, however, still lacking as none of the controlled SCT studies was primarily powered for lung outcomes. Comparison between SCT studies and trials investigating immunosuppressive therapies in SSc-ILD is also limited due to differences in inclusion criteria and subsequently baseline characteristics, treatment regimens and clinical endpoints. Importantly, ILD was not a sole inclusion criterion in the SCT trials so as a consequence not all included patients in these studies had ILD at baseline, while in studies investigating the impact of immunosuppressive and antifibrotic therapies all participants had established SSc-ILD. Furthermore, no outcome measures on impact on symptoms and pulmonary performance in daily life for patients are collected in published SCT trials [56].

Currently, MMF is the first treatment choice for SSc-ILD as it has a favourable safety profile and was demonstrated to stabilise lung function after two years in the Scleroderma Lung study II [57]. Also, CYC still has a place in the treatment of SSc-ILD, when followed by other disease-modifying anti-rheumatic drug therapies [58-60]. Biologics such as rituximab have demonstrated benefits in SSc-ILD by improving both restriction and diffusion capacity in a meta-analysis [16], and subcutaneous tocilizumab showed a trend towards stabilisation of FVC [61, 62]. Particularly, tocilizumab seems to stabilise lung function decline in patients with early SSc-ILD and elevated acute-phase reactants [63], and in patients with positive anti-topoisomerase antibodies [64]. Nintedanib managed to slow down FVC decline, and can be a potential addition to immunosuppressive therapies such as MMF [65, 66].

Although new immunomodulating and combined treatment with antifibrotic therapies are emerging into the clinics and will be first-line therapy for most patients with SSc-ILD, SCT remains a potent treatment that could prevent progression of SSc-ILD on the long-term in patients with early rapidly progressive dcSSc. International guidelines recommend SCT in a careful selection of SSc patients in highly experienced centres [67]. Accordingly, the recent European consensus guidelines adopted SCT as an escalation treatment for a subset of patients with SSc-ILD [17]. Unfortunately, details about this selected subset that could guide treatment decisions are not mentioned in this guideline. Based on the existing literature there is only evidence for SCT in dcSSc-ILD patients as lcSSc-ILD patients were not included in most studies. Furthermore, SCT trials included patients with rapidly progressive and early disease rather than refractory cases, as is suggested by the European consensus guideline. We therefore recommend that SCT is used in line with eligibility criteria of the ASTIS and SCOT trials only in dcSSc-ILD patients. Caution should be taken in patients with extensive, refractory ILD because of the risk of (pulmonary) complications related to SCT procedures and infections as described in this review, and the lack of evidence of efficacy of SCT in this group of patients. Future research is needed to refine treatment strategies in patients with lcSSc-ILD and patients

with extensive disease and subsequent high risk of complications, to establish impact of SCT on patient-reported outcomes and identification of predictors for response. Also, the ongoing UPSIDE trial may shed light on the impact of upfront SCT on SSc-ILD as this trial also evaluates lung outcomes measures comprehensively.

In conclusion, autologous SCT in dcSSc is a powerful treatment option which can stabilise and even improve lung involvement in a selected group of patients with dcSSc; however, more research is needed to further determine its role in the management of SSc-ILD.

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Chapter 7

Association of endothelial to mesenchymal transition and cellular senescence with fibrosis in skin biopsies of systemic sclerosis patients: a cross-sectional study

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Abstract

Objective

Fibrosis is the dominant hallmark of systemic sclerosis (SSc). Several mechanisms have been proposed to drive the disease process, but how these relate to skin fibrosis is poorly understood.

Methods

We performed a cross-sectional study on archival skin biopsies from 18 SSc patients and four controls. Dermal fibrosis and inflammatory cell infiltration were scored in HE and Masson's Trichrome-stained sections. The presence of senescence was defined by P21 and/or P16 positivity in Ki-67 negative cells. Endothelial to mesenchymal transition (EndMT) was identified by co-localization of CD31 and α-SMA in immunofluorescent double-stained sections, and by an enclosure of ERG positive endothelial cell nuclei by α-SMA stained cytoplasm in immunohistochemical double staining.

Results

The histological dermal fibrosis score of SSc skin biopsies was correlated with the modified Rodnan skin score (rho 0.55, $p = 0.042$). Staining for markers of cellular senescence on fibroblasts was correlated with fibrosis score, inflammatory score, and CCN2 staining on fibroblasts. Moreover, EndMT was more abundant in skin from patients with SSc ($p < 0.01$) but did not differ between groups with different fibrosis severity. The frequency of these EndMT features increased with the abundance of senescence markers and CCN2 on fibroblasts and dermal inflammation.

Conclusion

EndMT and fibroblast senescence were more abundant in skin biopsies from SSc patients. This finding indicates that both senescence and EndMT are involved in the pathway leading to skin fibrosis and might be valuable biomarkers and/or possible targets for novel therapeutic interventions.

Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterised by a triad of inflammation, vasculopathy and fibrosis. Fibrosis, particularly prominent in skin and lungs, is a pivotal process in SSc [1]. Fibrogenesis is mediated by the activation and proliferation of fibroblasts to myofibroblasts which leads to excessive extracellular matrix production. Several mechanisms may be involved in fibrogenesis, including response to inflammation and vasculopathy, but its pathogenesis still remains poorly understood.

As mentioned earlier, endothelial to mesenchymal transition (EndMT) and cellular senescence may be involved in the pathogenesis of SSc [2, 3]. EndMT is a transition process of endothelial cells acquiring more mesenchymal characteristics, including expression of α-SMA and various extracellular matrix (ECM) molecules [2]. In physiological and pathological conditions, EndMT can be induced by transforming growth factor beta (TGF-β), cellular communication network factor 2 (CCN2, also known as CTGF or Connective Tissue Growth Factor), and Endothelin-1 [4, 5]. Both CCN2 and TGF-β are also phenotypic markers in SSc and contribute to the cellular senescence-associated secretory phenotype (SASP) that stimulates fibrosis and inflammation [6-8]. Cellular senescence is a permanent status of cell cycle arrest. It can occur in response to various stimuli, including telomere shortening, oxidative stress, genotoxic injury and inflammation [6, 9]. Dermal fibroblasts from patients with SSc have been shown to have less abundant minichromosome maintenance helicase proteins, lower autophagic capacity, and increased senescence features, including β-galactosidase activity and SASP [10, 11]. To further explore the potential of histopathological assessment of skin biopsies for EndMT and cellular senescence features in SSc as biomarkers of fibrosis severity and identification of novel therapeutic targets, we studied archival skin tissue biopsies from SSc patients and controls in relation to skin fibrosis, reflected by the modified Rodnan skin score (mRSS).

Materials and methods

Patients

We performed a cross-sectional biobank study on formalin-fixed paraffin-embedded (FFPE) skin biopsies from patients meeting the ACR/EULAR 2013 classification criteria for SSc. Demography, comorbidity, medication, autoantibodies, disease duration, disease subset and mRSS at the time of biopsy were retrieved from medical records. Healthy controls were acquired from normal skin in adult resection material for unrelated indications. This study was approved by the institutional review board at University Medical Centre Utrecht (TcBio 22-014).

Histopathology

Slides were observed under a photo microscope (Nikon Eclipse E800) or fluorescence microscope (Leica DM 5500 B). Fibrosis and inflammatory cell infiltration of the dermis were scored in haematoxylin and eosin stain (HE) and Masson's Trichrome stained 3 µm sections from FFPE skin biopsies. Fibrosis severity was semi-quantified by the extent of dermal fibrosis and scored from one to three [12], and fibrosis activity was estimated from CCN2-staining (see below). Dermal inflammatory score was estimated from lymphocyte infiltration in the dermis and scored from zero to two. All semi-quantitative scores were evaluated independently in the superficial and deep dermis. The resulting score was recorded by agreement of two researchers (MRD and YHC). Immunohistochemical staining for P16, P21, and CCN2 in fibroblasts and endothelial cells was scored as absent (0), weak (1), or strong (2). The percentage of P16/P21 positive fibroblasts was counted in 10 random high-power-fields. The presence of senescence was defined as P21 or P16 positive without co-localized Ki-67 [13]. EndMT was detected by co-localization of CD31 and α-SMA by immunofluorescence double staining (α-SMA-CD31 positive) and enclosure of ERG positive endothelial cell nuclei by α-SMA stained cytoplasm in immunohistochemically double-stained (α-SMA-ERG positive) sections. EndMT was quantified by the percentage of vessel cross-sections detected EndMT feature in dermis. The endothelial cells of both blood vessel and lymphatic ducts express CD31 and ERG, whereas only the lymph endothelial cells express D2-40 (podoplanin) [14]. Therefore, numbers of lymph vessels/mm2 were determined in D2-40 stained sections.

Primary antibodies used were rabbit anti-P21 (Cell Signaling Technologies 2947, 1:100 dilution), mouse anti-P16 (Immunologic VWRKILM0632-C05, 1:800 dilution), rabbit anti-Ki-67 (Thermo Scientific RM9106S, 1:400 dilution), rabbit anti-CCN2 (Cell Signaling Technology 86641, 1:600 dilution), mouse anti-α-SMA (Sigma A2547, 1:8000 dilution), rabbit anti-α-SMA (Abcam ab5694, 1:1000 dilution), rabbit anti-CD31 (LSBio LS-B4737, 1:50 dilution), rabbit anti-ERG (Dako M7314, 1:200 dilution), and mouse anti-D2-40 (Biolegend 916602, 1:400 dilution).

Digital analysis

The slides were scanned and imported in QuPath 0.4.1 [15]. Figures were colour deconvoluted with vectors in haematoxylin, target staining (immunohistochemistry staining and blue in Masson's Trichrome staining) and residual (unwanted or confounding colour, including Ki-67 in the double staining, debris and hemosiderin deposition). After manually annotating the dermis, excluding hair follicles, muscle, glands, fat, and vessels (with a wall of more than 3 cell layers), the percentage of positive staining pixels in the annotation was determined by a pixel classifier.

Statistical analysis

The difference of continuous variables between groups was determined using Kruskal-Wallis test and Wilcoxon rank-sum test. The correlation between ordinal and/or continuous variables was examined with Spearman's rank correlation rho. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using R 4.0.3.

Results

Patient characteristics

In total, skin biopsies from 18 SSc patients were identified in the archive of the department of pathology of the UMC Utrecht and investigated together with skin samples from four adult healthy controls. The healthy controls had a median age of 38 (34–47) years, and three of the four (75 %) healthy controls were females. The control skin was taken two from lower limb and two from trunk. Patients' characteristics are summarised in Table 1.

Table 1. Clinical features of 18 patients with systemic sclerosis

Characteristics	All, n = 18	Limited SSc, n = 13	Diffuse SSc, n = 5
Age, median (IQR)	47 (36–56)	46 (36–54)	49 (36–68)
Female, n (%)	11 (61)	7 (53)	4 (80)
Disease duration month, median (IQR)	5.5 (0.0–29.0)	5 (0–26)	6 (2–30)
Biopsy site, n (%)			
Upper limb	3 (17)	2 (16)	1 (20)
Trunk	6 (33)	5 (38)	1 (20)
Lower limb	8 (44)	5 (38)	3 (60)
mRSS, median (IQR)	7 (3–12)	3 (2–10)	27 (22–30)
Autoantibodies, n (%)			
Anti-topoisomerase I antibody	3 (17)	1 (8)	2 (40)
Anti-RNA polymerase III antibody	2 (11)	1 (8)	1 (20)
Anti-centromere antibody	3 (17)	3 (23)	0
Negative on autoantibodies	1 (6)	1 (8)	0
Immunosuppressants, n (%)			
Mycophenolate mofetil	1 (6)	0	1 (20)
Methotrexate	2 (11)	1 (8)	1 (20)
Disease complications, n (%)			
ILD	2 (11)	1 (8)	1 (20)
PAH	3 (17)	2 (15)	1 (20)
Digital ulcers	3 (17)	2 (15)	1 (20)

SSc, systemic sclerosis; IQR, interquartile range; mRSS, modified Rodnan skin score; ILD, interstitial lung disease; PAH, pulmonary arterial hypertension. Biopsy site was not available in one patient.

Relation of histopathological fibrosis and inflammatory scores with mRSS

Patients with higher histological scores for the extent of dermal fibrosis had higher mRSS (rho 0.55, p = 0.042) (Figure 1). The fibrosis score was correlated with percentage of positive pixels in Masson's Trichrome staining (rho 0.78, p < 0.001). Dermal inflammatory score did not correlate with the serum inflammatory markers C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). The correlation between dermal fibrosis and inflammatory score was 0.55 (p = 0.008). CCN2 staining of fibroblasts correlated with the dermal inflammatory score (rho 0.58, p < 0.001) and the histopathological fibrosis score (rho 0.44, p = 0.041).

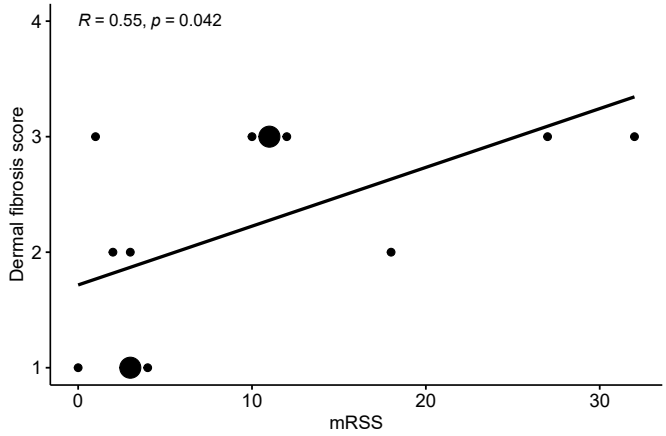

Figure 1. The modified Rodnan skin score (mRSS) was higher in skin biopsies with severe fibrosis. The point size is increased with overlapping.

Cellular senescence

Senescence markers was more abundant in dermal fibroblasts in skin from SSc patients with elevated histopathological fibrosis score than in healthy control skin (Figure 2). The correlation between fibrosis score and the score of senescence markers on fibroblasts was 0.43 for P16 (p = 0.048) and 0.55 for P21 (p = 0.008); the fibrosis score was also correlated with percentage of positive senescence pixels, including P16 (rho 0.49, p = 0.022) and P21 (rho 0.44, p = 0.040). There was hardly any Ki-67 co-localization with senescence markers on fibroblasts or endothelial cells (Figure S1). In addition, increased senescence markers were associated with a higher CCN2-abundance on fibroblasts and increased dermal inflammatory score. The correlation between the abundance of CCN2 and senescence markers on fibroblasts was 0.63 for P16 (p < 0.001) and 0.52 for P21 (p < 0.001). The percentage of positive CCN2 pixels was also correlated with positive

senescence pixels, including P16 (rho 0.54, p = 0.010) and P21 (rho 0.44, p = 0.042). The correlation between the dermal inflammatory score and senescence markers on fibroblasts was 0.45 for P16 (p = 0.002) and 0.37 for P21 (p = 0.014.) Moreover, there was only minimal abundance of senescence markers in endothelial cells, and this was not correlated with fibrosis severity (Table S1).

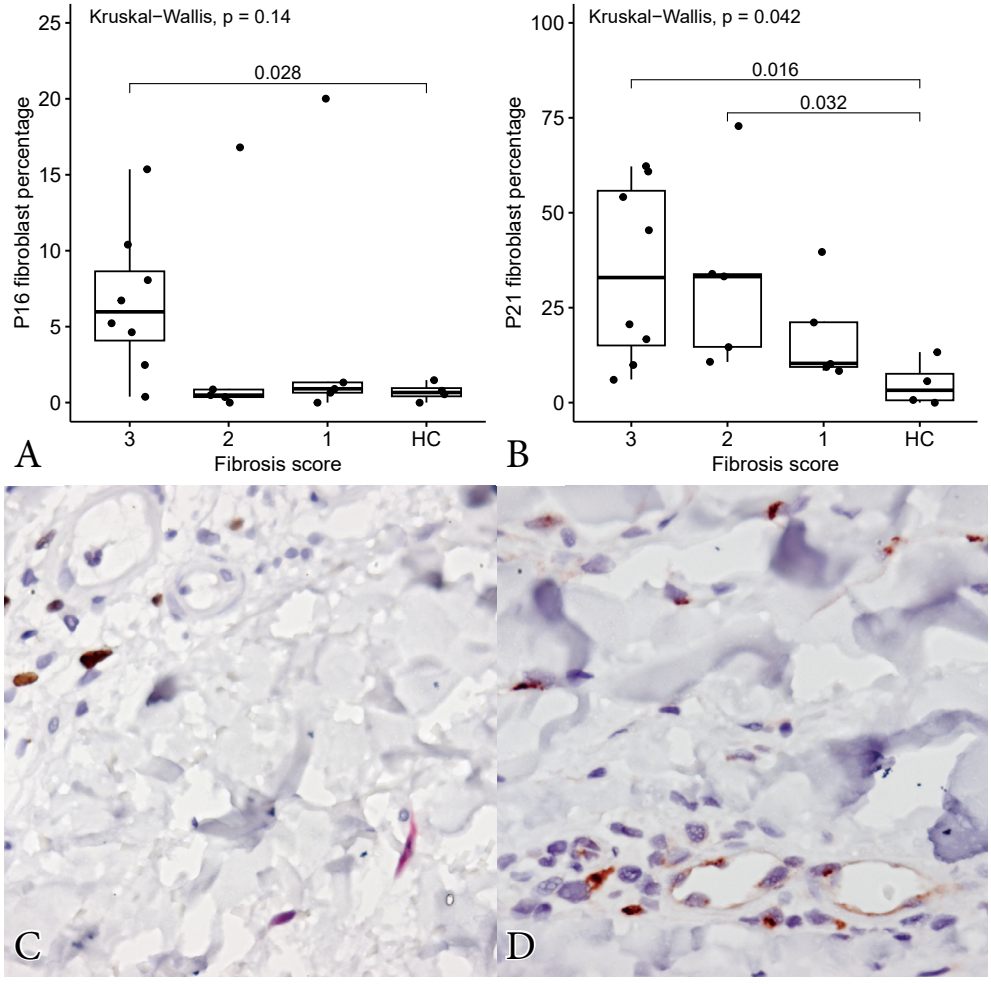

Figure 2. Cellular senescence markers in fibrotic systemic sclerosis (SSc) skin biopsies. (A) P16 is increased in fibroblasts from the highly fibrotic dermis, but not in most of the milder cases. (B) P21 is more abundantly expressed than p16, and also increased in moderately fibrotic SSc biopsies. (C) P16 (bright red) expressed on fibroblasts without co-localization of Ki-67 (brown). (D) CCN2 (brown) was expressed on fibroblasts and endothelium.

Endothelial to mesenchymal transition

The frequency of EndMT was significantly higher in patients with SSc but did not differ between groups with different fibrosis severity according to mRSS and histopathological fibrosis score (Figure 3A, B). The median (IQR) percentage of α-SMA-CD31 positive vessels was 6.4 (4.2–8.3) in SSc and 0.4 (0.0–1.4) in healthy controls ($p = 0.006$). The median (IQR) percentage of α-SMA-ERG positive vessels was 9.1 (6.7–14.5) in SSc and 2.8 (1.7–3.9) in healthy control ($p = 0.003$). Furthermore, the frequency of these EndMT features increased with the abundance of senescence markers and CCN2 on fibroblasts (Figure 3C, D), as well as dermal inflammation. The percentage of α-SMA-ERG positive vessels was correlated with the percentage of CCN2 positive pixels (rho 0.52, $p = 0.014$). The correlation between senescence and EndMT was observed in the percentage of α-SMA-ERG positive vessels and the percentage of P16 positive pixels (rho 0.53, $p = 0.010$) as well as the percentage of α-SMA-CD31 positive vessels and the percentage of P21 positive pixels (rho 0.44, $p = 0.039$). The median (IQR) percentage of CD31 and α-SMA co-localization on vessels was 0.7 (0.0–3.5) in tissue free from inflammatory cells infiltration and 6.4 (4.0–8.4) in tissue with inflammatory cells infiltration ($p = 0.015$). The median (IQR) percentage of α-SMA-ERG positive vessels was 9.8 (6.9–14.9) and 3.1 (2.1–3.5) in tissue with and without inflammatory cells infiltration, respectively ($p < 0.001$). EndMT was not observed in lymphatic vessels (i.e. there was no co-localisation of D2-40 and α-SMA). The density of blood and lymphatic vessels did not correlate with inflammatory cell infiltration score, mRSS or histopathological fibrosis score.

Discussion

In this cross-sectional study of SSc skin biopsies, we observed more EndMT and fibroblast senescence in SSc skin biopsies compared to healthy control skin. The abundance of fibroblast senescence was also associated with the abundance of CCN2 on fibroblasts, and with the degree of fibrosis and inflammation in SSc skin. Our data reinforce the notion that EndMT and fibroblast senescence contribute to the pathogenesis of inflammation and fibrosis in SSc skin.

Senescence markers were increased in dermal fibroblasts from highly fibrotic SSc skin and were associated with CCN2. Senescent cells are in permanent cell cycle arrest and resist apoptosis [6]. They undergo epigenetic changes resulting in altered, metabolically active phenotypes, including SASP, characterized by production and release of pro-fibrotic and pro-inflammatory cytokines [3]. Studies with isolated dermal SSc fibroblasts have revealed SASP-like characteristics. They produced more mitochondrial reactive oxygen species (ROS) and displayed higher levels of phosphorylated TGF-β-activated kinase 1(TAK1) and downstream IKKβ–IRF5 signalling than healthy fibroblasts [11].

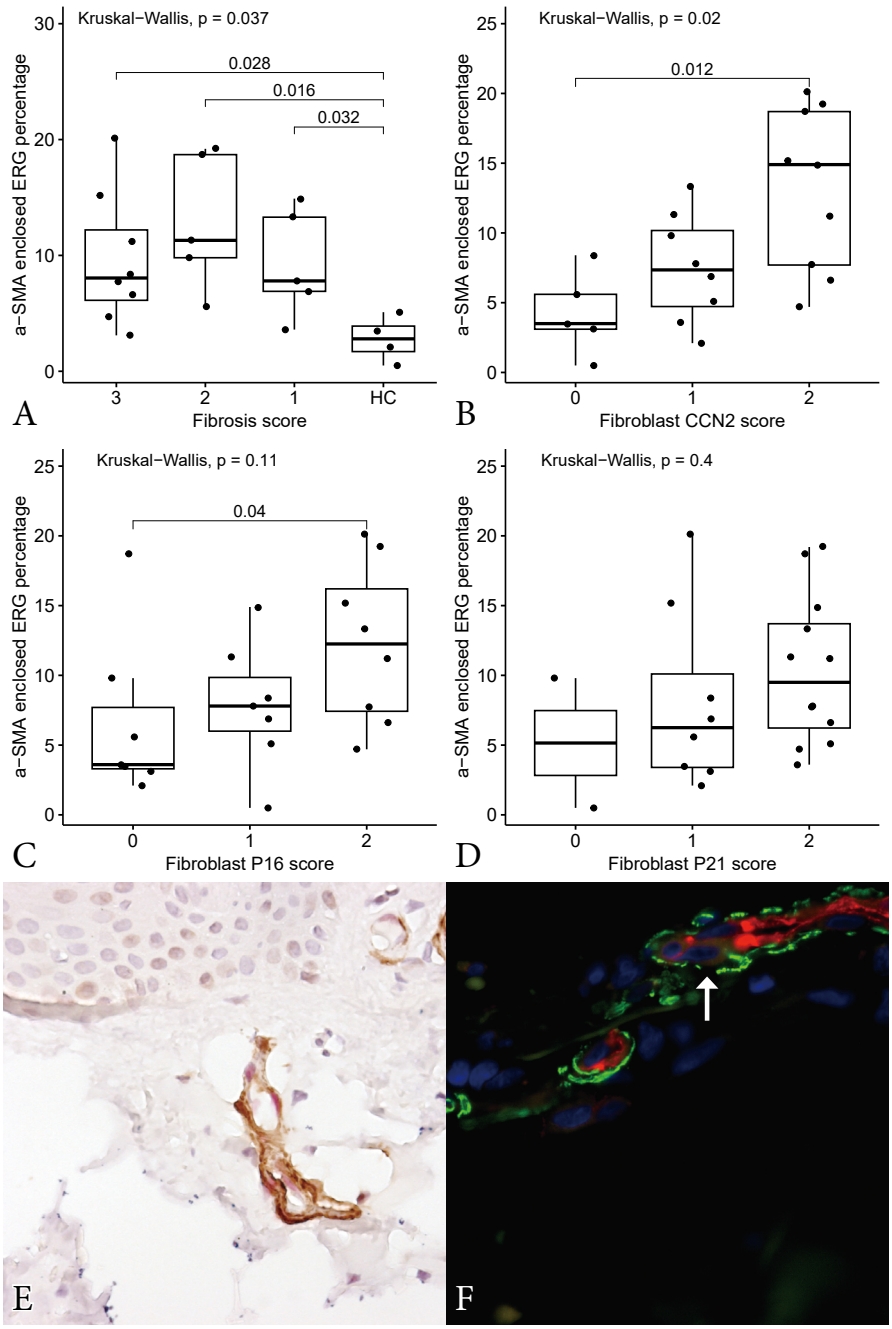

Figure 3. (A) Endothelial to mesenchymal transition (EndMT) was more prominent in systemic sclerosis (SSc) skin. Still, within the SSc group, there was no difference between biopsies with less or more severe fibrosis. (B, C, D) The frequency of EndMT increased with CCN2 and senescence markers on fibroblasts. (E) EndMT was detected by enclosure of ERG (bright red) positive endothelial cell nuclei by α-SMA (brown) stained cytoplasm under bright field. (F) EndMT was detected by co-localization of CD31 (red) and α-SMA (green) immunofluorescent double staining.

The IRF5 pathway also regulated type 1 collagen α1, α2, MMP13, selectin P, selectin E, intercellular adhesion molecule 1, IL-6, PDGF-B and CCN2 in dermal fibroblasts [16]. Although senolytic therapy with dasatinib, a tyrosine kinase inhibitor, did not achieve a significant clinical effect on SSc-ILD and mRSS in an open-label phase 2a trial [17], the SSc patients with response to dasatinib showed a higher SASP signature of gene expression at baseline and a significant SASP expression reduction after senolytic therapy [3].

Our results also demonstrated that fibroblast senescence was associated with increased abundance of CCN2 and EndMT, possibly reflecting crosstalk between (senescent) fibroblasts and endothelial cells in SSc. TGF-β and CCN2 are not only iconic SASP but also capable of inducing EndMT, especially in response to oxidative stress in SSc [6-8]. Senescent fibroblasts with SASP generate a paracrine effect on endothelia and aggravate fibrosis. In addition, fibroblasts have also been reported to interact with endothelial cells via NOTCH3 signalling in rheumatoid arthritis synovium, which accelerated IL-6 associated inflammation and fibroblast activation [18]. The crosstalk between fibroblasts and endothelial cells thus points to a vicious circle of progressive fibrosis in SSc.

The mRSS was correlated with the dermal semi-quantitative fibrotic score. However, neither lymph nor blood vessel density correlated with dermal fibrosis in our study, while in another small study of SSc forearm skin biopsies, a decrease in dermal lymph density was noted [19]. Moreover, EndMT was not observed on lymph vessels. Although both blood and lymph endothelial cells are capable of transdifferentiation to a more mesenchymal phenotype, the plasticity of remaining in a transition state between phenotypes may influence the detectability of EndMT in the snapshot of a skin biopsy [2, 20]. Fully mesenchymal-transited endothelial cells would lose the typical cobblestone morphology, cell-cell junctions and EC markers, including CD31 and ERG, and therefore cannot be detected by double staining [21]. Furthermore, CCN2 was also detected in fibroblasts and endothelium in healthy controls, especially in superficial dermis. Of note: CCN2 is not only involved in regulation of fibrosis but also in angiogenesis, chondrogenesis, and osteogenesis in soft tissue homeostasis [22]. The production of CCN2 may be stimulated by pressure, sun light or chemicals exposure, especially in superficial dermis.

We utilised both digital analysis and semiquantitative scoring in this study. Digital analysis with colour deconvolution and pixel classification generates objective and reproducible data. On the other hand, semiquantitative scoring evaluates abundance of markers on specific target, including fibroblasts and vessels, which has yet to be satisfactorily proceed in digital analysis. In addition, confounding, including fat infiltration, glands, hair follicles, muscles, background staining and fissures, were relatively more manageable by experienced pathologists in semiquantitative scoring than de-annotation in the software. An incorporation of both digital analysis and semiquantitative scoring connects the correlation of observed markers and possible origins.

Our study investigated histopathological features of skin biopsies taken for regular diagnostic purposes. Since skin biopsies are not an integral procedure for the diagnosis of SSc, our biopsies were derived from patients with apparently less complete or less typical clinical symptomatology. Despite the above, we only included patients meeting the ACR/EULAR 2013 classification criteria for SSc. Our study population was relatively heterogeneous because the biopsies were taken from various sites. Differences between normal skin characteristics in various areas of the body might have reduced the power of our analyses. For example, in sun-exposed skin, ultraviolet light can deregulate the TGF-β/SMAD pathway and induce cellular senescence [23, 24]. Nevertheless, we found no significant difference of investigated markers between biopsy sites in our study; only for P16 and CCN2, there was a trend suggesting potentially higher abundance on dermal fibroblasts from upper limbs.

Conclusions

In summary, we observed EndMT and fibroblast senescence to be more abundant in SSc skin than in healthy control skin. Moreover, the abundance of fibroblast cellular senescence was associated with EndMT, dermal inflammation and fibrosis. Our data provide further support for the notion that fibroblast senescence and EndMT is implicated in the pathogenesis of skin fibrosis and inflammation in at least a subset of SSc patients. We propose that skin biopsies may serve as a valuable source for companion diagnostic biomarker assessment in further exploring (alternative) senolytics therapies in SSc.

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Supplementary

Methods

Haematoxylin and Eosin staining

1. Deparaffinize and rehydrate in serial xylene-ethanol wash
2. Incubate in Mayer's Haematoxylin for 8 minutes followed by running tap water for 10 minutes
3. Wash in demi-water for 1 minute
4. Incubate in eosin for 30 seconds
5. Wash in demi-water for 1 minute
6. Dry and mount coverslip

Immunohistochemistry (IHC) and Immunofluorescence (IF) staining

1. Deparaffinize and rehydrate slides in serial xylene-ethanol wash
2. Block endogenous peroxidase with incubating in 1.5% H2O2 for 30 minutes (if proceed with peroxidase-reacted substrate, ex. Nova red and DAB)
3. Wash with demi water for 5 minutes
4. Antigen retrieval in boiled citrate (10 mM, pH 6) or EDTA (1 mM, pH 9) buffer for 20 minutes and then cool to room temperature
5. Incubate with primary antibody for 1 hour at room temperature
6. Wash with PBS for 5 minutes 3 times
7. Incubate in secondary antibody (peroxidase, alkaline phosphatase or fluorescence labelled) for 45 minutes
8. Wash with PBS for 5 minutes 3 times

(For IF staining, skip step 9 to 13)

9. Incubate with substrate (peroxidase or alkaline phosphatase reacted) for 10 minutes
10. Wash with demi water for 1 minute twice

(Repeat step 5 – 10 for double staining)

11. Counterstain with Mayer's Haematoxylin (1:3 diluted) for 10 seconds
12. Wash with running tap water for 10 minutes
13. Dehydrate and mount coverslip

(Counterstain in IF staining)

14. Counterstain with DAPI for 5 minutes
15. Wash with BPS for 5 minutes twice
16. Mount with VECTASHIELD® Antifade Mounting Medium
17. Seal/fasten coverslips with nail polish

Masson's Trichrome staining was performed automatically with Dako Artisan Link Pro according to the manufacturer's instructions.

Table S1. Summary of staining markers

Markers	All	SSc	Healthy control
Dermal fibrotic score, n			
1		5	
2		5	
3		8	
Tissue inflammation score, n			
Superficial dermis			
0	7	3	4
1	13	13	0
2	2	2	0
Deep dermis			
0	15	11	4
1	6	6	0
2	1	1	0
Senescence at endothelia, n			
P16, superficial dermis			
0	21	17	4
1	1	1	0
P16, deep dermis			
0	22	18	4
P21, superficial dermis			
0	8	6	2
1	13	11	2
2	1	1	0
P21, deep dermis			
0	16	12	4
1	6	6	0
Senescence at fibroblasts			
$P16^+$ percentage, median (IQR)	1.1 (0.5—6.3)	1.9 (0.5—7.7)	0.7 (0.4—1.0)
P16, superficial dermis			
0	9	7	2
1	8	6	2
2	5	5	0
P16, deep dermis			
0	13	9	4
1	4	4	0
2	5	5	0
$P21^+$ percentage, median (IQR)	15.7 (9.5—38.1)	20.8 (10.4—43.9)	3.3 (0.6—7.6)*
P21, superficial dermis			
0	2	1	1
1	9	7	2
2	11	10	1
P21, deep dermis			
0	8	5	3
1	9	8	1
2	5	5	0

(continued)

Markers	All	SSc	Healthy control
EndMT, median (IQR)%			
αSMA/CD31	6.2 (3.1—7.7)	6.4 (4.2—8.3)	0.4 (0.0—1.4)*
αSMA/ERG	7.8 (4.8—12.8)	9.1 (6.7—14.5)	2.8 (1.7—3.9)*
CCN2 on endothelia, superficial dermis			
0	2	2	0
1	15	13	2
2	5	3	2
CCN2 on endothelia, deep dermis			
0	11	9	2
1	11	9	2
CCN2 on fibroblasts, superficial dermis			
0	7	5	2
1	10	8	2
2	5	5	0
CCN2 on fibroblasts, deep dermis			
0	11	7	4
1	4	4	0
2	7	7	0
Lymph density, median (IQR) (/mm^2)	6.9 (4.5—9.4)	7.2 (4.5—9.6)	5.5 (4.4—7.0)

SSc, systemic sclerosis; EndMT, endothelial to mesenchymal transition; IQR, interquartile range. A significant difference between SSc and healthy control skin were labelled with *.

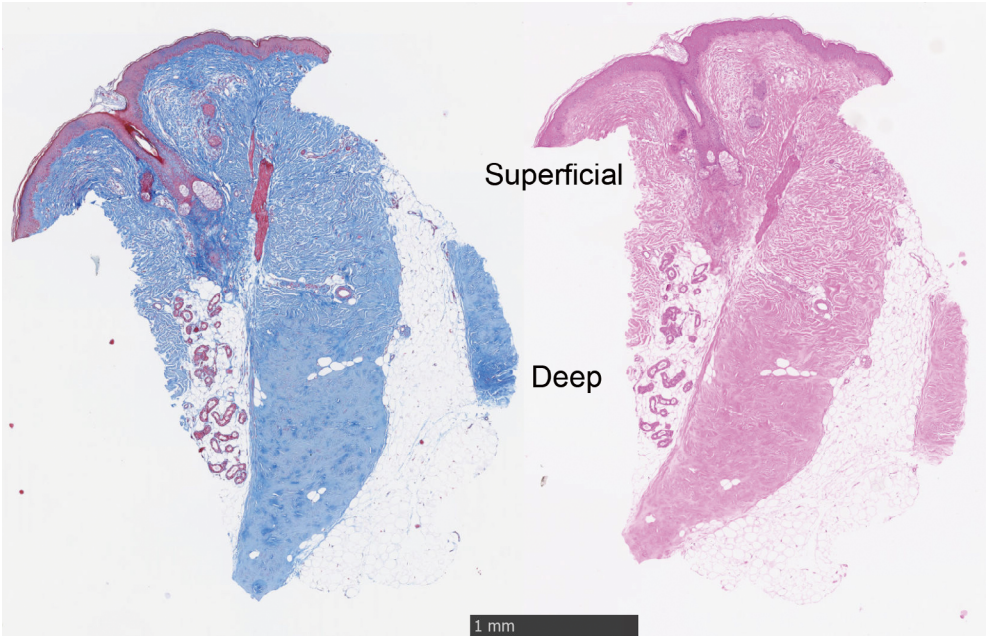

Figure S1. Full view of a Masson's Trichrome stained (left) and haematoxylin and eosin stained (right) skin biopsy. This biopsy had fibrosis score 1 in superficial dermis and 3 in deep dermis.

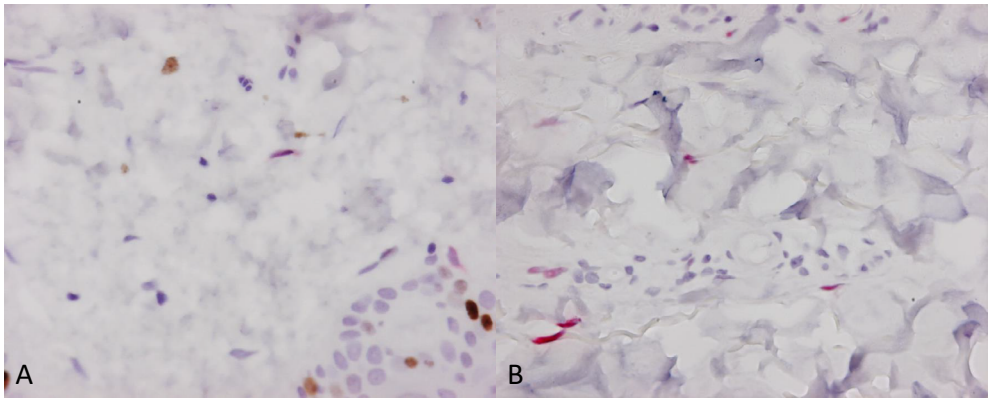

Figure S2. Senescence markers, P16 (A) and P21(B) was stained in bright red. The presence of senescence was observed from P21 or P16 positive without co-localized Ki-67 (brown).

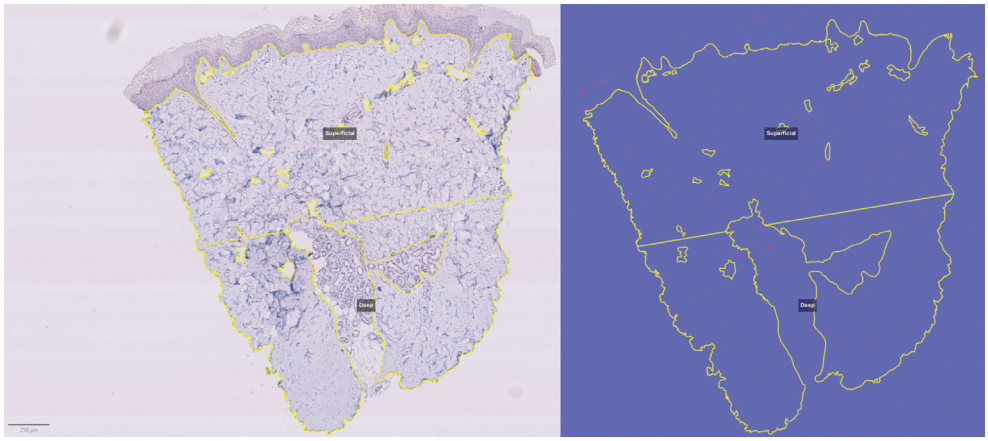

Figure S3. A CCN2 stained skin biopsy with annotation on superficial and deep dermis by the yellow line (left). The result of pixel classification on the CCN2 staining with red for positive pixels and blue for negative pixels (right).

Chapter 8
Summary and general discussion

Summary

Connective tissue diseases-associated interstitial lung disease (CTD-ILD) is a heterogeneous condition, not only in clinical presentation but also in underlying pathogenetic inflammatory mechanisms. Therefore, early identification and risk stratification remain challenging but essential for optimal management.

This thesis aimed to identify risk factors and biomarkers for progressive pulmonary fibrosis (PPF) and mortality in patients with CTD-ILD. We investigated prognostic factors for CTD-ILD in **Chapters 2** and **3**. In general, older age, smoking, increased extent of fibrosis on high-resolution computed tomography (HRCT) and poor lung function were associated with mortality. In **Chapter 2**, we investigated a cohort of predominantly patients with systemic sclerosis (SSc) and high extent of fibrosis on HRCT. In this cohort, glucocorticoid use, elevated C-reactive protein (CRP) and PPF were also associated with mortality. Patients with fibrotic HRCT patterns at baseline, diabetes mellitus, and glucocorticoid use were more likely to develop PPF. We validated the risk factors for PPF in a different cohort, which is described in **Chapter 3**.

We compared three different PPF criteria in **Chapter 3**. The prognostication of the three PPF criteria did not differ significantly. Still, the performance of these criteria in predicting mortality via the time-dependent receiver operator characteristic curve (ROC) was better in the cohorts from **Chapter 2** (predominantly SSc and high extent of fibrosis on HRCT) than **Chapter 3** (predominantly rheumatoid arthritis (RA) and low extent of fibrosis on HRCT). The risk of mortality increased during the first 3 years after PPF occurred and achieved a plateau thereafter. The proportions of patients identified as having PPF vary according to the three criteria; therefore, the benefits and costs of which patients require antifibrotic treatment need further consideration.

In **Chapter 4**, serum biomarkers were examined in CTD-ILD patients at baseline and one year. The decrease in the chemokine C-X-C motif ligand 11 (CXCL11), connective tissue growth factor (CTGF), and Krebs von den Lungen 6 (KL-6) serum concentrations during treatment were associated with inflammatory HRCT patterns and better pulmonary outcomes. Interestingly, baseline biomarkers, especially cytokines and chemokines, were determined more by underlying CTD than ILD manifestations. In **Chapter 5,** we describe a biomarker study in primary Sjögren syndrome (pSS)-associated ILD. KL-6 was found to be a valuable biomarker in identifying preclinical ILD in patients with newly diagnosed pSS.

SSc-ILD can be progressive with high morbidity and increased mortality. For SSc patients with rapid progression, intensive immunomodulation such as haematopoietic stem cell transplantation (HSCT) are considered to ameliorate lung and skin involvement. We reviewed the place of autologous HSCT, mechanism of action and future perspective in

SSc-ILD in **Chapter 6**. HSCT resets the immune system and provides a chance to reshape the immune system by antigenic selection that may differ from the first triggering of diseases. As a highly intensive therapy with related morbidity and mortality, HSCT should be provided by experienced multidisciplinary teams in carefully selected patients. The benefits and risks of early HSCT are investigated in the ongoing randomised UPSIDE trial.

The profibrotic pathophysiology in SSc, including cellular senescence and endothelial-to-mesenchymal transition (EndMT), was evaluated in **Chapter 7**. Progression of skin fibrosis is highly correlated with PPF in SSc patients. Moreover, acquiring skin samples is less invasive than lung samples. EndMT and fibroblast senescence were found to be more abundant in SSc than in healthy controls. The abundance of fibroblast senescence was correlated with EndMT, dermal fibrosis and inflammation.

General discussion

The unmet needs

ILD contributes significantly to the disease burden in CTD patients as it leads to reduced quality of life and increased mortality. Uncertainty and controversy at the interface between CTD and ILD have aroused our interest to carry out the studies described in this thesis, including biomarkers in identifying CTD-ILD and PPF, potential effective therapies and optimal patient classification for future trial design.

In 2019, the INBUILD trial has shed light on treatments for CTD-ILD with PPF, but more questions have emerged. For example, what are useful biomarkers for early identification of PPF in CTD-ILD? What is the place and timing of antifibrotics? Could early identification and intervention with antifibrotics prevent PPF and decrease mortality? This thesis contributes to the understanding of CTD-ILD and PPF.

Pulmonary monitoring and identifying PPF

ILD may be underestimated in CTD patients, especially those without respiratory symptoms. Although all CTD patients are at risk for developing ILD [1, 2], sensitivity, invasiveness and cost-effectiveness (in association with disease prevalence and prognostic implication) of screening tools are considered in who should be screened for ILD. For example, in a small (n=37) Austrian study, more than 60% of asymptomatic pSS patients with normal chest X-ray had abnormalities on HRCT and/or pulmonary function tests (PFT) [3]; however, these HRCT findings consisted mainly of septal thickening and micronodules, and pSS-ILD may be less progressive than other CTD [4, 5]. A biomarker assisted screening policy may be more appropriate than to screen all pSS patients with

HRCT and PFT. The upcoming 2023 American College of Rheumatology (ACR) CTD-ILD recommendation suggests screening PFT and HRCT in all pSS patients with one or more risk factors, including positive anti-Ro52, positive antinuclear antibodies (ANA), Raynaud phenomenon, older age and lymphopenia [6]. In Chapter 5, we report that an elevated serum KL-6 predicted development of ILD in pSS patients. KL-6 measurement for ILD screening is also recommended to be considered in the 2023 Japanese guideline [2]. In addition, there is consensus on routinely screening ILD in patients with poor prognosis. Screening for all SSc patients, especially diffuse cutaneous type, is therefore recommended by American, British, European and Japanese experts [2, 7-9]. Likewise, routine screening is recommended in idiopathic inflammatory myopathies (IIM) patients with anti-aminoacyl transfer ribonucleic acid (tRNA) synthetase (ARS) or anti-melanoma differentiation-associated gene 5 (MDA5) autoantibodies according to the Japanese guideline [2].

We identified that increased age, tobacco exposure, higher extent of fibrosis on HRCT and poor lung function are associated with mortality in Chapters 2 and 3. Our findings were in line with the general risk factors in ILD, including the GAP (gender, age and pulmonary physiology) and SADL (smoking, age and diffusing capacity for carbon monoxide [DLCO]) models, which have been described in Chapter 1 [10-12]. Patient characteristics, pulmonary function and HRCT remain the cornerstones of risk stratification.

PPF was associated with increased mortality in CTD-ILD, which is in line with previous studies [13-17]. In particular, the association between PPF and mortality was more robust in the cohort with high extent of fibrosis on HRCT in Chapter 2 than the cohort with low extent of fibrosis in Chapter 3. The prognostic value of the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Asociación Latinoamericana de Tórax (ATS/ERS/JRS/ALAT) 2022 criteria, the INBUILD criteria and the simplified progressive fibrosing criteria did not differ significantly throughout the whole disease course on the time-dependent ROC for predicting mortality. Moreover, the association between PPF and mortality was the strongest in the first five years after the presence of PPF (Chapter 3 Figure 2). Interestingly, the ATS/ERS/JRS/ALAT 2022 criteria showed a nuance of the lowest plateau of predictability in this riskiest period, the first five years after PPF. The ATS/ERS/JRS/ALAT 2022 criteria may be less prognosticated in real-time disease monitoring.

The course of ILD in patients with CTDs varies widely across individuals, and PPF can occur at any time in the disease course [18, 19]. Regular monitoring, with reduced frequency once a patient is stable, is recommended in the 2020 and 2023 Japanese guidelines and the upcoming 2023 ACR recommendation [2, 6, 20]. Intensive monitoring is needed in a progressive/acute stage of CTD-ILD. The 2023 Japanese guideline recommends X-ray and/or HRCT every few days (especially for those with anti-MDA5^{+}

IIM-ILD) to every month in acute/subacute CTD-ILD with progression and HRCT (consider with serum KL-6 and SP-D measurements) every 1 to 3 months in patients with acute/subacute CTD-ILD [2]. The early released summary of the upcoming 2023 ACR guideline recommends PFT monitoring every 3–6 months for patients with SSc-ILD and IIM-ILD in the first year after ILD diagnosis, while 3–12 months is recommended for the other CTD subtypes associated with ILD [6].

The frequency of monitoring after stabilisation of ILD depends on the risk of progression. Fibrotic HRCT patterns were associated with PPF in CTD-ILD (Chapter 2). Similarly, fibrotic non-specific interstitial pneumonia (fNSIP) was found to be associated with PPF in RA patients (Chapter 3). Also, patients with a usual interstitial pneumonia (UIP) pattern on HRCT were more likely to develop PPF than patients with non-UIP patterns [19]. In addition, other clinical features such as old age, male, smoking history, lower lung function and history of gastroesophageal reflux disease were associated with PPF in a large cohort of fibrotic ILD, of which 902 (33%) were CTD-ILD [21]. Male sex, higher skin fibrosis score and reflux were also associated with forced vital capacity (FVC) decline over 5 years in a large SSc cohort (EUSTAR) [22].

The 2023 Japanese guideline recommends HRCT every 6 to 12 months and PFT at least once per year in patients with CTD-ILD [2]. Less frequent PFT and HRCT follow-up may result in delayed identification/management of PPF and therefore increase mortality risk. In Chapter 3, we observed that patients with PFT monitoring had a better prognosis than those with missing data in the first two years. Although there may be selection bias that patients in poor condition were more likely missing PFT follow up, the increased mortality risk of infrequent follow-up remained after PPF identification (Chapter 3 Figure S2). There are tools for home PFT monitoring which may strengthen monitoring frequency and have been utilised in obstructive lung disease, ILD and the coronavirus pandemic [23-28]. However, due to the variability of unsupervised spirometry (home PFT), supervised clinic spirometry remains the benchmark of ILD monitoring [29]. In addition, documentation of respiratory symptoms in regular practice is mostly descriptive and inconsistently reported in electronic medical records. Furthermore, it is unclear which symptom is important in prognostication. Therefore, early detection of PPF could be challenging.

Respiratory symptoms can be quantified by several tools, including functional status and patient report outcome (PRO) tools. However, the clinical significance is more difficult to be determined in these PROs than in physiological measurements. In the nintedanib trials (SENSCIS and INBUILD), there was a significant difference in FVC decline between the treatment and placebo groups, whereas the difference in PROs between the two groups was not significant [30, 31]. Interestingly, in the RECITAL trial (rituximab versus cyclophosphamide in CTD-ILD), patients in both arms had improvement in both FVC and

PROs [32]. Symptom improvement requires a strong PFT response over the treatment course, i.e., an improvement in FVC is more appreciable than simply a reduction in FVC decline. Moreover, these PRO questionnaires are not routinely collected in regular clinics, and patients' PRO scores can vary from day to day.

The threshold and criteria for a minimal clinically meaningful progression varied across studies (Chapter 1, Table 1). The underlying patient characteristics and background therapy also influence prognostication of the criteria threshold. For example, the post-hoc analysis of INBUILD trial placebo group, including CTD-ILD patients meeting INBUILD criteria without baseline immunosuppressants, forced vital capacity (FVC) decline ≥10% predicted a higher FVC loss (-241.9 mL/year) than FVC decline ≥5–<10% with symptoms and/or HRCT progression (-133.1 mL/year) and the combination of HRCT and symptoms progression (-115.3 mL/year, p=0.0002 for subgroup-by-time interaction) [33]. In the multi-centre cohort with CTD-ILD patients, HRCT progression of fibrosis (alone or in combination with symptoms, FVC or diffusing capacity for carbon monoxide (DLCO) decline) was associated with the largest subsequent FVC decline [19]. Moreover, the criteria threshold also contributes to the number needed to treat with antifibrotics. Although the mortality prognostication of the three PPF criteria was similar (Chapter 3), the ATS/ERS/JRS/ALAT 2022 criteria, which used absolute decline in PFT compared to relative decline in other PPF criteria, identified the fewest patients. PPF was identified in 23% of patients with the ATS/ERS/JRS/ALAT 2022 criteria, 27% with INBUILD criteria and 54% with simplified PF criteria. Therefore, the balance between missed diagnosis and overdiagnosis for the PPF criteria needs more consideration [34]. While the benefit of upfront antifibrotics in CTD-ILD with PPF is unclear, the balance between cost and effectiveness on patient selection for antifibrotic therapy needs further investigation. Nevertheless, progression, whether clinically significant or not, can only be observed if patients are monitored.

Biomarkers

The clinical implementation of biomarkers depends on accuracy, accessibility and cost-effectiveness. As mentioned in Chapter 1, myriads of biomarkers have been investigated for diagnosis, monitoring and prognostication in CTD-ILD. New biomarkers are emerging in the context of research and are gradually finding their way into clinical practice. We evaluated the clinical utility of serum KL-6 in identifying ILD in pSS patients in Chapter 5 and the association between PPF and biomarkers, including autoantibodies, clinically available physiological and serum tests, and 38 novel biomarkers in Chapters 2,3 and 4.

Blood biomarkers that are available in usual care are autoantibodies, which can be used for disease classification and risk stratification [35]. In general, the presence of autoantibodies is associated with specific autoimmune CTD diseases. In the two

cohorts we described in this thesis (Chapters 2 and 3), we looked at ILD in a mixed CTD population. In our studies, we found that anti-Ro and anti-centromere were associated with increased mortality risk in univariate analysis but lost significance after multivariate adjustment (Chapter 2). Anti-centromere autoantibodies are associated with limited cutaneous SSc (lcSSc), and ILD in this disease subset can occur but is less progressive compared to diffuse cutaneous SSc (dcSSc) [36]. Anti-U1-RNP was associated with PPF, using ATS/ERS/JRS/ALAT 2022 criteria (Chapter 3), whereas anti-dsDNA was associated with lower PPF risk (Chapter 2). In our cohorts, we did not observe an increased risk of PPF in anti-Scl-70 positive patients, possibly because all patients had ILD. In other studies, anti-Scl-70 positivity was associated with dcSSc and ILD, compared to lcSSc [37]. Despite dcSSc patients having a higher risk of severe organ involvement and mortality, the presence of major organ complication is a more dominant mortality risk factor than the extent of skin involvement in SSc [38].

Another autoantibody associated with PPF is anti-MDA5, which can be seen in patients with IIM [39]. However, anti-MDA5 was only positive in 5 (3%) patients in Chapter 2 and 1 (0.4%) in Chapter 3. High proportion (20–75%) of anti-MDA5$^+$ IIM-ILD patients, especially in East Asia, had worsening dyspnoea and cough, with radiographic deterioration causing hypoxia within 3 months from respiratory symptom onset, also known as rapidly progressive ILD (RP-ILD) [40, 41]. Therefore, the Japanese CTD-ILD guideline suggests intensive monitoring of patients with anti-MDA5$^+$ IIM-ILD [2].

We validated the value of serum KL-6 in screening and monitoring CTD-ILD. Serum KL-6 was found to be associated with ILD in newly diagnosed pSS patients (Chapter 5). The area under the ROC (AUC) of KL-6 predicting ILD was 0.810 in enzyme-linked immunosorbent assay and 0.669 in latex particle-enhanced turbidimetric immunoassay. A decrease in serum KL-6 levels during follow-up was associated with improvement of inflammatory HRCT patterns under immunosuppressive therapy (Chapter 4). The value of using KL-6 in diagnosing and monitoring ILD is in line with previous studies in CTD-ILD and other ILDs [42-44]. Moreover, elevated serum KL-6 may also predict PPF development in CTD-ILD. A serum KL-6 of more than 1000 U/mL revealed a hazard ratio 2.9 in PPF development in a CTD-ILD cohort [45]. As a glycoprotein in response to pneumocyte remodelling, KL-6 can be detected in both blood and bronchoalveolar lavage [46]. However, cost and accessibility of the biomarkers are as important as sensitivity and specificity in clinical implication. Currently, KL-6 is clinically approved and available for use in routine practice in Japan and Taiwan, but not yet in the Netherlands.

With multiplex immunoassays, we observed a reduction of CXCL11 (interferon-inducible T cell α chemoattractant) and CTGF (also known as cellular communication network factor 2, CCN2) in CTD-ILD patients who had inflammatory HRCT patterns and responded to immunosuppressive treatment (Chapter 4). CXCL11 is a chemokine in response to

interferon γ and is associated with inflammation, anti-angiogenesis and defensin-like antimicrobial properties [47]. Elevated baseline CXCL11 was also correlated with response to treatment with FVC improvement in a Japanese treatment- naïve CTD-ILD cohort [48]. In addition, the abundance of CTGF on fibroblasts of SSc skin biopsies was correlated with inflammation, fibrosis, fibroblast senescence and EndMT (Chapter 7). CTGF enhances receptor binding of transforming growth factor-β 1 and implicates multiple cellular events, including angiogenesis, skeletogenesis and tissue remodelling like wound healing [49]. Elevated serum CTGF has been shown to be associated with ILD in RA and fibrosis severity in SSc [50, 51]. Nevertheless, chemokines and cytokines can be heterogenous across underlying CTD and inflammatory pathways. More research is needed to validate clinical relevance of chemokines and cytokines in subgroups of CTD-ILD.

Personalised treatment

Regular monitoring of patients is key to identifying PPF. Once risk factors are identified, monitoring treatment response and disease progression is essential for a personalised treatment strategy. In general, the therapeutic goal in CTD-ILD is to control the underlying inflammation according to the treat-to-target, e.g. remission and low disease activity, in each CTD subtype. Add-on antifibrotics may be considered if pulmonary fibrotic progression is present despite systemic inflammation control. The therapeutic options for CTD-ILD have been described in Chapter 1. Figure 1 summarises the pivotal randomised controlled trials (RCTs) in CTD-ILD.

Glucocorticoids are a double-edged sword in the treatment of CTD. We identified that glucocorticoid use is a risk factor for PPF (Chapter 2). Glucocorticoids are potent immunosuppressants with rapid onset, which are often used in combination with other immunosuppressants as induction therapy in CTD and CTD-ILD. Therefore, a short course and then tapering glucocorticoids as low as possible are strongly recommended [6, 52]. Risks of prolonged glucocorticoids include renal crisis in SSc, glucose intolerance, diabetes, cardiovascular disease, osteoporosis, peptic ulcer disease, glaucoma and recurrent infections [53]. In addition, prolonged glucocorticoid use raised profibrotic concern in addition to systemic side effects. The association between glucocorticoid use and PPF was also observed in a recently published Korea CTD-ILD cohort [45]. Glucocorticoid use was associated with a larger annual FVC decline in the disease course of non-idiopathic pulmonary fibrosis (IPF) PPF (the placebo group in INBUILD trial) and increased the risk of death and hospitalisation in patients with IPF (PANTHER-IPF trial) [54, 55]. Because of the risk of scleroderma renal crisis, the upcoming 2023 ACR guideline gives a strong recommendation against glucocorticoids as first-line treatment in SSc-ILD [6].

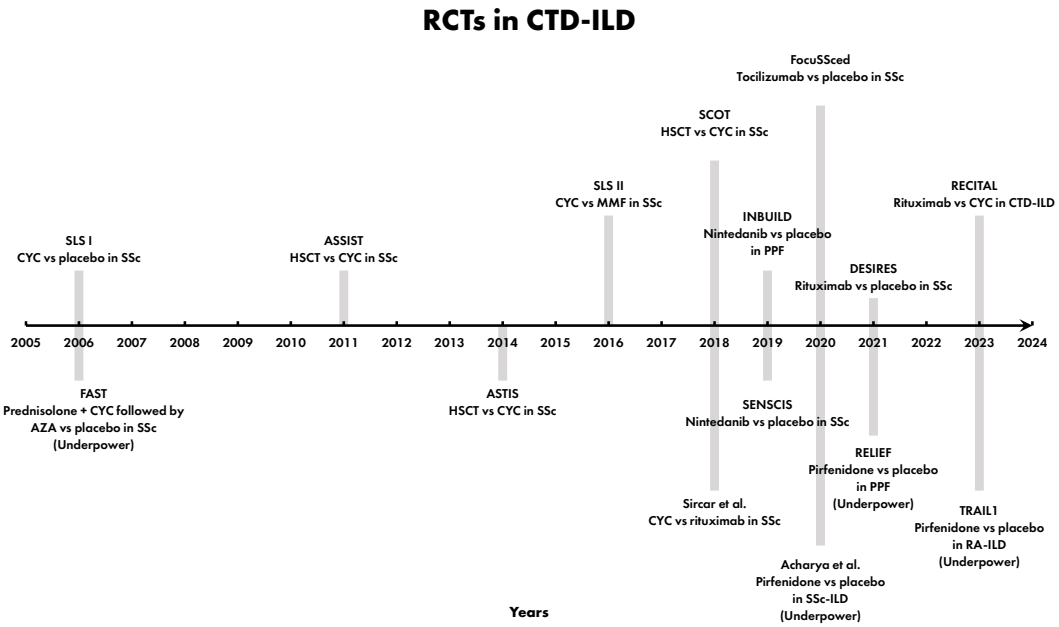

Figure 1. Timeline of milestone randomised controlled trials (RCTs) in managing connective tissue diseases-associated interstitial lung disease (CTD-ILD). CYC, cyclophosphamide; AZA, azathioprine; SSc, Systemic sclerosis; RA, rheumatoid arthritis; SLS, Scleroderma Lung Study; FAST, Fibrosing Alveolitis in Scleroderma Trial; ASSIST, American Scleroderma Stem Cell versus Immune Suppression Trial; HSCT, hematopoietic stem-cell transplantation; PPF, progressive pulmonary fibrosis; ASTIS, Autologous Stem Cell Transplantation International Scleroderma trial; SCOT, Scleroderma: Cyclophosphamide Or Transplantation trial; INBUILD, Nintedanib in Progressive Fibrosing Interstitial Lung Diseases trial; SENSCIS, Safety and Efficacy of Nintedanib in Systemic Sclerosis trial; focuSSced, A Study of the Efficacy and Safety of Tocilizumab in Participants With Systemic Sclerosis; DESIRES, Safety and efficacy of rituximab in systemic sclerosis trial; RELIEF, Exploring efficacy and safety of oral Pirfenidone for progressive, non-IPF lung fibrosis; RECITAL, Rituximab versus intravenous cyclophosphamide in patients with connective tissue disease-associated interstitial lung disease in the United Kingdom; TRAIL1, Treatment for Rheumatoid Arthritis and Interstitial Lung Disease 1.

The efficacy of CYC, MMF, rituximab, tocilizumab and HSCT in treating CTD-ILD has been shown in RCTs (Figure 1). Mycophenolate mofetil (MMF), rituximab and cyclophosphamide have shown benefits in both systemic disease activity control and attenuation of pulmonary progression [6, 32]. In patients with elevated acute-phase reactants and intolerant to the above-mentioned medications, tocilizumab can be an alternative medication [7-9, 56, 57]. HSCT may be considered in carefully selected patients at risk of progression and organ failure [58-60]. In addition, the efficacy of calcineurin inhibitors (CNI), including cyclosporin and tacrolimus, was reported only in observational studies and a small-scale open-label RCT in IIM-ILD [61-63]. Nevertheless, further research is necessary to identify biomarkers that can predict patients' response to specific treatments (or treatment combinations) and assist in precisely selecting therapeutic options.

There is yet to be clear evidence on the benefit of an upfront combination of antifibrotics with immunosuppressants in CTD-ILD in preventing PPF. Only a few patients received antifibrotics in our cohorts (Chapters 2 and 3). The early terminated SLS III trial revealed a trend towards a faster improvement in FVC in SSc patients receiving the upfront combination of pirfenidone and mycophenolate mofetil (MMF) than MMF alone prior to PPF onset [64]. However, due to underpowering and short follow-up periods, the effectiveness of upfront antifibrotics in preventing PPF is still elusive. Moreover, although antifibrotics alone can alleviate the FVC decline in ILD with PPF, the benefit of antifibrotics reducing mortality in CTD-ILD patients was not clear [65]. Furthermore, the current on-market antifibrotics (nintedanib and pirfenidone) do not improve but just slow down pulmonary progression, whereas FVC improvement can be observed in patients treated with immunosuppressive therapy [66, 67]. For this reason, antifibrotics should not be used as monotherapy in CTD-ILD. More research is needed to determine the effectiveness of early combination of antifibrotics and immunosuppressants, as well as which patients may benefit from these combinations and which patients do well with immunosuppressants alone. Nonetheless, treatment strategy and monitoring should be personalised, especially according to the underlying CTD.

Future research and clinical perspective

In addition to conventional and biological disease-modifying antirheumatic drugs (c and bDMARDs), Janus kinase inhibitors and antifibrotics, there are new therapeutic targets under investigation in mitigating inflammation and fibrosis [68]. Pathophysiology targets include the transforming growth factor beta (TGF-β) signalling pathway, peroxisome proliferator-activated receptors (PPAR, especially the γ receptor), reactive oxygen species and stromal developmental pathways (WNT, Notch and sonic hedgehog) and others [69]. We have shown that fibroblast senescence and EndMT may play a role in the inflammation and fibrosis in SSc patients (Chapter 7). Spatial transcriptomics of SSc skin biopsies also revealed a correlation between senescence signature and extracellular matrix signature [70]. Eliminating senescent cells may interrupt the vicious cycle of inflammation and fibrosis. Several novel therapeutic compounds interfere with senescence and facilitate senescent cells undergoing apoptosis (senolytic), including the combination of dasatinib and quercetin, navitoclax, phosphodiesterase 4 (PDE4) inhibitors and even chimeric antigen receptor (CAR) T cells targeting therapy. Dasatinib is a tyrosine kinase inhibitor targeting Src family kinases (SFK), Abl family kinases and Lck, which is approved in treating chronic myeloid leukaemia [71]. Quercetin is a naturally occurring flavonoid molecules and induces apoptosis in senescent endothelial cells [72]. Navitoclax, targeting BCL-XL/BCL-2, induces myofibroblast apoptosis and attenuates fibrosis in bleomycin-induced mice fibrosis and human myelofibrosis (not clinically approved yet) [73, 74]. Moreover, CAR-T cells that target either urokinase plasminogen

activator receptor (uPAR) or fibroblast-activation peptide (FAP) have been shown to eliminate senescent cells and therefore attenuate fibrosis in cellular and animal studies [75-77]. However, dasatinib alone did not show efficacy in skin and lung fibrosis in a phase 2a open-label single-arm trial in SSc patients [78]; interestingly, the cellular senescence-associated secretory phenotype (SASP) gene signature in skin biopsies from SSc patients with response to dasatinib was higher than non-responders at baseline and reduced after senolytic therapy [79]. Senescence abundance in skin biopsies may serve as a valuable source for diagnostic and prognostic biomarkers in further exploring therapies, especially senolytics in SSc.

PDE4 may be another potential therapeutic target. Inhibiting PDE4 suppresses the hydrolysis of the second messengers, cyclic adenosine monophosphate (cAMP) and interferes with multiple signalling pathways, including β-adrenoceptor and N-methyl-D-aspartic acid receptor (NMDAR), 5-hydroxytryptamine (5-HT) receptor, receptors for activated C kinase 1 (RACK1), A-kinase-anchoring proteins (AKAPs), cAMP-responsive element binding protein (CREB), activating transcription factor 1 (ATF-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and B-cell lymphoma 6 protein (Bcl-6) [80-82]. Therefore, PDE4 inhibitors (PDE4i) may contribute to anti-inflammatory, antifibrotic and senolytic effects [83-85]. Inhibition of PDE4 alleviates skin fibrosis and inflammation by directly suppressing the activation of macrophages and T cells in SSc animal models [86, 87]. In a phase 2 RCT, PDE4i (BI 1015550) reduced the FVC decline (even slightly improved) in patients with IPF [88]. The median change in FVC was +5.7 mL in patients with PDE4i without antifibrotics, +2.7 mL in PDE4i with antifibrotics, −81.7 mL in placebo without antifibrotics and −59.2 mL in placebo with antifibrotics. The safety and efficacy of BI 1015550 in PPF (using INBUILD criteria) is investigating in an ongoing RCT (NCT05321082) [89]. While there are clinically approved oral PDE4i with adequate safety profiles (apremilast in psoriatic arthritis and roflumilast in chronic obstructive pulmonary disease), investigating the safety and efficacy of repurposing marketed PDE4i in CTD-ILD is warranted.

Personalised therapy relies on accurately classifying patients for the optimal treatment. The current PPF criteria predict mortality in CTD-ILD, but the prognostication works better in patients with high extent of fibrosis on HRCT than in those with low extent. Further real-world studies in patients treated with immunosuppressants and antifibrotics can pave the way to optimise PPF criteria. Regarding biomarkers, the key to translating research into clinical practice is cost-effectiveness. For example, if KL-6 can be measured as cheaply as CRP with preserved accuracy, it's more likely to be approved for clinical use and reimbursement. Moreover, investigating biomarkers that identify patients who need early intensive therapy, including HSCT or combination of immunosuppressants and antifobrotics, is needed. Furthermore, RCTs on safety and efficacy of other novel targeted

therapies that reduce inflammation and fibrosis may expand therapeutic choices in CTD-ILD. Last but not the least, multidisciplinary discussion and strict monitoring of CTD-ILD patients may contribute to better outcomes.

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Nederlandse Samenvatting

Bindweefselziekten-geassocieerde interstitiële longziekte (CTD-ILD) is een heterogene aandoening, niet alleen in klinische presentatie maar ook in onderliggende pathogenetische ontstekingsmechanismen. Daarom blijft vroege identificatie en risicostratificatie een uitdaging, maar essentieel voor een optimale management.

Dit proefschrift is gericht op het identificeren van risicofactoren en biomarkers voor progressieve longfibrose (PPF) en mortaliteit bij patiënten met CTD-ILD. In hoofdstuk 2 en hoofdstuk 3 hebben we prognostische factoren voor CTD-ILD onderzocht. Over het algemeen werden oudere leeftijd, roken, een grotere mate van fibrose op hoge-resolutie computertomografie (HRCT) en een slechte longfunctie geassocieerd met mortaliteit. In hoofdstuk 2 onderzochten we een cohort van voornamelijk patiënten met systemische sclerose (SSc) en een hoge mate van fibrose op HRCT. In dit cohort waren corticosteroïdgebruik, verhoogd CRP en PPF ook geassocieerd met mortaliteit. Patiënten met fibrotische HRCT-patronen bij aanvang, diabetes mellitus en corticosteroïdgebruik hadden een grotere kans om PPF te ontwikkelen. We hebben de risicofactoren voor PPF gevalideerd in een ander cohort, dat wordt beschreven in hoofdstuk 3.

In hoofdstuk 3 hebben we drie verschillende PPF-criteria vergeleken. De prognosticatie van de drie PPF-criteria verschilde niet significant. Toch was de prestatie van deze criteria in het voorspellen van mortaliteit via de tijdsafhankelijke receiver operator characteristic curve (ROC) beter in de cohorten uit hoofdstuk 2 (voornamelijk patiënten met systemische sclerose (SSc) en een hoge mate van fibrose op HRCT) dan in hoofdstuk 3 (voornamelijk patiënten met reumatoïde artritis (RA) en een lage mate van fibrose op HRCT). Het sterfterisico nam toe gedurende de eerste 3 jaar na het optreden van PPF en bereikte daarna een plateau. Het percentage patiënten met PPF varieert volgens de drie criteria; daarom moeten de voordelen en kosten van welke patiënten een antifibrotische behandeling nodig hebben, verder worden onderzocht.

In hoofdstuk 4 werden serum biomarkers onderzocht bij CTD-ILD patiënten op baseline en na één jaar. De afname van de serumconcentraties van het chemokine C-X-C motief ligand 11 (CXCL11), bindweefselgroeifactor (CTGF) en Krebs von den Lungen 6 (KL-6) tijdens de behandeling werd geassocieerd met inflammatoire HRCT-patronen en betere pulmonale uitkomsten. Interessant genoeg werden baseline biomarkers, met name cytokinen en chemokinen, meer bepaald door onderliggende CTD dan door ILD manifestaties. In hoofdstuk 5 beschrijven we een biomarkerstudie in primair Sjögren-syndroom (pSS)-geassocieerde ILD. KL-6 bleek een waardevolle biomarker te zijn voor het identificeren van preklinische ILD bij patiënten met nieuw gediagnosticeerde pSS.

SSc-ILD kan een progressief beloop hebben met een verhoogde morbiditeit en mortaliteit. Voor SSc-patiënten met snelle progressie wordt intensieve immunomodulatie

zoals hematopoëtische stamceltransplantatie (HSCT) overwogen om de long- en huidbetrokkenheid te verbeteren. In hoofdstuk 6 bespreken we de plaats van autologe HSCT, het werkingsmechanisme en het toekomstperspectief bij SSc-ILD. HSCT reset het immuunsysteem en biedt een kans om het immuunsysteem opnieuw vorm te geven door antigene selectie die kan verschillen van de eerste presentatie van de ziekte. Gezien de zeer intensieve therapie met bijbehorende morbiditeit en mortaliteit, moet HSCT worden uitgevoerd door ervaren multidisciplinaire teams bij zorgvuldig geselecteerde patiënten. De voordelen en risico's van vroege HSCT wordt heden onderzocht in de lopende gerandomiseerde UPSIDE studie.

De profibrotische pathofysiologie bij SSc, inclusief cellulaire senescentie en endotheliale naar mesenchymale transitie (EndMT), werd geëvalueerd in hoofdstuk 7. Progressie van huidfibrose is sterk gecorreleerd met PPF bij SSc-patiënten. Bovendien zijn biopten van de huid minder invasief dan longbiopten. Met de cross-sectionele benadering van SSc huidbiopten werd vastgesteld dat EndMT en fibroblast senescentie overvloediger aanwezig waren bij SSc dan bij gezonde controles. De overvloed aan fibroblastsenescentie was gecorreleerd met EndMT, huidfibrose en ontsteking. Over het algemeen levert het onderzoek waardevolle inzichten op in de complexiteit van CTD-ILD, met identificatie van risicofactoren, biomarkers en potentiële behandelingen voor een betere patiëntenuitkomst.

Appendix

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- **Yu-Hsiang Chiu**, Julia Spierings, Pim A de Jong, Firdaus Mohamed Hoesein, Jan C Grutters, Jacob M van Laar, Mareye Voortman*. Predictors for progressive fibrosis in patients with connective tissue disease associated interstitial lung diseases. *Respir Med*. 2021 Aug 18;187:106579. **[Thesis]**

- **Yu-Hsiang Chiu**, Chen-Chih Chu Chun-Chi Lu, Feng-Cheng Liu, Shin-En Tang, Shi-Jye Chu, San-Yuan Kuo, Hsiang-Cheng Chen*. KL-6 as a Biomarker of Interstitial Lung Disease Development in Patients with Sjögren Syndrome: A Retrospective Case-Control Study. *J Inflamm Res*. 2022 Apr 8;15:2255-2262. **[Thesis]**

- **Yu-Hsiang Chiu**, Maaike F. Koops, Mareye Voortman, H. Wouter van Es, Lucianne C. Langezaal, Paco M. Welsing, Anna Jamnitski, Anne E. Wind, Jacob M. Van Laar, Jan C. Grutters, Julia Spierings*. Prognostication of progressive pulmonary fibrosis in connective tissue disease-associated interstitial lung diseases: a cohort study. *Front Med (Lausanne).* 2023 Feb 27;10:1106560. **[Thesis]**

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- **Yu-Hsiang Chiu**, Anouk Drijver, Rick Admiraal, Anna van Rhenen, Stefan Nierkens, Jacob M van Laar, Julia Spierings*. Anti-thymocyte globulin exposure in patients with diffuse cutaneous systemic sclerosis undergoing autologous haematopoietic stem cell transplantation. *J Scleroderma Relat Disord.* 2023 Oct;8(3):241-246.

Curriculum Vitae

Yu-Hsiang Chiu was born on November 7, 1985, in Kaohsiung in Taiwan. As a child, he loved fantasy epics and immortal hero stories, which raised his interest in the mystery of creatures (mortals) and beyond (immortals). Out of curiosity about life and partly for financial reasons, he studied medicine at a military-funded medical school, the National Defense Medical Center, in 2004. After achieving a medicine degree in 2011, he served in the Air Force as a flight surgeon for two years. After that, he received residence training in internal medicine and a rheumatology and immunology fellowship at Tri-Service General Hospital. He encountered challenging cases and struggled with the unmet needs in managing patients with connective tissue disease associated interstitial lung disease. He was inspired by Prof. Jacob van Laar's lecture in the 2018 EULAR and raised the idea of pursuing a PhD after specialist training. Luckily, he won the full scholarship for studying abroad after becoming a rheumatologist in 2019. However, a haze of a new severe acute respiratory syndrome endemic was raised, and he was assigned to the emergency room dealing with COVID crisis for a year. In 2020, he moved to the Netherlands and began his PhD project in the Department of Rheumatology and Clinical Immunology at University Medical Center Utrecht under the supervision of Prof. Jacob van Laar, Dr. Julia Spierings and Dr. Mareye Voortman. He had the great opportunity to perform translational research in collaboration with the Pathology Department under the supervision of Prof. Roel Goldschmeding. He built up research collaboration with Leiden UMC, St. Antonius Hospital and UMC Groningen and was involved in the multicentre randomised trial, the UPSIDE trial. During his PhD years, he presented at various international conferences and published in peer-reviewed journals. He will continue to dedicate himself to patient care and research in Taiwan.

Abbreviations

Abbreviation	Meaning
6MWD	Six minutes walk test distance
AAV	Antineutrophil cytoplasmic antibody-associated vasculitis
ACR	American College of Rheumatology
aHSCT	Autologous hematopoietic stem cell transplantation
ALAT	Asociación Latinoamericana de Tórax
ANA	Antinuclear antibody
ANCA	Antineutrophil cytoplasmic antibody
ARS	Anti-aminoacyl transfer ribonucleic acid synthetase autoantibodies
ASCT	Autologous stem cell transplantation
ASSIST	American Scleroderma Stem Cell versus Immune Suppression Trial
ASTIS	Autologous Stem Cell Transplantation International Scleroderma Trial
ATA	Anti-topoisomerase I autoantibodies
ATG	Anti-thymocyte globulin
ATS	American Thoracic Society
AUC	Area under curve
AZA	Azathioprine
Breg	B regulatory cell
C3	Complement 3
C4	Complement 4
CCN2	Cellular communication network factor 2
CI	Confidence interval
CLEIA	Chemiluminescence enzyme immunoassay
CNI	Calcineurin Inhibitors
cNSIP	Cellular non-specific interstitial pneumonia
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CTD	Connective tissue disease
CTD-ILD	Connective tissue disease associated interstitial lung disease
CTGF	Connective tissue growth factor
CXCL	The chemokine C-X-C motif ligand
CYC	Cyclophosphamide
dcSSc	Diffuse cutaneous systemic sclerosis
DESIRES	Safety and efficacy of rituximab in systemic sclerosis trial
DIP	Desquamative interstitial pneumonia
DLCO	Diffusing capacity for carbon monoxide
ECM	Extracellular matrix
EFS	Event free survival
ELISA	Enzyme-linked immunosorbent assay
EndMT	Endothelial to mesenchymal transition
ERG	Erythroblast transformation specific family-related gene transcription factor
ERS	European Respiratory Society
ESR	Erythrocyte sedimentation rate
ESSDAI	European League Against Rheumatism Sjögren's syndrome disease activity index

(Continued)

Abbreviation	Meaning
EULAR	European League Against Rheumatism
EUSTAR	European Scleroderma Trials and Research
FBN1	Fibrillin 1
FFPE	Formalin-fixed paraffin-embedded
FLI1	Friend leukemia integration 1
fNSIP	Fibrotic non-specific interstitial pneumonia
focuSSced	A Study of the Efficacy and Safety of Tocilizumab in Participants With Systemic Sclerosis
FRA2	Fos-related antigen 2
FU	Follow-up
FVC	Forced vital capacity
Gal3	Galectin-3
G-CSF	Granulocyte colony-stimulating factor
GGO	Ground-glass opacity
GMCSF	Granulocyte-macrophage colonystimulating factor
GRCS	Global rank composite scores
HE	Haematoxylin and eosin stain
HR	Hazard ratios
HRCT	High-resolution computed tomography
HSCT	Hematopoietic stem cell transplantation
IIM	Idiopathic Inflammatory myopathy
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
ILD	Interstitial lung disease
INBUILD	Nintedanib in progressive fibrosing interstitial lung diseases trial
INF	Interferon
INFγ	Interferon gamma
IPAF	Interstitial pneumonia with autoimmune features
IPF	Idiopathic pulmonary fibrosis
IQR	Interquartile range
ITT	Intention-to-treat
IV	Intravenous
JAKi	Janus kinase inhibitors
JRS	Japanese Respiratory Society
KCNA5	Potassium voltagegated channel 5
KL-6	Krebs von den Lungen 6
lcSSc	Limited cutaneous systemic sclerosis
LETIA	Latex particle-enhanced turbidimetric immunoassay
LIP	Lymphocytic interstitial pneumonia
MCTD	Mixed connective tissue disease
MDA5	Melanoma differentiation-associated protein 5
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MMP	Matrix metalloproteinase
mNSIP	Mixed non-specific interstitial pneumonia
mRSS	Modified Rodnan skin score
MTX	Methotrexate

(Continued)

Abbreviation	Meaning
NLH	Nodular lymphocytic hyperplasia
NSAID	Non-steroidal anti-inflammatory drugs
NSIP	Non-specific interstitial pneumonia
OP	Organising pneumonia
PDGF-B	Platelet derived growth factor subunit B
PF-ILD	Progressive fibrosing interstitial lung disease
PFS	Progression free survival
PFT	Pulmonary function tests
PPF	Progressive pulmonary fibrosis
PPFE	Pleuroparenchymal fibro-elastosis
PRO	Patient report outcome
Psel	P-selectin
pSS	Primary Sjögren syndrome
RA	Rheumatoid arthritis
RECITAL	Rituximab versus intravenous cyclophosphamide in patients with connective tissue disease-associated interstitial lung disease in the United Kingdom
RELIEF	Exploring efficacy and safety of oral Pirfenidone for progressive, non-IPF lung fibrosis
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SAA1	Serum amyloid A1
SASP	Senescence-associated secretory phenotype
SCOT	Scleroderma: Cyclophosphamide Or Transplantation Trial
SCT	Stem cell transplantation
SD	Standard deviation
SENSCIS	Safety and Efficacy of Nintedanib in Systemic Sclerosis trial
sICAM	Soluble intercellular adhesion molecule-1;
SLE	Systemic lupus erythematosus
SLS	Scleroderma lung study
SpA	Spondyloarthritis
SPARC	Secreted protein acid rich in cysteine
SPD	Surfactant protein D
sPD-1	Soluble programmed death-1
SS	Sjögren syndrome
SSc	Systemic sclerosis
sVCAM	Soluble vascular cell adhesion molecule-1
TAK1	Transforming growth factor beta-activated kinase 1
TBI	Total body irradiation
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TNFi	Tumor necrosis factor alfa inhibitors
TNFα	Tumor necrosis factor alpha
TRAIL1	Treatment for Rheumatoid Arthritis and Interstitial Lung Disease 1
tRNA	transfer ribonucleic acid
UCTD	Undifferentiated connective tissue disease
UIP	Usual interstitial pneumonia
UMCU	University Medical Centre Utrecht

(Continued)

Abbreviation	Meaning
uPAR	Urokinase-type plasmogen activator receptor
UPSIDE	UPfront autologous hematopoietic Stem cell transplantation versus Immunosuppressive medication in early DiffusE cutaneous systemic sclerosis trial
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
α-SMA	Alpha smooth muscle actin