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Aus der Klinik für Pneumologie, Ruhrlandklinik

**Azathioprine in connective tissue disease-associated interstitial lung  
disease: data from a retrospective single centre study**

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# 1 INTRODUCTION

## 1.1 Definition and classification of interstitial lung disease (ILD)

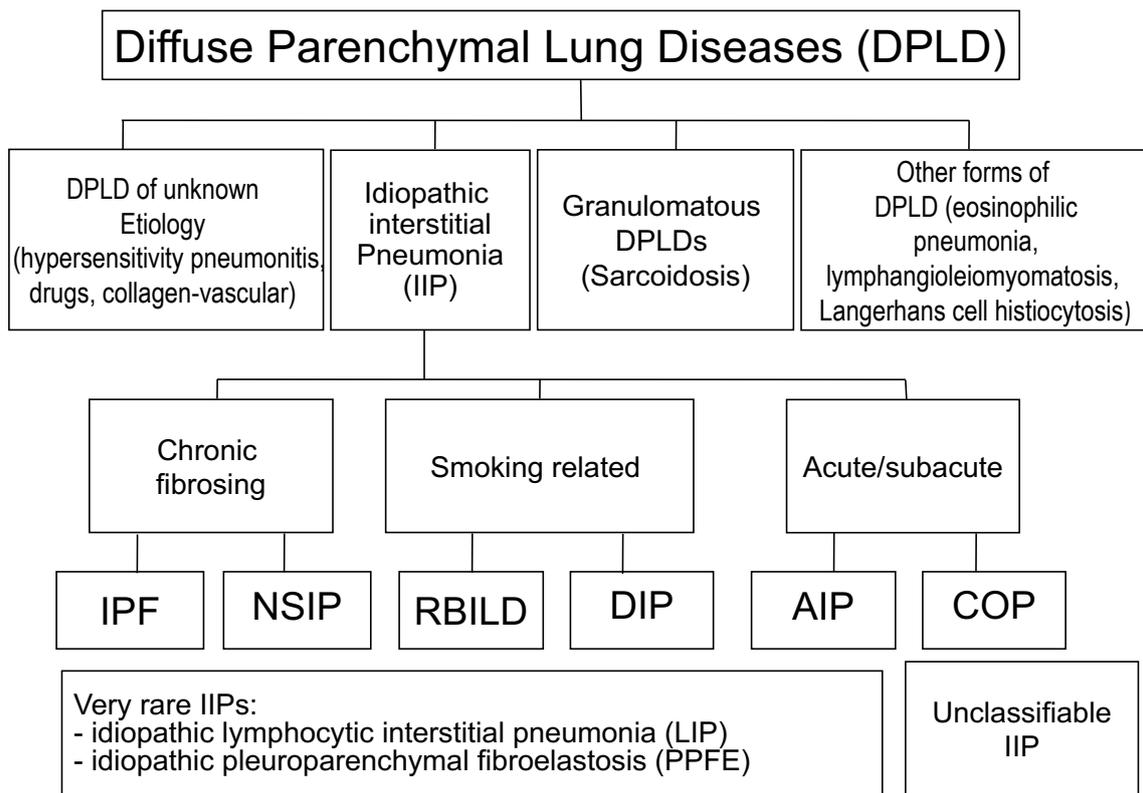
Interstitial lung diseases are a heterogeneous group of acute or chronic disorders which involve the lung parenchyma and cause various degrees of inflammation and/or fibrosis of the lung. This spectrum of diseases involves not only the interstitium but also the alveoli and bronchi. Therefore the term 'diffuse parenchymal lung diseases' (DPLD) is also used. The classification of ILDs depends on the underlying aetiology or histomorphology.

Idiopathic interstitial pneumonias (IIP) are a group of diffuse parenchymal lung diseases with unknown cause or association (Travis et al., 2013), **Figure 1**. Before an ILD is diagnosed as 'idiopathic', other underlying casualties such as connective tissue diseases (CTD), ILD caused by inhalation exposure such as silicosis or hypersensitivity pneumonitis and drug-induced ILD should be excluded.

ILDs cause progressive fibrosis and impaired pulmonary function with ensuing respiratory insufficiency. Restrictive ventilatory impairment and reduced diffusion capacity are the usual findings of ILDs in the pulmonary function test.

A precise medical history of co-existing diseases such as connective tissue diseases, occupational exposure to dusts, smoking history, exposure to feathers and mould is essential for the differential diagnosis of the ILDs, while the treatment options differs.

Furthermore, clinical signs of connective tissue diseases as well as biomarkers play a crucial role in the differential diagnosis of CTD associated ILDs and exclusion of other ILDs.



Travis et al., Am J Respir Crit Care Med 2013

IPF: Idiopathic pulmonary fibrosis, NSIP: Nonspecific interstitial pneumonia, RBILD: Respiratory bronchiolitis-interstitial lung disease, DIP: Desquamative interstitial pneumonia, AIP: Acute interstitial pneumonia, COP: Cryptogene organizing pneumonia

**Figure 1** Idiopathic interstitial pneumonias (IIPs)

CTD-related ILDs (CTD-ILDs) can vary in their clinical, radiological and histological appearances. The treatment and prognosis of CTD-ILDs differs from the IIPs, albeit the radiological and histopathological patterns are similar. Whereas in IIPs the most common form is UIP (47% to 62%), followed by NSIP (14% to 36%), in CTD-ILD the most frequent pattern is NSIP (Nicholson et al., 2000; Park et al., 2007). Other histopathological patterns which can be observed in CTD-ILDs include organising pneumonia (OP), lymphoid interstitial pneumonia (LIP), and diffuse alveolar damage (DAD). The patterns vary in frequency in the different CTDs (Tzelepis et al., 2008) **Table 1.**

**Table 1** Types of interstitial lung disease encountered in connective tissue diseases

	<b>SSc</b>	<b>PM/DM</b>	<b>PSS</b>	<b>RA</b>	<b>SLE</b>	<b>MCTD</b>
Usual interstitial pneumonia	++	++	+	++	+	+
Nonspecific interstitial pneumonia	++++	++++	+	+	++	++
Organizing pneumonia	+	++	+	+	+	-
Diffuse alveolar damage	+	++	+	+	++	-
Lymphocytic interstitial pneumonia	-	-	+++	-	-	-

SSc: systemic sclerosis, PM: polymyositis, DM: dermatomyositis, PSS: primary Sjögren's syndrome, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, MCTD: mixed connective tissue disease, +: lowest frequency, ++++: highest frequency, -: rare pulmonary involvement.

## 1.2 Spectrum of CTDs with associated ILD

CTDs or collagen vascular diseases are a heterogeneous group of autoimmune diseases which involve connective tissues, skin, muscles, joints, lungs and other organs. Antibody related inflammatory reactions trigger organ damage due to autoimmunity with consecutive fibrotic changes (Fischer and Richeldi, 2014). Inflammatory changes can manifest in different parts of the pulmonary system, from airways to the lung parenchyma, and also in the pleura and the pulmonary vasculature (Fischer and du Bois, 2012), **Table 2**.

**Table 2** Pulmonary manifestations in CTD

	<b>ILD</b>	<b>Airways</b>	<b>Pleural</b>	<b>Vascular</b>	<b>DAH</b>
Systemic sclerosis	+++	-	-	+++	-
Rheumatoid Arthritis	++	++	++	+	-
Primary Sjögren's syndrome	++	++	+	+	-
Mixed CTD	++	+	+	++	-
Polymyositis/dermatomyositis	+++	-	-	+	-
Systemic lupus erythematosus	+	+	+++	+	++

The signs show prevalence of each manifestation (-= no prevalence, +=low prevalence, ++=medium prevalence, +++high prevalence). DAH=diffuse alveolar damage.

The CTDs consist of scleroderma (SSc, systemic sclerosis), poly-/dermatomyositis (PM/DM), primary Sjögren syndrome (SjS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), mixed CTD (MCTD), and undifferentiated CTD (UCTD) (Olson et al., 2012) **Table 3**.

**Table 3** CTDs and other autoimmune diseases with lung involvement

<b>Connective tissue diseases</b>
Rheumatoid arthritis
Systemic sclerosis (scleroderma)
Poly-/dermatomyositis (Anti-synthetase syndrome)
Sjögren's syndrome
Systemic lupus erythematosus
Mixed connective tissue disease
Undifferentiated connective tissue disease
<b>Other autoimmune disorders</b>
Polyangiitis with granulomatosis (Wegener's syndrome)
Churg-Strauss vasculitis
Antiphospholipid syndrome
Behcet's disease
Relapsing polychondritis
Spondyloarthropathy

The pathophysiologic evolution of the CTDs is triggered by an inflammatory reaction with consequent fibrotic changes of connective tissue in multiple organs. Endothelial damage, which might be triggered by autoantibodies, viral agents or reactive oxygen radicals is the earliest step in the development of the inflammatory cascade and fibrosis. Endothelial damage generates vasodilatation, increased capillary permeability and expression of vasoactive mediators such as endothelial leukocyte adhesion molecule 1, VCAM-1. Activated endothelial cells release endothelin-1, which stimulates leukocyte adhesion to endothelium, fibroblast activation and smooth muscle cell proliferation. Fibroblasts and myofibroblasts

are playing an essential role in the development of fibrosis and derangement of the extracellular matrix. One of the pivotal mediators in the fibrotic process is transforming growth factor- $\beta$  (TGF- $\beta$ ) (Varga and Abraham, 2007).

Transforming growth factor- $\beta$  is a potent stimulus which activates the fibrotic cascade via connective tissue accumulation and myofibroblast proliferation. TGF- $\beta$  activates (phosphorylates) the intracellular signalling transduction system SMAD2, SMAD3 and SMAD4 in the nucleus which triggers DNA transcription of type I collagen, connective tissue growth factor (CTGF) and fibronectin (Varga and Abraham, 2007). Smad independent signal pathways such as c-Abl, TAK1, phosphatidylinositol 3 kinase and mitogen activated protein kinases are also triggered by TGF- $\beta$  and play a role in the pathogenesis of fibrosis, cancer, and autoimmunity (Castelino and Varga, 2014).

Another transcription factor playing a role in the development of fibrosis is the early growth response-1 (Egr-1), a zinc finger DNA binding protein, which is activated at sites of injury and regulates tissue response to acute injury by activating the synthesis of CTGF, plasminogen activator inhibitor-1, platelet derived growth factor and TGF- $\beta$ . Increased Egr-1 expression correlates with increased collagen accumulation in the bleomycin induced scleroderma mice and proliferation of myofibroblasts (Wu et al., 2009). Hence, it is associated with progression of fibrosis.

### **1.2.1 Rheumatoid Arthritis (RA)**

Rheumatoid arthritis (RA) usually manifests symmetrically as a progressive and destructive polyarthritis. It affects approximately 1% of the world population (Brown, 2007). It is the most frequent CTD. The incidence varies between 12 and 70 per 100.000 in the male population. It is seen twice as much in the female population.

Extraarticular manifestations are usually vascular, cardiac and pulmonary. They occur in up to 40% of the patients and are associated with higher mortality and morbidity. Their incidence has not declined over the decades (Gabriel et al., 2003; Turesson et al., 2003). On HRCT, ILD can be detected in up to 50% of patients with RA, but clinically significant RA-ILD is less frequent and affects approximately 10% of patients. Although approximately 40% of the RA patients die from cardiovascular diseases (Sihvonen et al., 2004), RA-ILD causes 10% to 20% of mortality in individuals with RA (Olson et al., 2011).

### **1.2.2 Systemic sclerosis (SSc)**

Systemic sclerosis shows features of progressive skin induration and sclerosis, fibrosis and small vessel vasculopathy. Depending on the extension of skin involvement SSc is divided into limited cutaneous scleroderma (LcSSc), diffuse cutaneous scleroderma (DcSSc) and SSc without skin involvement. The prevalence of SSc varies between 50 and 300/million (Chiffot et al., 2008).

The clinical manifestation of SSc include skin thickening of fingers spreading to metacarpophalangeal joints, fingertip lesions, telangiectasia, abnormal nailfold capillaries, interstitial lung disease, pulmonary hypertension, Raynaud's phenomenon and SSc related autoantibodies (van den Hoogen et al., 2013).

Autoantibodies against topoisomerase I, also called anti-Scl-70 are usually detected in patients with DcSSc and often associated with development of SSc-ILD and ensuing lung fibrosis. On the other hand detection of anticentromere antibody is less likely to be associated with SSc-ILD and usually correlates with pulmonary hypertension (Bonella and Costabel, 2014).

SSc-ILD and pulmonary hypertension are the most common causes of SSc related mortality (Steen and Medsger, 2007). Non-specific interstitial pneumonia (NSIP) is the most frequent histological pattern in SSc-ILD. The mortality correlates

rather with decreased DLCO and FVC and the presence of pulmonary hypertension than with the histomorphological pattern (Bouros et al., 2002).

### **1.2.3 Polymyositis/Dermatomyositis (PM/DM) - (Anti-synthetase syndrome)**

PM/DM are subacute inflammatory diseases which manifest in proximal muscles, DM affects also the skin. Clinical presentation of PM/DM is mainly progressive muscle weakness. However other organ manifestations such as pulmonary involvement, cardiac involvement and dysphagia are common. The incidence of PM/DM ranges from 1 to 6 per 1 million and these diseases predominantly affect the female population (Dalakas and Hohlfeld, 2003).

Anti-synthetase syndrome is a subgroup of PM/DM and characterized by positivity of an anti-tRNA synthetase autoantibody and the features of myositis, inflammatory arthralgias or arthritis, Raynaud's phenomenon (RP), 'mechanic's hands' (roughened skin over the tips of hands), ILD and esophageal dysmotility. Patients with anti-synthetase syndrome present usually clinical features of ILD with dyspnoea at the early stages of the clinical course; the symptoms of myositis may remain subclinical (Fischer et al., 2009).

Pulmonary complications are the major cause of mortality and morbidity of PM/DM. Hypoventilation related atelectasis or pneumonia due to thoracic muscle weakness occurs in 5% (Fathi et al., 2007). Furthermore 11% of individuals suffer from opportunistic infections and 17% of PM/DM patients develop aspiration pneumonia due to pharyngeal muscle weakness during the course of disease (Marie et al., 2005). PM/DM related ILD is present in approximately 30% of the patients (Schnabel et al., 2003). NSIP is the most common histopathological ILD pattern in PM/DM, followed by UIP, organizing pneumonia, diffuse alveolar damage and lymphocytic interstitial pneumonia (LIP) (Connors et al., 2010).

### **1.2.4 Sjögren Syndrome (SjS)**

Sjögren syndrome (SjS) is an autoimmune disease which manifests with inflammation of the exocrine glands due to lymphocytic infiltration. Especially the salivary and lacrimal glands are frequently affected with consequent symptoms of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) (Goules et al., 2014).

The incidence of Sjögren syndrome is 6.92 per 100 000 and the prevalence is 60.82 cases per 100 000 population (Qin et al., 2015). Two of the following three diagnostic criteria are defining a Sjögren Syndrome:

1. Focal lymphocytic sialadenitis with a focus score  $1 \geq \text{focus}/4 \text{ mm}^2$  in a salivary gland biopsy.
2. Positive antibody levels of serum anti-SSA/Ro and/or anti-SSB/La or rheumatoid factor and elevated antinuclear antibody (ANA) titres  $> 1:320$ .
3. Evidence of keratoconjunctivitis sicca with ocular staining score  $\geq 3$  (under the exclusion of current use of eye drops for glaucoma and exclusion of ocular operations in the last 5 years) (Shiboski et al., 2012).

Sjögren syndrome can be primary or secondary to another connective tissue disease, such as rheumatoid arthritis or SLE (Kreider and Highland, 2014). The most frequent interstitial lung involvement is NSIP. Further lung manifestations are UIP, LIP, bronchiolitis, bronchiectasis, amyloid changes and pulmonary lymphoma (Ito et al., 2005; Kreider and Highland, 2014).

### **1.2.5 Mixed connective tissue disease (MCTD)**

Mixed connective tissue disease (MCTD) is an overlap syndrome between at least two autoimmune diseases including SLE, SSc, PM/DM and rheumatoid arthritis. MCTD is associated with elevated levels of serum antibodies to U1 ribonuclear protein component of the spliceosome (U1RNP), which is seen as the hallmark of the disease. Common clinical manifestations include Raynaud's

syndrome, swollen hands, arthritis, sclerodactyly, polymyositis, interstitial lung fibrosis, pulmonary hypertension and nephritis (Aringer and Smolen, 2007). Presence of pulmonary hypertension and ILD is associated with increased morbidity and mortality. ILD is detected in 52% of patients. The most common interstitial lung change on HRCT is reticular pattern (35%) and severe lung fibrosis is seen in 19% of the individuals (Gunnarsson et al., 2012).

### **1.2.6 Undifferentiated connective tissue disease (UCTD)**

Undifferentiated connective tissue disease is a distinct entity, which does not fulfil all criteria of a defined CTD, however shows some signs and symptoms of CTD with serological changes.

A broader definition of UCTD-ILD was initially set up by Kinder with wider inclusion criteria of symptoms and serological markers (Kinder et al., 2010). These criteria were narrowed down to more specific symptoms and serological parameter by Corte (Corte et al., 2012).

Recently a new term, interstitial pneumonia with autoimmune features (IPAF), was proposed for patients with ILD without fulfilling definitive CTD criteria (Fischer et al., 2015). The new definition of this term includes the presence of an ILD without alternate aetiology, and the absence of a defined CTD, and at least one feature suggestive of autoimmunity from at least two of three main domains: the clinical, serological and morphological domain. The clinical domain includes extrathoracic features suggestive of CTD, the serological domain a panel of autoantibodies associated with CTDs, and the morphological domain radiological or histomorphological patterns such as organising pneumonia and/or NSIP or multicompartiment involvement. Multicentre prospective trials are necessary to validate these criteria (Strek and Costabel, 2016).

### **1.3 Treatment Options for CTD-ILDs**

Immunosuppressive therapies constitute the main treatment options in CTD-ILDs. The selection of agents, combination of medications, time to commencement of therapy and duration of treatment usually depend on clinical expertise rather than evidence base recommendations.

The few existing randomised controlled trials were conducted only in SSc –ILD. They showed the efficacy and tolerability of cyclophosphamide versus placebo and the non-inferiority of mycophenolate mofetil to cyclophosphamide in SSc-ILD. The need for further randomised controlled trials in CTD-ILDs is high.

#### **Corticosteroids**

A small retrospective study of corticosteroid monotherapy for SSc-ILD showed an improvement of forced vital capacity (FVC) (158 ml) in comparison to a non-treatment group (-61 ml), but there was no significant survival difference between both groups (Ando et al., 2013).

In cases of organizing pneumonia, often seen in polymyositis, the treatment is usually started with higher doses of oral corticosteroids, often in the range of 0.5-1 mg/kg body weight of prednisone. In very severe cases also pulse therapy (500-1000 mg/day i.v.) is applied, despite the lack of prospective trials (Aggarwal and Oddis, 2012). Corticosteroids are usually combined with other immunosuppressive agents as steroid sparing agents (Fischer and Chartrand, 2015).

#### **Cyclophosphamide**

Cyclophosphamide is an alkylating agent with the highest level of evidence in the treatment of CTD-ILDs. There are two randomized placebo controlled trials with cyclophosphamide in the treatment of SSc-ILD.

Tashkin et al. showed an improvement of FVC (2.53%,  $p=0.03$ ) after one-year treatment with cyclophosphamide (2mg/kg body weight per day) in scleroderma patients in comparison to placebo. This improvement lasted for 18 months, however declined after 24 months (Tashkin et al., 2007). Hence, there is a demand for maintenance treatment with sequential immunosuppressive therapy due to the dose-limiting toxicity of cyclophosphamide, mainly haemorrhagic cystitis, gonadal dysfunction, bladder cancer and hematologic malignancies (Oggenovski et al., 2004; Somers et al., 2005). Hoyles et al. showed a slight improvement of FVC ( $p=0.08$ ) in SSc-ILD patients, who received low dose corticosteroid in combination with initial cyclophosphamide (600 mg/m<sup>2</sup> body surface area) intravenously 6 times in monthly intervals, followed by substitution of cyclophosphamide with azathioprine (2.5 mg/kg/d maximal dosage 200 mg) in comparison to placebo for a total treatment duration of one year (Hoyles et al., 2006). Beside these randomised controlled studies several retrospective studies showed a similar tendency of improvement of lung functions (Poormoghim et al., 2012).

### **Mycophenolate Mofetil**

Mycophenolate mofetil is an inhibitor of monophosphate dehydrogenase, which acts through inhibition of purine synthesis and consequently reduced T cell proliferation. The efficacy and safety of MMF in the treatment of SSc have been shown in several retrospective studies (Tzouvelekis et al., 2012). Fischer et al. could demonstrate a stabilisation or an improvement of FVC in different CTD-ILD groups (44 SSc-ILD, 32 PM/DM-ILD, 18 RA-ILD) under MMF in a retrospective study (Fischer et al., 2013). Tashkin et al. compared MMF versus oral cyclophosphamide in a randomised controlled trial. MMF showed fewer toxicity and better tolerability, however a superiority of efficacy against cyclophosphamide was not shown. The course of % FVC did not vary significantly between the treatment

arms after 24 months of treatment ( $p=0.24$ ) (Tashkin et al., 2016). Meanwhile MMF is recommended by the Canadian Scleroderma Research Group as maintenance therapy for SSc-ILD (Walker and Pope, 2012).

### **Azathioprine**

Azathioprine is a prodrug of mercaptopurine and broadly used as an immunosuppressant agent in different medical fields, especially in autoimmune diseases, transplantation medicine, inflammatory bowel diseases, and interstitial lung diseases (Maltzman and Koretzky, 2003).

Azathioprine is a purine analog and inhibits the enzyme amidophosphoribosyltransferase also called glutamin phosphoribosylpyrophosphate amidotransferase which is required for DNA synthesis, via its activated metabolite methyl-thioinosine monophosphate. Due to the inhibition of the DNA synthesis, fast proliferating cells such as haematopoietic cells, especially T and B-lymphocytes, are depleted. Furthermore azathioprine causes T cell apoptosis by blocking the stimulation of CD28 via Rac1 (Poppe et al., 2006).

Azathioprine is administered orally and its bioavailability varies between 5%–37% (Zimm et al., 1983). Plasma half time is 26 to 80 minutes and of its metabolites 3 to 5 hours (Hardman and Gilman, 2001). After digestion azathioprine is metabolised to 6-mercaptopurine. 6-mercaptopurine can be methylated to methyl-mercaptopurine and S-adenosylhomocystine by thiopurine-S-methyltransferase (TPMT). Almost 15 to 20% of patients show an altered metabolism of azathioprine, favouring a metabolization of 6-mercaptopurine to methyl-mercaptopurine instead of thioguanine nucleotides, consequently causing increased thiopurine hypermethylation and increased hepatotoxicity, myelosuppression and non-response to treatment (Dubinsky et al., 2002). Approximately 0,3% of the population has decreased to absent enzyme activity due to inheritance of

(TPMT<sup>L</sup>/TPMT<sup>L</sup>) gene mutation. Almost 11% of the population has a heterozygote mutation and 89% has a homozygote wild type with normal to increased activity (Osterman et al., 2006).

There are two retrospective studies on azathioprine in CTD-ILDs, randomized controlled trials are still missing. Oldham et al. performed a retrospective study comparing azathioprine with MMF in CTD-ILD patients which showed yearly improvement of FVC by 1.5% and of DLCO by 4.9% over four years in the azathioprine group. 27% of the patients had to discontinue treatment in the azathioprine group, compared to only 5% of patients in the MMF group. 11% of the patients had a disease progression in the azathioprine group, and 9% in the MMF group (Oldham et al., 2016). A small retrospective case series with 11 patients supports the efficacy of azathioprine in combination with low dose corticosteroid in scleroderma-ILD. In this study, FVC improved in 5 patients and remained stable in 3 patients after 18 months. 3 patients had to discontinue azathioprine because of side effects (1 nausea, 1 leukopenia, 1 tuberculosis) (Dheda et al., 2004).

### **Rituximab**

Rituximab is a monoclonal chimeric antibody against the CD20 surface antigen on B-lymphocytes which causes depletion of B cells for 6 to 9 months from the peripheral blood. Rituximab is broadly applied in the treatment of rheumatoid arthritis, immune thrombocytopenic purpura and antineutrophil cytoplasmic antibody (ANCA) associated vasculitis.

A retrospective study with 50 non-IPF patients (33 of the cases with CTD-ILD) with severe lung function impairment (mean FVC 44% pred and mean DLCO 24.5% pred) and a significant decline in pulmonary function (13.3% FVC and 18.8% DLCO) 6-12 months before rituximab showed an 8.9% improvement of FVC and no change in DLCO 6-12 months after initiation of rituximab therapy. In this study 2 patients developed pneumonia and 10 patients died due to progression of ILD (Keir

et al., 2014). In another retrospective study of 24 CTD-ILD patients under rituximab treatment, there was no change in pulmonary function (Chartrand et al., 2016). A further retrospective study of 24 CTD-ILD patients showed an improvement of FVC (+4.1%) and stabilisation of DLCO (+2.1%); the effect was most pronounced in the myositis group of patients. 13 of 22 patients showed improvement or stabilisation of radiology, whereas 9 of 22 patients showed worsening (Sharp et al., 2016).

Randomized controlled trials and their data with long term effects of rituximab in CTD-ILD are not available. Hence an evidence based recommendation for rituximab in CTD-ILDs is not given.

### **Other Immunomodulatory Treatments**

Calcineurin antagonists, tacrolimus and cyclosporine, inhibit the activation of T-cells with ensuing dampening of inflammation. These drugs are widely used in transplantation medicine, whereas in the treatment of CTD-ILDs randomized controlled trials are lacking. Several retrospective case studies showed their efficacy in PM/DM induced ILDs (Takada et al., 2005).

Anti-tumor necrosis factor (TNF)- $\alpha$  antibodies, such as infliximab and etanercept, are widely and effectively used in the treatment of rheumatoid arthritis as well as inflammatory bowel disease, on the other hand these are often associated with drug induced ILDs. Despite some case reports showing the efficacy of TNF- $\alpha$  inhibitors in CTD-ILDs (Horai et al., 2012), their application in therapy of CTD-ILDs with pre-existing parenchymal changes is rather limited.

Imatinib is a tyrosine kinase inhibitor, which inhibits selectively the activity of BCR-Abl. It is mainly applied in the treatment of chronic myeloid leukaemia. Due to its antifibrotic effects on lung fibroblast in vitro and in vivo studies, several studies were performed to test its tolerability and efficacy in CTD-ILDs. Spiera et al. demonstrated an improvement of FVC of 6.4% compared with initial FVC after 12

months treatment in a phase IIa, open label, single arm study with SSc patients under imatinib 400 mg daily (Spiera et al., 2011). In another phase IIa study under a higher dose of imatinib 600mg daily, a high rate of adverse events with a 60% withdrawal rate was reported in 20 patients with SSc-ILD (Khanna et al., 2011). A newer phase II study in 22 SSc-ILD patients demonstrated an improvement or stabilisation of lung function in 55% of patients under 200mg imatinib daily for 6 months in cyclophosphamide refractory cases (Fratlicelli et al., 2014). So far there is no evidence based recommendations for the usage of imatinib in CTD-ILDs.

#### **1.4 KL-6 as a biomarker to assess ILD severity**

Krebs von den Lungen-6 (KL-6) is a human MUC1 mucin protein released by alveolar epithelial cells. Regenerating Typ II alveolar epithelial cells are the main source of KL-6 in lungs affected by inflammation and fibrosis. Serum KL-6 values are elevated in more than 70% of ILD patients, including IIPs, hypersensitivity pneumonia, CTD-ILD, acute respiratory distress syndrome, sarcoidosis, pulmonary alveolar proteinosis and radiation pneumonitis (Ishikawa et al., 2012). In regard to CTD-ILDs the serum levels of KL-6 have been found to be elevated in 89% of patients with RA-ILD, but in only 0.6% of RA patients without interstitial pneumonia (Oyama et al., 1997).

KL-6 has been shown to correlate with disease severity in various CTD-ILDs. In patients with PM/DM an inverse correlation of KL-6 values with FVC, TLC and DLCO was shown (Fathi et al., 2012). In patients with SSc the fibrosis score on HRCT correlated with increased KL-6 levels (Bonella et al., 2011). Taken together, KL-6 is a valuable biomarker to assess disease severity in ILD, especially in IPF and CTD-ILD, correlating with pulmonary function tests, DLCO and being an independent predictor of disease progression (Bonella and Costabel, 2014).

## **1.5 Aim of the Study**

This retrospective study aimed to investigate the efficacy and tolerability of azathioprine in CTD-ILD patients. There are no prospective randomised controlled trials with azathioprine for the treatment of CTD-ILDs. A further aim was to explore the role of KL-6 as predictor of response to treatment in these patients.

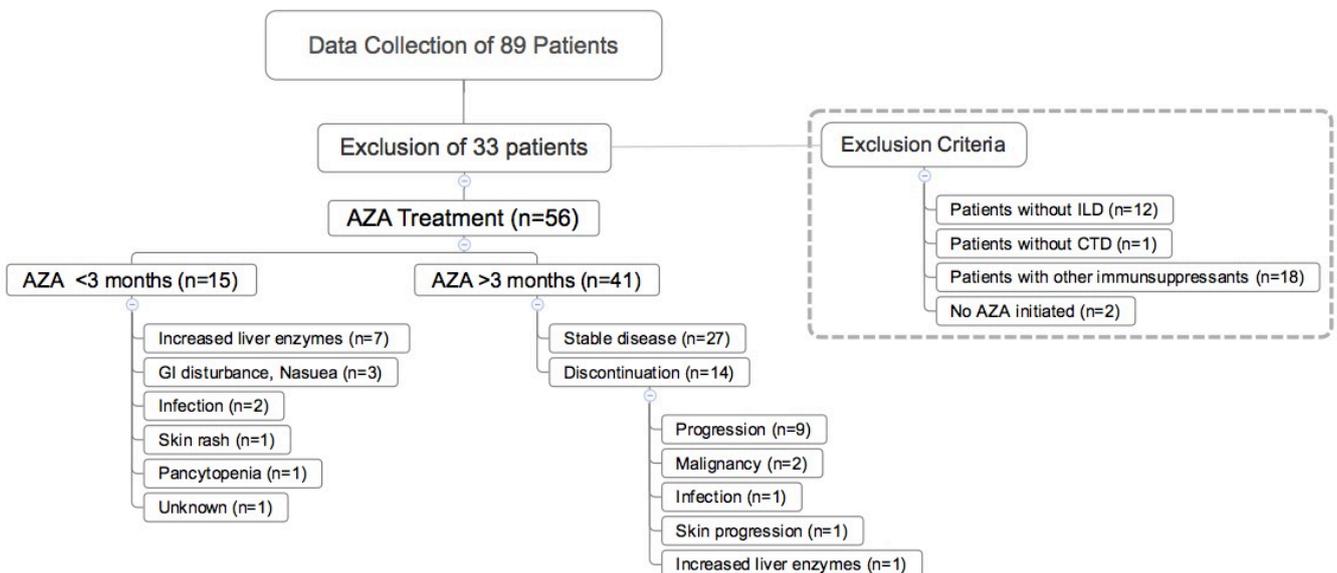
## 2 METHODS

### 2.1 Study Cohort

Based on a retrospective chart review, 56 patients admitted to the Ruhrlandklinik between 2003 and 2014 with the diagnosis of a CTD-ILD and treated with azathioprine were included in this analysis, 33 patients were excluded due to various reasons (**Figure 2**).

The study was approved by the Ethic Committee of the University of Duisburg-Essen (approval number 15-6613-BO). Data of the patients were collected in pseudo-anonymised form.

In 15 of the 56 patients azathioprine was discontinued due to side effects within the first 3 months, and these patients were excluded from the efficacy analysis. A diagram of the study cohort is shown in **Figure 2**.



**Figure 2** Flow chart of patients

## **2.2 Diagnostic Criteria**

The diagnosis and the classification of the ILDs were made mostly based on HRCT findings (Raghu et al., 2011; Travis et al., 2013). A histological examination was usually not performed. Only one patient underwent surgical biopsy and one patient transbronchial biopsy.

A diagnosis of CTD was based on history, physical examination, and laboratory assessment of autoantibodies related to disease. Patients were usually referred to our institution from a rheumatologist for the evaluation of lung involvement of a CTD and treatment options. If a CTD was first suspected in our hospital, patients were referred to a rheumatologist for the confirmation of a CTD and for further follow up.

## **2.3 Azathioprine Intake**

After a diagnosis of CTD-ILD the patients received immunosuppressive treatment. If another immunosuppressant than azathioprine had been commenced prior to referral to the Ruhrlandklinik, this therapy was not changed as long as the patient had a stable disease (18 patients). Only in 2 cases (1 patient with hepatitis C, 1 patient with allopurinol intake) azathioprine was not initiated due to possible side effects to avoid toxicity. Azathioprine was commenced in 56 patients in combination with prednisone as a corticosteroid-sparing agent to reduce side effects of corticosteroids.

The initial corticosteroid dose was 0,5 mg/kg body weight per day, approximately 40 mg per day on average. The dose was reduced by 10 mg per month until a maintenance dosage of 10 mg was achieved. In the long term, after 9 to 12 months, the patients received 5 to 10mg prednisone per day.

The initial azathioprine dose was 50 mg per day, which was increased by 50 mg per week up to the maintenance dose of 2.0 mg/kg bodyweight per day, with a

range between 100mg and 200mg per day. The serum levels of thiopurine-S-methyltransferase were not measured prior to azathioprine initiation because it was not routinely available in our laboratory. Regular laboratory assessments of liver enzymes and blood cell counts were recommended weekly for the first 4 weeks and then every 4 weeks for surveillance of drug toxicity.

## **2.4 Pulmonary Function Tests**

Measurements included maximum inspiratory vital capacity (IVC), forced vital capacity (FVC) and diffusion capacity of the lung for carbon monoxide (DLCO), total lung capacity (TLC), partial pressure of oxygen in arterial blood (PaO<sub>2</sub>), partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>), alveolar-arterial oxygen gradient (AaDO<sub>2</sub>) and arterial oxygen saturation (SaO<sub>2</sub>).

## **2.5 Laboratory measurements**

Serum KL-6 was measured by using NANOPIA® KL-6 assay (SEKISUI Diagnostics, UK), for the quantitative measurement (Latex agglutination turbidimetric method) on a chemistry analyser (ADVIA 1800 Chemistry System, Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). The upper limit of normal was 458 U/mL as determined in 142 Caucasian healthy subjects (Bonella et al., 2016).

Serum lactate dehydrogenase (LDH) was measured routinely as activity marker of ILDs. The normal value for LDH in our laboratory is < 240 IU/L.

## **2.6 Follow up**

The patients were seen in the ILD outpatient clinic every 3 months. Beside the medical history and physical examination, chest X –ray, pulmonary function test, and blood sampling were performed at each follow up visit.

## **2.7 Definition of ILD progression and improvement**

ILD progression was defined as decrease of FVC  $\geq 5\%$  pred and/or DLCO  $\geq 10\%$  pred. Patients who had decline in FVC  $< 5\%$  pred and/or DLCO  $< 10\%$  pred were considered stable. Improvement was defined as positive change in FVC and DLCO at follow up.

## **2.8 Statistical analysis**

Continuous variables were evaluated for a normal distribution with the Kolmogorov-Smirnov test. Normal distributive parametric data were presented as mean  $\pm$  standard error of the mean (SEM) or standard deviation (SD), when indicated; non-normal distributive data were presented as median and interquartile range). Categorical variables, such as sex, age group, frequency of ILD pattern or CTD, were presented as either a percentage of the total or numerically, as appropriate. Comparison between two groups was done with Student's t-test or Wilcoxon's rank test for continuous variables, Chi-squared or Fischer's exact test for categorical variables. Multiple comparisons were performed by Kruskal-Wallis test and stepwise step-down multiple comparisons. Spearman's or Pearson's correlation coefficient was obtained for linear correlations. Receiver operating characteristic (ROC) analysis was used to test the role of serum KL-6 and LDH as predictors of disease progression. P values lower than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

## 3 RESULTS

### 3.1 Patients' distribution and clinical characteristics

#### 3.1.1 Demographics and clinical characteristics

56 patients with CTD-ILD received azathioprine between 2003 and 2014 at our institution. Demographic and clinical characteristics of the subjects are summarized in the **Table 4**.

The mean follow up time was 35 (range: 3-109 months) and the mean treatment duration was 34 (range: 3-105 months). The median time to ILD onset after CTD diagnosis was 3.3 years (range: 0-31 years). In two patients ILD preceded the diagnosis of CTD for 3 and 7 years.

The most frequent ILD pattern was non-specific interstitial pneumonia (NSIP) with 70% (n=39), followed by usual interstitial pneumonia (UIP) with 16% (n=9) and organizing pneumonia (OP) with 4% (n=2). In 6 patients (11%) lung fibrosis was unclassifiable.

With regard to the smoking history, 8 % of patients were current smokers, 47% ex-smokers and 45% non-smokers. The patients had mild to moderate restriction (FVC of  $66 \pm 18\%$  pred) and a moderate to severe impairment of DLCO ( $42 \pm 19\%$  pred). The degree of respiratory insufficiency was mild.

At treatment start both KL-6 and LDH were moderately elevated (**Table 4**).

**Table 4** Demographics and characteristics of the studied subjects at baseline

	<b>N=56</b>
<b>Gender</b>	
Female/Male	31/25
<b>Smoking History</b>	38
Current Smoker/Ex-Smoker/Non-Smoker	3/18/17
<b>Age</b>	64 ± 14
Female	63 ± 11
Male	65 ± 17
<b>Pulmonary function</b>	
FVC, % pred (n=41)	66 ± 18
DLCO, % pred (n=38)	42 ± 19
<b>Blood gas analysis</b>	
PaO <sub>2</sub> , mmHg (n=55)	74 ± 10
(A-a)DO <sub>2</sub> , mmHg (n=49)	29 ± 10
<b>Serum biomarkers†</b>	
LDH, U/L (n=56)	294 ± 71 (153-563)*
KL-6, U/mL (n=53)	1655±821 (313-6228)*

N= number of patients.  
Data are presented as mean ± SEM unless otherwise stated  
\* Data are mean ± ± SEM (min-max)  
† Reference values for serum biomarkers are indicated in the methods.

### 3.1.2 Demographics and clinical characteristics according to CTD

The most frequent CTD was RA (37.5%) followed by SSc (25%), PM/DM (18%), UCTD (7%), MCTD (5%), Sjögren syndrome (4%), and psoriatic arthritis (4%) (**Table 5**).

There was a prevalence of female subjects in all CTD-groups. The SSc patients were the youngest with a mean age of 55 ± 4 years, while the RA group was the oldest with a mean age of 71 ± 2.5 years.

The NSIP pattern was the most common ILD pattern in all CTD-Subgroups. With regard to the pulmonary function impairment across the CTD groups, some differences were seen, but without statistical significance. RA patients had the

mildest restriction (FVC  $70 \pm 19\%$  pred), while UCTD patients the most severe (FVC  $51 \pm 19\%$  pred). The mildest impairment of DLCO was seen in RA patients ( $46 \pm 15\%$  pred), while UCTD patients showed the lowest DLCO levels ( $24 \pm 19\%$  pred) (**Table 5**).

The highest serum KL-6 levels were measured in UCTD patients with  $3742 \pm 2067$  U/mL, while RA patients showed the lowest KL-6 levels ( $1560 \pm 1028$  U/mL) ( $p=0.001$  vs UCTD patients). No significant differences were seen in serum LDH levels among CTD patients (**Table 5**).

**Table 5** Demographics and clinical characteristics according to CTD at baseline

Characteristics of subgroups	Rheumatoid Arthritis (N=21)	Systemic sclerosis (N=14)	PM/DM (N=10)	UCTD (N=4)	Others* (N=7)
Gender (F/M), n	11/10	8/6	7/3	2/2	5/2
Age, years	$71 \pm 2.5$	$55 \pm 4^{**}$	$63.5 \pm 5$	$61.5 \pm 7$	$67 \pm 2$
<b>ILD Pattern, n</b>					
UIP	4	2	1	1	1
NSIP	14	12	6	2	5
OP	1	0	1	0	0
Unclassifiable	2	0	2	1	1
<b>Pulmonary function</b>					
FVC, % pred	$70 \pm 19$	$67 \pm 14$	$64 \pm 22$	$51 \pm 19$	$60 \pm 8$
DLCO, % pred	$46 \pm 15$	$44 \pm 24$	$41 \pm 16$	$24 \pm 19$	$41 \pm 16$
<b>Blood gas analysis</b>					
PaO <sub>2</sub> , mmHg	$74 \pm 10$	$75 \pm 7$	$69 \pm 14$	$71 \pm 7$	$76 \pm 8$
(A-a)DO <sub>2</sub> , mmHg	$32 \pm 9$	$26 \pm 8$	$29 \pm 12$	$33 \pm 9$	$25 \pm 5$
<b>Serum biomarkers†</b>					
LDH, U/L	$291 \pm 62$	$275 \pm 47$	$332 \pm 112$	$333 \pm 78$	$258 \pm 42$
KL-6, U/mL	$1560 \pm 1028^{***}$ (n=21)	$2500 \pm 1543$ (n=14)	$2692 \pm$ 1256	$3742 \pm 2067$ (n=4)	$3077 \pm 2232$ (n=5)

Otherwise indicated, data are expressed as mean  $\pm$  SD.

\* sjögren syndrome (n=2), mixed connective tissue disease (n=3), psoriatic arthritis (n=2)

\*\* $p < 0.05$  vs RA

\*\*\* $p = 0.001$  vs UCTD

† Reference values are indicated in the methods.

### 3.2 Analysis of Treatment Response

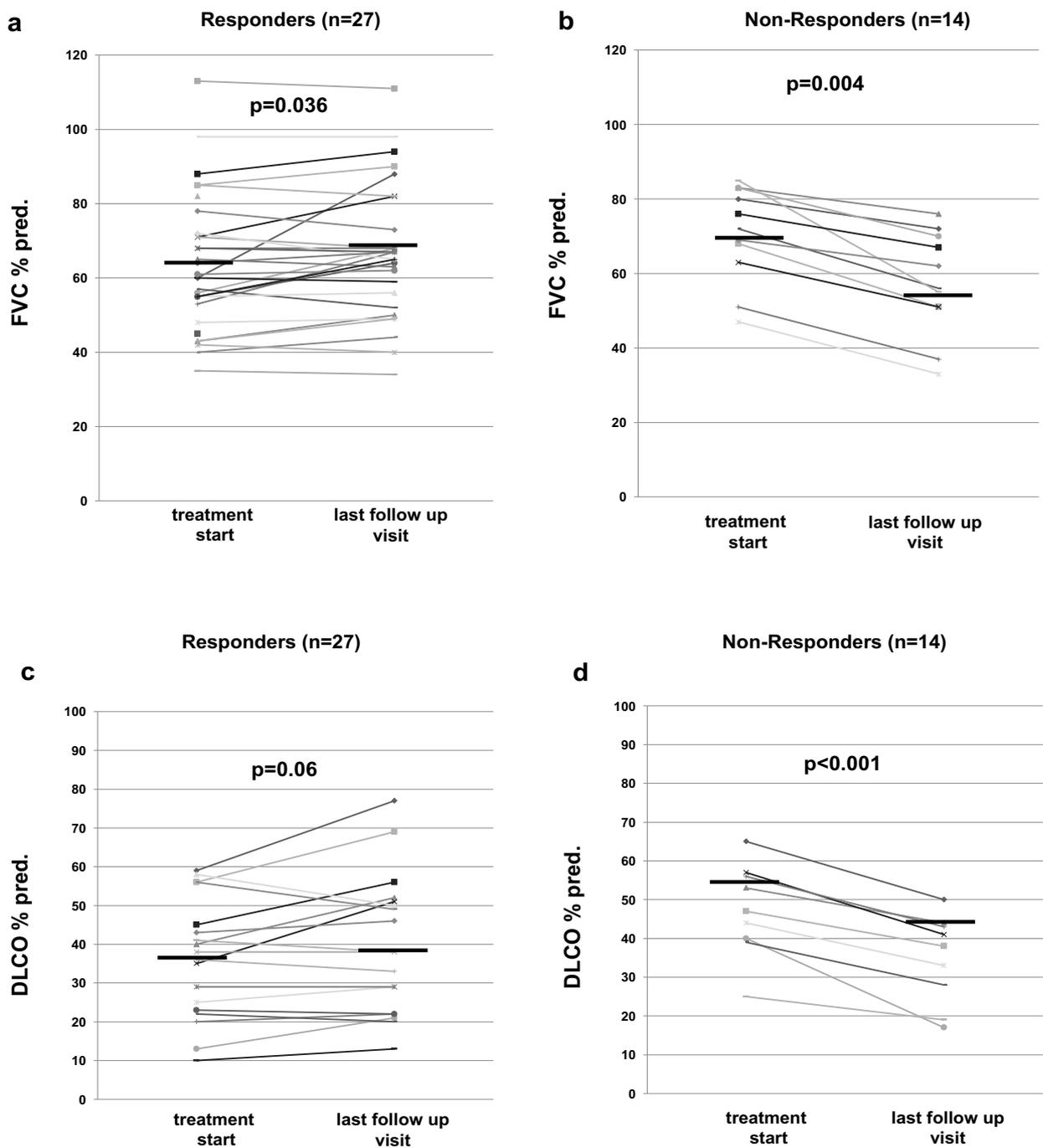
Patients who remained stable or improved, were considered as treatment responders, those who progressed as non-responders (for definition of progression, stable disease, improvement see methods).

In all patients, the mean FVC was  $66 \pm 18$  % pred at the initiation of treatment and  $63 \pm 18$  % pred at the last follow up visit ( $p=0.2$ ), and the mean DLCO was  $42 \pm 19$  % pred at the initiation of treatment and  $40 \pm 17$  % pred at the last follow up visit ( $p=0.25$ ). There were 14/41 (34%) patients with progression of ILD and 27/41 (66%) who remained stable or improved.

In patients, who remained stable or improved the mean FVC was  $62 \pm 17$  % pred at the initiation of treatment and  $65 \pm 17$  % pred at the last follow up visit ( $p=0.036$ ), and the mean DLCO was  $38 \pm 16$  % pred at the initiation of treatment and  $39 \pm 17$  % pred at the last follow up visit ( $p=0.06$ ) (**Figure 3**).

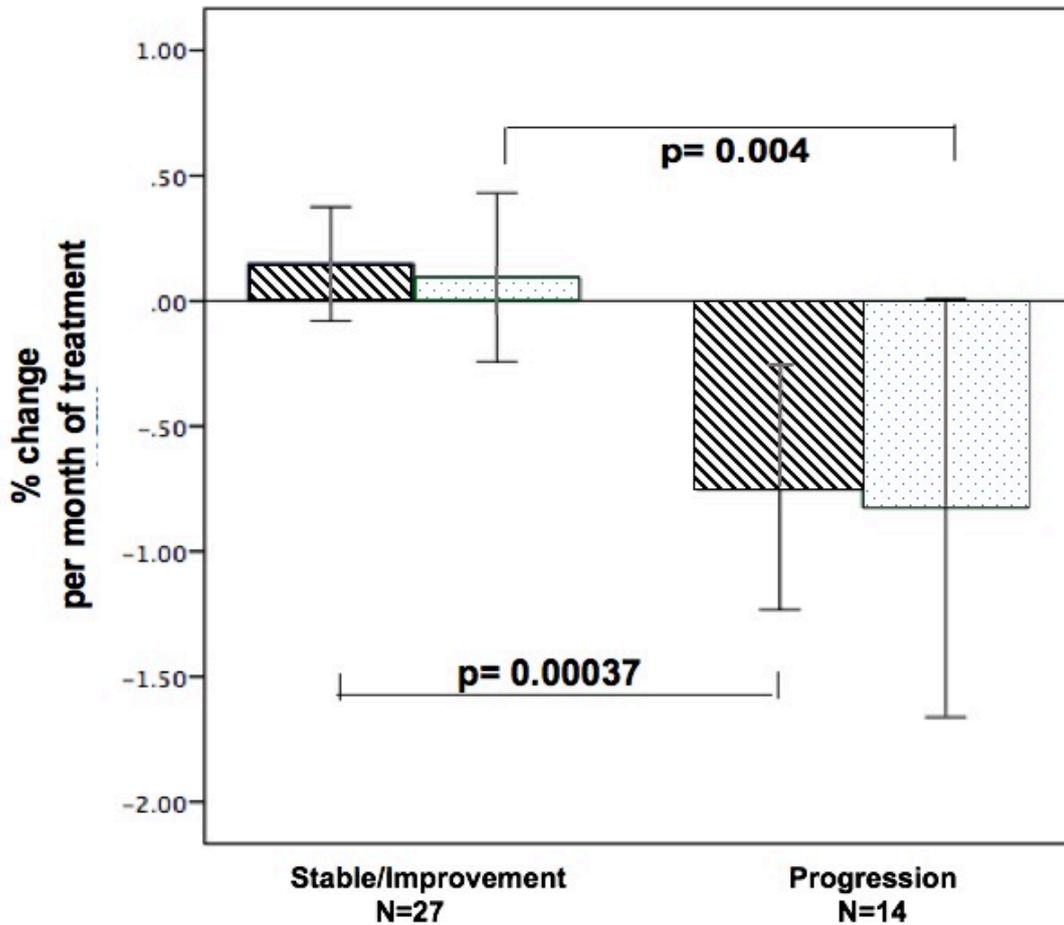
In those who had ILD progression the mean FVC was  $72 \pm 19$  % pred at the initiation of treatment and  $58 \pm 18$  % pred at the last follow up visit ( $p=0.004$ ), and the mean DLCO was  $55 \pm 15$  % pred at the initiation of treatment and  $44 \pm 15$  % pred at the last follow up visit ( $p<0.001$ ) (**Figure 3**).

Since treatment duration was different between the patients, FVC and DLCO changes were adjusted for azathioprine intake per time. In the patients who remained stable or improved, the percent change of FVC per month was  $+0.14 \pm 0.05$  % and of DLCO was  $+0.09 \pm 0.08$  %. In those who had progression, the percent change of FVC per month was  $-0.76 \pm 0.6$  % and of DLCO was  $-0.82 \pm 1.16$  % (**Figure 4**).



**Figure 3** Pulmonary function tests under azathioprine treatment

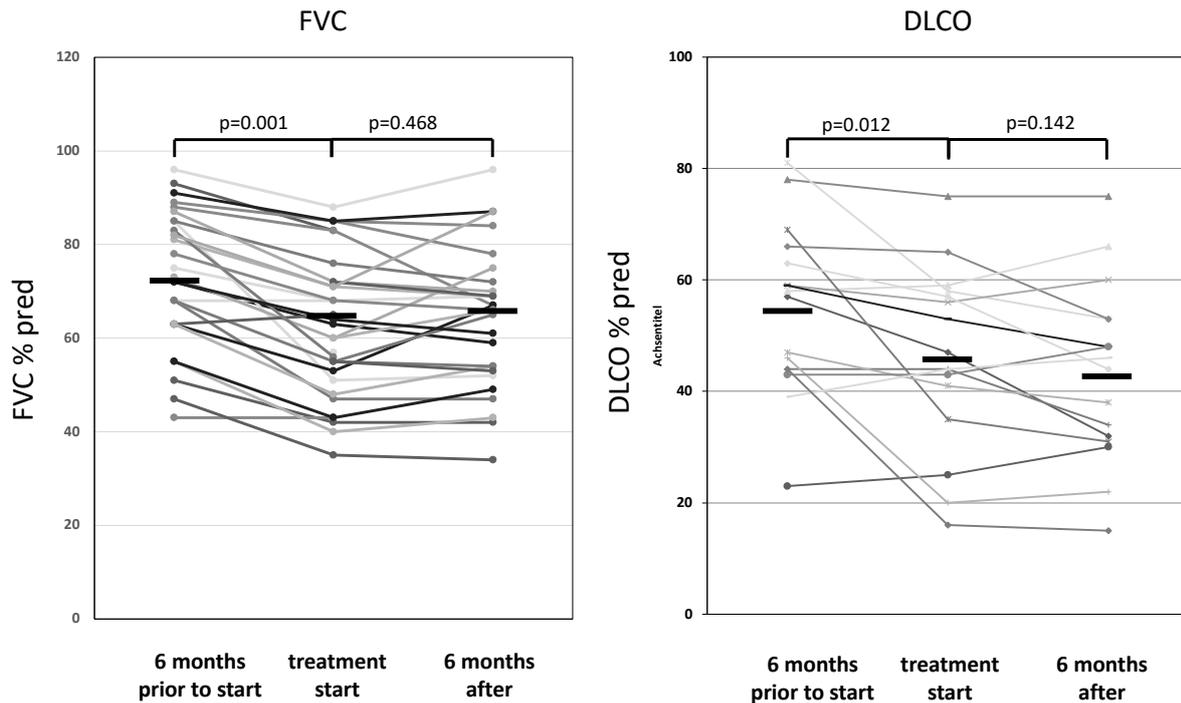
Pulmonary function tests (FVC and DLCO) at start of therapy and end of follow up in patients who remained stable or improved (responders) (a, c) and those who had disease progression (non-responders) (b, d). Dots represent single values. Bold black lines represent mean values.



**Figure 4** Percent change of lung function per month of treatment. Data present mean  $\pm$  SEM.  = FVC,  = DLCO

Of the 41 patients treated with azathioprine for longer than 3 months, FVC data 6 months prior to treatment with azathioprine were available in 21 patients, and DLCO data in 16 patients.

**Figure 5** shows the mean values of FVC and DLCO at 6 months prior to start of treatment, at start of treatment, and 6 months after treatment.



**Figure 5** Changes in FVC in 21 patients and DLCO in 16 patients from 6 months prior to start of treatment until 6 months after. Each line represents a patient. For the comparison of the means, represented by bold black lines, paired t-test was used.

During the 6 months prior to initiating azathioprine, the mean decline in FVC was  $-9 \pm 2\%$  pred ( $p < 0.001$ ) and the decline in DLCO  $-8.5 \pm 2\%$  pred ( $p = 0.012$ ). 6 months after treatment start, the mean change in FVC was  $1 \pm 1.4\%$  pred ( $p = 0.468$ ) and for DLCO was  $-2.6 \pm 1.8\%$  pred ( $p = 0.142$ ).

### 3.3 Subgroup Analysis

#### 3.3.1 Changes of pulmonary function over time according to HRCT pattern

There were no significant differences in change of FVC or DLCO over time between patients with UIP and NSIP pattern. In subjects with UIP pattern on HRCT ( $n = 7$ ) the mean FVC was  $68 \pm 16\%$  pred at initiation of treatment and  $63 \pm 16\%$

pred at the end of follow up. Mean DLCO was  $51 \pm 15\%$  pred at the initiation of treatment and  $41 \pm 05\%$  pred at the end of follow up.

In subjects with NSIP pattern on HRCT (n=28) the mean FVC was  $67 \pm 43\%$  pred at initiation of treatment and  $63 \pm 19\%$  pred at the end of follow up. Mean DLCO was  $43 \pm 18 \%$  pred at initiation of treatment and  $41 \pm 15\%$  pred at the end of follow up.

### 3.3.2 Changes of pulmonary function over time according to CTD subtypes

There were no significant differences in pulmonary function tests at baseline and at end of follow up across the CTD-subgroups. In patients with RA, there was a significant difference in the change of FVC adjusted for azathioprine intake per time under treatment in comparison to the other CTD subgroups ( $-0.5 \pm 0.8$  vs.  $+0.05 \pm 0.6 \%$  pred per month,  $p=0.016$  respectively). The same was seen for DLCO ( $-0.6 \pm 1$  vs.  $+0.1 \pm 0.9$  pred per month,  $p=0.047$ ).

### 3.4 Outcome

During the treatment 2 patients developed malignancy (one case developed lung cancer and one non-Hodgkin lymphoma). Three patients died during follow up (two from ILD progression, one from acute exacerbation of the ILD) (**Table 6**).

**Table 6** Outcome of the studied subjects

Outcome	N (%)
Improvement or stability of ILD*	27/41 (66%)
Progression of ILD*	14/41 (34%)
Malignancy	2/56 (4%)
Death	3/56 (5%)

\* Patients with follow up or with azathioprine intake < 3 months were excluded from this analysis

### 3.5 Safety, Tolerability and Side Effects

All patients (n=56) were included in the safety and tolerability analysis independent of intake time. 27% of the patients (n=15) had to discontinue treatment within 3 months due to side effects. All discontinuations were definitive. 73% of the patients (n=41) received azathioprine for longer than 3 months and had a mean treatment duration of 34 months.

Side effects are reported in the **Table 7**. Infection 11/56 (20%) was the most common side effect, however increased liver enzymes with 8/56 (14%) was the most common cause of discontinuation. Other side effects were gastrointestinal disturbance, anaemia, leukocytopenia, pancytopenia, skin rash. In one case azathioprine was discontinued due to a non-specified adverse event.

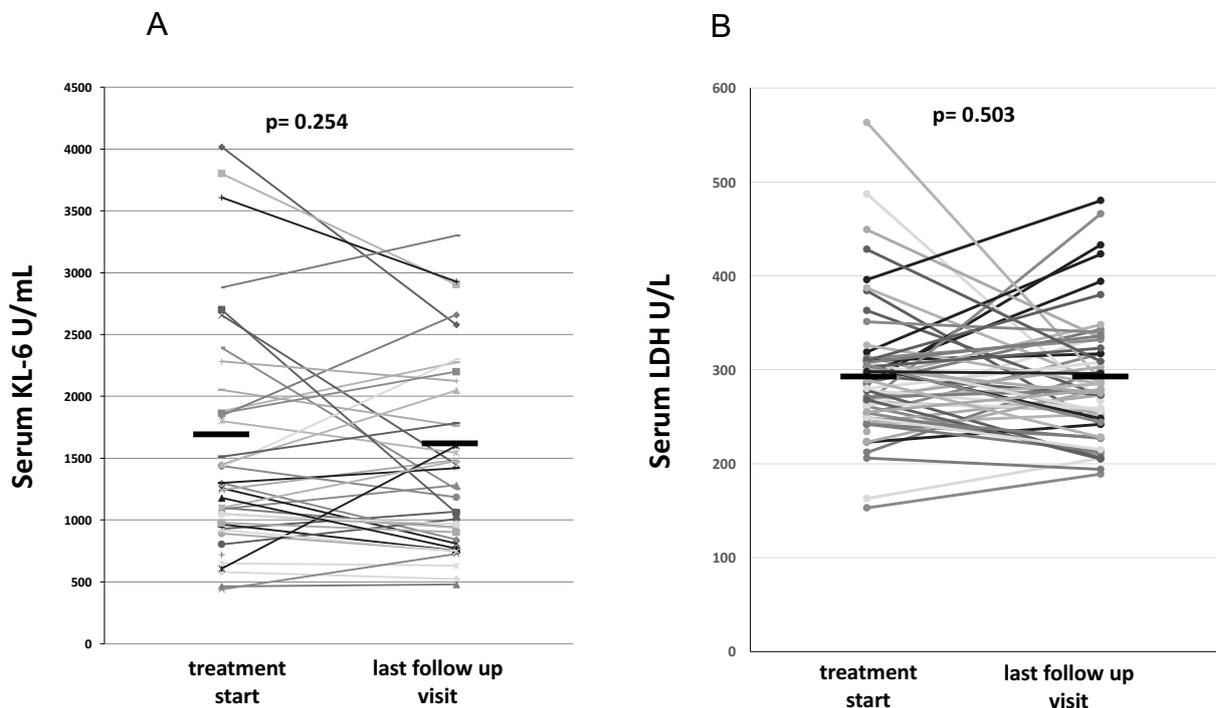
**Table 7** Tolerability and safety of azathioprine

<b>Tolerability</b>	
<b>Azathioprine discontinuation</b>	<b>29/56 (52%)</b>
Side effects	17/56 (30%)
ILD-Progression	9/56 (16%)
Malignancy	2/56 (4%)
Skin progression	1/56 (2%)
<b>Total Side Effects</b>	<b>31/56 (55%)</b>
Infection	11/56 (20%)
Liver enzyme increase	9/56 (16%)
Nausea/stomach discomfort	5/56 (9%)
Skin rash	1/56 (2%)
Pancytopenia	1/56 (2%)
Anaemia	2/56 (4%)
Leukocytopenia	2/56 (4%)

Among the patients who received azathioprine for longer than 3 months (n=41) the progression of the lung fibrosis was the most common reason for discontinuation (22%) (n=9), followed by infection (n=1), skin progression of CTD (n=1), liver enzyme elevation (n=1), and malignancy (n=2). The majority of these cases (66%) (n=27) could maintain the treatment with stable lung function.

### 3.6 Serum KL-6 and LDH as biomarkers

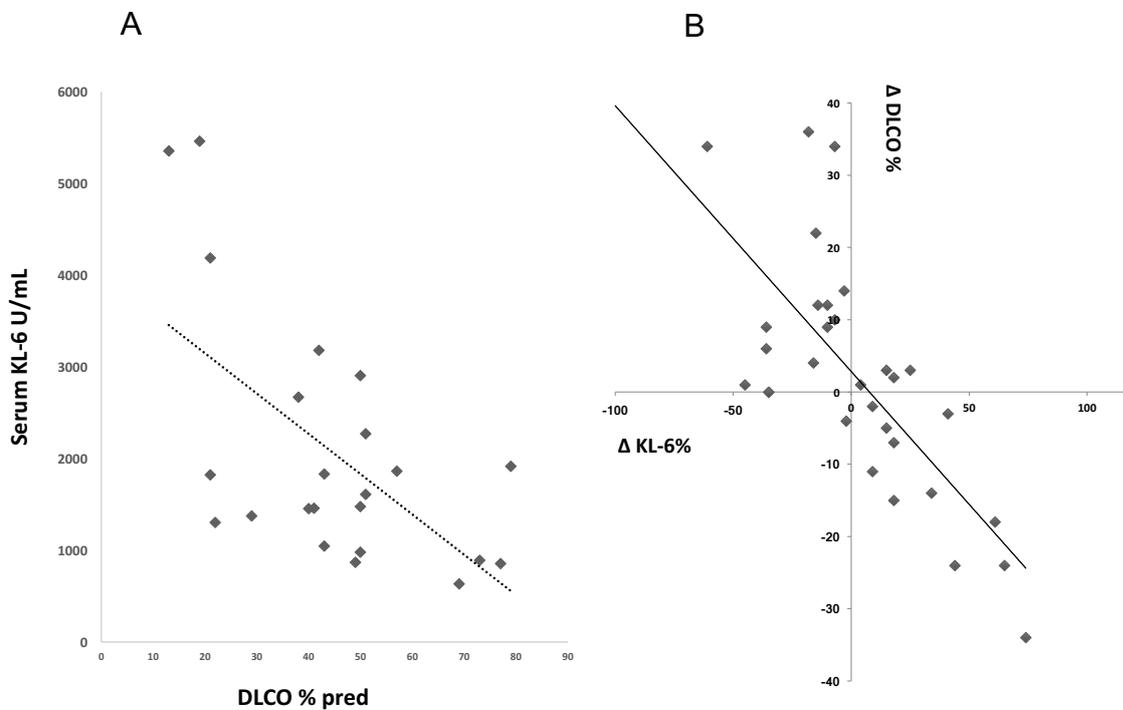
Serum KL-6 levels were measured at start of treatment with azathioprine and at the last follow up visit in 31 patients. The mean KL-6 serum level at start of treatment was  $1655 \pm 821$  U/mL and  $1589 \pm 744$  at the last follow up visit ( $p=0.254$ ), as shown in **Figure 6A**. Serum LDH was measured at start of treatment and at the last follow-up visit in 40 patients. The mean LDH serum level at start of treatment was  $294 \pm 71$  U/mL and  $287 \pm 66$  at the last follow up visit ( $p=0.503$ ) **Figure 6B**.



**Figure 6** Serum KL-6 (A) and LDH (B) at start of treatment and last follow-up visit in the studied subjects. Dots represent single values, black lines are the mean.

No correlation was seen between serum KL-6 levels and gender, age or smoking history. Patients with UIP pattern at HRCT tended to have higher KL-6 levels at start of treatment than NSIP patients ( $3272 \pm 1226$  vs  $2256 \pm 1630$  U/L,  $p=0.086$ ), although the two groups did not differ in terms of FVC and DLCO at baseline (see page 31 and 32).

Moreover, patients with a UIP pattern showed a greater decrease in serum KL-6 levels over time in comparison to NSIP patients ( $-34 \pm 21\%$  vs  $-0.5 \pm 7\%$ ,  $p=0.027$ ). This was not associated with the percent change of FVC and DLCO over time, which did not differ between the groups (see page 31 and 32).

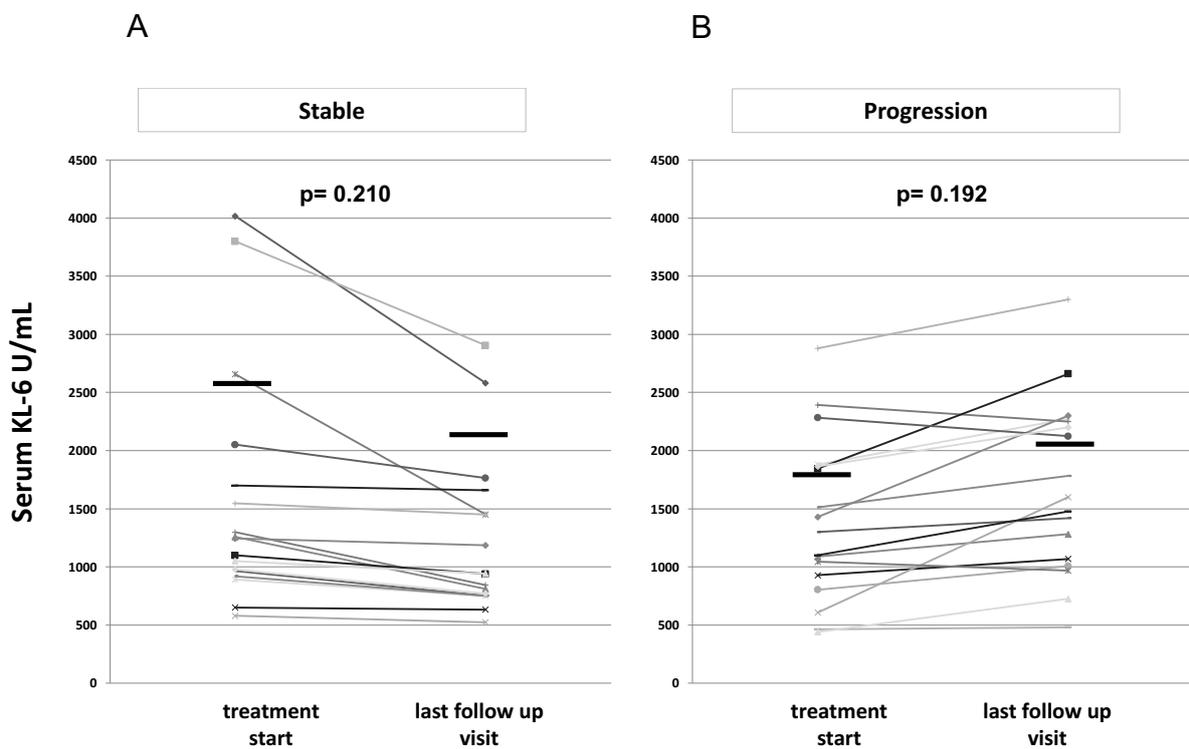


**Figure 7** Correlation between serum KL-6 levels and DLCO % pred at start of treatment (A) and correlation between changes in serum KL-6 % and changes in DLCO% over time (B) in patients under azathioprine treatment.

In those patients who received azathioprine for longer than 3 months, a weak negative correlation was seen between initial serum KL-6 and FVC at end of follow up ( $r=-0.46$ ,  $p=0.031$ ). A negative correlation was seen between initial serum KL-6 and DLCO at the start of the treatment ( $r= -0.603$ ;  $p= 0.003$ ) (**Figure 7A**) as well

as between changes in serum KL-6 % and changes in DLCO% over time ( $r = -0.733$ ;  $p = 0.001$ ) (**Figure 7B**).

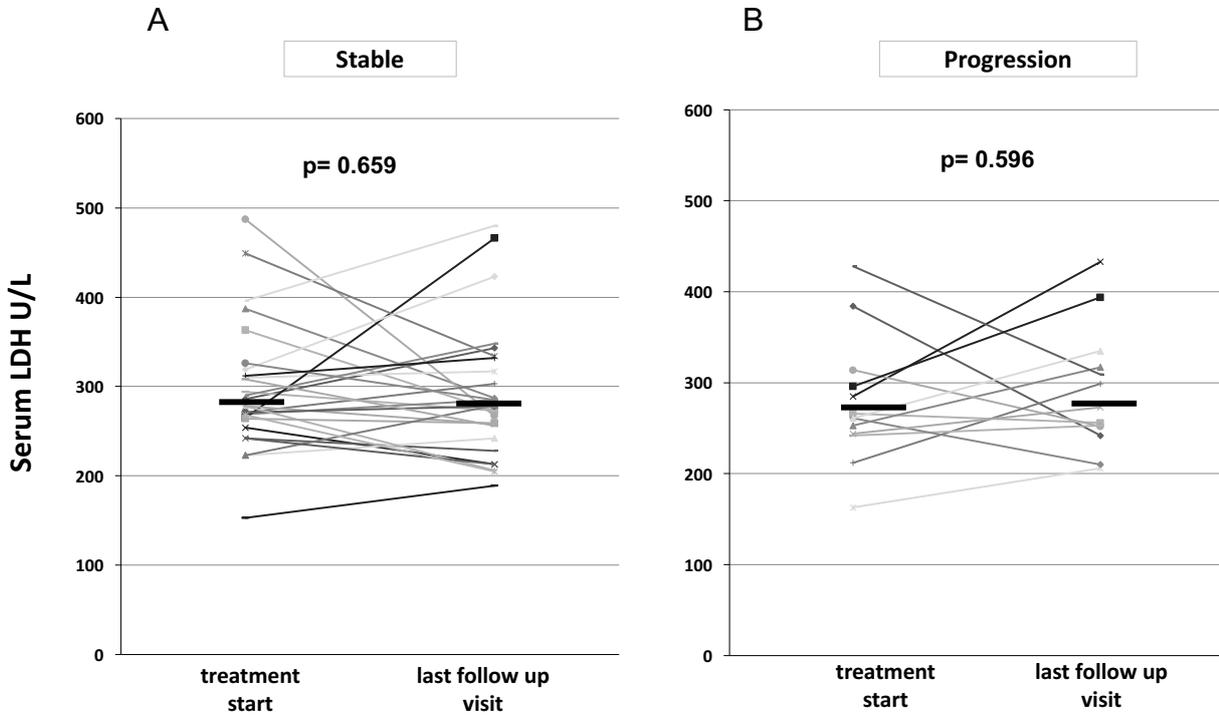
Serum KL-6 levels showed a decline in stable or improved patients during treatment, but this was not significant ( $p = 0.210$ ) (**Figure 8A**). In patients who progressed KL-6 levels increased, but this was not significant ( $p = 0.192$ ) (**Figure 8B**).



**Figure 8** Serum KL-6 levels at start of treatment and last follow up visit in patients who remained stable or improved ( $n = 17$ ) A) and in those who progressed ( $n = 14$ ) B). Dots represent single values, black lines are the mean.

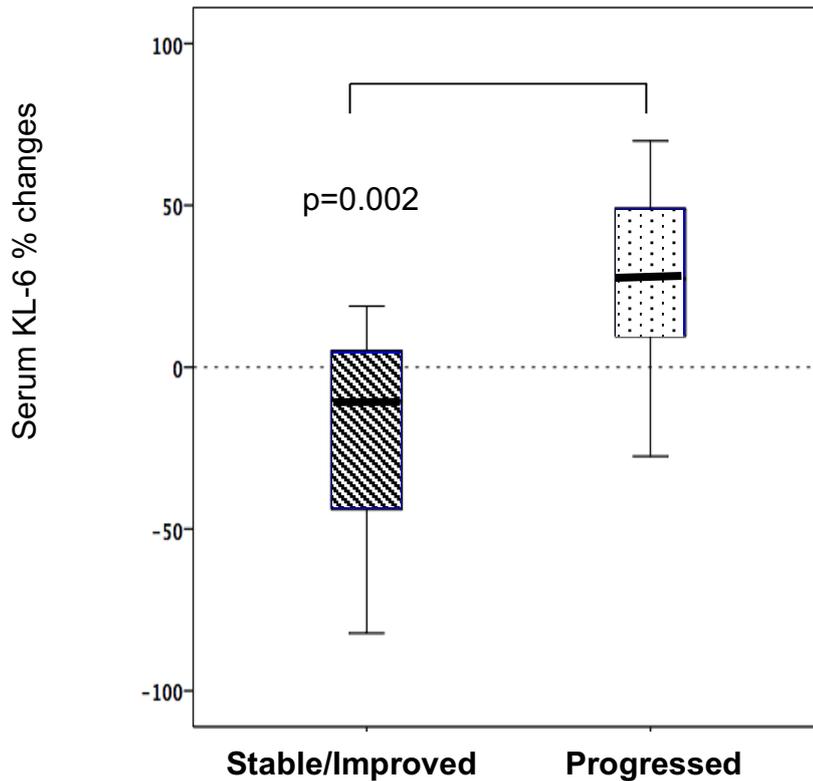
Serum LDH levels did not change during treatment, neither in those patients who remained stable or improved ( $p=0.659$ ) (**Figure 9A**) nor in those who progressed ( $p=0.596$ ) (**Figure 9B**).

8



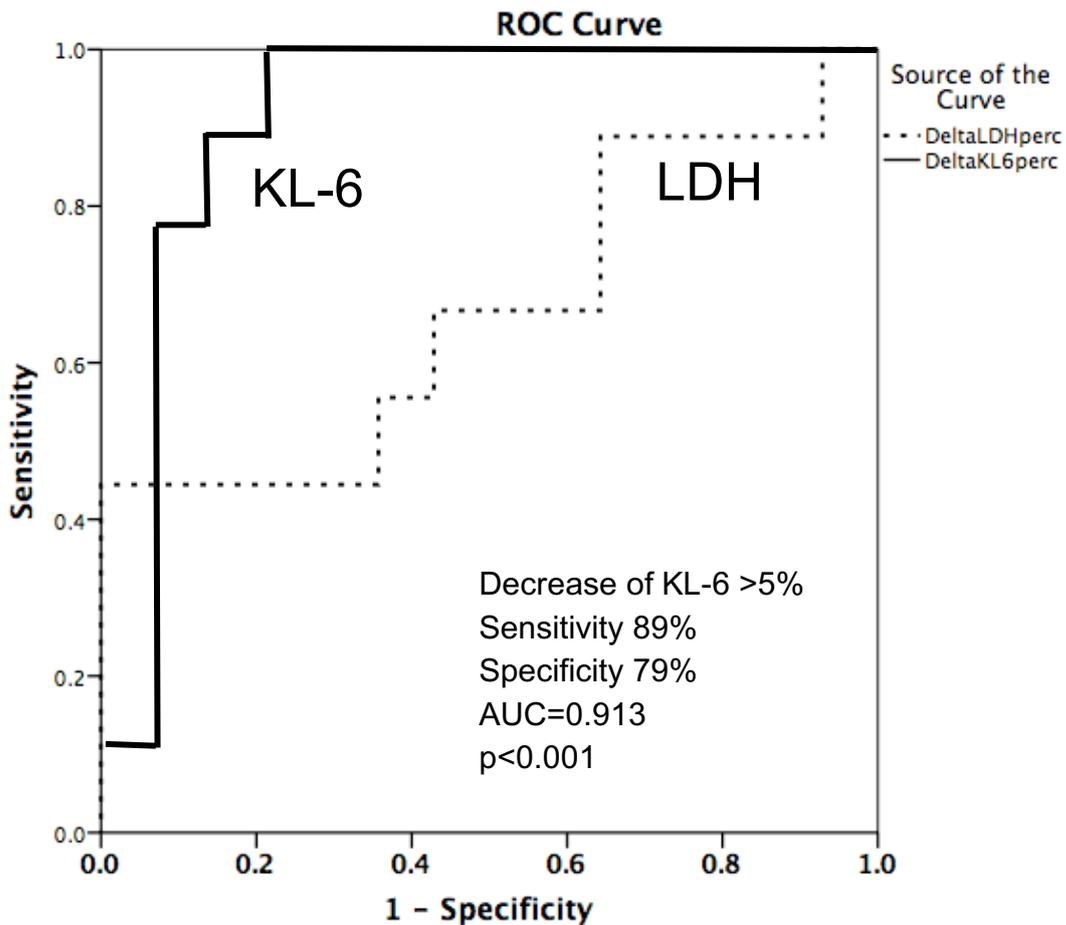
**Figure 9** Serum LDH levels at start of treatment and last follow up visit in patients who remained stable or improved ( $n=27$ ) (A) and in those who progressed ( $n=13$ ) (B). Dots represent single values, black lines are the mean.

In order to correct for the inter-individual variability of serum KL-6 levels, the change over time was normalized and expressed as percent change from baseline. The responders to the azathioprine treatment showed a decrease of KL-6 by  $17\pm 20\%$  over time, and non-responders an increase by  $31\pm 35\%$  ( $p=0.002$ ) (**Figure 10**).



**Figure 10** Percent change of serum KL-6 baseline under azathioprine treatment. Percent change of serum KL-6 from baseline in patients who remained stable or improved (N=17) and those who had disease progression (N=14). Black lines represent the median. The box represents the interquartile range (middle 50% of values). The whisker plots show bottom and top quartile (lowest and highest 25% of values respectively).

In order to determine the best cut-off for change of KL-6 over time associated with response to treatment, a ROC analysis was performed. At a cut-off level of >5% decrease from baseline, serum KL-6 levels showed a sensitivity of 89%, specificity of 79% and accuracy of 84% for association with response to treatment, defined as stability or improvement in FVC (AUC 0.913;  $p < 0.001$ ). For Serum LDH no significant cut off was identified (**Figure 11**).



**Figure 11** Receiver operating characteristic analysis showing change in serum KL-6 and LDH from baseline associated with response to treatment in those patients who received azathioprine treatment for longer than 3 months at the cut off of >5% change. AUC: area under the curve.

## 4 DISCUSSION

This retrospective study shows that azathioprine for CTD-ILD stabilised or ameliorated lung function in the majority of patients. This finding was supported by the relationship between changes of serum levels of KL-6, a well-established biomarker for assessing ILD severity, and the treatment response.

Azathioprine has been broadly applied as corticosteroid-sparing immunosuppressant in the treatment of idiopathic interstitial pneumonia, especially NSIP, chronic hypersensitivity pneumonitis, and sarcoidosis (Baughman and Lower, 2015; Morisset et al., 2017; Poletti et al., 2012; Vorselaars et al., 2013). For the treatment of CTD-ILD data on the efficacy of azathioprine are scarce, albeit it is often used in daily practice. The efficacy of azathioprine in combination with corticosteroids in CTD-ILD has not been studied in any prospective randomized controlled trial.

A small case series suggests efficacy of azathioprine for CTD-ILD (Dheda et al., 2004). This was a retrospective study with azathioprine in combination of low dose corticosteroid in 11 scleroderma-ILD patients showing that FVC improved in 5 patients and remained stable in 3 patients after 18 months. 3 patients had to discontinue azathioprine because of side effects (1 nausea, 1 leukopenia, 1 tuberculosis).

Oldham et al. compared azathioprine versus MMF in a retrospective study of fibrotic CTD-ILD patients during long-term follow up. 27% of patients discontinued azathioprine due to non-respiratory side effects, versus 5% of MMF treated patients. The most common side effect of azathioprine was increased liver enzymes (7%). Disease progression was seen in 11% of patients in the azathioprine group and in 9% of the MMF group. FVC increased yearly by 1.53% and DLCO by 4.91% over four years under azathioprine. In our study 8 of 56 patients (14%) had to discontinue treatment due to elevated liver enzymes.

Although infection 11/56 (20%) was the most common side effect, only 3/56 (5%) patients had to discontinue azathioprine due to infections. Progression of ILD was seen in 14 of our 41 patients (34%) who were treated for longer than 3 months.

In our cohort there was a mild to moderate restriction with a mean FVC of 66% pred. DLCO showed a mean of 42% pred at baseline. 73% of our patients received azathioprine for longer than 3 months with a mean intake time of 34 months. After adjustment for azathioprine intake per time, stable patients showed a significant improvement in FVC ( $+0.14 \pm 0.05$  %) compared to those who progressed ( $-0.76 \pm 0.6$  %), for DLCO a similar trend was seen ( $+0.09 \pm 0.08$  % versus  $-0.82 \pm 1.16$  %).

In a retrospective study of MMF treatment in a cohort of 125 CTD-ILD patients, the average decline of FVC was -2% pred and of DLCO was -11% pred before treatment start. At weeks 52, 104 and 156 after the implementation of treatment an improvement in FVC of 5%, 6%, and 7% pred respectively was observed ( $p < 0.05$  for all time points) (Fischer et al., 2013). In our study 6 months prior to azathioprine intake, the mean decline in FVC had been  $-9 \pm 2\%$  pred ( $p < 0.001$ ) and the decline in DLCO  $-8.5 \pm 2\%$  pred ( $p = 0.012$ ). 6 months after treatment start, the mean change in FVC was  $1 \pm 1.4\%$  pred ( $p = 0.468$ ) and for DLCO was  $-2.6 \pm 1.8\%$  pred ( $p = 0.142$ ).

In our study the changes of FVC or DLCO over time were not different between patients with a UIP or a NSIP pattern on HRCT. Solomon et al. observed a shorter survival in RA-UIP patients than in RA-NSIP patients ( $p = 0.02$ ) (Solomon et al., 2016). In our study, patients with RA showed a significant difference in the change of FVC adjusted for azathioprine intake per time under treatment in comparison to the other CTD subgroups ( $-0.5 \pm 0.8$  vs.  $0.05 \pm 0.6$  % pred per month),  $p = 0.016$  respectively, indicating that they might not respond as well to treatment as the other CTD subgroups.

KL-6 is a reliable serum biomarker to assess disease activity of ILDs and is widely used in Japan in the clinical daily practice. Oyama et al. showed increased

levels of KL-6 in 90% of RA patients with ILD manifestation, compared to only 0.6% of patients without ILD manifestation. In their study elevated KL-6 was not associated with the activity of RA in other organs but strongly correlated with the manifestation of interstitial pneumonia (Oyama et al., 1997). Bonella et al. showed a correlation between increased KL-6 values and decreased FVC or DLCO and a correlation between KL-6 and HRCT-fibrosis score in SSc patients (Bonella et al., 2011).

In the present study KL-6 values were elevated in all CTD-ILD groups, the highest value was seen in the UCTD group. No correlation was detected between serum KL-6 levels and gender, age or smoking history. Regarding HRCT patterns, patients with the UIP pattern had higher KL-6 levels at start of treatment than NSIP patients, although the two groups did not differ in term of FVC and DLCO at baseline. Regarding pulmonary function, the serum KL-6 levels showed a nonsignificant decline in stable or improved patients and a nonsignificant increase in patients, who progressed under treatment. These results indicate a potential utility of serum KL-6 to assess disease activity under treatment but this needs to be validated in further studies. Fathi et al. demonstrated inverse changes of KL-6 values with FVC or DLCO in their PM/DM cohort as well (Fathi et al., 2012). Taken together these data support the potential utility of KL-6 as a biomarker to measure treatment response in CTD-ILDs.

The mean serum LDH levels were elevated in all CTD-ILD groups at the start of treatment and did not decline at the end of the treatment. Regarding pulmonary function, the serum LDH levels did not change during treatment, neither in those patients, who remained stable or improved, nor in those who progressed. Hence LDH does not seem to have the same potential as a biomarker as KL-6 to indicate disease activity or progression.

This study has several limitations. First, the design was retrospective and there was no comparison group with another immunosuppressive treatment and

also no control group without treatment. Hence we can not quantify the magnitude of treatment efficacy in comparison to other immunosuppressive drugs or placebo. Second, the CTD cohort was heterogeneous in terms of diagnosis, disease stage and severity. Third, we did not perform the thiopurine S-methyltransferase enzyme activity test with the bias that we have a higher incidence of side effects in comparison to other published cohorts.

In conclusion, in the present study we could show that azathioprine has the potential to stabilise CTD-ILD for long-term in those patients who can tolerate the drug during the first 3 months. We could also show that KL-6 may have promise as a biomarker to assess response to treatment in CTD-ILD. Further randomised clinical trials are necessary to define the role of azathioprine in the treatment of CTD-ILD.

## 5 SUMMARY

Immunosuppressive agents and corticosteroids are the standard treatments for patients with connective tissue disease associated ILD (CTD-ILD). This retrospective study aimed to provide data on tolerability and efficacy of azathioprine in CTD-ILDs.

56 CTD-ILD patients treated with azathioprine between 2003 and 2014 were included in the study. Azathioprine was added to oral corticosteroids as a corticosteroid sparing agent. Patients were assessed every 3 months in the outpatient clinic.

15 patients (27%) discontinued treatment due to side effects, mostly due to elevated liver enzymes, within the first 3 months. The mean treatment duration was 34 months with a range of 3 to 105 months. 41 patients were treated for longer than 3 months, and 27 of those (66%) had stable or improved pulmonary function during treatment. In patients who remained stable or improved, the mean FVC was  $62 \pm 17$  % pred at the initiation of treatment and  $65 \pm 17$  % pred at the last follow up visit ( $p=0.036$ ), and the mean DLCO was  $38 \pm 16$  % pred at initiation of treatment and  $39 \pm 17$  % pred at the last follow up visit ( $p=0.06$ ). The mean serum level of Krebs von den Lungen-6 (KL-6) was elevated in all patients, it was  $1655 \pm 821$  U/mL at start of treatment and  $1589 \pm 744$  U/mL at the last follow up visit. Serum KL-6 levels showed a decline in stable or improved patients during treatment, but this was not significant ( $p= 0.210$ ).

In conclusion, our findings confirm the role of azathioprine in treatment of CTD-ILD. While early drug intolerance is frequent, most patients who tolerated the drug well achieved long-term stabilisation or improvement of lung function. Randomised controlled trials are still needed to validate these results. KL-6 seems to be a promising biomarker for monitoring CTD-ILDs under treatment.

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## 7 ABBREVIATIONS

AaDO <sub>2</sub>	alveolar-arterial oxygen gradient
ANA	antinuclear antibody
COP	Cryptogenic organizing pneumonia
CTD	connective tissue disease
CTD-ILD	CTD with associated ILD
CTGF	connective tissue growth factor
DM	dermatomyositis
DLCO	diffusion capacity of the lung for carbon monoxide
DcSSc	diffuse cutaneous scleroderma
DPLD	diffuse parenchymal lung disease
Egr-1	early growth response-1
DNA	deoxyribonucleic acid
FVC	forced vital capacity
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HRCT	high resolution computed tomography
IIP	idiopathic interstitial pneumonia
ILD	interstitial lung disease
IPAF	interstitial pneumonia with autoimmune features
IPF	idiopathic pulmonary fibrosis
IVC	inspiratory vital capacity
KL-6	Krebs von der Lunge 6
LcSSc	limited cutaneous scleroderma
LDH	lactate dehydrogenase
LIP	lymphocytic interstitial pneumonia

MCTD	mixed connective tissue disease
MMF	mycophenolate mofetil
MTX	methotrexate
NSIP	non-specific interstitial pneumonia
OP	organizing pneumonia
PaCO <sub>2</sub>	partial pressure of carbon dioxide in arterial blood
PaO <sub>2</sub>	pressure of oxygen in arterial blood
PM	polymyositis
RA	rheumatoid arthritis
RF	rheumatoid factor
RNP	ribonucleoprotein
ROC	receiver operating characteristic
SaO <sub>2</sub>	arterial oxygen saturation
SSc	systemic sclerosis
SjS	Sjögren syndrome
SLE	systemic lupus erythematosus
TGF-β	transforming growth factor-β
TPMT	thiopurine-S-methyltransferase
UCTD	undifferentiated connective tissue disease
UIP	usual interstitial pneumonia
U1RNP	ribonuclear protein component of the spliceosome

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## **9 CURRICULUM VITAE**

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

## 10 PUBLICATIONS

### Original Article

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