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Azathioprine for Connective Tissue Disease-Associated Interstitial Lung Disease

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Connective tissue disease · Azathioprine · Interstitial lung disease · Krebs von den Lungen-6

Abstract
Background: Immunosuppressive therapy still is the standard treatment for patients with connective tissue disease-associated interstitial lung disease (CTD-ILD). ***Objectives:*** This retrospective study aimed to provide data on the tolerability and efficacy of azathioprine in progressive CTD-ILDs. ***Methods:*** A total of 56 patients with CTD-ILD treated with azathioprine between 2003 and 2014 were included in the study. The patients were assessed every 3 months during follow-up. ***Results:*** The mean treatment duration was 34 months, with a range of 3–105 months. Fifteen patients (27%) discontinued treatment due to side effects, mostly due to elevated liver enzymes, within the first 3 months. Forty-one patients were treated for longer than 3 months, and 27 of those (66%) had stabilization or improvement of pulmonary function during treatment. In patients who remained stable or improved, the mean FVC was 62 ± 17% predicted (% pred) at initiation of treatment and 65 ± 17% pred at the last follow-up visit ($p = 0.036$), and the mean DLCO was 38 ± 16% pred at initiation of treatment and 39 ± 17% pred at the last follow-up visit ($p = 0.06$). ***Conclusions:*** Azathioprine can stabilize or improve CTD-ILD. While early drug intolerance is frequent, most patients who have tolerated the drug well achieve long-term stabilization or improvement of lung function.

Introduction

Immunosuppressive therapy still constitutes the main treatment option for connective tissue disease-associated interstitial lung diseases (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 on evidence-based recommendations.

Randomized controlled trials with immunosuppressants were conducted mostly on systemic sclerosis-associated ILD (SSc-ILD), demonstrating the efficacy and tolerability of cyclophosphamide versus placebo and the noninferiority of mycophenolate mofetil (MMF) to cyclophosphamide in SSc-ILD [1, 2]. MMF use was also as-

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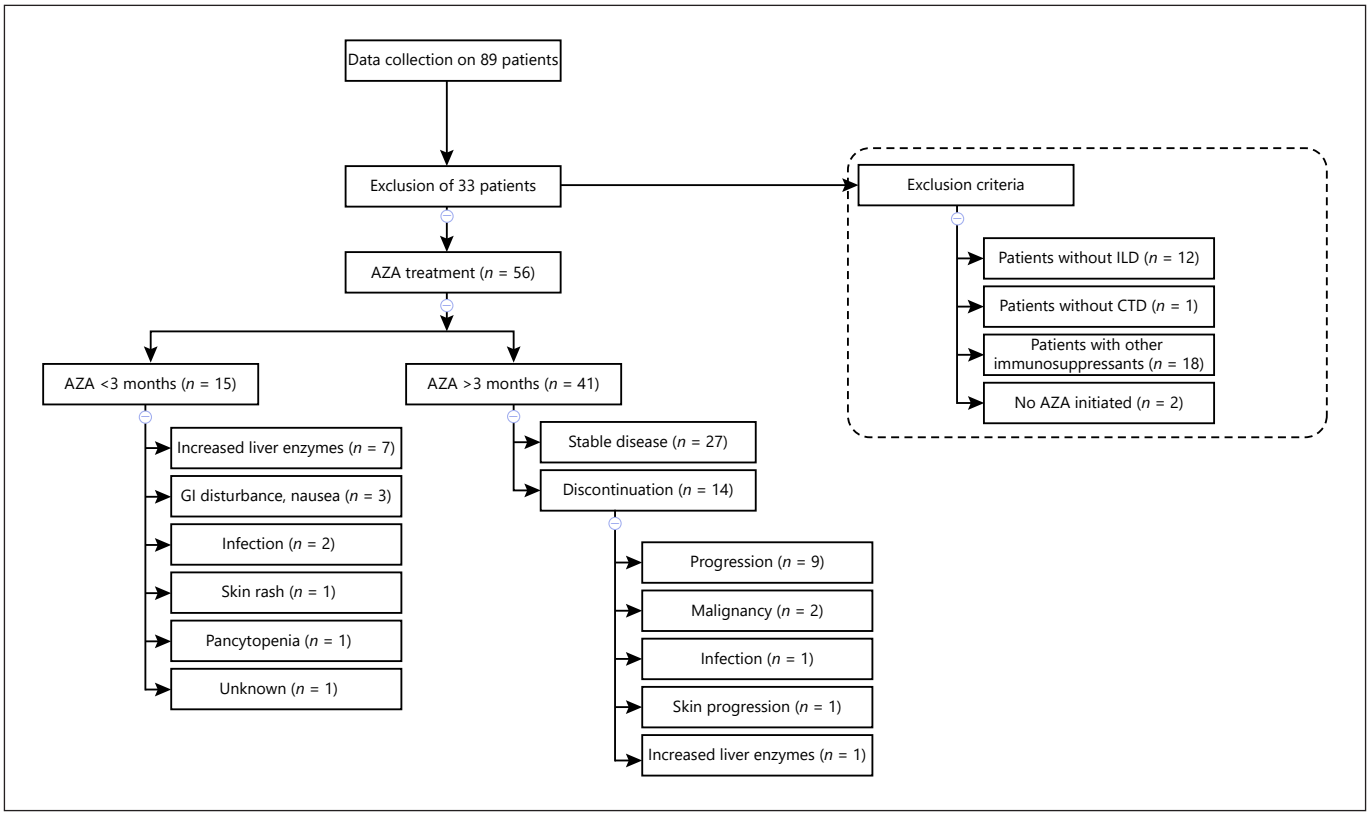

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Fig. 1. Flowchart of patient recruitment. AZA, azathioprine; ILD, interstitial lung disease; CTD, connective tissue disease; GI, gastrointestinal.

sociated with a smaller decline in lung function alone or in combination with nintedanib, an antifibrotic drug, in the SCENSIS trial [3]. Tocilizumab and rituximab are promising immunosuppressive treatments for SSc-ILD [4–6]. Nintedanib has shown efficacy in slowing decline in forced vital capacity (FVC) in autoimmune-related ILD with progressive fibrosis [7, 8].

There are three retrospective studies on azathioprine in CTD-ILD. One study was based on a case series of 11 patients with SSc [9]. The two other studies compared azathioprine to MMF [10, 11], which showed similar effects, with stabilization of pulmonary function in both groups. Adverse effects leading to discontinuation were seen less commonly with MMF.

Krebs von den Lungen-6 (KL-6) is a human MUC1 mucin protein released by alveolar epithelial cells. Although weak-to-moderate expression was observed in several cancer tissues such as stomach, colon, and hepatocellular tumors, serum KL-6/MUC1 levels are significantly increased in more than 70% of ILD patients [12]. In regard to CTD-ILDs, serum levels of KL-6/MUC1 have been found to be elevated in patients with rheumatoid arthritis (RA)-ILD, polymyositis/dermatomyositis (PM/DM)-ILD and SSc-ILD [12, 13], and to correlate with disease severity.

This retrospective study aimed to investigate the efficacy and tolerability of azathioprine in CTD-ILD patients. A further aim was to explore the role of KL-6/MUC1 as a predictor of response to treatment in these patients.

Patients and Methods

Study Cohort

We retrospectively studied 89 consecutive patients admitted to the Ruhrlandklinik between 2003 and 2014 with the diagnosis of a CTD-ILD and having started treatment with azathioprine. Thirty-three patients were excluded from the analysis for various reasons, among them 18 who were stable under various immunosuppressive treatments (9 receiving methotrexate, 5 cyclophosphamide, and 4 anti-TNF-α biologics). Fifteen of the 56 recruited patients discontinued azathioprine due to side effects within the first 3 months, and these were included in the safety analysis only (Fig. 1).

The diagnosis of CTD was based on history, physical examination, and specific autoantibodies and confirmed by rheumatolo-

Table 1. Demographics and characteristics of the studied subjects at baseline ($N = 56$)

Gender	
Female/male	31/25
Smoking history	38
Current smoker/ex-smoker/ nonsmoker	3/18/17
Age, years	64±14
Female	63±11
Male	65±17
Pulmonary function, % pred	
FVC ($n = 41$)	66±18
DLCO ($n = 38$)	42±19
Blood gas analysis, mm Hg	
PaO_2 ($n = 55$)	74±10
(A-a)DO_2 ($n = 49$)	29±10
Serum biomarkers[1]	
LDH, U/L ($n = 56$)	294±71 (153–563)[2]
KL-6/MUC1, U/mL ($n = 53$)	1,655±821 (313–6,228)[2]

Data are presented as mean ± SD unless otherwise stated. *N*, number of patients. [1] Reference values for serum biomarkers are indicated in Patients and Methods. [2] Data are mean ± SD (min.–max.).

gists. The diagnosis and the classification of the ILDs were mainly based on high-resolution computed tomography (HRCT) findings [14, 15]. A histological examination was usually not performed, since it is not considered a mandatory procedure by ILD experts. Only 1 patient underwent surgical biopsy and 1 patient transbronchial biopsy.

The patients were evaluated every 3 months. Besides medical history-taking and a physical examination, a chest X-ray, a pulmonary function test, and blood sampling were performed at each follow-up visit.

Definition of ILD Progression and Improvement

ILD progression was defined as a decrease in FVC of ≥5% predicted (pred) and/or in DLCO of ≥10% pred over a 6-month period, corresponding to 2 follow-up visits. Patients who had a decline in FVC of <5% pred and/or in DLCO of <10% pred were considered stable. Improvement was defined as any increase in FVC and DLCO at follow-up. In the absence of lung function tests, worsening of chest X-ray or HRCT findings and clinical symptoms related to ILD were considered sufficient to define progression. No patients were defined as having ILD progression on the basis of symptom worsening only.

Drug Administration

Azathioprine was started at 50 mg/day and was weekly increased by 50 mg/day up to the maintenance dose of 2.0 mg/kg body weight/day, with a range between 100 and 200 mg/day. Serum levels of thiopurine S-methyltransferase were not measured prior to azathioprine initiation, because it was not routinely available at 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.

Corticosteroids, mostly prednisone, were generally administered at an initial dose of 0.5 mg/kg body weight/day, except to patients with SSc-ILD and those with contraindications to high doses (mostly diabetes, obesity, and ocular problems). After 1 month, the dose was reduced by 10 mg each month until a maintenance dose of 10 mg was reached. In the long term, the patients received 5–10 mg/day.

Pulmonary Function Tests and Blood Gas Analysis

Measurements including FVC, forced expiratory volume in 1 s, total lung capacity, DLCO, arterial oxygen tension, arterial carbon dioxide tension, arterial oxygen saturation, and alveolar-arterial oxygen tension difference were performed at the time of blood sample collection. Values are expressed as percentage of predicted normal values.

Laboratory Measurements

Serum KL-6/MUC1 was measured with the NANOPIA® assay (Sekisui Diagnostics, Maidstone, UK), a latex agglutination turbidimetric method on a chemistry analyzer (ADVIA 1800 Chemistry System; Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). The upper limit of normal (458 U/mL) was determined in 142 healthy Caucasian subjects [16]. Serum lactate dehydrogenase (LDH) was measured routinely as an activity marker of ILDs (local normal laboratory range <240 IU/L).

Statistical Analysis

Variables were evaluated for by Kolmogorov-Smirnov test. Normally distributed data are presented as mean ± SEM or SD, nonnormally distributed data as median and interquartile range (IQR). Comparison between two groups was done with Student's *t* test or Wilcoxon's rank test for continuous variables; the χ^2 or Fisher's exact test was used to compare frequency between groups. Multiple comparisons were performed by Kruskal-Wallis test. Spearman's or Pearson's correlation coefficient was obtained for linear correlations. p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Demographics and Clinical Characteristics

The demographic and clinical characteristics of the subjects at baseline are summarized in Table 1. The median follow-up time was 35 months (IQR 3–109) and the median treatment duration was 34 months (IQR 3–105).

The median time to ILD onset after CTD diagnosis was 3.3 years (IQR 0–31). In 2 patients, ILD had preceded the diagnosis of CTD for 3 and 7 years, respectively. The most frequent ILD pattern on HRCT was nonspecific interstitial pneumonia (NSIP) at 70% ($n = 39$), followed by usual interstitial pneumonia (UIP) at 16% ($n = 9$), and organizing pneumonia at 4% ($n = 2$). In 6 patients (11%), the lung fibrosis was unclassifiable.

Table 2. Demographics and clinical characteristics according to CTD at baseline

	Rheumatoid arthritis (n = 21)	Systemic sclerosis (n = 14)	PM/DM (n = 10)	UCTD (n = 4)	Others[1] (n = 7)
Gender (F/M), n	11/10	8/6	7/3	2/2	5/2
Age, years	71±3	55±4[2]	63.5±5	61.5±7	67±2
ILD pattern, n					
UIP	4	2	1	1	1
NSIP	14	12	6	2	5
OP	1	0	1	0	0
Unclassifiable	2	0	2	1	1
Pulmonary function, % pred					
FVC	70±19 (n = 16)	67±14 (n = 10)	64±22 (n = 8)	51±19 (n = 3)	60±8 (n = 4)
DLCO	46±15 (n = 14)	44±24 (n = 9)	41±16 (n = 8)	24±19 (n = 2)	41±16 (n = 4)
Blood gas analysis, mm Hg					
PaO_2	74±10 (n = 20)	75±7 (n = 13)	69±14 (n = 10)	71±7 (n = 4)	76±8 (n = 7)
(A-a)DO_2	32±9 (n = 20)	26±8 (n = 10)	29±12 (n = 9)	33±9 (n = 4)	25±5 (n = 7)
Serum biomarkers[3]					
LDH, U/L	291±62 (n = 21)	275±47 (n = 14)	332±112 (n = 11)	333±78 (n = 4)	258±42 (n = 6)
KL-6/MUC1, U/mL	1,560±1,028[4] (n = 21)	2,500±1,543 (n = 14)	2,692±1,256 (n = 9)	3,742±2,067 (n = 4)	3,077±2,232 (n = 5)

Unless indicated, data are expressed as mean ± SD. CTD, connective tissue disease; PM/DM, polymyositis/dermatomyositis; UCTD, undifferentiated CTD; ILD, interstitial lung disease; UIP, usual interstitial pneumonia; NSIP, nonspecific interstitial pneumonia; OP, organizing pneumonia. [1] Sjögren syndrome (n = 2), mixed CTD (n = 3), psoriatic arthritis (n = 2). [2] $p < 0.05$ vs. rheumatoid arthritis. [3] Reference values are indicated in Subjects and Methods. [4] $p = 0.001$ vs. UCTD.

Table 3. Outcomes of the studied subjects

Outcome	n (%)
Improvement or stability of ILD[1]	27/41 (66)
Progression of ILD[1]	14/41 (34)
Malignancy[2]	2/56 (4)
Death	3/56 (5)

ILD, interstitial lung disease. [1] Patients with follow-up or with azathioprine intake <3 months were excluded from this analysis. [2] One lung cancer, one non-Hodgkin lymphoma.

The most frequent CTD was RA (38%), followed by SSc (25%), PM/DM (18%), undifferentiated CTC (UCTD; 7%), mixed CTD (5%), Sjögren syndrome (4%), and psoriatic arthritis (4%) (Table 2). 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 ± 3 years. NSIP was the most common ILD pattern in all CTD subgroups. With regard to pulmonary function impairment across the CTD groups, some differences were seen, but without statistical significance. The UCTD patients had the lowest FVC (51 ± 19% pred) and DLCO (24 ± 19% pred) (Table 2).

Three patients died during follow-up (2 from ILD progression and 1 from acute exacerbation of the ILD) (Table 3).

Efficacy Analysis

Among all patients, the mean FVC was 66 ± 18% pred at initiation of treatment and 63 ± 18% pred at the last follow-up visit ($p = 0.2$), and the mean DLCO was 42 ± 19% pred at initiation of treatment and 40 ± 17% pred at the last follow-up visit ($p = 0.25$). There were 27/41 patients (66%) who remained stable or improved and 14/41 patients (34%) with progression of ILD (Table 3).

Among the patients who remained stable or improved (n = 27), the mean FVC was 62 ± 17% pred at initiation of treatment and 65 ± 17% pred at the last follow-up visit ($p = 0.036$), and the mean DLCO was 38 ± 16% pred at initiation of treatment and 39 ± 17% pred at the last follow-up visit ($p = 0.06$) (Fig. 2).

Among those who had ILD progression (n = 14), the mean FVC was 72 ± 19% pred at initiation of treatment and 58 ± 18% pred at the last follow-up visit ($p = 0.004$), and the mean DLCO was 55 ± 15% pred at initiation of treatment and 44 ± 15% pred at the last follow-up visit ($p < 0.001$) (Fig. 2).

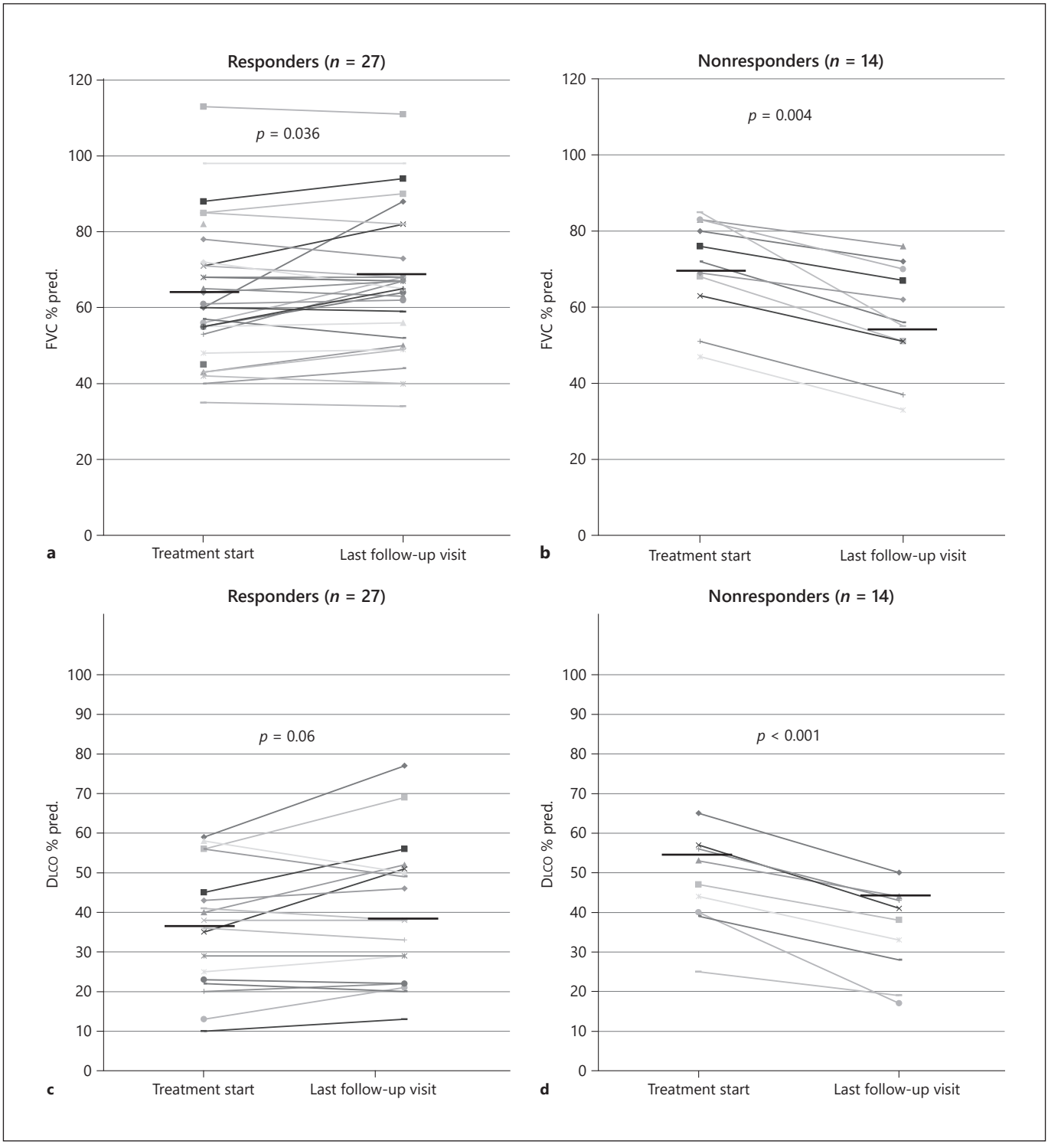

Fig. 2. Pulmonary function tests under azathioprine treatment. Pulmonary function test results (FVC and DLCO) at the start of therapy and at the end of follow-up for patients who remained stable or improved (responders; **a**, **c**) and those who had disease progression (nonresponders; **b**, **d**). Dots represent single values. Bold black lines represent mean values.

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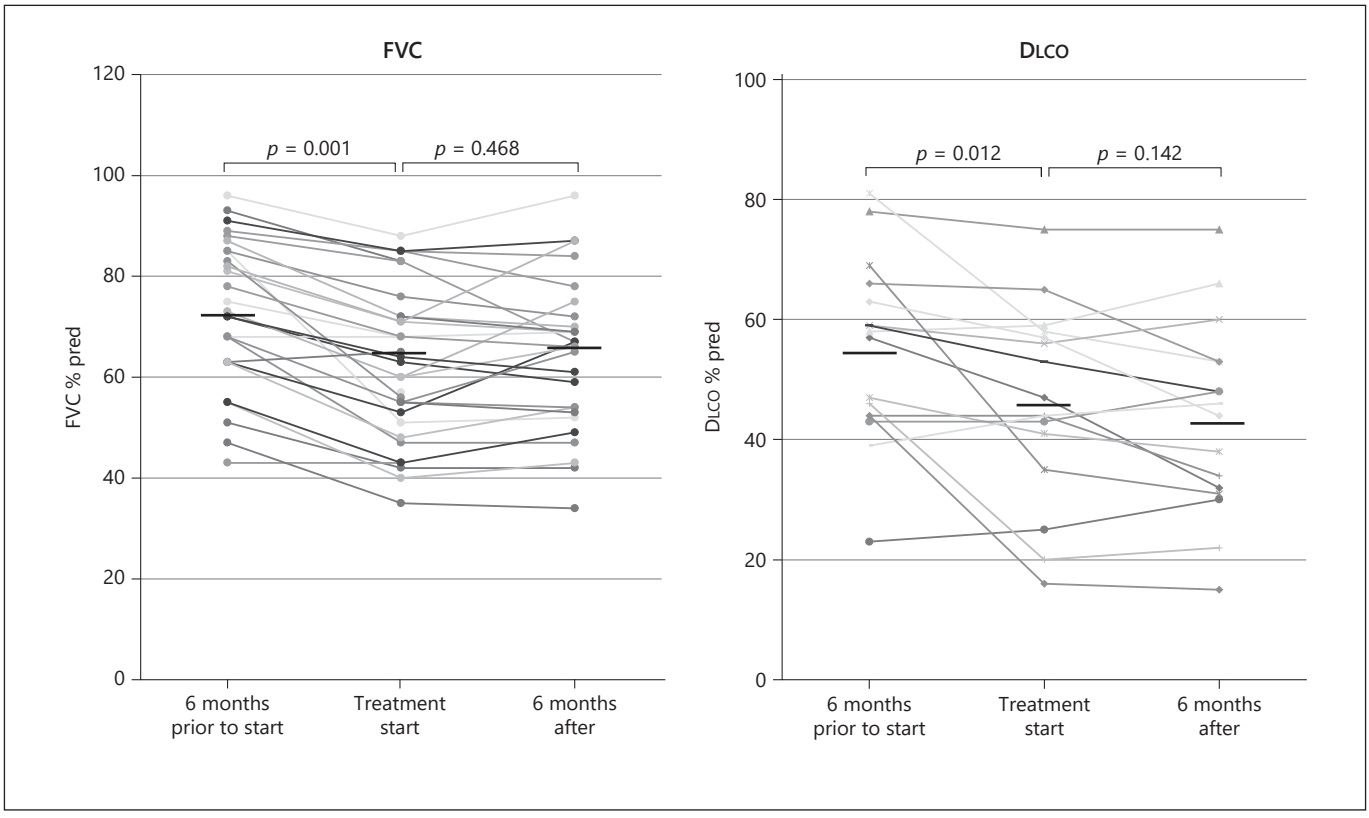

Fig. 3. Changes in FVC in 21 patients and in DLCO in 16 patients from 6 months prior to the start of treatment until 6 months after. Each line represents a patient. For comparison of the means, represented by bold black lines, the paired *t* test was used.

After adjustment for treatment duration, the percent change in FVC per month was +0.14 ± 0.05% and that in DLCO was +0.09 ± 0.08% among the patients who remained stable, while the percent change in FVC per month was –0.76 ± 0.6% and that in DLCO was –0.82 ± 1.16% among those who had ILD progression.

In a subgroup of patients, we were able to compare changes in FVC (n = 21) and in DLCO (n = 16) before and after initiation of azathioprine. Six months prior to treatment with azathioprine, the mean decline in FVC was –9 ± 2% pred ($p < 0.001$) and that in DLCO was –8.5 ± 2% pred ($p = 0.012$). Six months after treatment initiation, the mean change in FVC was 1 ± 1.4% pred ($p = 0.468$) and that in DLCO was –2.6 ± 1.8% pred ($p = 0.142$) (Fig. 3).

Among the patients with RA, there was a significant difference in change in FVC adjusted for azathioprine intake per time under treatment in comparison to the other CTD subgroups (–0.5 ± 0.8 vs. +0.05 ± 0.6% pred per month; $p = 0.016$, respectively). The same was seen for DLCO (–0.6 ± 1 vs. +0.1 ± 0.9% pred per month; $p = 0.047$). There were no significant differences in change in FVC or DLCO over time according to the HRCT pattern.

Safety and Tolerability

All patients (n = 56) were included in the safety and tolerability analysis independently of treatment duration. The most common side effects were infections, occurring in 11/56 patients (20%; Table 4). Side effects were the most common cause of azathioprine discontinuation (17/56; 30%), and the major side effect leading to discontinuation was increased liver enzymes (8/56; 14%; Table 4). Fifteen patients (27%) had to discontinue treatment within 3 months due to side effects. All discontinuations were definitive.

Serum KL-6/MUC1 and LDH Levels as Biomarkers

Serum KL-6/MUC1 levels and LDH levels were measured at the start of treatment with azathioprine and at the last follow-up visit in 31 and 40 patients, respectively.

The mean KL-6/MUC1 serum level was 1,655 ± 821 U/mL at the start of treatment and 1,589 ± 744 U/mL at the last follow-up visit (p = 0.254; online suppl. Fig. 1A; see www.karger.com/doi/10.1159/000508540 for all online suppl. material). The mean LDH serum level was 294 ± 71 U/mL at the start of treatment and 287 ± 66 U/mL at the last follow-up visit (p = 0.503; online suppl. Fig. 1B). The highest serum KL-6/MUC1 levels were measured in the UCTD patients at 3,742 ± 2,067 U/mL, while the RA patients showed the lowest KL-6/MUC1 levels (1,560 ± 1,028 U/mL; p = 0.001 vs. UCTD patients). The serum LDH levels were not significantly different between CTD patients (Table 2).

No correlation was seen between serum KL-6/MUC1 levels and gender, age, or smoking history. Patients with the UIP pattern on HRCT tended to have higher KL-6/MUC1 levels at the start of treatment than those with the NSIP pattern (3,272 ± 1,226 vs. 2,256 ± 1,630 U/L; p = 0.086), although the two groups did not differ in terms of FVC and DLCO at baseline.

Serum KL-6/MUC1 levels tended to decrease in stable or improved patients during treatment (p = 0.210; online suppl. Fig. 2A) and tended to decline in patients who progressed (p = 0.192; online suppl. Fig. 2B).

Serum LDH levels did not change during treatment, neither in those patients who remained stable or improved (p = 0.659; online suppl. Fig. 3A) nor in those who progressed (p = 0.596; online suppl. Fig. 3B).

Discussion

This retrospective study shows that azathioprine stabilized or ameliorated lung function in the majority of patients with CTD-ILD. Azathioprine has been broadly applied as a corticosteroid-sparing immunosuppressant in the treatment of idiopathic interstitial pneumonia, especially NSIP, chronic hypersensitivity pneumonitis, and sarcoidosis [17–20]. For the treatment of CTD-ILD, data on the efficacy of azathioprine are scarce, even though 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 the efficacy of azathioprine for CTD-ILD [9]. This was a retrospective study of azathioprine in combination with low-dose corticosteroid in 11 scleroderma-ILD patients showing that FVC improved in 5 patients and remained stable in 3 patients after 18 months.

Table 4. Tolerability and safety of azathioprine

Side effects	31/56 (55%)
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%)
Anemia	2/56 (4%)
Leukocytopenia	2/56 (4%)
Azathioprine discontinuation	29/56 (52%)
Side effects	17/56 (30%)
ILD progression	9/56 (16%)
Malignancy	2/56 (4%)
Skin progression	1/56 (2%)

ILD, interstitial lung disease.

Oldham et al. [10] compared azathioprine with MMF in a retrospective study of fibrotic CTD-ILD patients during long-term follow-up. The drug was discontinued in 27% of the azathioprine-treated versus 5% of the MMF-treated patients due to nonrespiratory side effects. Disease progression was seen in 11% of the patients in the azathioprine group and in 9% of the patients in the MMF group. FVC increased yearly by 1.53% and DLCO by 4.91% over 4 years under azathioprine. The most common side effect of azathioprine was increased liver enzymes (7%) [10].

Owen et al. [11] in a retrospective study (n = 71) showed similar stabilization of FVC in both groups (azathioprine and MMF); however, adverse events (especially gastrointestinal side effects) leading to early discontinuation (<12 months of treatment) were seen less commonly in the MMF group (4/22 vs. 13/49).

In our study, 8 of the 56 patients (14%) had to discontinue treatment due to elevated liver enzymes. In all, 73% of our patients received azathioprine for longer than 3 months, with a mean intake time of 34 months. Progression of ILD was seen in 34% of these patients. After adjustment for azathioprine intake per time, stable patients showed a significant improvement in FVC (+0.14 ± 0.05%) compared to those who progressed (–0.76 ± 0.6%); for DLCO, a similar trend was seen (+0.09 ± 0.08 vs. –0.82 ± 1.16%). With regard to safety, infection (11/56; 20%) was the most common side effect, but only 3/56 patients (5%) had to discontinue azathioprine due to infections.

In a retrospective study of MMF treatment in a cohort of 125 CTD-ILD patients, the average decline in FVC was –2% pred and that in DLCO was –11% pred before the start of treatment. At weeks 52, 104, and 156 after the imple-

mentation of treatment, an improvement in FVC of 5, 6, and 7% pred, respectively, was observed ($p < 0.05$ for all time points) [21]. In a recent trial on nintedanib for progressive fibrosing ILD, data on disease progression before treatment were not available, so that a direct comparison to immunosuppressive treatment is not possible [7, 8].

In our study, during the 6 months before the start of azathioprine, the mean decline in FVC was –9 ± 2% pred ($p < 0.001$) and that in DLCO was –8.5 ± 2% pred ($p = 0.012$). During the 6 months of treatment with azathioprine, the mean change in FVC was 1 ± 1.4% pred ($p = 0.468$) and that in DLCO was –2.6 ± 1.8% pred ($p = 0.142$).

In our study, the changes in FVC or DLCO over time were not different between patients with a UIP and those with an NSIP pattern on HRCT. Solomon et al. [22] observed shorter survival among RA-UIP patients than among RA-NSIP patients ($p = 0.02$). In our study, the patients with RA showed a significant difference in change in FVC adjusted for azathioprine intake per time under treatment in comparison to the other CTD subgroups (–0.5 ± 0.8 vs. 0.05 ± 0.6% pred per month; $p = 0.016$, respectively), indicating that they might not respond to treatment as well as the other CTD subgroups.

In order to support the efficacy data, we included serum KL-6/MUC1 as a disease activity marker. Serum KL-6/MUC1 is routinely used in Japan to assess disease activity of ILDs [12]. Oyama et al. [13] showed increased levels of KL-6/MUC1 in 90% of RA patients with ILD manifestation, compared to only 0.6% of patients without ILD manifestation. In their study, elevated KL-6/MUC1 was not associated with the activity of RA in other organs, but strongly correlated with the manifestation of interstitial pneumonia. Fathi et al. [23] demonstrated an inverse relationship of changes in KL-6/MUC1 values with FVC or DLCO in their PM/DM cohort as well. Bonella et al. [24] showed an inverse relationship between serum KL-6/MUC1 and FVC or DLCO values and a correlation between KL-6/MUC1 and HRCT fibrosis score in SSc-ILD patients. In the present study, KL-6/MUC1 values were elevated in all CTD-ILD groups, with the highest value in the UCTD group, and there was a tendency for serum KL-6/MUC1 to decline in stable or improved patients and a tendency for it to increase in patients who progressed under treatment. In consideration of the wide range of KL-6 levels observed and the weak tendency to reflect clinical response to immunosuppressive treatment, validation of these results is needed.

This study has several limitations. First, the design was retrospective and there was no comparison group with another immunosuppressive treatment or a control group without treatment. Hence, we cannot 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, and ILD was usually not confirmed histologically, although this is not considered mandatory in the presence of a definite CTD diagnosis [25]. Third, we did not perform the thiopurine S-methyltransferase enzyme activity test with the bias that we have a higher incidence of side effects than in other published cohort studies. Finally, due to the low number of serum samples collected during follow-up, the study was not powered for biomarker validation.

In conclusion, in the present study we could show that azathioprine has the potential to stabilize CTD-ILD long term in those patients who can tolerate the drug during the first 3 months. Further, randomized clinical trials are necessary to define the role of azathioprine in the treatment of CTD-ILD.

Statement of Ethics

This investigation complies with the guidelines for human studies and should include evidence that the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The study protocol was approved by the local IRB (approval No. 15-6616-BO, Ethics Committee of the Medical Faculty, University of Duisburg-Essen).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

E.B.B. and F.B. contributed to the conception and design of the study; to the collection of patient data and blood samples; to the analysis and interpretation of the data; and to drafting and finalizing the manuscript. M.C. collected patient data and samples and contributed to finalizing the manuscript. D.T. and S.O. contributed to the analysis and interpretation of the data; to the performance of statistics; and to finalizing the manuscript. U.C. contributed to the conception and design of the study; to the interpretation of the data; and to finalizing the manuscript. All authors have read and approved the final manuscript.

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