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Role of anti-DFS70 antibodies in idiopathic interstitial pneumonia and in connective tissue disease associated interstitial lung disease

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1. INTRODUCTION

Connective tissue disease (CTD), also named collagen vascular disease, are various systemic autoimmune diseases characterized by autoimmune phenomena and immune-mediated organ dysfunction (Olson et al., 2012). The primary target of pathology is the connective tissue of the body. The connective tissue functions as the structural portion of our body and is composed of two major structural proteins, collagen and elastin. The abnormal immune system directed against one's own body tissues (autoimmunity) causes chronic inflammatory injury to tissues which may even prove lethal (Janeway et al., 2001). According to the heterogeneous clinical features, CTDs are classified into systemic lupus erythematosus (SLE), systemic sclerosis (scleroderma, SSc), primary Sjögren's syndrome (SS), polymyositis/dermatomyositis (PM/DM), rheumatoid arthritis (RA), antisynthetase syndrome (ASS), mixed CTD (MCTD) and undifferentiated CTD (UCTD) (Fischer and du Bois, 2012).

The lung is a vulnerable target of autoimmune mediated injury in patients with CTD because of its abundant vasculature and large content of connective tissue. Pulmonary involvement may be the first manifestation of the disease (Yazisiz et al., 2010). Every component of the respiratory tract can be affected, including the interstitium, the large and small airways, the pleura and the pulmonary vasculature (de Lauretis et al., 2011). Interstitial lung diseases (ILD) is a common respiratory involvement which has been proved to occur in all kinds of CTDs (overall incidence of 15%), especially in patients with SSc and RA (de Lauretis et al., 2011; Fischer and du Bois, 2012). High-resolution computed tomography (HRCT) demonstrated that about two-thirds of the SSc patients have interstitial lung involvement, and postmortem examination validated that nearly all cases had developed pulmonary fibrosis (Launay et al., 2006). In patients with RA, up to 30% were found to have ILD, even though it was clinically apparent in only 5 to 10% of patients (Froidevaux-Janin et al., 2011). ILD is seen less frequently in SLE, but still 4% of patients with SLE have been found to develop pulmonary fibrosis (Park et al., 2007).

ILDs, also called diffuse parenchymal lung diseases (DPLDs), are characterized by inflammation and/or fibrosis of the lung parenchyma (Travis et al., 2013). They are a heterogeneous group of disorders typically presenting with progressive dyspnea and restrictive ventilatory impairment of pulmonary function. The diagnosis is mainly based on clinical and HRCT findings, sometimes histopathological confirmation is necessary (Travis et al., 2013). ILDs are divided into two main groups according to etiology: the idiopathic interstitial pneumonia (IIP), whose underlying cause is unknown, including idiopathic pulmonary fibrosis (IPF), idiopathic nonspecific interstitial pneumonia (iNSIP), and cryptogenic organizing pneumonia (COP). Other groups of ILDs are those of known origin like hypersensitivity pneumonitis (HP) or the granulomatous disorders such as sarcoidosis (ATS/ERS, 2002).

IPF is a chronic, progressive and ultimately fatal disease of unknown aetiology (Costabel, 2003; Raghu et al., 2011). It is the most common form of IIPs, the annual incidence is estimated at 3-9 cases per 100,000 for Europe and North America (Hutchinson et al., 2015). IPF is mainly diagnosed in older adults and has a predominance in men and ex/current smokers (Raghu et al., 2011). The median survival time of IPF is 3 to 5 years following diagnosis. Its prognosis is even worse than for many cancers (Fernandez et al., 2010; Ley et al., 2011; Raghu et al., 2011). Although the pathogenetic mechanisms of IPF remain to be determined, recurrent injury to the alveolar epithelium is believed to be the early pathogenic event in IPF (Katzenstein and Myers, 1998; Wynes et al., 2001; Phan, 2008), and then the migration and proliferation of fibroblasts and extracellular matrix remodeling result in irreversible distortion of the lung's architecture which is characterized by the histopathologic and/or radiologic pattern of usual interstitial pneumonia (UIP) (Selman et al., 2001; Harari and Caminati, 2010).

NSIP is the second most frequent cause of IIP, about 14%-36% of patients with IIP receive a diagnosis of NSIP (Kligerman et al., 2009). When no underlying causes and associations have been identified the diagnosis of idiopathic NSIP (iNSIP) is made, but more commonly NSIP occurs in a wide variety of clinical contexts, including toxic effects of drugs,

hypersensitivity pneumonitis, occupational exposure, and CTD (Travis et al., 2008). The histology of NSIP may range from a predominantly inflammatory process (i.e. cellular NSIP) to predominant fibrosis (i.e. fibrotic NSIP), but cellular NSIP is much less common than fibrotic NSIP (Katzenstein and Fiorelli, 1994). In contrast to IPF, NSIP shows a relatively homogeneous involvement of the alveolar walls by inflammation and fibrosis; bilateral ground-glass opacity is the most common HRCT pattern (Silva et al., 2008; Travis et al., 2013). NSIP has a considerably better prognosis with a 5-year survival of 80% compared to approximately 30% for IPF (Tafti et al., 2008; Kligerman et al., 2009).

In CTDs the manifestations of ILDs vary widely, with certain patterns more frequent than others in individual CTDs (**Table 1**). The most prevalent histologic pattern complicating SSc is fibrotic NSIP followed by the UIP pattern (Kim et al., 2002; Fischer et al., 2008). NSIP is also seen frequently in PM/DM and MCTD, whereas RA is more frequently associated with a UIP pattern (Kocheril et al., 2005). The ILD presenting in CTDs is a significant cause of mortality, especially in patients with SSc where ILD has become the leading cause of death. In RA, ILD contributes to increased mortality in up to 10% of patients (Olson et al., 2012).

Table 1. Types of ILD encountered in CTDs (Tzelepis et al., 2008)

Diagnosis	SSc	PM/DM	SS	RA	SLE	MCTD
Usual interstitial pneumonia	++	++	+	++	+	+
Nonspecific interstitial pneumonia	++++	++++	+	+	++	++
Cryptogenic organising pneumonia	+	++	+	+	+	−
Diffuse alveolar damage	+	++	+	+	++	−
Lymphocytic interstitial pneumonia	−	−	+++	−	−	−

+: lowest frequency; ++++: highest frequency; −: rare pulmonary involvement.

When ILDs occur in association with CTDs, they may present before, during or after the full manifestation of the defined criteria for a given CTD (Tzelepis et al., 2008). If presenting before, ILDs may be the only manifestation and precede the extrapulmonary CTD manifestation by several months, sometimes by more than 5 years (Homma et al., 1995).

The radiological and histological characteristics of CTD-ILDs are similar to their idiopathic counterparts (Hwang et al., 2009). There are, however, significant differences in survival and management between patients with CTD-ILD and those with IIP. Although CTD patients presenting with ILDs have an increased mortality compared to those without ILD, they have a better survival than patients with IIP. CTD patients with a chronic ILD usually experience a stable or slowly progressive course (Antoniou et al., 2009). CTD-ILD may be responsive to anti-inflammatory or immunosuppressive treatment which can improve lung function and health-related quality of life (Tashkin et al., 2006), whereas anti-inflammatory therapy has been shown to be ineffective for IPF, and is not recommended by the ATS/ERS international guideline (Raghu et al,. 2011). By contrast with IIPs, the patients with CTD-ILD need more surveillance and guiding management decisions for specific extrathoracic disease manifestations (Fischer et al., 2012). Along these lines it is crucially important to distinguish CTD-ILD from IIP. If patients with occult CTD-ILD and with no consistent evidence of autoimmune disease are misdiagnosed as IIP, they may be deprived of the appropriate therapeutic regimen or subjected to unwarranted therapies.

Since it is sometimes impossible for the physician to determine the diagnosis only based on the lung features, autoimmune screening should be done in all patients with apparent idiopathic ILDs. According to the recent guidelines for diagnosis and management of IPF, serologic tests for CTD should be performed in the majority of IPF patients (Raghu et al., 2011). The serologic evaluation should include rheumatoid factor, anti-cyclic citrullinated peptide, and antinuclear antibody (ANA) titer and pattern.

The presence of autoantibodies directed against cytoplasmic and nuclear autoantigens of protein or nucleic acid nature is considered as a serological hallmark of systemic autoimmune rheumatic disease. In 1958, Friou first described an indirect immunofluorescence (IIF) assay for ANA detection (Friou et al., 1958). Initially the ANA IIF test relied on rodent tissue substrates but contemporary the human larynx epithelioma-HEp-2 cells (human epithelioma type 2-CCl 23 ATCC clone) are used as ideal substrate in this assay (Plebani et al., 2009). HEp-2 cells have a large nucleus and consist

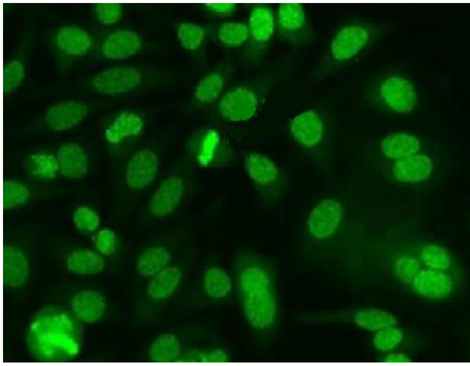
of hundreds of potential autoantigens which significantly improves the sensitivity of ANA detection. The IIF assay on HEp-2 cells was recommended as the “gold standard” assay to screen for ANAs in sera by a task force of the American College of Rheumatology (ACR) (Meroni and Schur, 2010).

The IIF used for ANA determination includes more than 25 patterns, many of them are directed against nuclear components and are usually specific for a particular CTD, greatly aiding the diagnostic process (de Lauretis et al., 2011). There are three major ANA patterns: the homogeneous pattern (associated with ANAs against double-stranded DNA (dsDNA) [SLE] and histones), the speckled/peripheral pattern (less specific) and the nucleolar pattern (most often associated with limited scleroderma).

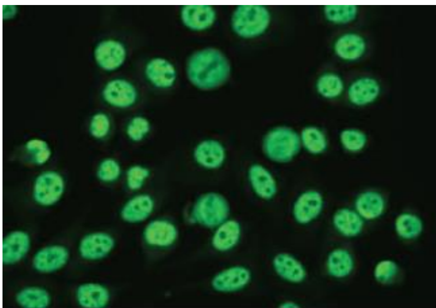
Specific serum ANA subsets have been shown to be related with pulmonary manifestations in CTD patients. For instance, in SSc, antibodies reactive with topoisomerase I (anti-ATA antibodies; also termed Scl-70) are associated with a greater risk of ILD, while anticentromere antibodies (ACAs) are rarely seen in SSc-ILD (Fischer and du Bois, 2012). Among patients with SS, pulmonary complications arise particularly in the anti-La/Ro antibody positivity individuals (Davidson et al., 2000; Yazisiz et al., 2010). Ferreira et al reported that anti-Ro52 antibodies are very sensitive (100%) for ILD diagnosis in MCTD, UCTD and SS (Ferreira et al., 2012).

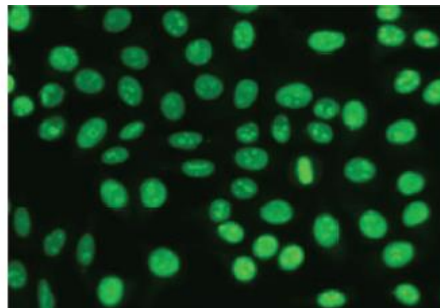
ANAs are not specific for CTDs, since approximately 20% of apparently healthy individuals have a positive IIF ANA test (Watanabe et al., 2004). The limited specificity has been one of the most important drawbacks of the HEp-2 IIF assay as a screening test for CTD (Mariz et al., 2011). The main target of the positive ANA pattern seen in healthy individuals is the dense fine speckled 70 antigen (DFS70) (Watanabe et al., 2004; Mariz et al., 2011). The recognition of this dense fine speckled ANA pattern is important to discriminate healthy individuals from CTD patients.

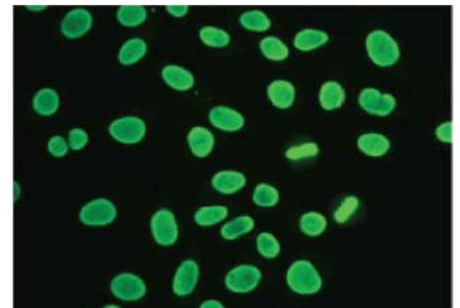
(a) DFS pattern



(b) Patterns to be differentiated from DFS pattern







Speckled pattern | Homogenous + nucleolar pattern | Homogenous pattern

Figure 1. Characteristic features of various ANA patterns. DFS pattern is characterized by dense fine speckles in the nucleus of interphase cells and by strong chromatin staining in the metaphase of mitotic cells (a), the DFS pattern has to be differentiated from speckled patterns and homogeneous patterns (b) (Mahler et al., 2011; Mahler and Fritzler, 2012).

The DFS pattern on HEp-2 cells is characterized by dense fine speckles in the nucleus of interphase cells and by strong chromatin staining in the metaphase of mitotic cells. It is a common ANA-IIF pattern, in recent studies 0.8% to 37% of samples showed the typical DFS-IIF pattern in consecutive samples tested for ANA (Ochs et al., 2016). Identification of the DFS pattern is not always easy, the discrimination between DFS and a homogeneous pattern can be a challenge for routine diagnostic laboratories (**Figure 1**). Recently, Mahler and colleagues developed an immunoadsorption IIF assay, a method that dilutes the patient serum samples in a buffer containing recombinant DFS70 antigen before the routine process of IIF, in this way anti-DFS70 antibodies can be prevented from binding to their target on HEp-2 substrate and producing the DFS pattern. They believe this novel assay can significantly improve the performance characteristics of ANA by IIF (Mahler, et

al., 2011). Besides IIF, the presence of anti-DFS70 antibodies can be analyzed by other techniques including enzyme-linked immunosorbent assay (ELISA), chemiluminescent assay, addressable laser bead assay, and immunoblot. Anti-DFS70 antibodies are predominantly IgG (Ochs et al., 2000; Mahler et al., 2012).

The anti-DFS70 target autoantigen DFS70 was initially identified in the 1990s during a survey of ANAs in patients with interstitial cystitis and was termed for a 70-kDa protein recognized by immunoblotting (Ochs et al., 1994). Later the complete DFS70 DNA was sequenced and shown to be identical to the lens epithelium-derived growth factor (LEDGF) and DNA binding transcription coactivator p75 (Ge et al., 1998; Singh et al., 1999). DFS70 consists of 15 exons and 14 introns (Singh et al., 1999). Its N-terminal region contains a proline-tryptophan-tryptophan-proline domain, charged regions, AT-hook motifs and a nuclear localization signal, all these sequences act as chromatin-binding elements cooperating to bind DFS70 to active transcription sites in the chromatin (Ochs et al., 2016). The C-terminal region contains the autoepitope (residues 347-435) recognized by the autoantibodies. This autoepitope is absent in the short splice variants of DFS70 (i.e. LEDGF/p52) which can explain why anti-DFS70 antibodies consistently recognize a single band of 70-75 kD and not the LEDGF/p52 in immunoblots of cell lysates (Ogawa et al., 2004).

Although originally presumed to be a lens epithelial cell growth factor, compelling evidence indicates that the main function of DFS70 is to promote cell survival against cellular oxidative stress which can be induced by alcohol, UVB irradiation, serum starvation, certain viruses, cytotoxic drugs and other environmental stressors (Basu et al., 2012; Leitz et al., 2014). Both the N- and C-terminal regions contribute to its stress survival functions (Ochs et al., 2016). DFS70 transcripts are expressed in various human tissues and cells, including lens epithelial cells, keratinocytes, fibroblasts, cancer cells and most laboratory transformed cell lines (Singh et al., 2000; Huang et al., 2007). The transcriptional and function of DFS70 are regulated by many factors. Increased cellular expression of transcription factor Sp1 can lead to upregulation of DFS70 via a TATA-less promoter

activation (Desfarges et al., 2011). Transforming growth factor beta 1 (TGF-β1) and micro-RNAs (miRNAs) can also influence the expression of DFS70 (Sharma et al., 2003; Swaminathan et al., 2012). During apoptosis induced by caspase-3, DFS70 is cleaved to several fragments; this apoptotic cleavage impairs DFS70's stress survival activity (Wu et al., 2002). Taken together, DFS70 is a key protein in maintaining the delicate balance between the crossroads of cell death and survival in response to stress through transcriptional activation of antioxidant and other protective genes (Ochs et al., 2016).

In inflammatory conditions, there is a regulatory crosstalk between DFS70 and the inflammatory pathways. In HaCaT skin cells, overexpression of DFS70 has been shown to induce the IL-6/STAT3 signaling pathway (Takeichi et al., 2010). DFS70 can also stimulate the release of tumor necrosis factor and IL-8 from keratinocytes (Takeichi et al., 2011). The link between DFS70 and the inflammatory pathway may contribute to inflammatory processes. Along these lines, anti-DFS70 antibodies have been detected in patients with a variety of chronic inflammatory conditions, in cancer patients, and also in apparently healthy individuals (Watanabe et al., 2004; Daniels et al., 2005; Meroni and Schur, 2010). The highest prevalence of anti-DFS70 antibodies has been reported in patients with Vogt-Harada syndrome (66.7%) (Yamada et al., 2001) and atopic dermatitis (30%) (Ochs et al., 2000; Ayaki et al., 2002). In contrast, the prevalence of anti-DFS70 antibodies in autoimmune diseases such as CTD is significantly lower (~2-3%) (Ganapathy and Casiano, 2004; Mahler et al., 2011). Anti-DFS70 autoantibodies could play a pathogenic role and might function in the removal of DFS70 and its cleavage fragments from cellular debris to prevent the release of intracellular self-antigens and danger signals (Wu et al., 2002).

The higher prevalence of anti-DFS70 antibodies in healthy subjects than in autoimmune diseases supports the hypothesis that these autoantibodies can serve as protective or natural autoantibodies (Miyara et al., 2013). On the other hand, the presence of anti-DFS70 antibodies in apparently healthy individuals may be indicative of an undetected chronic inflammatory response.

The low frequency of anti-DFS70 autoantibodies in systemic autoimmune diseases has been confirmed by several comprehensive studies. Dellavance et al. evaluated over 13,000 ANA positive samples of a general clinical laboratory by IIF and immunoblot and found that anti-DFS70 autoantibodies were seen in 37% of samples and more commonly associated with nonautoimmune conditions than with systemic autoimmune disease (Dellavance et al., 2005). Another study examined 500 systemic autoimmune rheumatic disease sera for the presence of anti-DFS70 antibodies and found that only 22 (4.4%) were positive. The majority, 18/22 (86%), of these anti-DFS70 antibody positive patients were also positive for other autoantibodies such as anti-dsDNA, anti-SS-A, or anti-U1-RNP (Muro et al., 2008). Interestingly, in regard to the long term outcome, after an average follow-up of 4 years, none of the healthy individuals with isolated anti-DFS70 antibody reactivity later developed a systemic autoimmune disease (Mariz et al., 2011). Taken together these findings indicate that anti-DFS70 antibodies are rarely observed in systemic autoimmune diseases, and when they are, they are usually accompanied by additional autoantibodies. Individuals producing only anti-DFS70 antibodies, and no other autoantibody, have a low likelihood to develop a systemic autoimmune disease.

Anti-DFS70 antibodies have been investigated in only a few lung disorders. In a study of patients with atopic dermatitis, 16% of the patients with asthma were DFS70 positive compared with 30% of atopic dermatitis and 0% of healthy subjects (Ochs et al., 2000). Yamada et al. found that in patients with panuveitis, including also sarcoidosis patients, anti-DFS70 positivity was seen in 67% of patients versus 22% of healthy controls. Investigations on anti-DFS70 antibodies in ILD patients are lacking in the literature.

Since NSIP and IPF are characterized by inflammation and fibrosis, and in consideration of the transcript expression of DFS70 in fibroblasts and the essential function of DFS70 in inflammatory conditions, we hypothesized that DFS70 is associated with the pathophysiology of these two ILDs, and anti-DFS70 antibodies might be abnormally expressed compared to healthy individuals. Furthermore, by comparing the expression of anti-DFS70 antibodies between ILD patients with and without CTD the present study was

conducted to investigate the potential value of anti-DFS70 antibody as a biomarker to exclude the development of CTD in ILDs.

2. AIM OF THE STUDY

The aim of the current study was to investigate the frequency of serum anti-DFS70 antibodies and a possible correlation with ANA status in patients with IPF and NSIP. Additionally, we investigated the potential role of anti-DFS70 antibody to distinguish between CTD-ILD and IIP and to predict CTD development in patients diagnosed initially as IIP. Finally, we explored the potential correlations between anti-DFS70 antibodies and clinical parameters of pulmonary involvements.

3. METHODS

3.1. Study population

260 patients with ILDs were investigated, including 100 patients with NSIP and 160 patients with IPF. These patients were admitted to the Ruhrlandklinik from April 2007 to August 2013. Two groups of controls were included: the healthy control (HC) group was composed of 49 blood donors; 35 SSc-ILD patients (13 from the Ruhrlandklinik, 22 from the Department of Internal Medicine, University of Verona, Italy) served as negative controls for anti-DFS70 antibodies (Karsten et al., 2015).

Written informed consent was obtained according to institutional guidelines. The study was approved by the local IRB. Demographic and laboratory characteristics of the enrolled subjects are shown in **Table 2**.

The IPF and NSIP patients were diagnosed based on clinical, radiographic, and histopathologic findings according to ATS/ERS criteria 2011 and 2013 respectively (Raghu et al., 2011; Travis et al., 2013). The SSc patients were diagnosed based on current criteria (van den Hoogen et al., 2013).

During follow-up of the IPF and NSIP patients, the diagnosis of CTD was made by referring rheumatologists according to current criteria for the respective disease (Tan et al., 1982; Aletaha et al., 2010; Shiboski et al., 2012; van den Hoogen et al., 2013). All patients had a follow-up time of at least 2 years.

Table 2. Demographics and patents' characteristics of the studied groups.

Variables	NSIP n=100	IPF n=160	SSc-ILD n=35	HC n=49
<u>**Demographics**</u>				
Age, yrs	65±1$^{*\#\#\S\S\S}$	68±0.7$^{\#\#\#\S\S\S}$	57±2§§§	34±2
Gender male/female (n)	47/53$^{***\#\S\S}$	128/32$^{\#\#\#}$	9/26§§§	36/13
Smoking status‡ current/ex/non (n)	2/39/29$^{**\#\#\S\S\S}$	5/100/30$^{\#\#\#\S\S\S}$	1/7/23§	7/4/22
BMI	28±0.4	28±0.5	27±1	-
<u>**Pulmonary function**</u>				
FVC, % pred.	70±2	70±1	78±5	-
FEV_1, % pred.	69±2	72±1	71±5	-
DLco, % pred.	48±4	42±1$^{\#\#}$	55±4	-
TLC, % pred.	68±2$^{\#\#}$	67±1$^{\#\#}$	78±4	-
<u>**Blood gas analysis**</u>				
PaO_2, mmHg	74±1	72±1	75±3	-
$PaCO_2$, mmHg	37±0.4	38±0.3	37±1	-
$AaDO_2$, mmHg	29±1	30±1	27±3	-
SaO_2, %	95±0.3	95±0.2	96±0.6	-

Unless otherwise indicated, values are expressed as mean ± standard error of mean (SEM).
Independent-t test or Chi-square test was used to calculate p value.
*: p<0.05, **: p<0.01, ***: p<0.001 vs. IPF.
$^{\#}$: p<0.05, $^{\#\#}$: p<0.01, $^{\#\#\#}$: p<0.001 vs. SSc-ILD.
§: p<0.05, §§: p<0.01, §§§: p<0.001 vs. HC.
‡: Smoking status was not available in all patients.

3.2. Pulmonary function tests and blood gas analysis

Pulmonary function tests and blood gas analysis measurements included forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), total lung capacity (TLC), diffusing capacity of the lung for carbon monoxide (DLco), partial pressure of oxygen in arterial blood (PaO_2), arterial carbon dioxide tension ($PaCO_2$), alveolar-arterial oxygen gradient ($AaDO_2$) and arterial oxygen saturation (SaO_2).

3.3. Indirect immunofluorescence assay for antinuclear antibody detection

Serum samples were obtained at the initial presentation, and then were stored at -80°C until analyzed. Lactate dehydrogenase (LDH) in serum was routinely measured in the serological laboratory of our institution.

The ANA-IIF assay was performed at the central laboratory of the University Hospital Essen according to the following protocol: The serum samples were diluted in 0.15M NaCl and 10 mM phosphate buffered saline (PBS), pH 7.4, and incubated with HEp-2 cells in two different dilutions, 1:80 and 1:320 (Euroimmun AG, BIOCHIPS TITERPLANE®-technique, EUROPLUS®, ANA-global test, FA1510-2010-1, Lübeck, Germany) at room temperature for 30 minutes. After washing twice with PBS-Tween for 10 minutes, the slides were incubated with goat anti-human immunoglobulin fluorescein isothiocyanate in a dark chamber at room temperature for another 30 minutes. After washing twice as before, the slides were embedded with buffered glycerol and coverslips (Euroimmun AG, ZF1200-0120, Lübeck, Germany). Analysis was performed by expert observers using a fluorescence microscope at 488nm (EUROSTAR, Euroimmun, Lübeck, Germany, magnification of 400x). Internal positive and negative controls (provided by the manufacturer, Euroimmun AG) were used. ANA titers were determined by testing successive 2-fold dilutions of the serum and were considered as elevated when higher than 1:80.

3.4. Enzyme-linked immunosorbent assay

The concentrations of serum anti-DFS70 antibodies at baseline were measured using a commercially available ELISA kit (Medical and Biological Laboratories Co., Ltd., Fujioka City, Japan). The assay is currently intended for research use only. The ELISA was performed according to the manufacturer’s instructions.

Each serum was diluted 1:101 by adding 10µl serum to 1ml Assay Diluent. 100µl Assay

Diluent (for blank), 100μl Calibrator and 100μl of each diluted serum sample were added to the microwell coated with the antigen DFS70. The plate was incubated at room temperature (20-30℃) for 60 minutes and then washed 4 times with solution to remove any unbound material. After adding 100μl working conjugate solution to each well, the plate was incubated at room temperature for another 60 minutes. After washing 4 times to remove any unbound conjugate, 100μl of substrate was added to each well. The plate was incubated for 60 minutes at room temperature and then the reaction was stopped by adding 100μl stop solution to each well. The optical density value of the plate was read by a spectro-photometer immediately, setting the test wavelength at 450nm and the reference at 620nm. Finally, the concentration of anti-DFS70 antibodies in the samples was calculated through a formula provided by the instructions.

The cut-off for positivity was set at 400 U/ml in this study, which was twice the standard deviation of the average of the negative control group (36 SSc-ILD patients sera).

3.5. Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were evaluated for a normal distribution with the Kolmogorov-Smirnov test. Normally distributed parametric data were presented as mean ± SEM; nonnormally distributed data were presented as median (interquartile, IQR). Categorical variables, such as sex, age, prevalence of anti-DFS70 antibodies, 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. P values lower than 0.05 were considered statistically significant.

4. RESULTS

4.1. Expression of investigated serum biomarkers

4.1.1 Prevalence of ANA positivity

Within the control groups, 20 of 35 (83%) SSc-ILD patients were ANA positive and all 49 healthy controls were ANA negative. The prevalence of ANA positivity in both the NSIP and IPF groups was significantly lower than in SSc-ILD and significantly higher than in healthy controls (p<0.001, for all comparisons). Within the ILD groups, ANA was positive in 47 of 100 (47%) NSIP patients and 51 of 160 (32%) IPF patients. The ANA positive percentage of NSIP patients was significantly higher than that of IPF patients (p<0.05) (**Table 3**).

Table 3. The prevalence of investigated serum biomarkers in studied groups.

Biomarkers	NSIP n=100	IPF n=160	SSc-ILD n=35	HC n=49
ANA (+), n (%)	47 (47)*###§§§	51 (32)###§§§	29 (83)§§§	0 (0)
Anti-DFS70 (+), n (%)	12 (12)§§	31 (19)§	2 (6)§§	17 (35)
Serum anti-DFS70 antibody levels‡, median (IQR) U/ml	111 (134)§§§	123 (265)§§§	97 (140)§§§	320 (253)

Data expressed as number (n) or median (IQR).

Chi-square or Mann-Whitney test was used to calculate p value.

*: p<0.05 vs. IPF.

###: p<0.001 vs. SSc-ILD.

§: p<0.05, §§: p<0.01, §§§: p<0.001 vs. HC.

‡: Anti-DFS70 antibody levels of SSc-ILD were normally distributed, but were also expressed as median (IQR) to compare with other groups.

In the total individuals of ILD patients, ANA positivity had the same prevalence in men and in women (66, 38% vs. 32, 38%, p>0.05). We divided the age of ILD patients into 3 groups: ≤60 yrs, 61～70 yrs and >70 yrs, and found that the frequency of ANA positivity was stable across these age groups (p>0.05) (**Table 4**).

Table 4. The prevalence of ANA in ILD patients categorized by gender and age groups.

Variables	**ANA (+)**, n (%)	**ANA (-)**, n (%)	**p value**
Gender			
male (n=175)	66 (38)	109 (62)	n.s.
Female (n=85)	32 (38)	53 (62)	
Age			
⩽60 yrs (n=59)	26 (44)	33 (56)	n.s.
60~70 yrs (n=92)	31 (34)	61 (66)	
>70 yrs (n=109)	41 (38)	68 (62)	

Data expressed as number (n) and percentage (%).
Chi-square test was used to calculate p value.
n.s.= not significant

4.1.2 Prevalence of anti-DFS70 antibody positivity

Within the control groups, anti-DFS70 antibodies were positive in 17 of 49 (35%) healthy controls and 2 of 35 (6%) SSc-ILD patients, the difference was significant (p<0.01). The prevalence of anti-DFS70 antibody positivity in both the NSIP and IPF groups was significantly lower than in HC (p<0.05, respectively), and tended to be higher than in SSc-ILD (p>0.05, respectively). Within the ILDs groups, anti-DFS70 antibodies were positive in 12% of NSIP patients and 19% of IPF patients, the difference was not significant (p>0.05) (**Table 3**).

In the total individuals of ILD patients, the prevalence of anti-DFS70 antibodies was not different between men and women (17% vs. 15%, p>0.05) and also not different between various age groups (p>0.05) (**Table 5**).

Table 5. The prevalence of anti-DFS70 antibodies in ILD patients categorized by gender and age groups.

Variables	**Anti-DFS70 (+),** n (%)	**Anti-DFS70 (-),** n (%)	**p value**
Gender			
Male (n=175)	30 (17)	145 (83)	n.s.
Female (n=85)	13 (15)	72 (85)	
Age			
≤60 yrs (n=59)	10 (17)	49 (83)	n.s.
60~70 yrs (n=92)	15 (16)	77 (84)	
>70 yrs (n=109)	18 (17)	91 (83)	

Data expressed as number (n) and percentage (%).
Chi-square test was used to calculate p value.
n.s.= not significant

4.1.3 Serum anti-DFS70 antibody levels

Within the control groups, the serum anti-DFS70 antibody levels in SSc-ILD and in HC were 97 U/ml (IQR: 140 U/ml) and 320 U/ml (IQR: 253 U/ml), respectively, the difference was significant ($p<0.05$). Both the NSIP and IPF patients had significantly lower serum anti-DFS70 antibody levels than HC ($p<0.001$, respectively); the levels were similar to SSc-ILD ($p>0.05$, respectively) and not different between NSIP and IPF patients (**Table 3** and **Figure 2**).

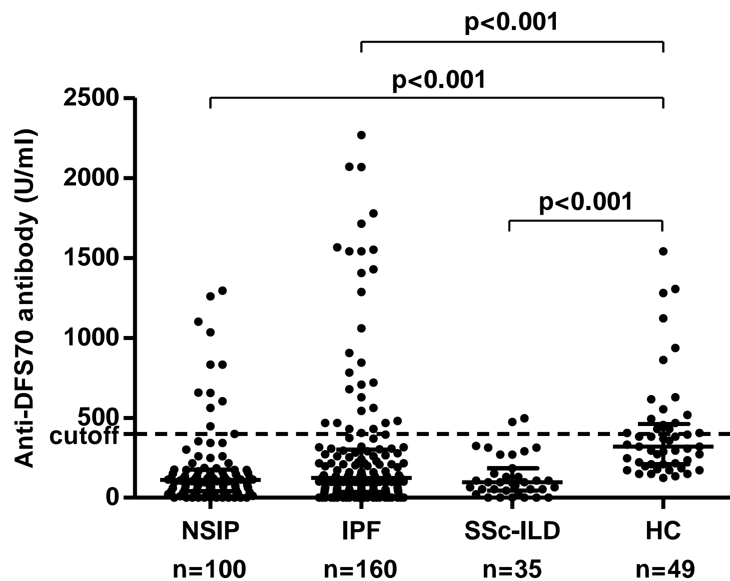


Figure 2. Comparisons of serum anti-DFS70 antibodies levels between ILD patients and control groups. Dots represent single patients, the central line represents the median and the whiskers represent the interquartile range. Kruskal-Wallis and stepwise step-down multiple comparisons test were used to calculate p value.

In the total population of ILD patients, the serum anti-DFS70 antibody levels in men tended to be higher than in women, but the difference was not significant (123 U/ml, IQR: 227 U/ml vs. 98 U/ml, IQR: 216 U/ml, p>0.05) (**Figure 3a**), and there was no difference according to smoking status (**Figure 3b**).

No association between anti-DFS70 antibody levels and age was observed (**Figure 3c**). Also the levels of anti-DFS70 antibodies were stable across the age groups: 94 U/ml (IQR: 222 U/ml) in ≤60 yrs; 130 U/ml (IQR: 183 U/ml) in 61～70 yrs; 114 U/ml (IQR: 249 U/ml) in >70 yrs; p>0.05 (**Figure 3d**).

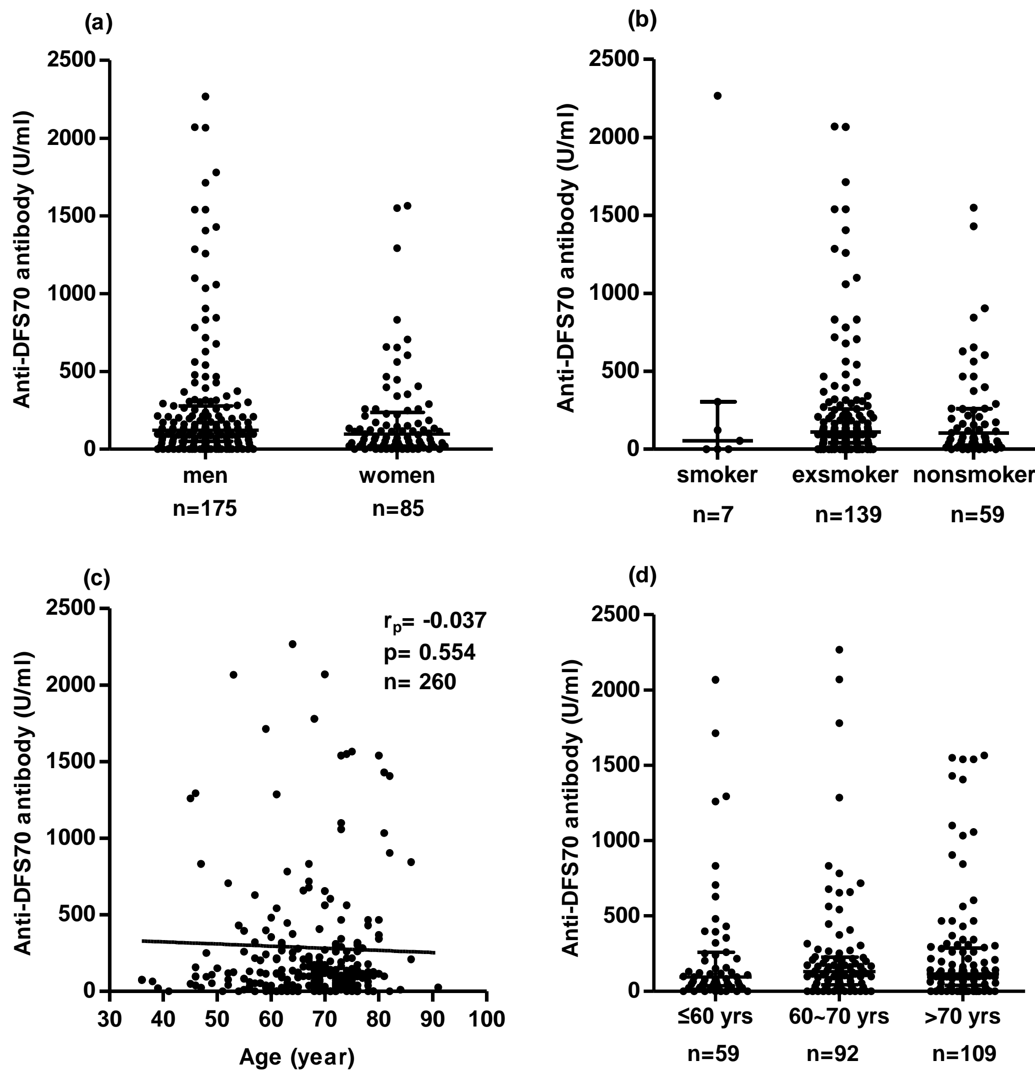


Figure 3. Within all the ILD patients, comparison of serum anti-DFS70 antibody levels categorized by gender (a), smoking status (b) and age groups (d). Dots represent single patients, the central line represents the median and the whiskers represent the interquartile range. Mann-Whitney or Kruskal-Wallis test was used to calculate p value. Correlation between serum anti-DFS70 antibody levels and age (c). Spearman's correlation test was used to calculate p value.

4.2. Expression of investigated serum biomarkers according to development of CTD

During 24 months of follow-up, 37 ILD patients (34 NSIP and 3 IPF) developed CTD: 22 RA (59%), 7 SSc (19%), 4 ASS (11%), 2 SS (5%), 1 MCTD (3%), 1 UCTD (3%). All the 3 CTD-UIP patients developed RA. The patients' demographics and characteristics according to CTD development are shown in **Table 6**.

Table 6. Demographics and characteristics of patients according to CTD development.

Variables	IIP n=223	CTD-ILD n=37	SSc-ILD n=35	HC n=49
Demographics				
Age, yrs	67±1$^{###§§§}$	64±2$^{#§§§}$	57±2§§§	34±2
Gender, male/female (n)	160/63$^{***###}$	15/22§§	9/26§§§	36/13
Smoking habits‡, current/ex/non (n)	7/122/49$^{###§§§}$	0/17/10$^{##§§§}$	1/7/23§	7/4/22
BMI	28±0.4	27±0.6	27±1	-
Pulmonary function				
FVC, %pred.	70±1	69±4	78±5	-
FEV_1, %pred.	72±1*	65±3	71±5	-
DLco, %pred.	43±1$^{##}$	53±9	55±4	-
TLC, %pred.	67±1$^{##}$	70±3	78±4	-
Blood gas analysis				
PaO_2, mmHg	73±1	74±2	75±3	-
$PaCO_2$, mmHg	38±0.2	37±0.7	37±1	-
$AaDO_2$, mmHg	30±1	30±2	27±3	-
SaO_2, %	95±0.2	95±0.6	96±0.6	-
Biomarkers				
ANA (+), n (%)	73 (33)$^{***###§§§}$	25 (68)§§§	29 (83)§§§	0(0)
Anti-DFS70 (+), n (%)	37 (17)§§	6 (16)§	2 (6)§§	17 (35)
Serum anti-DFS70 levels, median (IQR) U/ml	119 (232)§§§	104 (175)§§§	97 (140)§§§	320 (253)

Unless otherwise indicated, values are expressed as mean ± SEM.

Independent-t test, Mann-Whitney test or Chi-square test was used to calculate p value.

*: p<0.05, ***: p<0.001 vs. CTD-ILD.

#: p<0.05, ##: p<0.01, ###: p<0.001 vs. SSc-ILD.

§: p<0.05, §§: p<0.01, §§§: p<0.001 vs. HC.

‡: Smoking status and BALF were not available in all individuals.

Among the 260 ILD patients, 98 (38%) were ANA positive at baseline. The frequency of CTD development was significantly higher in those ILD patients who were ANA (+) (25/98, 26%) than ANA (-) (12/162, 7%), p<0.001. 24 of the 25 (96%) ANA (+) patients who developed CTD had NSIP, only one had UIP. Among the ANA (-) CTD-ILDs, 10/12 (83%) were RA compared to 12/25 (48%) of ANA (+) CTD-ILDs.

The prevalence of ANA positivity in the IIP group (33%) was significantly lower than in CTD-ILD (68%) and significantly higher than in healthy controls (0%, $p<0.05$ respectively) (**Table 6**). Within the NSIP group, ANA were positive in 35% iNSIP patients which was a significantly lower percentage than in CTD-NSIP (71%) and SSc-ILD (83%) patients ($p<0.001$, respectively). The ANA positive percentage in IPF-UIP (32%) was also significantly lower than in SSc-ILD ($p<0.001$), but similar to CTD-UIP (33%, $p>0.05$). There were no significant differences between iNSIP and IPF-UIP, or CTD-UIP ($P>0.05$, respectively) (**Figure 4**). Although the difference between CTD-UIP and SSc-ILD was significant, when considering the small number of CTD-UIP (n=3), the result is questionable.

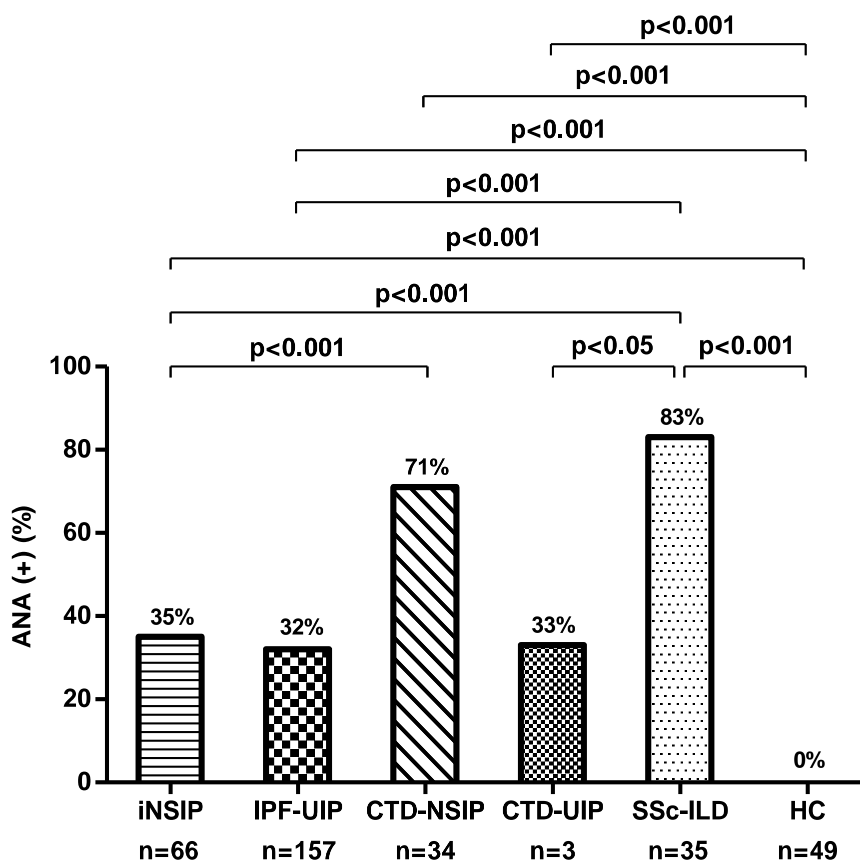


Figure 4. Prevalence of ANA positivity according to CTD development. Data expressed as percentage (%). Chi-square test was used to calculate p value.

The prevalence of anti-DFS70 antibody positivity in the IIP group (17%) was significantly lower than in healthy controls (35%, $p<0.05$) and not significantly different from CTD-ILD (16%, $p>0.05$) (**Table 6**). Anti-DFS70 antibodies were positive in 11% iNSIP patients, significantly less than in HC ($p<0.05$) and not significantly different from CTD-NSIP (15%) and SSc-ILD (6%), $p>0.05$ respectively. The anti-DFS70 antibody positive percentage of IPF-UIP (19%) was also significantly lower than HC ($p<0.05$) and similar to CTD-UIP (33%, $p>0.05$) but higher than in SSc-ILD ($p=0.06$). The prevalence of anti-DFS70 antibody positivity in CTD-NSIP was significantly lower than in healthy controls ($p<0.05$) and was similar to SSc-ILD ($p>0.05$). No significant difference was seen between CTD-UIP and SSc-ILD/HC, as well as between iNSIP and IPF-UIP, CTD-NSIP and CTD-UIP ($P>0.05$ for all comparisons) (**Figure 5**).

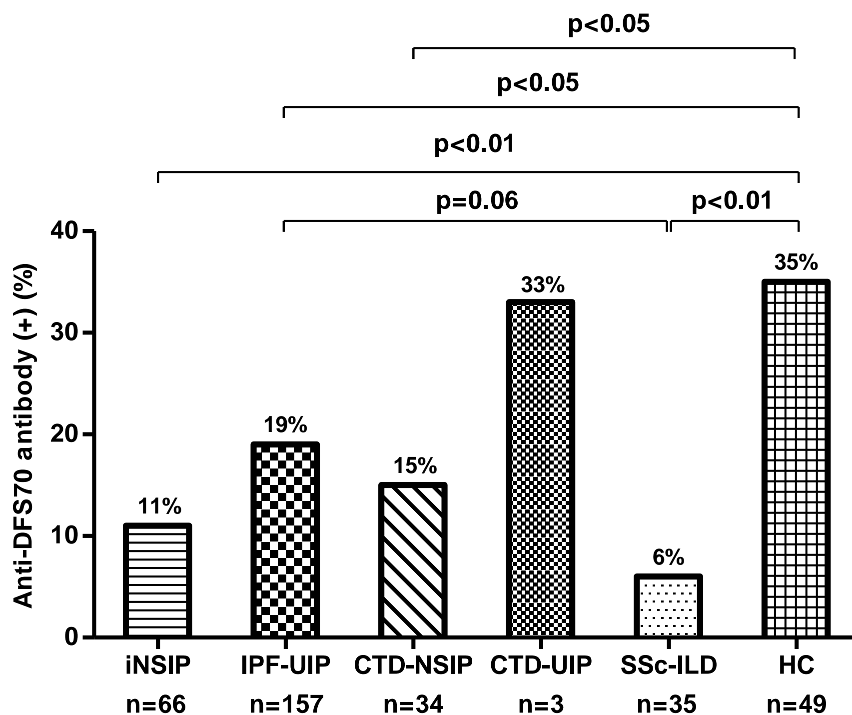


Figure 5. Prevalence of anti-DFS70 antibody positivity according to CTD development. Data expressed as percentage (%). Chi-square test was used to calculate p value.

The serum anti-DFS70 antibody levels in the IIP group (119 U/ml, IQR: 232 U/ml) were significantly lower than in healthy controls (320 U/ml, IQR: 253 U/ml, p<0.001), but not significantly different from CTD-ILD (104 U/ml, IQR: 175 U/ml, p>0.05) (**Table 6**). Anti-DFS70 antibody levels in iNSIP patients (119 U/ml, IQR: 125 U/ml), in IPF-UIP (123 U/ml, IQR: 263 U/ml) and in CTD-NSIP (101 U/ml, IQR: 140 U/ml) were all significantly lower than in healthy controls (p<0.05 for all comparisons), but were similar to SSc-ILD (97 U/ml, IQR: 140 U/ml, p>0.05 for all comparisons). No significant difference was seen between CTD-UIP and SSc-ILD/HC, as well as between iNSIP and IPF-UIP, CTD-NSIP or CTD-UIP (P>0.05 for all comparisons) (**Figure 6**).

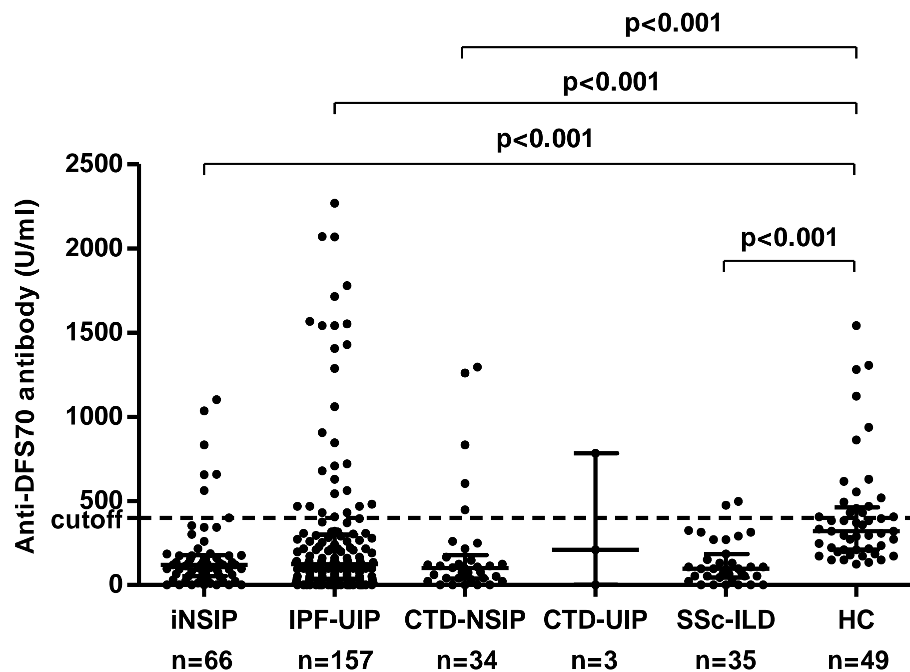


Figure 6. Comparison of serum anti-DFS70 antibody levels according to CTD development. Dots represent single patients, the central line represents the median and the whiskers represent the interquartile range. Kruskal-Wallis and stepwise step-down multiple comparisons test were used to calculate p value.

4.3. Relationship between ANA and anti-DFS70 antibodies

Among the ANA positive ILD patients, 20% were anti-DFS70 antibody positive. This was not significantly different from the ANA negative individuals who were positive in 14% (**Figure 7a**).

The serum anti-DFS70 antibody level in ANA positive ILD patients was 119 U/ml (IQR: 232 U/ml), not significantly different from ANA negative patients (97 U/ml, IQR: 162 U/ml, p>0.05) (**Figure 7b**).

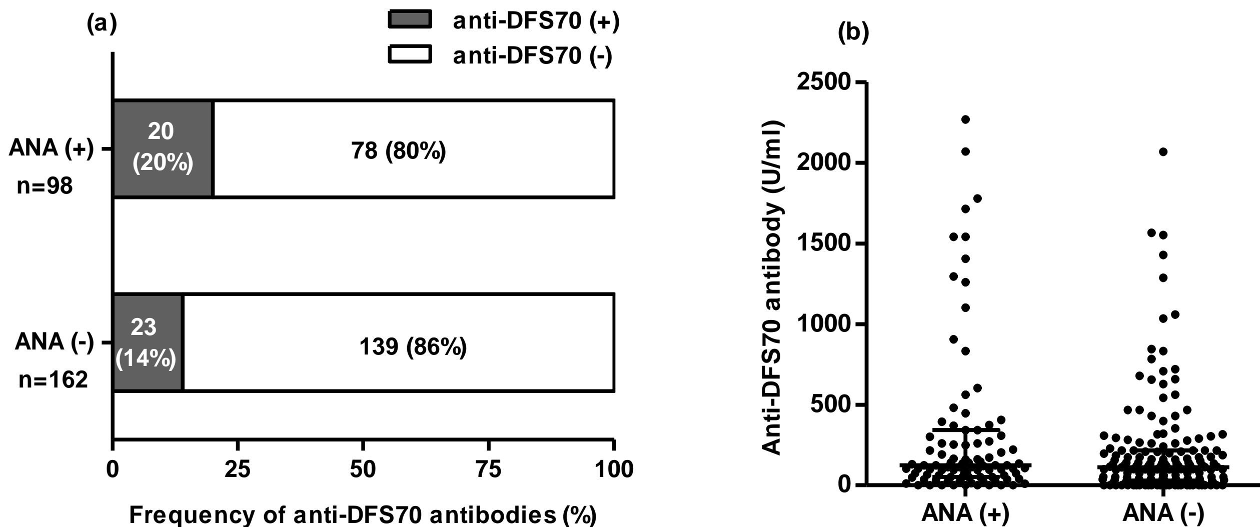


Figure 7. Relationship between ANA and anti-DFS70 antibodies in all ILD patients. Frequency of anti-DFS70 antibody positivity according to ANA status (a). Data expressed as percentage (%). Chi-square test was used to calculate p value. Serum anti-DFS70 antibody levels according to ANA status (b). Dots represent single patients, the central line represents the median and the whiskers represent the interquartile range. Mann-Whitney test was used to calculate p value.

At baseline, among the ANA positive ILD patients, the prevalence of anti-DFS70 antibody positivity in IPF (25%) was significantly higher than in SSc-ILD (3%, p<0.05) and was also higher, but not significantly, than in NSIP (15%, p>0.05). Among the ANA negative subjects,

HC had the highest percentage of anti-DFS70 antibody positivity (35%), significantly higher than NSIP or IPF patients (9% and 17%, $p<0.05$ respectively) (**Figure. 8**).

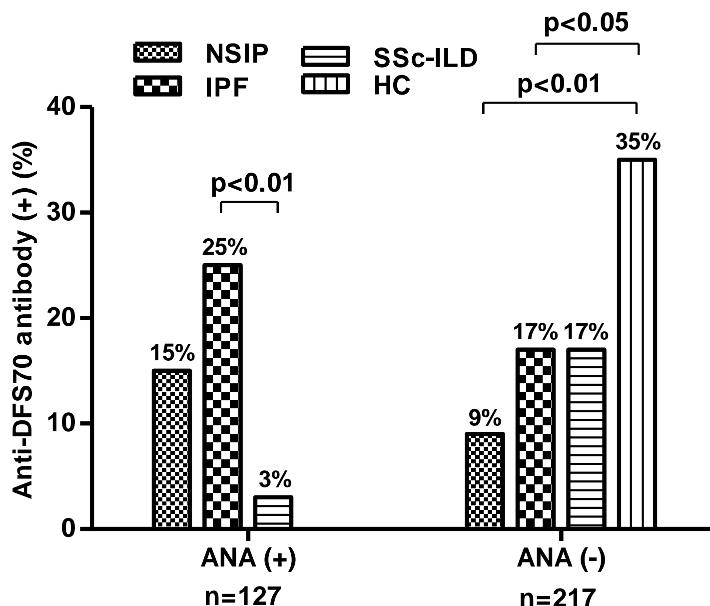


Figure 8. Frequency of anti-DFS70 antibody positivity according to ANA status at baseline. Data expressed as percentage (%). Chi-square test was used to calculate p value.

After follow-up, among the ANA positive subjects, the prevalence of anti-DFS70 antibody positivity in IIP (22%) tended to be higher than in CTD-ILD (16%, $p>0.05$). Among the ANA negative subjects, the prevalence of anti-DFS70 antibody positivity was similar in IIP (14%) and in CTD-ILD (17%), $p>0.05$ (**Figure. 9**).

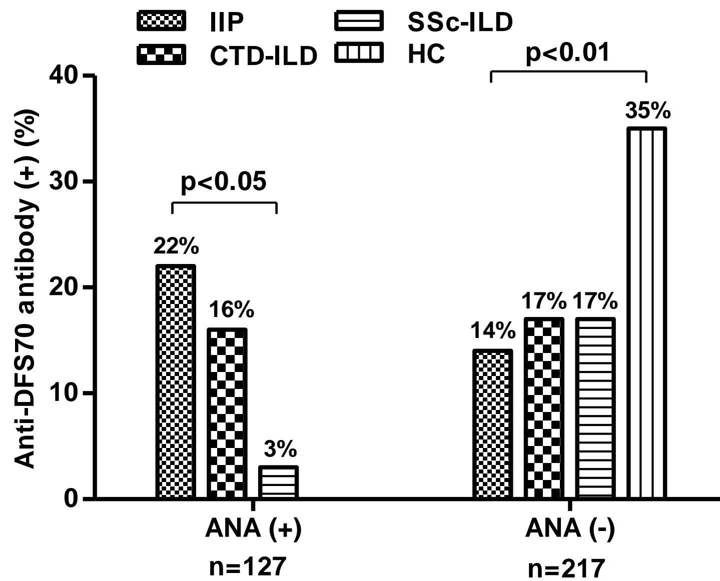


Figure 9. Frequency of anti-DFS70 antibody positivity according to ANA status at baseline after CTD development. Data expressed as percentage (%). Chi-square test or Fisher's exact test was used to calculate p value.

4.4. Frequency of CTD development according to ANA and anti-DFS70 antibody status

The distribution of the various categories of ANA/DFS70 status in all ILD patients at baseline is shown in **Figure 10**.

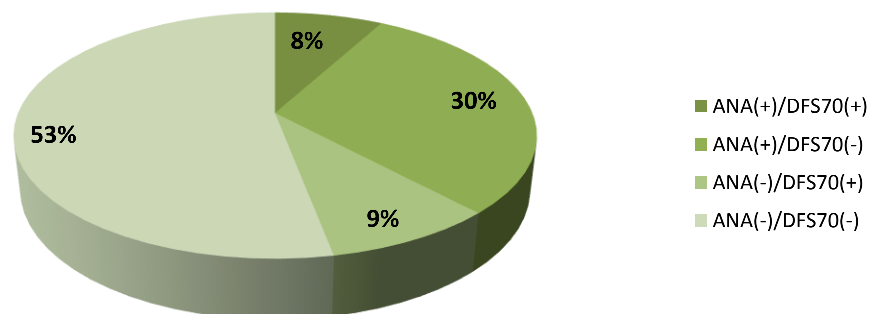


Figure 10. Frequency of ANA and anti-DFS70 antibody status at baseline in all ILD patients.

The patients with ANA (+)/DFS70 (-) status showed the highest frequency of CTD development (27%) which was significantly higher than in patients with ANA (-)/DFS70 (-) status (7%), p<0.05, and higher than in patients with ANA (-)/DFS70 (+) status (9%), p=0.07 (**Table 7**).

Table 7. Association of ANA and anti-DFS70 antibody status at baseline with development of CTD.

diagnosis	**ANA (+)/DFS70 (+)** n=20	**ANA (+)/DFS70 (-)** n=78	**ANA (-)/DFS70 (+)** n=23	**ANA (-)/DFS70 (-)** n=139
IIP, n (%)	16 (80)	57 (73)***	21 (91)	129 (93)
CTD-ILD, n (%)	4 (20)	21 (27)***	2 (9)	10 (7)

Chi-square test was used to calculate p value.
***: p<0.001 vs. ANA (-)/DFS70 (-).

4.5. Relationship of anti-DFS70 antibodies with clinical/laboratory parameters

4.5.1 Clinical/laboratory parameters according to anti-DFS70 antibody status

Among the ILD patients, the serum levels of LDH were significantly lower in patients with anti-DFS70 antibody (+) than in patients with anti-DFS70 antibody (-) (254±8 vs. 287±5, p<0.01). Also in IIP patients, the LDH levels were significantly lower in anti-DFS70 antibody (+) than in anti-DFS70 antibody (-) patients (253±9 vs. 288±6, p<0.01) (**Table 8**). There was no significant difference in pulmonary function and blood gas analysis parameters between patients with and without anti-DFS70 antibodies in any patient group (p>0.05 for all comparisons) (**Table 8**).

Table 8. Clinical parameters of patient groups according to anti-DFS70 antibody status.

Variables	anti-DFS70 status of all *ILD*		anti-DFS70 status of *IIP*		anti-DFS70 status of *CTD-ILD*	
	(+) n=43	**(-)** n=217	**(+)** n=37	**(-)** n=186	**(+)** n=6	**(-)** n=31
Pulmonary function						
FVC, %pred.	70±3	70±1	70±3	70±1	72±10	69±4
FEV_1, %pred.	72±3	71±1	72±3	72±1	70±10	64±3
DLco, %pred.	45±2	44±2	46±3	42±1	37±3	57±11
TLC, %pred.	68±2	67±1	68±2	66±1	71±5	69±4
Blood gas analysis						
PaO_2, mmHg	75±2	73±1	74±2	73±1	78±5	73±2
$PaCO_2$, mmHg	38±0.5	38±0.3	38±0.5	38±0.3	35±1	38±1
$AaDO_2$, mmHg	27±2	30±1	27±2	30±1	28±4	31±2
SaO_2, %	96±0.3	95±0.2	96±0.3	95±0.2	96±1	95±1
Biomarker						
LDH, U/l	**254±8****	**287±5**	**253±9****	**288±6**	266±19	279±14

Values are expressed as mean ± SEM.
Independent-t test was used to calculate p value.
*: p<0.05, **: p<0.01 vs. anti-DFS70 (-).

In the NSIP group, no significant difference in serum LDH was seen between patients with and without anti-DFS70 antibodies (p>0.05), though there was a numerical trend towards lower levels in anti-DFS70 antibody (+) patients (**Table 9**). Except for significantly lower levels of $PaCO_2$ (mmHg) in anti-DFS70 antibody (+) patients than in anti-DFS70 antibody (-) patients (35±0.8 vs. 38±0.4, p<0.05), there were no differences in clinical characteristics between the NSIP groups according to anti-DFS70 antibody status (**Table 9**).

Table 9. Clinical parameters of NSIP patients according to anti-DFS70 antibody status.

Variables	anti-DFS70 status of *NSIP*		anti-DFS70 status of i*NSIP*		anti-DFS70 status of *CTD-NSIP*	
	(+) n=12	(-) n=88	(+) n=7	(-) n=59	(+) n=5	(-) n=29
Pulmonary function						
FVC, %pred.	65±6	70±2	62±7	71±2	70±13	69±4
FEV_1, %pred.	64±6	69±2	61±7	72±3	68±12	64±3
DLco, %pred.	37±3	49±4	39±5	45±2	34±3	57±12
TLC, %pred.	66±4	68±2	62±4	67±2	72±6	69±4
Blood gas analysis						
PaO_2, mmHg	75±3	74±1	71±3	75±2	81±2	73±2
$PaCO_2$, mmHg	**35±0.8***	**38±0.4**	35±1	37±1	35±1	38±1
$AaDO_2$, mmHg	31±2	29±1	35±3	28±2	26±4	31±2
SaO_2, %	96±0.5	95±0.4	95±0.6	96±0.6	97±0.4	95±0.8
Biomarker						
LDH, U/l	261±12	288±9	263±17	290±12	258±21	285±14

Values are expressed as mean ± SEM.
Independent-t test was used to calculate p value.
*: $p<0.05$ vs. anti-DFS70 (-).

In IPF patients, the levels of LDH were significantly lower in anti-DFS70 antibody (+) than in anti-DFS70 antibody (-) patients (252±10 vs. 286±6, $p<0.05$) (**Table 10**). In regard to pulmonary function parameters, DLco (%pred.) was significantly higher in anti-DFS70 antibody (+) than in anti-DFS70 antibody (-) IPF patients (48±3 vs. 41±1, $p<0.05$), and the $AaDO_2$ gradient was significantly lower in anti-DFS70 antibody (+) than in anti-DFS70 antibody (-) IPF patients (26±2 vs. 31±1, $p<0.05$) (**Table 10**).

Table 10. Clinical parameters of IPF patients according to anti-DFS70 antibody status.

Variables	anti-DFS70 (+) n=31	anti-DFS70 (-) n=129
Pulmonary function		
FVC, %pred.	72±3	70±2
FEV_1, %pred.	75±3	71±2
DLco, %pred.	**48±3***	**41±1**
TLC, %pred.	69±2	66±1
Blood gas analysis		
PaO_2, mmHg	75±2	71±1
$PaCO_2$, mmHg	39±0.5	38±0.3
$AaDO_2$, mmHg	**26±2***	**31±1**
SaO_2, %	96±0.4	95±0.3
Biomarker		
LDH, U/l	**252±10***	**286±6**

Values are expressed as mean ± SEM.
Independent-t test was used to calculate p value.
*: p<0.05 vs. anti-DFS70 (-).

4.5.2 Correlations between serum anti-DFS70 antibody levels and clinical/laboratory parameters

In all ILD patients, anti-DFS70 antibody levels weakly inversely correlated with LDH (r_p=-0.125, p<0.05), the same weak correlation was seen in IIP patients (r_p=-0.153, p<0.05). There was no other association between the clinical parameters and anti-DFS70 antibody levels (**Table 11**).

Table 11. Linear correlation of anti-DFS70 antibodies levels and clinical characteristics according to diagnosis. The table shows the Spearman's correlation coefficients (r_p).

Variables	ILD n=260	IIP n=223	CTD-ILD n=37
Pulmonary function			
FVC, %pred.	-0.011	-0.059	0.201
FEV_1, %pred.	0.017	-0.037	0.245
DLco, %pred.	0.031	0.066	-0.217
TLC, %pred.	0.011	-0.010	0.135
Blood gas analysis			
PaO_2, mmHg	0.042	0.024	0.139
$PaCO_2$, mmHg	0.052	0.117	-0.307
$AaDO_2$, mmHg	-0.079	-0.081	-0.073
SaO_2, %	0.057	0.057	0.001
Biomarker			
LDH	**-0.125***	**-0.153***	0.062

*: p<0.05

In iNSIP patients, anti-DFS70 antibody levels inversely correlated with FVC (%pred.) (r_p=-0.320, p<0.05), FEV_1 (%pred.) (r_p=-0.393, p<0.01), TLC (%pred.) (r_p=-0.360, p<0.01), and positively with $AaDO_2$ (mmHg) (r_p=0.263, p<0.05) (**Figure 11**), indicating more marked impairment of lung function and gas exchange in patients with higher anti-DFS70 antibody levels.

In IPF patients, anti-DFS70 antibody levels weakly inversely correlated with LDH (r_p=-0.196, p<0.05) and $AaDO_2$ (mmHg) (r_p=-0.199, p<0.05) (**Figure 12**).

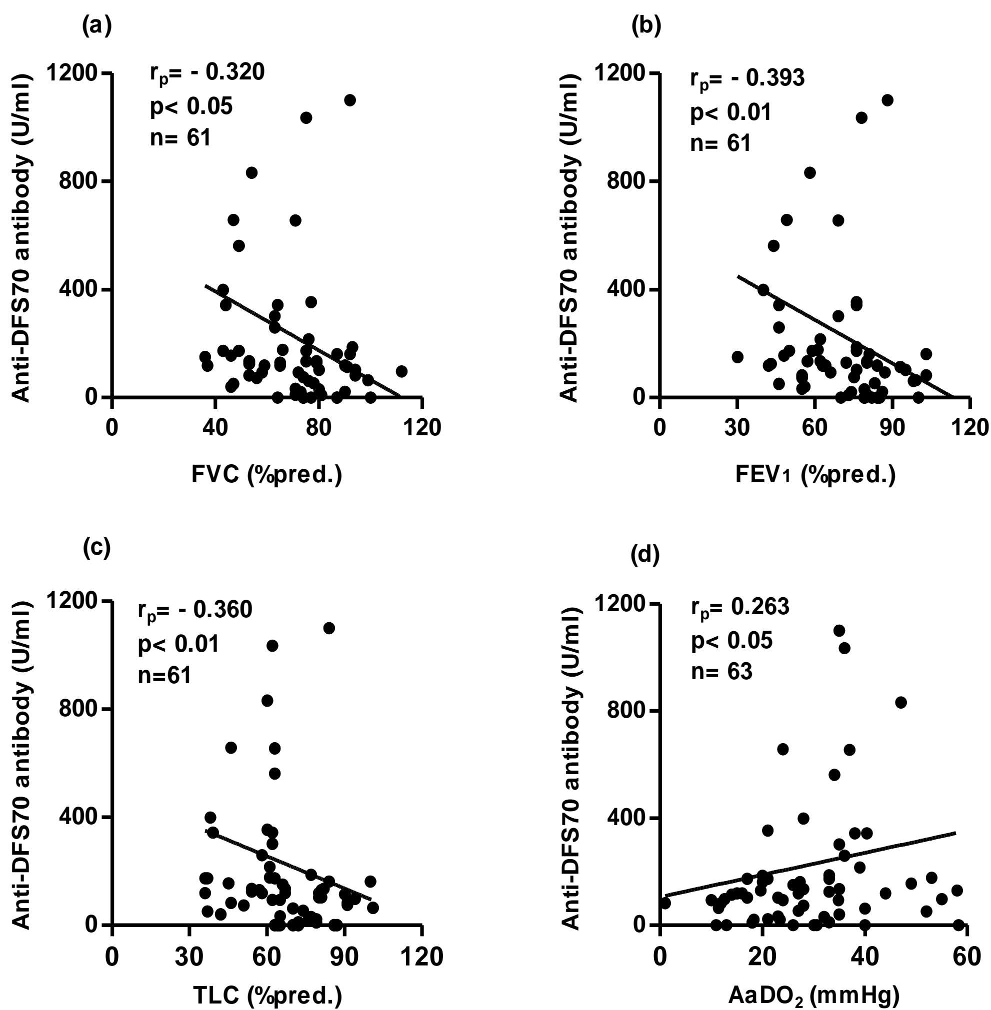


Figure 11. Correlation of serum anti-DFS70 antibody levels with FVC (%pred.) (a), FEV_1 (%pred.) (b), TLC (%pred.) (c), and $AaDO_2$ (mmHg) (d) in iNSIP patients. Dots represent single patients. Spearman's correlation test was used to calculate p value.

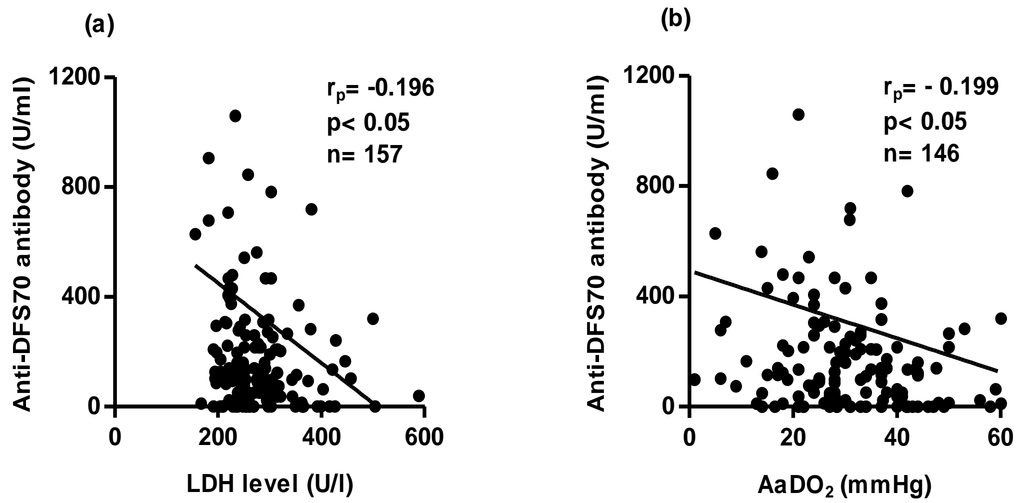


Figure 12. Correlation of serum anti-DFS70 antibody levels with LDH (a) and $AaDO_2$ (mmHg) (b) in IPF patients. Dots represent single patients. Spearman's correlation test was used to calculate p value.

5. DISCUSSION

In the current study, we found that serum anti-DFS70 antibodies are less prevalent in ILD and SSc-ILD patients than in healthy controls. There was no significant difference in anti-DFS70 antibody expression according to the ANA status of ILD patients. During follow-up, ANA (+) ILD patients with negative anti-DFS70 antibody developed CTD more frequently. In patients with iNSIP a weak relationship was seen between serum anti-DFS70 antibody levels and more pronounced impairment of lung function. To the best of our knowledge, this is the first study that investigated the role of anti-DFS70 antibodies in ILD patients.

In the 1960s, Turner-Warwick was the first to describe that ANA are positive in nearly 50% of ILD patients (Turner-Warwick and Doniach, 1965). Later studies confirmed this, e.g. the study of Mittoo et al. who found 54% of patients were positive for at least one autoantibody (Mittoo et al., 2009). In our study, positive ANAs were observed in 47% of NSIP and 32% of IPF patients. In our CTD-ILD patients, ANA positivity was seen in 68% which is relatively low for a CTD cohort. This may be due to the composition of our CTD-ILD population, with 59% being RA patients. RA is not considered to be an ANA-associated disease. Despite the fact that the prevalence of ANAs has been reported to be higher in healthy women than in healthy men (Fernandez et al., 2003; Hayashi et al., 2008) and higher in elderly than in younger healthy individuals (Candorec et al., 1997; Hurme et al., 2007), in the ILD subjects of our study we did not see a difference in the frequency of ANA positivity between gender and age groups.

To avoid the low specificity of ANA-IIF test as screening test for systemic autoimmune rheumatic disease, subsequent immunoassays for specified antibodies are usually done. But there is no consistency in the recommendations for the use of autoantibodies and diagnostic algorithms in different serological laboratories (Mahler and Fritzler, 2012). It has been recommend to include Ro/SSA, La/SSB, Jo-1, Scl-70, Th/To, RNA-Polymerase III, Sm, dsDNA (SLE) and CCP in the serological examination of ILD patients (Bahmer et al.,

2016). Since by now more than 150 different autoantibodies have been discovered, in consideration of feasibility and economic costs, it is impossible to include all CTD associated ANAs into ANA screening ELISA tests. Therefore, it would be useful to have a biomarker which would exclude CTD in patients with ILD and positive ANA serology but without symptoms of CTD. Such a biomarker could result in considerable cost-savings by adding accuracy to the ANA screening test. As such, Anti-DFS70 antibody is one of the best candidates since it seems to be helpful in discriminating between subjects with and those without autoimmune disease among ANA positive patients.

The prevalence of anti-DFS70 antibodies has been reported to vary but consistent findings are that they are present in healthy ANA (+) individuals even when ANA reach moderate or high titers, and that they are rarely found in patients with CTD (Dellavance et al., 2005; Pazini et al., 2010; Miyara et al., 2013). Fitch-Rogalsky et al. investigated 643 patients with a positive ANA and found that 15% were anti-DFS70 antibody positive and 91% of these did not have an autoimmune disease (Fitch-Rogalsky et al., 2014). In a study of routine ANA screening DFS and traditional fine speckled patterns were the major ANA-IIF patterns, and 61% of DFS positivity was seen in nonautoimmune conditions (Dellavance et al., 2005). Our study confirms this for patients with ILDs. Among the ANA positive ILD patients in our study, 20% of anti-DFS70 antibody positive patients developed CTD, in contrast to 27% of anti-DFS70 antibody negative. This suggests that, compared with other ANAs, anti-DFS70 positivity decreases the likelihood of a CTD-ILD.

Anti-DFS70 antibodies are not completely absent in CTD, and the coexistence with other autoantibodies should be taken into account. In a group of SLE patients, approximately 6% had anti-DFS70 antibodies, and all the anti-DFS70 antibody positive patients had additional autoantibodies which were included in the classification criteria for SLE (Muro et al., 2008). Similar data were reported in another study where the prevalence of anti-DFS70 antibodies in SLE was 2.6%, and only one of the 7 anti-DFS70 (+) patients did not show other autoantibody reactivity (Mahler et al., 2012).

The sensitivity of the ANA-IIF is influenced by the chosen cut off value for ANA titers. In the current study we set the positive value at the dilution of 1:160 as recommended by the ANA Subcommittee of the International Union of Immunological Societies Standardization Committee (Tan et al., 1997). We noted that 23 of the 43 anti-DFS70 antibody positive patients identified by ELISA were ANA negative on HEp-2 slides. Some investigators attribute such inconsistent results between various assays to different HEp-2 substrate preparations, the type of DFS70 epitope exposed in various assays, and the interobserver variation in the interpretation of the ANA-IIF pattern (Mahler et al., 2008; Bizzaro et al., 2011). Fortunately, since anti-DFS70 antibodies are not associated with CTD, the low sensitivity in the ANA-IIP test will not affect the final diagnosis, because in most laboratories the patients with ANA negativity are ruled out to have a CTD. There is growing consensus that it is inadequate to use the ANA-IIF test as the sole method for the detection of autoantibodies (Mahler and Fritzler, 2012; Miyara et al., 2013), and the confirmatory ELISA test should include anti-DFS70 autoantibody. In our study, patients with both ANA and anti-DFS70 antibody negativity had a low likelihood of only 7% for developing CTD, and those with ANA positivity and anti-DFS70 antibody negativity had an increased likelihood of 27% for developing CTD during follow-up.

In some studies, investigators observed that anti-DFS70 autoantibodies were more prevalent or had higher titers in females than in males (Ochs et al., 2000; Watanabe et al., 2004). Our study, in agreement with others, did not find any such difference in gender groups (Mahler et al., 2012). Differences of cohort collection and disease composition may explain the discrepancy. An age dependency was shown in a study of 918 healthy individuals, more than 90% of the DFS patterns were observed in people younger than 50 years (Mariz et al., 2011). However this was not confirmed in another study (Mahler et al., 2012). Also in our study, the status of anti-DFS70 antibodies was similar across 3 age strata and showed no correlation with age. Smoking history is a risk factor for the development of some ILDs, including RA associated ILD (Saag et al., 1996; Gochuico et al., 2008). In the current study we did not find an association between smoking history/habit and anti-DFS70 antibodies in any patient group.

The clinical and biological significance of anti-DFS70 autoantibody is still puzzling. Although the autoantibodies can be induced by aberrant tissue manifestations of DFS70 (i.e. overexpression, extracellular release, cell death cleavage), there is no apparent relationship between the levels of these antigens and antibodies in some human diseases. Daniels et al. observed a strong expression of DFS70 in benign prostatic hyperplasia tissue but an absent expression of anti-DFS70 autoantibodies in the patients' sera. They speculated that the discrepancy could be due to the relatively low rates of cell death and decreased DFS70 cleavage fragments in the benign prostatic hyperplasia tissue which would be insufficient to induce the antibodies (Daniels et al., 2005). In another study, 36 children with chronic nonspecific complaints (CFS) had much higher titers and a higher prevalence of anti-DFS70 antibodies than autoimmune fatigue syndrome, Kawasaki disease, SS, atopic dermatitis and normal human sera. It seems that CFS patients have some anti-stress dysfunction, such as sensitivity against UV exposure, infection, physical exercise, and even emotional crisis (Kuwabara et al., 2009). It is possible that in diseases characterized by anti-DFS70 antibody reduction, the anti-DFS70 antibodies which accumulate under 'normal' condition, are cleared or neutralized by the overexpressed DFS70 antigen.

In the current study we found that in all groups of ILD patients the anti-DFS70 antibody negative patients showed higher levels of LDH levels which is a nonspecific marker of tissue injury widely used to monitor the course of acute lung injury, indicating that the higher degree of tissue injury is possibly associated with a reduction of anti-DFS70 antibodies. Interestingly, in iNSIP patients, anti-DFS70 antibody levels inversely correlated with lung function parameters, such as FVC (%pred.), FEV_1 (%pred.) and TLC (%pred.), indicating that in this disease higher anti-DFS70 antibody levels are related with more marked functional impairment. Whether DFS70 has a pathogenic role or is merely a bystander in the pathogenesis of ILDs is unclear at present.

In fibrotic ILDs TGF-β1 is a cytokine with many functions, including regulation of inflammation, cell growth and tissue fibrosis (Tahira et al., 2002). In lung fibrosis, TGF-β1

plays a significant role in fibrogenesis through the induction of myofibroblasts and the regulation of the synthesis and degradation of extracellular matrix (Sharma et al., 2003; Bonniaud et al., 2005). Based on HRCT scores, higher levels of TGF-β1 have been found to be associated with progressive lung disease in various kinds of ILD (Szlubowski et al., 2010). In the context of DFS70, TGF-β1 has been shown to down regulate DFS70 expression by diminishing its affinity for DNA binding in human lens epithelial cells (Sharma et al., 2003). In a Prdx6-/- knockout mouse cell line, increased TGF-β1 levels were observed combined with reduced DFS70, and in the lenses of diabetic and galactosemic cataractous rats, an inverse relationship between the expression of TGF-β1 and DFS70 was seen (Fatma et al., 2005). Based on these observations, the overexpression of TGF-β1 in ILDs might inhibit the induction of DFS70 (Miki et al., 2000; Honda et al., 2010).

MiRNAs are small RNA molecules and play important roles in many physiological processes such as cellular proliferation, tissue development, differentiation and repair (Lau et al., 2001). The miRNAs can also influence the expression of DFS70 at the transcriptional level, such as shown for miR-155 which reduced DFS70 levels (Swaminathan et al., 2012). The expression of miR-155 has been shown to highly correlate with profibrotic gene expression (Christmann et al., 2016). In the intratracheal bleomycin murine lung model, miR-155 was upregulated, and miR-155-deficient mice developed milder lung fibrosis (Christmann et al., 2016). in IPF, upregulation of miR-155 has been observed (Pandit et al., 2010). Taken together, DFS70 may be downregulated by increased miR-155 in patients with IPF.

Our study has several limitations, First, the exact ANA-IIF pattern was not available in all individuals, and we cannot analyze which autoantibodies coexist with anti-DFS70 antibody in CTD-ILD patients. Second, the number of CTD-UIP patients was very small (n=3), because few IPF patients developed CTD. Third, measurement of DFS70 levels in BALF/tissue was not performed and should be done in further studies. Comparing lung tissue expression of DFS70 versus circulating levels of anti-DFS70 antibodies would be

important to explore the potential function of this antigen-antibody system in the pathogenesis of ILDs.

In conclusion, the prevalence and serum levels of anti-DFS70 antibodies were highest in healthy subjects and decreased in patients with ILDs and SSc-ILD. Most of the ILD patients who developed CTD during follow-up were shown to be characterized by ANA positivity combined with anti-DFS70 antibody negativity. The potential function of DFS70 and the anti-DFS70 antibody system in the pathogenesis of ILDs needs to be further investigated.

6. SUMMARY

Anti-DFS70 antibodies, corresponding to the dense fine speckled ANA indirect immunofluorescence pattern in HEp-2 substrates, have been observed in chronic inflammatory conditions, cancer and in healthy individuals but only in a small percentage of patients with systemic autoimmune rheumatic diseases. The aim of this study was to investigate the role of anti-DFS70 antibodies as biomarker in ILD. We evaluated its value to distinguish connective tissue disease associated interstitial lung disease (CTD-ILD) from idiopathic interstitial pneumonia (IIP). In addition, we explored potential correlations between anti-DFS70 antibodies and clinical parameters.

Methodologically, serum samples were collected from 49 healthy controls, 35 scleroderma-ILD (SSc-ILD) patients as negative controls for anti-DFS70 antibody, and 260 patients with ILDs. The ILD patients included 100 nonspecific interstitial pneumonia (NSIP) and 160 idiopathic pulmonary fibrosis (IPF) patients. The serum anti-DFS70 antibodies were assessed by enzyme-linked immunosorbent assay.

The frequency and levels of serum anti-DFS70 antibodies were lower in ILD and SSc-ILD patients compared to healthy controls. Thirty-seven patients developed CTD during 24 months of follow-up (3 initial IPF and 34 initial idiopathic NSIP patients), most of them combined with ANA positivity and anti-DFS70 antibody negativity. Anti-DFS70 antibodies were not significantly different between CTD-ILD and idiopathic ILD. Anti-DFS70 antibody concentrations were inversely correlated with pulmonary functions in iNSIP.

In conclusion, the frequency and levels of serum anti-DFS70 antibodies are markedly decreased in patients with ILDs. Anti-DFS70 antibodies may play a role to predict CTD development in ILD patients.

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8. ABBREVIATIONS

$AaDO_2$	alveolar-arterial oxygen gradient
ACA	anticentromere antibody
ACR	American College of Rheumatology
ANA	antinuclear antibody
ANA-IIF	Indirect immunofluorescence assay for antinuclear antibody detection
ASS	antisynthetase syndrome
CFS	chronic fatigue syndrome
COP	cryptogenic organizing pneumonia
CTD	connective tissue disease
CTD-ILD	connective tissue disease associated interstitial lung disease
DFS70	dense fine speckled 70 antigen
DLco	diffusing capacity of the lung for carbon monoxide
DPLD	diffuse parenchymal lung disease
dsDNA	double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
HC	healthy control
HEp-2	human epithelioma type 2-CCl 23 ATCC clone
HP	hypersensitivity pneumonitis
HRCT	high resolution computed tomography;
IIF	indirect immunofluorescence
IIP	idiopathic interstitial pneumonia
IL	interleukin
ILD	interstitial lung disease
iNSIP	idiopathic nonspecific interstitial pneumonia
IPF	idiopathic pulmonary fibrosis

IQR	interquartile
LDH	Lactate dehydrogenase
LEDGF	lens epithelium-derived growth factor
MCTD	mixed connective tissue disease
miRNA	micro-RNA
NSIP	nonspecific interstitial pneumonia
PaO_2	arterial oxygen tension
PBS	phosphate buffered saline
$PaCO_2$	arterial carbon dioxide tension
PM/DM	polymyositis/dermatomyositis
RA	rheumatoid arthritis
SaO_2	arterial oxygen saturation
SEM	standard error of mean
SLE	systemic lupus erythematosus
SS	sjögren’s syndrome
SSc	systemic sclerosis
TGF-β1	transforming growth factor beta 1
TLC	total lung capacity
UCTD	undifferentiated connective tissue disease
UIP	usual interstitial pneumonia

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10. CURRICULUM VITAE

"The biography is not included in the online version for reasons of data protection"

11. PUBLICATIONS

1. Bonella, F., Lyu, Y., Theegarten, D., Boerner, E., Wessendorf, T. E., Shinichiro, O., Guzman, J., Costabel, U., Kreuter, M. (2016): Detection of anti DFS70 antibodies in patients with interstitial lung disease (ILD) with and without connective tissue disease (CTD) (abstract). Eur. Respir. J. 48, PA4877.

2. Bonella, F., Lyu, Y., Theegarten, D., Boerner, E., Wessendorf, T. E., Shinichiro, O., Guzman, J., Costabel, U., Kreuter, M. (2017): Potential utility of anti-DFS70 antibodies to exclude systemic autoimmune rheumatic disease (SARD) in patients with interstitial lung disease (ILD) (abstract). Pneumologie. 71 (in press).

3. Bonella, F., Lyu, Y., Theegarten, D., Boerner, E., Wessendorf, T. E., Shinichiro, O., Guzman, J., Costabel, U., Kreuter, M. (2017): Potential utility of anti DFS70 antibodies to exclude systemic autoimmune rheumatic disease (SARD) in patients with interstitial lung disease (ILD) (abstract). ATS (submitted).

4. Bonella, F., Lyu, Y., Theegarten, D., Boerner, E., Wessendorf, T. E., Guzman, J., Costabel, U., Kreuter, M. (2017): Serum anti DFS70 antibody titer and lung functional impairment in patients with interstitial lung disease (ILD) (abstract). ERS (submitted).

5. Lyu, Y., Yun, L., Wei, X. Q., Zhang, X. Z., Li, S. S., He, J. K.. (2017): AIDS knowledge and sexual behaviour status of female sex workers in Tangshan. Occupation and Health (Chinese) (submitted).

6. Lyu, Y., Long, X. P., Boerner, E., Wessendorf, T. E., Theegarten, D., Guzman, J., Kreuter, M., Costabel, U., Bonella, F. (2017): Detection of anti-dense fine speckled 70 antibodies in patients with interstitial lung disease (in preparation).