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Clinical correlates of a subset of anti-fibroblast antibodies in systemic sclerosis

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ABSTRACT

Anti-fibroblast antibodies (AFA) have been reported in systemic sclerosis (SSc) and are known to promote fibroblast activation. Aim of this study was to characterize the fine specificity of AFA and to analyze any correlations with clinical parameters associated to fibrosis. To this end, AFA were affinity-purified from a patient with diffuse cutaneous SSc (dcSSc) and interstitial lung disease (ILD). Panning of a phage display peptide library with purified AFA identified the motif <KxywxQ>. The peptide p121, bearing the AFA-specific motif, was used in ELISA to screen sera from 186 SSc patients and 81 healthy donors. Anti-p121 Ab serum levels were statistically higher in SSc than in healthy groups, and directly associated with dcSSc, reduced FVC (FVC < 70), and ILD. Given these clinical correlates, this study lays the groundwork for the identification of the antigen recognized by anti-p121 Ab, which might represent a novel therapeutic target for ILD.

1. Introduction

Systemic sclerosis (SSc) is a chronic, multisystem connective tissue disorder characterized by vascular abnormalities, activation of the immune system, and collagen deposition [1,2]. The disease causes fibrosis of the skin and internal organs, but its clinical manifestations and severity are highly heterogeneous. Recent data have highlighted the poor efficacy of currently available drugs for skin sclerosis, interstitial lung disease and pulmonary hypertension, the latter two conditions being the major causes of death [3,4]. In the last 20 years, a slight decrease of mortality has been recorded, and 10-year survival rate is now 75% as compared to around 60% before 1990 [4]. This is mostly due to early recognition of the disease, careful follow-up and the use of angiotensin-converting enzyme inhibitors, which have markedly reduced the risk of onset of a renal crisis [5]. Even so, internal organ involvement remains untreatable and there are no drugs available to control or block fibrosis. Very little is known about the pathogenesis of SSc, progressing from early inflammatory events to fibrosis of the skin and internal organs. Fibrosis is considered a late and irreversible event in the disease, likely resulting from a previous uncontrolled immune response to an unidentified auto-antigen/s.

The link between autoimmunity and fibrosis in SSc has been supported by the findings of an abnormal oligoclonal fibroblast-specific T and B cell response [6–8] and by the presence of autoantibodies in the

Abbreviations: Ab, antibodies; AFA, Anti-fibroblast antibodies; ACA, anti-centromere antibodies; ATA, anti-topoisomerase 1 antibodies; BSA, bovine serum albumin; dcSSc, diffuse cutaneous systemic sclerosis; FVC, forced vital capacity; DLCO, diffusing lung capacity for carbon monoxide; HBD, healthy blood donors; HRCT, high-resolution computed tomography; HRP, horseradish-peroxidase; ILD, interstitial lung disease; OPD, *o*-phenylenediamine; PDPL, phage display peptide library; pt46, patient 46; pVβ19, TCR Vβ19-derived peptide; ROC, receiver operating characteristics..

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sera of these patients against fibroblasts (AFA) [9,10]. AFA have been detected in 26–58% of SSc patients [9] and great attention has been paid to them, based on a number of in vitro effects induced upon fibroblast binding, including their internalization [11], induction of pro-adhesive and pro-inflammatory phenotypes [9], increased expression of type I collagen and several other extracellular matrix components [12], and of reactive oxygen species, with the activation of collagen genes [10].

AFA have been demonstrated to recognize multiple antigens in SSc, including *platelet-derived growth factor receptor* [13], as well as proteins playing key roles in cell biology and the maintenance of homeostasis such as glucose-6-phosphate dehydrogenase, heat-shock protein 27, phosphatidylinositol 3-kinase and α-enolase [14]. Furthermore, α-enolase-specific AFA, along with anti-topoisomerase 1 antibodies (ATA) have been associated to interstitial lung disease (ILD) in SSc [15]. However, these investigators evaluated AFA using whole sera from SSc patients and not affinity purified antibody populations, with the impossibility to clarify whether AFA is one antibody population with a single specificity, cross-reacting with different antigens, or consists of different antibody populations each with a distinct antigenic specificity.

Thus, the definition of the exact AFA epitope-specificity and its correlation with clinical parameter related to fibrosis, is an essential step to better understand any pathogenetic mechanism/s triggered by AFA, their possible prognostic significance and to identify novel fibroblast-associated antigens which might be potential sites for immune intervention.

In this study, the fine specificity of a subset of AFA, and their association with fibrosis-associated clinical features has been evaluated.

2. Materials and methods

2.1. Patients and clinical data

One hundred eighty-six patients with SSc, fulfilling both the 1980 ACR and the 2013 ACR/EULAR classification criteria [16], were recruited from the Rheumatology Units of the Universities of Bari, Naples, Rome, L'Aquila, Foggia and Catanzaro from 2016 to 2020. At presentation, blood was drawn and gender, age, age at diagnosis (measured from the onset of the first Raynaud's phenomenon [17] and first non Raynaud manifestation) were recorded, together with laboratory tests, including erythrocyte sedimentation rate, C-reactive protein, total serum protein (by capillary electrophoresis), autoantibody profile. Limited or diffuse type SSc was defined according to the classification system described by LeRoy [13,18]. Assessing pulmonary function, ILD was diagnosed with high-resolution computed tomography (HRCT), using a 0 to 3 scale [0 = normal, 1 = mild fibrosis (initial interstitial thickening), 2 = moderate fibrosis (lower and middle lobe fibrosis), and 3 = severe fibrosis (up to apical lobes fibrosis, ground-glass, reticular or honeycomb patterns)]. Forced vital capacity (FVC) and diffusing lung capacity for carbon monoxide (DLCO) were determined and expressed as percentages of predicted values. The study was approved by the Ethics Committees of the Universities of Bari, L'Aquila, Naples, Foggia, Rome, and Catanzaro, and written informed consent was obtained from each participants.

2.2. Serum samples and reagents

Serum samples from the 186 SSc patients and from 81 age-matched healthy blood donors (HBD) were stored at −80 °C until use.

Electrophoresis reagents were purchased from Bio-Rad Laboratories (Segrate, Milan, Italy). Horseradish-peroxidase (HRP) xeno-Abs to human IgG (Fc portion) were purchased from Jackson Immunoresearch Laboratories (Avondale, PA, USA). HRP-conjugated mouse mAb to bacteriophage M13 major coat protein product of gene VIII was purchased from Thermofisher Scientific (Monza, Italy). The anti-HLA class I mAb HC-10-specific peptide Qp1a (sequence NH2-QEGPEYWDRNTQ-COOH) was previously characterized [19]. The T cell receptor (TCR) beta variable 19-derived peptide pVβ19 (sequence NH2-AEGYSVSREKKESFPL-COOH), was available in our lab. Polyclonal human IgG preparations for intravenous use (IVIG, Intratec®) were purchased from Biotest (Dreieich, Germany).

The 12-mer Phage Display Peptide Library (PDPL) was purchased from New England Bio Lab (Ipswich, MA, USA). Peptides were synthesized by Primm (Milan, Italy) and their purity (>90%) was assessed by analytical reverse phase chromatography and mass spectrometry. Peptides were coupled to bovine serum albumin (BSA), as previously described [20]. Unless otherwise specified, other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Cells

Dermal fibroblasts from skin biopsies of healthy individuals and SSc patients were grown at 37 °C in a 5% CO2 atmosphere in DMEM supplemented with 20% FCS, 4 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and used from passage 2 to 8.

2.4. Affinity purification of AFA

Serum samples from Patient 46 (pt46) (with SSc and lung fibrosis), were longitudinally collected over a period of 2 years, and pooled as the source of AFA. SSc and HBD fibroblasts were pooled and used in all the following procedures.

Proteins from fibroblasts lysates were obtained by solubilizing cells in NP-40 lysis buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40 containing protease inhibitors; $1x10^7$cell/ml). Nuclei and insoluble material were removed by centrifugation [21,22]. Extracted proteins were conjugated to a mixture of AffiGel 10 and Affi-Gel 15 resin (Bio Rad laboratories, Hercules, CA, USA) (10 mg/ml of packed resin), following the manufacturer's instructions. Fibroblast proteins-conjugated Affi-Gel 10/15, obtained from pt46 pooled-serum samples, was packed in column for purification by affinity chromatography of AFA, as previously described [23]. BSA-conjugated Affi-Gel 10/15 resin was used in a pre-absorption step to remove non-specifically reactive IgG from total SSc IgG.

Eluted IgG were extensively dialyzed against PBS and their concentration was assessed by UV absorption. AFA purity was assessed by SDS-PAGE, as previously described [24].

2.5. Cellular assays

Cell-based ELISA binding assay to detect the reactivity of pt46 serum or AFA to fibroblasts was performed as previously described [9], with minor modifications. Briefly, serum (diluted 1:25) or different concentrations of AFA were added for 2 h to confluent fibroblast monolayers on 96-well plates. After washing, the plates were incubated for 1 h with HRP-conjugated goat anti-human IgG. The reaction was detected by the addition of *o*-phenylenediamine (OPD)-substrate solution and the absorbance was read at 490 nm. Samples were tested in duplicate, and experiments repeated 3 times.

Inhibition assay to determine the ability of p121 to inhibit AFA binding to fibroblasts was performed as previously described [21], with minor modifications. Briefly, 50 μL of a PBS solution containing 2-fold serial dilutions of p121 were mixed with an equal volume of purified AFA (5 μg/ml). After a 2-h incubation at 4 °C, the mixture was added to confluent target fibroblasts for 2 h at 4 °C, and bound Ab was detected by sequential addition of HRP-avidin and OPD-substrate solution. Absorbance was read at 490 nm. Binding of purified AFA in the presence of the unrelated peptide Qp1a was included as a specificity control. Results are expressed as percentage inhibition of AFA binding compared with binding in the absence of the inhibitor.

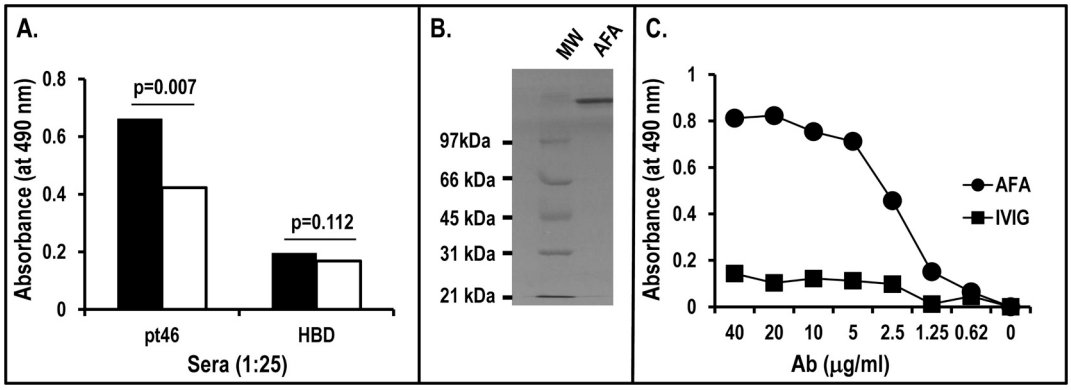

Fig. 1. Purity and specificity of anti-fibroblast antibody (AFA) purified from systemic sclerosis (SSc) pt46 serum.
(A.)The reactivity of pt46 serum with fibroblasts from SSc (black) or healthy blood donors (HBD) (white) was assessed by indirect cell-based ELISA assay, incubating confluent fibroblasts for 2 h with serum diluted 25-fold. Pooled sera from 16 HBD were used as controls. Data were expressed as mean of duplicate wells. *t*-test was considered statistically significant at $p < 0.05$. (B.) AFA were purified from pt46 serum by affinity-chromatography on fibroblast-derived lysate proteins-conjugated Affi-Gel 10/15 columns. AFA (5 µg/lane) were run onto SDS-PAGE under non-reducing conditions and stained with Coomassie brilliant blue. (C.) The reactivity of purified AFA with fibroblasts was assessed by indirect binding assay, incubating confluent fibroblasts for 2 h with different concentrations of purified AFA (closed circle). Human immunoglobulins for intravenous use (IVIG) (closed square) were used as negative control. Bound IgG was revealed with HRP-conjugated anti-human IgG and *o*-phenylenediamine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Panning of a phage display peptide library and nucleotide sequence analyses

To define the amino acid motif recognized by purified AFA, panning of the peptide library expressing 12-mer linear peptides with fibroblast specific-IgG was performed according to the procedure previously described [25]. AFA-selected phage colonies were amplified in *E.coli*, and phage supernatants tested in ELISA for specificity to AFA, as previously described [26]. Supernatants from positive clones were used as source for phage DNA purification and nucleotide sequence analysis.

Phage DNA from AFA-specific phage clones were sequenced at the Eurofins Genomics sequencing facility (Ebersberg, Germany). Nucleotide sequences corresponding to peptide inserts were analyzed with the ExPASy DNA translate tool (http://web.expasy.org/translate/) and the deduced amino acid sequences obtained. Antigenic motifs were determined by alignment of peptide sequences using the "Multalin Protein" tool at Pole Bio-Informatique Lyonnaise (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalin.html). Human proteins containing the antigenic motifs were searched for in the Swiss-Prot Protein Sequence Database using the PROSITE tool (http://www.expasy.ch/tools/scanprosite/).

2.7. p121-based ELISA

The indirect binding assay to test the reactivity of AFA with p121 was performed as previously described [26], with minor modifications. Briefly, plates were incubated with 50 µl of PBS solution containing p121 or the negative control pVβ19, both conjugated to BSA o/n at 4 °C. After 2 washings and blockage of free protein–binding sites, a 2-fold serial dilution of purified AFA or IVIG (negative control) was added to the plate and bound IgG was detected by sequential addition of HRP-conjugated xeno-antibodies to human IgG and OPD-substrate solution. The absorbance was read at 490 nm.

To quantify anti-p121 Ab serum levels, indirect ELISA was performed as previously described [27], with minor modifications. Briefly, plates were coated and blocked as above. Wells were washed and incubated for 4 h with serum samples (diluted 1:100 in PBS-BSA). A serum sample from pt46 was used as positive control. Bound IgG was revealed with HRP-conjugated anti-human IgG and OPD and absorbance read at 490 nm. Specific binding was determined by subtracting the background binding in wells coated with BSA-conjugated negative control pVβ19 from the binding in experimental wells. The levels of anti-p121 Ab in sera were expressed as the percentage of binding compared to the positive control. Samples were tested in duplicate, and experiments performed 3 times.

2.8. Statistical analyses

Statistical tests were performed using SPSS (v.21) and MedCalc (v. 7.6.0.0) software for Windows. *t*-test was used to compare means between two groups. Mann-Whitney and one-way ANOVA were used for continuous variables in comparisons between two and three or more groups, respectively. In cases of one-way ANOVA statistically significant differences, set at $p < 0.05$, Tukey HSD post-test was performed for comparisons. Spearman's rho test was used to analyze the correlation between two continuous variables, and multivariate analysis to define independent associations between two or more variables. Receiver operating characteristics (ROC) analysis was used to define cut-off values that best discriminate two groups. A p value <0.05 was considered significant.

3. Results

3.1. Purification and characterization of AFA

AFA were detected in pt46 serum, as assessed by cell-based ELISA (Fig. 1A). pt46 serum IgG reactivity was higher with fibroblasts from SSc patients than those from HBD ($p = 0.007$). By contrast, pooled sera from 16 HBD equally reacted with both SSc and HBD fibroblasts ($p = 0.112$), with a lower binding than that of pt46 (Fig. 1A). AFA were purified from pooled SSc pt46 serum samples collected in a temporal range of 2 years (Fig. 1B). Purification was performed by affinity-chromatography on a fibroblast lysate-coupled Affigel column. The purified Ab were tested by ELISA for binding to fibroblasts. AFA reacted in a dose-dependent manner with pooled fibroblasts from SSc patients and HBD; by contrast, IVIG showed no significant binding (Fig. 1C).

3.2. Analysis of the fine specificity of AFA by means of PDPL

Purified AFA were used to pan a 12-mer PDPL to isolate specific phage clones. IVIG were used in each round to remove phage clones binding to isotypic and allotypic determinants. Immunoscreening by ELISA of 130 supernatants of randomly selected phage clones identified 39 (30%) of phage clones that specifically reacted with AFA. Nucleotide

Table 1
Definition of pt46 AFA motif.

Phage clone insert #	Clones, n (%)	Phage clone denomination	Deduced amino acid sequence [a)]	Binding specificity (A_{490nm}) [b)]	
				AFA	IVIG
1	9 (64)	pc46.100	XXX**K**X**YW**X**Q**XXX	1.77±0.12	0.09±0.01
2	1 (7)	pc46.121	X**K**X**YW**X**Q**XXXX	1.93±0.16	0.07±0.02
3	1 (7)	pc46.75	XXXX**K**X**YW**X**Q**XX	1.62±0.10	0.10±0.04
4	1 (7)	pc46.45	XX**K**X**HY**X**Q**XXX-	1.61±0.12	0.06±0.03
5	2 (14)	pc46.82	XXX**K**X**YF**X**Q**XXX--	1.65±0.14	0.07±0.01
pt46 motif			K-yw-Q----		

IVIG, intravenous human immunoglobulins for human use.
[a] Multiple alignments were performed with MULTALIN at Pole Bio-Informatique Lyonnaise (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_multalin.htmlhttp://npsa-pbil.ibcp.fr/cgi-bin/align_multalin.pl). Amino acids matching those of the motif are underlined and bold.
[b] Phage supernatants were diluted 16-folds. Data are expressed as mean (±SEM) of duplicate wells.

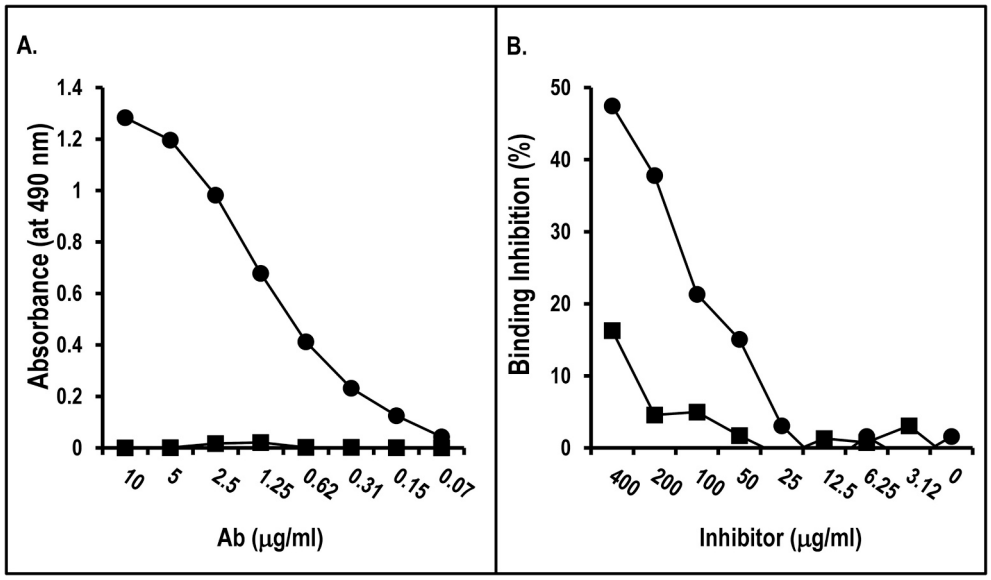

Fig. 2. Specificity of the reactivity of anti-fibroblast antibody (AFA) with the synthetic peptide p121 assessed by binding and inhibition ELISAs.
(A.) In the binding ELISA, microtiter plates were coated with 50 µL of PBS solution containing BSA-p121 (closed circle). After washings and blockage of free protein-binding sites, a 2-fold dilution of AFA was added to the plate and their binding to peptide was detected with HRP-conjugated xeno-antibodies to human IgG (Fc portion). Bindings of AFA to BSA-Qp1a (closed square) were included as negative controls. (B.) In the inhibition assay, different concentrations of p121 (closed circle) were mixed with the highest dilution of purified AFA, yielding 90% of the maximal binding (5 µg/mL). After a 2-h incubation at 4 °C, the mixture was added to the fibroblasts. AFA binding to fibroblasts was then detected with HRP-conjugated xeno-antibodies to human IgG (Fc portion). Binding of AFA to fibroblasts in the presence of Qp1a peptide (closed square) was included as negative control. Results are expressed as percentage of binding compared to binding without the inhibitor.

sequences of AFA-specific phage clones revealed 14 distinct sequences. The alignment of 5 sequences permitted the identification of the antigenic motif KxYWxQ (Table 1).

3.3. Peptide synthesis and its specific reactivity with AFA

The phage clone insert #2 bearing the motif KxYWxQ, displaying the highest binding with AFA (Table 1), was used as template for synthesizing the linear peptide p121, which was subsequently tested in ELISA for its reactivity with AFA. Since in preliminary experiments AFA did not bind to the free peptide (data not shown), p121 was conjugated to the carrier protein BSA and tested for its reactivity with different concentration of AFA. Fig. 2A showed that AFA reacted with the BSA-conjugated peptide p121 (BSA-p121) in a dose-dependent manner. The binding was specific since AFA did not react with the unrelated peptide Qp1a coupled to the same carrier protein BSA. To define whether p121 is complementary to the antigen combining site of AFA, its ability to inhibit AFA binding to fibroblasts was assessed. As shown in Fig. 2B, p121 dose-dependently inhibited the binding of AFA to fibroblasts. The inhibition was specific starting from 200 µg/ml of inhibitor since from this concentration herein the binding was not affected in the

Table 2
Clinical characteristics of 186 patients with systemic sclerosis.

Variable	Value
Female, n (%)	168 (90.3)
Age (mean ± SD)	57.7 ± 12.9
Age at RP onset (mean ± SD)	43.6 ± 15.8
Disease duration (time since RP), mean ± SD	21.1 ± 11.6
Diffuse disease, n (%)	32 (17.2)
ACA, n (%)	117 (62.9)
ATA, n (%)	48 (25.8)
dnAb, n (%)	10 (5.3)
Disease severity scale sub-items	
ILD[a], n (%)	59(31.7)[a]
FVC mean ± SD;<70% [n (%)]	101.8 ± 21.1; [13 (7)]
DLCO mean ± SD;<70% [n (%)]	73.83 ± 19.26; [75 (40.3)]

ACA, anti-centromere antibodies; **ATA**, anti-topoisomerase I antibodies; **DLCO**, diffusing lung capacity for carbon monoxide; **dnAb,** double negative for ACA and ATA; **ECG**, electrocardiogram; **FVC**, forced vital capacity; **ILD**, interstitial lung disease; **RP**, Raynaud's phenomenon; **SD**, standard deviation.
FVC was measured as percentage of predicted value.
[a] Defined at high resolution computed tomography (HRCT) > 0.

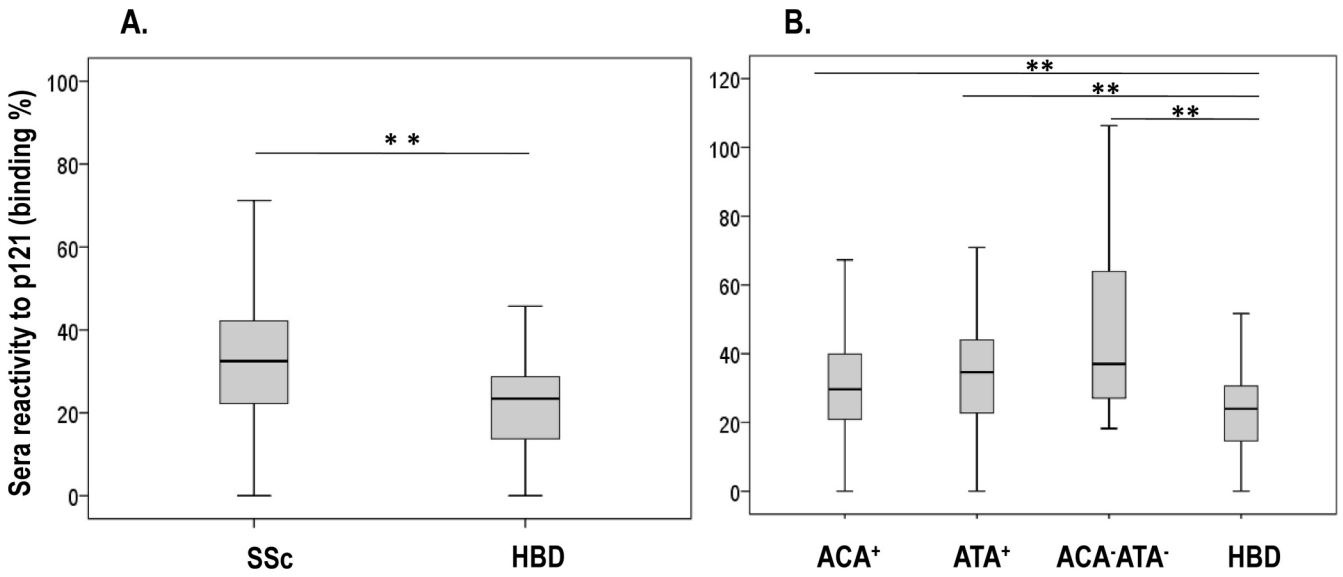

Fig. 3. Levels of anti-p121 Ab in systemic sclerosis (SSc) patients and healthy blood donors (HBD).
(A) Sera from 186 SSc patients and 81 healthy blood donors were screened for reactivity with p121 by indirect ELISA. Binding of anti-p121 Ab is expressed as a percentage of the binding obtained with positive control serum from SSc pt46. ** Mann-Whitney $p < 0.05$. (B) Anti-p121 Ab levels in SSc patients, grouped according to their Ab profile. Horizontal bars mark the medians and boxes indicate interquartile ranges. ** One-way ANOVA $p < 0.05$.

Table 3
Anti-p121 antibody levels are significantly associated to parameters reflecting interstitial lung disease.

		Kendall's tau test		Fisher's exact t-test	
Variable (cut off)	Patients, n	R	p[a]	OR (95% CI)	p[a]
dcSSc	186	0.154	0.011	3.11 (1.37–7.07)	0.009
HRCT>1	154	0.156	0.019	3.25 (1.36–7.74)	0.012
HRCT = 3	154	0.145	0.029	3.25 (1.36–7.74)	0.006
FVC < 70	186	0.146	0.015	4.77 (1.50–15.11)	0.009
ATA	176	0.104	0.093	2.40 (1.13–5.12)	0.025

ATA, anti-topoisomerase I antibodies; **dcSSc**, diffuse cutaneous systemic sclerosis; **FVC**, forced vital capacity; **HRCT**, high-resolution computed tomography.
[a] Kendall's tau and Fisher's exact t-test were considered statistically significant at $p < 0.05$.

presence of the unrelated peptide Qp1a.

3.4. Relationships between the reactivity profile of SSc sera with p121 and the corresponding clinical subsets

This retrospective study enrolled 186 patients affected by SSc, 117 (62.9%) positive for anti-centromere Ab (ACA), 48 (25.8%) positive for ATA, and 10 (5.3%) double negative for ACA and ATA (dnAb). The clinical characteristics of the study population are described in Table 2. The female to male ratio was 9.3:1, mean age ± SD was 57.7 ± 12.9 years, and mean age at onset of the first symptoms (Raynaud's phenomenon) was 43.6 ± 15.8 years. The mean disease duration was 21.1 years. dcSSc affected 32 patients (17.2%). Of the 186 patients, 59 (31.7%) had ILD, 13 patients (7%) had FVC values at < 70% of the predicted value, and 76 patients (40.9%) had DLCO <70%. Among patients with ILD, 31 patients (52.5%) had mild form, 5 patients (8.4%) had moderate ILD, and 26 patients (44%) had severe fibrosis. Reduced FVC (FVC < 70%) was recorded in 15.2% of patients with ILD.

Anti-p121 Ab levels were determined in sera of the 186 patients with SSc and of 81 age-matched HBD. As shown in Fig. 3A, the levels of anti-p121 in SSc patients were markedly higher than in HBD (Mann-Whitney $p = 0.001$). Specifically, significant differences in anti-p121 titers were detectable in ACA+ (one-way ANOVA $p = 0.005$), ATA+ (one-way ANOVA p = 0.001), and ACA-ATA- (one-way ANOVA p = 0.001) groups as compared with HBD (Fig. 3B).

To define the correlations between serum anti-p121 Ab levels and clinical variables reflecting fibrosis, a Kendall's tau test was performed.

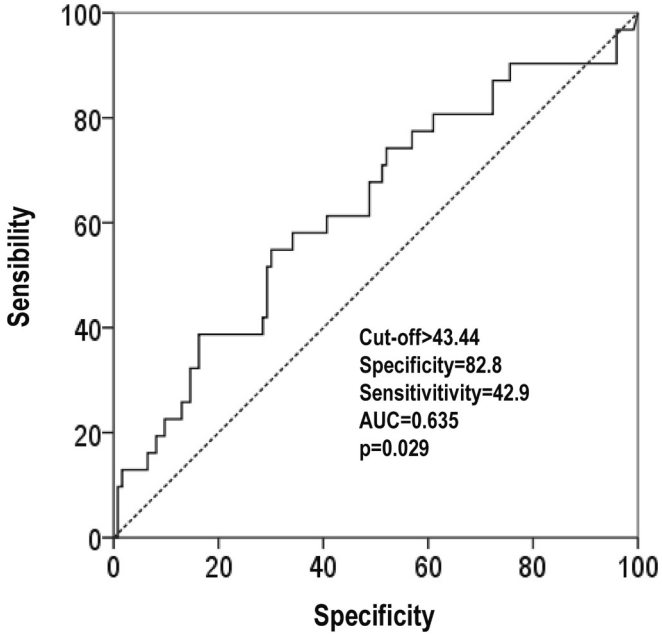

Fig. 4. Receiver operating characteristic (ROC) analysis to define levels of anti-p121 Ab (cut-offs) that distinguish SSc patients with HRCT>1 vs those with HRCT≤1.

Table 4
The association between anti-p121 antibody (Ab) positivity and fibrosis is independent of age and disease duration.

Outcome variable	Predictor	p	OR (95% CI)
HRCT>1	Anti-p121 Ab	0.012	3.24 (1.29–8.15)
HRCT = 3	Anti-p121 Ab	0.009	3.65 (1.39–9.59)
FVC < 70	Anti-p121 Ab	0.017	4.38 (1.29–14.83)

CI, confidence interval; **FVC**, forced vital capacity; **HRCT**, high-resolution computed tomography; **OR**, odds ratio.
Significance set at $p < 0.05$.

Table 5
Anti-p121 antibody (Ab) and anti-topoisomerase I Ab (ATA) are independently associated to low forced vital capacity (FVC).

Outcome variable	Predictor	p	OR (95% CI)
FVC < 70	Anti-p121 Ab	0.041	3.89(1.43–21.18)
	ATA	0.012	5.26 (1.44–19.20)
	Age	0.609	0.98 (0.94–1.03)
	Disease duration	0.551	0.98 (0.92–1.04)

ATA, anti-topoisomerase I antibodies; **FVC**, forced vital capacity. **OR**, odds ratio.
Significance set at $p < 0.05$.

As reported in Table 3, anti-p121 Ab levels were directly associated with dcSSc ($p = 0.011$), the presence of moderate to severe ILD (HRCT>1, $p = 0.019$; HRCT = 3, $p = 0.029$), and reduced FVC ($p = 0.015$). No significant association between anti-p121 Ab levels and ATA positivity was found at Kendall's tau test (Table 3).

To define the optimal anti-p121 Ab cut-off to discriminate SSc patients with moderate to severe ILD (HRCT>1) from those with mild or no ILD (HRCT≤1) a ROC curve was generated. As shown in Fig. 4, the best anti-p121 Ab cut-off discriminating patients with moderate to severe ILD from those with mild or no ILD was >43.44 of the binding percentage (AUC = 0.635, p = 0.029, 42.9% sensitivity, 82.8% specificity).

This cut-off was used to subdivide SSc patients into anti-p121 positive (anti-p121 levels>43.44; pts.#41, 22%) and negative patients (anti-p121 levels≤43.44; pts. #145, 78%). Fisher's exact-test was performed to further analyze the associations found significant at Kendall's tau test and to evaluate a possible association between anti-p121 Ab and ATA positivity. As shown in Table 3, anti-p121 Ab were directly associated with dcSSc (OR = 3.11, $p = 0.009$), HRCT>1 (OR = 3.25, $p = 0.012$), HRCT = 3 (OR = 3.73, $p = 0.006$), reduced FVC (OR = 4.77, $p < 0.009$), and ATA positivity (OR = 2.40, $p = 0.025$). Multivariate logistic regression analysis was performed to evaluate whether the association between anti-p121 Ab positivity and ILD was influenced by age and/or disease duration. As shown in Table 4, the associations between anti-p121 Ab with HRCT>1 (OR = 3.24, p = 0.012), HRCT = 3 (OR = 3.65, p = 0.009) and FVC < 70 (OR = 5.78, $p = 0.007$) were independent of age and disease duration.

To evaluate whether the association of anti-p121 Ab positivity with reduced FVC was independent of ATA positivity, multivariable regression analysis was performed. As shown in Table 5, anti-p121 Ab (OR = 3.89, $p = 0.041$) and ATA (OR = 5.26, p = 0.012) were significantly associated to FVC < 70. These results demonstrated that the association between anti-p121 Ab and FVC is independent of ATA positivity.

4. Discussion

In the present study, we identified a subgroup of AFA, defined by their reactivity with the peptide p121 (anti-p121 Ab), whose serum levels were directly associated with moderate to severe ILD in SSc. ILD is the major complication driving mortality in SSc, and SSc-ILD patients display a mortality risk almost three-fold higher than SSc patients without pulmonary fibrosis [28]. Among SSc-ILD patients, approximately 25–30% will develop progressive severe ILD [29]. Although several markers have been studied as possible predictors of ILD progression in SSc [30], validated biomarkers to predict SSc-ILD occurrence, prognosis, and response to therapy are still lacking.

Fibroblasts are recognized to play a pivotal role in the pathogenesis and progression of ILD [31], although AFA have not been comprehensively characterized in terms of association to a given clinical subset, and their contribution to the pathogenesis of fibrosis in SSc is still debated [32,33].

Terrier et al. previously demonstrated that AFA targeting α-enolase are associated with ILD and ATA [15], the latter conferring an increased risk of ILD in SSc patients, independently of skin involvement, in retrospective longitudinal studies [34]. α-enolase has been also demonstrated to be one of the main targets of natural endothelial Ab [35]. However, these Ab are not SSc-specific, and have been demonstrated in a wide spectrum of autoimmune and inflammatory disease [36–40].

The higher binding of pt46 sera to SSc fibroblasts as compared to normal fibroblasts could be the result of the recognition either of a naturally occurring surface antigen, whose expression is increased in SSc or else of a disease-related novel antigen. Whether this antigen is expressed by cells other than fibroblasts, including endothelial cells, remains to be assessed.

Unlikely α-enolase Ab, anti-p121 Ab positivity was strongly associated with diffuse type SSc, that is a well established risk factor for developing SSc-ILD [41]. Furthermore, an association between anti-p121 Ab and ATA positivity was found. In a large single-centre cohort retrospective longitudinal study, ATA positivity was demonstrated to be associated with low FVC (FVC≤70) and to be a predictive marker of FVC decline [42]. We could not evaluate the potential predictivity of anti-p121 Ab in predicting FVC decline because of the lack of follow-up data. However, we demonstrated that anti-p121 Ab positivity and ATA positivity are independently associated to reduced FVC (FVC < 70).

In systematic review [43], and retrospective longitudinal studies [44], low FVC is a predictor of poor outcome in SSc-ILD. The evidence that anti-p121 Ab positivity is associated with impaired FVC suggests that these Ab may be useful to define a subset of SSc patients at higher risk of ILD progression. Furthermore, the evidence that anti-p121 Ab serum levels were higher in ACA-ATA- SSc than in ATA+ patients suggests that these Ab may be helpful in seronegative SSc patients in the clinical setting of ILD.

The present study has some limitations. Firstly, the antigen recognized by anti-p121 Ab has yet to be identified; secondly, it remains to be evaluated whether anti-p121 are functional Ab. These findings, along with the assessment of anti-p121 in patients with idiopathic ILD and in other non-SSc connective tissue diseases, will be helpful to highlight similarities or differences in the pathogenic mechanisms of fibrosis in different clinical settings in humans. Experiments along this line are ongoing in our laboratory. Furthermore, prospective studies are needed to evaluate the potential predictive value of anti-p121 Ab for the onset and/or progression of ILD and/or response to therapy in SSc patients.

5. Conclusions

This study identified a subset of AFA, namely anti-p121 Ab, whose serum levels are associated with interstitial lung disease in SSc patients. Further studies will evaluate whether Anti-p121 Ab may represent an helpful biomarker for lung fibrosis in SSc.

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Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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