Novel biomarker for pulmonary vascular disease in systemic sclerosis patients

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Abstract

Objective

In systemic sclerosis (SSc) patients, pulmonary arterial hypertension (PAH), which is preceded by pulmonary vascular disease (PVD), is one the of major causes of morbidity and mortality. Given the higher risk of PAH among anti-CENP antibodies (ACA)+ patients, we previously characterised a subset of ACA+ patients, based on a differential reactivity of their ACA with the phage clone (pc4.2)-expressing peptide 4.2 (p4.2). There was a considerably greater prevalence of a low diffusing lung capacity for carbon monoxide (DLCO), an expression of PVD, among patients with high anti-pc4.2 Ab levels. Here we examine whether a similar clinical subgroup can be identified within a larger cohort of ACA+ patients, using the synthetic p4.2.

Methods

Clinical data and serum samples were collected from 134 ACA+ patients. Sera were screened for reactivity with p4.2 by indirect ELISA. Statistical analyses were performed to define any associations between anti-p4.2 Ab levels and PVD.

Results

Kendall's analysis showed that anti-p4.2 Ab were directly associated with both a reduced DLCO and the presence of pulmonary fibrosis (PF). These associations were confirmed by Fisher's exact test. At multivariate analysis, anti-p4.2 Ab was associated to DLCO<70, DLCO≤60, and PF. Moreover, multivariable analysis showed that only the association of anti-p4.2 Ab with DLCO<70, and not with DLCO≤60, was independent of PF.

Conclusion

Anti-p4.2 Ab are able to identify SSc patients at high risk of developing PVD even in the absence of PF. Patients with high anti-p4.2 Ab levels should be strictly monitored for PVD onset and eventually PAH.

Key words

systemic sclerosis, pulmonary vascular disease, pulmonary arterial hypertension, biomarkers, low diffusing lung capacity for carbon monoxide, anti-CENP-A antibody subsets, phage clones, peptides

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Received on December 14, 2021; accepted in revised form on February 28, 2022.

Funding: this work was supported by a grant from the 'Italian Group against Systemic Sclerosis', GILS. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: none declared.

Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disease characterised by autoimmunity, vasculopathy and progressive fibrosis affecting the skin and internal organs (1-3). Three SSc clinical forms are identified, based on the extent of cutaneous fibrosis, namely limited (lcSSc), diffuse cutaneous SSc (dcSSc), and SSc without skin involvement (sine scleroderma) (4).

Antinuclear antibodies (ANA) have been detected in more than 90% of SSc patients, and include the two mutually exclusive, highly SSc-specific antibody (Ab) populations, anti-topoisomerase 1 and anti-centromere (CENP) Ab (ACA) (5, 6). Additional highly specific Ab are those directed to RNA polymerase III (RNAP III). All these Ab populations have been linked to distinct clinical features, severity and prognosis in SSc (5). Specifically, anti-topoisomerase 1 and anti-RNP are strongly associated with dcSSc. The former auto-Ab are also associated with an increased risk of interstitial lung disease (ILD), while the latter are associated with a high risk of scleroderma renal crisis. By contrast, ACA, prevalent in lcSSc, predict a higher risk of developing pulmonary arterial hypertension (PAH), often in the absence of ILD (5, 7, 8).

PAH is one of the leading causes, along with advanced ILD, of SSc-related deaths. The diagnosis can be challenging because PAH is often asymptomatic in the early stages (9). As PAH occurs in about 10% to 20% of ACA positive SSc patients (10, 11), reliable biomarkers are urgently needed to identify a subset of ACA positive patients with an even higher risk of developing PAH.

Among previously defined markers associated to pulmonary vascular disease (PVD), a reduced diffusing lung capacity for carbon monoxide (DLCO) has been demonstrated to be one of the best long-term predictors of the development of PAH in SSc patients with mild or no ILD (12-14). Additional potential markers have also been described, consisting of two auto-Ab populations, namely anti-endothelin receptor type A (anti-ETaR), and anti-angiotensin II receptor type I Ab (anti-AT1R). Both were able to predict PVD in terms of DLCO<55% in SSc patients (15). Nevertheless, no clinical studies have confirmed or disputed their effective role in predicting PAH.

We previously characterised a phage clone (pc4.2) expressing peptide 4.2, isolated by panning a phage display peptide library with purified immunoglobulins (Ig) specific for the dominant amino terminus epitope of CENP-A (amino acid residues 1 to 17), obtained from a SSc patient (patient #4, pt4). The levels of anti-pc4.2 Ab were found to be heterogeneously expressed in sera from anti-CENP-A Ab positive patients (16), and in those patients expressing high Ab levels there was a significant association with a low DLCO (DLCO<70%), and hence with PVD severity (7).

Because of the technical difficulties in handling phage clones and because of their low stability, here we assessed whether, similarly to anti-pc4.2 Ab, the level of Ab against the chemically synthesised peptide p4.2 (derived from the peptide insert sequence expressed by the phage clone pc4.2) could define a subgroup of anti-CENP-Ab positive patients (anti-CENP-A and anti-p4.2 Ab positive group) with a high likelihood of developing pulmonary vascular disease (7).

Indeed, we found that anti-p4.2 Ab levels are strongly associated with low DLCO and ILD, and that the association with DLCO<70 was independent of pulmonary fibrosis, thus defining a subset of anti-CENP-Ab positive patients at higher risk of developing PVD, and hence eventually PAH.

Materials and methods

Patients and clinical data

From 2013 to 2018, 151 consecutive ACA positive SSc patients satisfying both the 1980 ACR and the 2013 ACR/EULAR criteria (17) were recruited at the Rheumatology Units of the Universities of Bari, Foggia, L'Aquila, Naples and Rome. For each patient, information was collected about gender, age at the time of enrolment, age at the onset of the first Raynaud's phenomenon, and SSc subset (limited or diffuse according to LeRoy *et al*. (18)). Disease duration was determined from the onset of Raynaud's phenomenon (7,

19, 20). Data related to disease severity scale sub-items for the lung, namely FVC, DLCO, sPAP, and the presence of ILD were recorded. Specifically, FVC and DLCO were measured and expressed as a percentage of the predicted value; different DLCO cut-offs were considered for the analysis as previously reported (14, 21). sPAP was estimated by means of transthoracic echocardiography (TTE) on the basis of the tricuspid regurgitant jet velocity and the right atrial pressure. 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 or middle lobe fibrosis), and 3=severe fibrosis (ground-glass, reticular or honeycomb patterns)]. The presence of electrocardiogram abnormalities, acral lesions or teleangectasias was also recorded. Patients with left heart-dependent hemodynamic dysfunction assessed on echo were excluded from the study. Patients with DLCO <60% and a disease duration of at least 3 years were also evaluated yearly (in the absence of clinical signs suggestive of PAH) by the DETECT score system for eligibility for right heart cardiac catheterisation (RHC) (22). This study was approved by the Ethics Committee of the University of Bari. All participants gave written informed consent to enrolment in the study, as part of a project on disease markers in immune-mediated diseases.

Reagents and peptides

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The chimeric anti-CD20 mAb Rituximab was from Roche Pharmaceuticals (Basilea, Switzerland). Horseradish-peroxidase (HRP)-conjugated goat anti-human IgG (Fc portion) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Peptides p4.2, CENP-A derived peptides Ap1-17 and Ap17-30 (5), and the Rituximab-specific peptide Rp5-L (23, 24) were synthesised by Primm (Milan) and characterised as described (25). Peptides purity was >90%, as assessed by analytical reverse phase chromatography and mass spectroscopy.

Serum samples

Serum samples obtained from the 151 SSc patients were aliquoted and stored at -80°C until use. Pt4-derived pc4.2-specific serum and sera from the anti-topoisomerase 1 positive/anti-CENP negative SSc patients ptA1 and ptA2 were available in our laboratory.

Peptide conjugation to a carrier protein

Peptides were coupled to bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH) by means of glutaraldehyde, as previously described (26).

Serological assays

The presence of ACA was evaluated with the commercially available kit purchased from Orgentec Diagnostika (GmbH, Germany). ACA positive patients were screened by indirect ELISA for the presence of anti-CENP-A Ab, using CENP-A derived synthetic peptides Ap1-17 and Ap-17-30, as previously described with minor modifications (16, 25). Briefly, polyvinylchloride (pvc) 96-well round-bottom plates were incubated with 50µl PBS containing 5 µg/ml BSA-conjugated Ap1-17 cross-linked to Ap-17-30 for 12 h at 4°C. Wells were washed once with PBS containing 0.05% Tween 20 (PBS-T20) and protein-binding sites blocked with PBS containing 0.5% BSA (PBS-BSA). Following a 1-hr incubation at 25°C, wells were then washed and incubated with 50 µl of serum samples (diluted 100 times in PBS-BSA) for 4 h at 25°C. Following three washings with PBS-T20, bound IgG were detected by sequential addition of an appropriate dilution of affinity-purified HRP-xeno-antibodies to human IgG (Fc portion; 60-minute incubation at 25°C) and a freshly prepared o-phenylenediamine-H_2O_2 (OPD)-substrate solution. The colour reaction was stopped with 100 µL/well of 2N H_2SO_4. Absorbance was read at 492 nm on a Multiscan plate reader (Benchmark; Bio-Rad).

In the indirect binding assay to evaluate serum Ab reactivity with p4.2, plates were coated with BSA-conjugated p4.2, Rp5-L or BSA alone. Serum samples from two anti-topoisomerase 1 positive SSc patients lacking anti-pc4.2 Ab (ptA1 and ptA2) and the mAb Rituximab were used as specificity controls. Background binding was determined from the absorbance generated in BSA-coated wells. Specific binding was determined by subtracting the background absorbance from the absorbance in experimental wells. The levels of anti-p4.2 Ab in sera were expressed as percentages of binding compared to that of pt4 serum (100% of binding). Samples were tested in duplicate, and the experiment was repeated at least 3 times.

Statistical analyses

Statistical analyses were performed using SPSS software (v. 21 for Windows). Kendall's Tau test was used to define correlations between continuous variables and ordinal/dichotomic variables. Receiver operating characteristics (ROC) analysis was used to determine cut-off values that best discriminated the two groups. Fisher's exact test was used to test for associations between dichotomised variables. Variables with statistically significant associations at Fisher's exact test were analysed by multivariate logistic regression, adjusting for age and disease duration as confounding variables. Multivariable logistic regression was performed to define an independent association between variables and anti-p4.2 Ab. For all tests, a p-value <0.05 was considered significant.

Institutional Review Board statement

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee (Ethical Committee of the University of Bari Medical School, n. A31580/DS) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects involved in the study.

Results

Reactivity of synthetic peptide p4.2 with anti-pc4.2 Ab

In a preliminary set of experiments we found that carrier-free synthetic peptide p4.2 bound weakly to 96-well pvc, re-

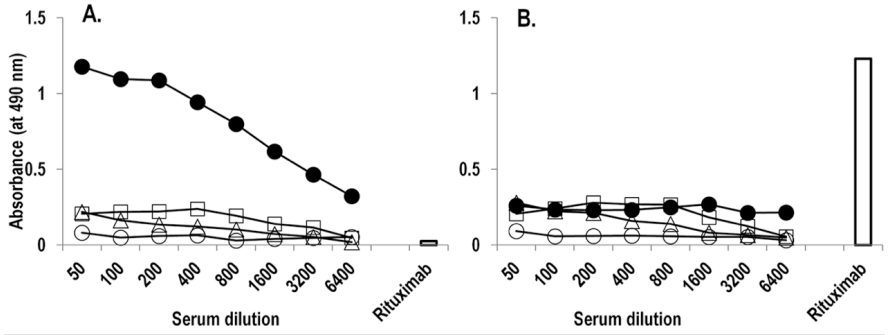

Fig. 1. The antigenicity of p4.2 is maintained outside the phage sequence.
Microtitre plates were coated with BSA-conjugated p4 (panel A) or BSA-conjugated negative control rituximab-specific peptide Rp5-L (panel B). Wells were incubated 2-fold serial dilutions of the anti-pc4.2 Ab positive serum before (closed circle) and after absorption (open circle) on a column packed with p4.2. Sera from the anti-topoisomerase 1 positive SSc patients ptA1 (open triangle) and ptA2 (open square), and the mAb rituximab (open bar) were used as specificity control. Each data point is the mean of duplicate wells (SEM <10%). The coating of plate with the Rp5-Lwas determined by its reactivity with mAb Rituximab (panel B, open bar).

sulting in a low detection of peptide-binding Ab, using anti-pc4.2 Ab positive serum from pt4) (data not shown). Therefore, to enhance the sensitivity of the assay, p4.2 was tested in ELISA conjugated either to BSA or to KLH. The former resulted more sensitive than the latter and was therefore used in all the following assays.

To evaluate whether the antigenic specificity of the synthetic peptide p4.2 was identical to that of the phage fused-peptide (pc4.2), ELISA binding assay was performed. As shown in Figure 1 (panel A), pt4-derived pc4.2-specific serum bound p4.2-coated plates. The binding was dose-dependent and specific since serum from anti-CENP negative patients (anti-topoisomerase 1 positive) did not react with p4.2, nor did pt4 serum react with the negative Rituximab-specific Rp5-L control (Fig. 1, panel B). In addition, anti-pc4 positive sera failed to react with p4.2 following extensive absorption on p4.2-conjugated columns. These data indicate that synthetic peptide on its own had an identical antigenic specificity to that of the same peptide expressed as a phage fusion protein.

Clinical associations of anti-p4.2 Ab in SSc

This retrospective study enrolled 151 ACA positive SSc patients, of which 134 (88.74%) scored positive for Ab against the two cross-linked peptides Ap1-17 and Ap17-30 (anti-CENP-A Ab), encompassing two immunodominant epitopes of CENP-A. The remaining 17 patients (12.68%), all CENP-A negative and CENP-B positive, were excluded from subsequent analyses. The clinical characteristics of the study population are described in Table I. The female to male ratio was 7.4:1, mean age ± SD was 59.8±12.36, whereas mean age at onset of the first symptoms (Raynaud's phenomenon) was 44.5±16.35. The mean disease duration was more than 22 years. Out of 134 patients, 124 (92.5%) had lcSSc, and 10 (7.5%) were with dcSSc. On the disease severity scale sub-items, 2 patients (1.5%) had FVC values at <70% of the predicted value,

Table I. Clinical characteristics of 134 patients with systemic sclerosis positive for anti-CENP-A antibody.

Variable	Whole cohort n=134	lcSSc n=124	dcSSc n=10
Female, n (%)	118 (88.1)	111 (89.5)	7 (70)
Age (mean±SD)	59.8 ± 12.36	60 ± 11.9	55.4 ± 19.1
Age at RP onset (mean±SD)	44.5 ± 16.35	45 ± 15.8	38.1 ± 23.3
Disease duration (time since RP), mean ±SD	22.3 ± 12.2	22.7 ± 12.4	17.5 ± 6.5
Disease severity scale sub-items	124 (92.5)		
FVC mean ±SD ;<70% [n (%)]	107.94 ± 19.5; [2 (1.5)]	107.8 ± 19.60; [2 (1.7)]	109.1 ± 19.1;[0]
DLCO mean ±SD;<70% [n (%)],	75.59 ± 19.75; [51 (38.1)]	75.8 ± 20.2 ; [45 (37.5)]	72.6 ± 12.4; [4 (40)]
sPAP (mmHg) mean ±SD;≥35mmHg [n (%)],	29.57 ± 9.22; [21 (15.7)]	29.9 ± 9.3; [21 (19.1)]	25.2 ± 6.3; [0]
ILD[a], n(%)	11 (9.1)[a]	8 (7.1)	3 (30)
LVEF (%)mean ±SD;<50% [n (%)]	60.92 ± 4.65; [10 (7.5)]	60.9 ± 4.6; [8 (6.5)]	61.1 ± 4.8; [0]
Abnormal ECG, n (%)	25 (18.7)	22 (17.74)	1 (10)
Arrhythmia, n (%)	10 (7.4)	10 (8.1)	0
RBBB, n (%)	10 (7.4)	9 (7.3)	1 (10)
AVB, n (%)	2 (1.49)	2 (1.6)	0
Right axis deviation, n (%)	1 (0.75)	1 (0.8)	0
Acral lesions	27 (20.2)	24 (19.3)	3 (30)
Teleangectasias, n (%)	44 (32.8)	41 (33.1)	3 (30)
Serum urate, mean ± SD	3.75 ± 1.6	3.75 ± 1.6	3.7 ± 1.5
NT-pro-BNP, mean ± SD	133 ± 246.1	135.2 ± 259.1	114 ± 80.9

AVB: atrioventricular block; DLCO: diffusing lung capacity for carbon monoxide; ECG: electrocardiogram; FVC: forced vital capacity; ILD: interstitial lung disease; LVEF: left ventricular ejection fraction; RBBB: right bundle branch block; RP: Raynaud's phenomenon; SD: standard deviation, sPAP: systolic pulmonary arterial pressure.
FVC and DLCO were measured as percentage of predicted value. sPAP was assessed by echocardiography.
[a]High-resolution computed tomography (HRCT)>1.

Table II. Anti-p4.2 antibody levels significantly correlate to anti-CENP-A antibody (Ab) and to parameters expression of pulmonary involvement.

Variable	Patients, n	Kendall's tau test			Fisher's exact t-test	
		Patients # (< or >cut-off)	R	p	OR (95% CI)	p^*
Anti-CENP-A Ab	134	NA	0.159	0.007	NA	NA
DLCO<70	128	51	0.16	0.027	2.44 (1.17-5.09)	0.025
DLCO≤60	128	26	0.17	0.019	2.50(1.04-6.01)	0.045
HRCT>1	121	11	0.22	0.003	17.5 (2.16-141.77)	<0.001
sPAP	118	NA	0.05	0.516	NA	NA
NT-pro-BNP	90	NA	0.05	0.643	NA	NA
Serum urate	88	NA	-0.05	0.569	NA	NA

DLCO: diffusing lung capacity for carbon monoxide; NA: not applicable; HRCT: high-resolution computed tomography.
OR (95% CI)p^*Kendall's tau and Fisher's exact t-test were considered statistically significant at $p<0.05$.

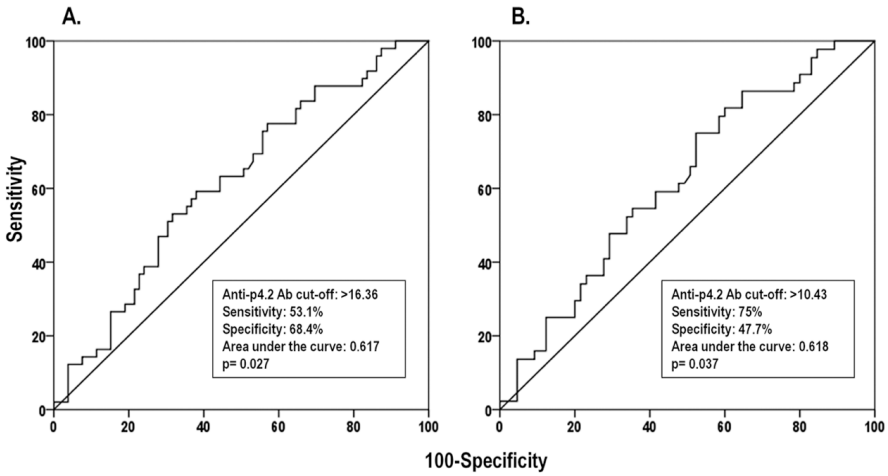

Fig. 2. Receiver operating characteristic (ROC) analysis to define the anti-p4.2 antibodies cut-off discriminating patients presenting DLCO<70.
A: Analysis performed on all 128 cohort patients; **B**: Analysis performed on the cohort after excluding the 11 patients with a high degree of fibrosis.

while 51 patients (38.1%) had DLCO <70%. There were 21 (17.4%) patients with only initial interstitial thickening (HRCT=1), and 11 (9.1%) patients with clear evidence of ILD (HRCT >1). ECG abnormalities were detected in 25 patients (18.7%), teleangectasias in 44 SSc patients (32.8%), and acral lesions in 27 patients (20.2%). The mean (± SD) of serum urate was 3.75±1.6, whereas that of NT-pro-BNP was 133±246.1. In addition, 60 patients (46.15%) had been treated with iloprost for Raynaud's phenomenon, 11 patients (8.46%) with bosentan for digital ulcers, and 4 patients (2.99%) with both.

To define the relationships between serum anti-p4.2 Ab levels, anti-CENP-A Ab, and clinical variables listed in Table I, including sPAP, a Kendall's correlation analysis was performed. As reported in Table II, anti-p4.2 Ab levels were directly associated with anti-CENP-A Ab (0=0.007), and with both a reduced DLCO (DLCO<70, p=0.027; DLCO≤60, p=0.019) and the presence of ILD (HRCT>1, p=0.003). On the other hand, anti-CENP-A Ab levels were not associated with low DLCO, nor with fibrosis, and therefore they were not included in further analyses.

A ROC curve was generated to define the optimal anti-p4.2 Ab cut-off to discriminate SSc patients with DLCO<70 from those with DLCO ≥70. As shown in Figure 2A, the ROC curve analysis outcome indicated an anti-p4.2 Ab-cut-off value of 16.36 as an acceptable level of discrimination (AUC=0.617, p=0.027, 53.1% sensitivity, 68.4% specificity) between patients with DLCO<70% and patients with DLCO≥70%.

A similar ROC curve was obtained when the 11 patients in the cohort with a high degree of fibrosis (HRCT>1) were excluded from the analyses (AUC=0.618 p=0.037, 75% sensitivity, 47.7% specificity) (Fig. 2B). These results suggested that in SSc patients, anti-p4.2 Ab are directly associated with pulmonary vasculopathy in the absence of an established fibrosis. The optimal anti-p4.2 Ab cut-off point able to discriminate SSc patients according to DLCO was used to subdivide SSc patients into an anti-p4.2 Ab positive group (anti-p4.2 Ab levels> 16.6; pts#53) and negative group (anti-p4.2 Ab levels≤16.6; pts #81) (Mann-Whitney p=0.027) (Fig. 3). Fisher's exact test was performed to further analyse the relationship between this cut-off and variables reflecting pulmonary disease. As shown in Table II, anti-p4.2 Ab were directly associated with DLCO<70 (OR=2.44, p=0.025), DLCO≤60 (OR=2.50, p=0.045), HRCT>1 (OR= 17.5, p<0.001).

Moreover, the relationships between anti-p4.2 Ab levels and lung disease, and the interdependency with age and/or disease duration, were analysed by multivariate forward logistic regression. Specifically, anti-p4.2 Ab levels, along with age and disease duration, were included as independent variables, while the outcome variables were DLCO<70, DLCO≤60, and HRCT>1. As shown in Table III, anti-p4.2 Ab was the only independent variable retained in the model for DLCO<70, and DLCO≤60, while both anti-p4.2 Ab and age were retained in the model for HRCT>1. These results indicate that the association between anti-p4.2 Ab and lung involvement is independent of age and disease duration.

Finally, to better evaluate whether the association between anti-p4.2 Ab and reduced DLCO was independent of pulmonary fibrosis, multivariable forward logistic regression was performed. As shown in Table IV, anti-p4.2 Ab were significantly associated with DLCO<70 (OR=2.46, p=0.030), HRCT>1 (OR=16.67, p=0.012), while the association with DLCO≤60 was lost. These results showed that the association of anti-p4.2 Ab with DLCO<70 was independent of pulmonary fibrosis, while

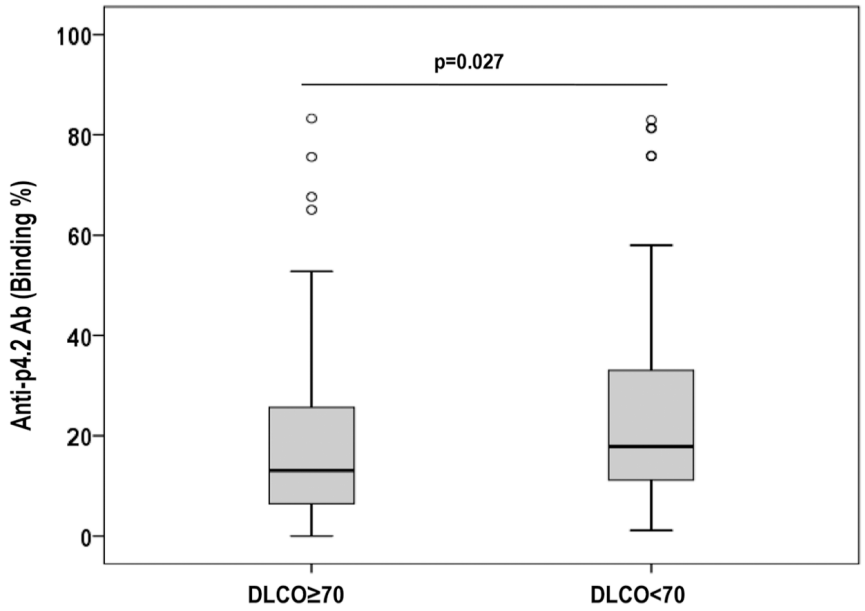

Fig. 3. Distribution of anti-p4.2 antibodies (Ab) in anti-CENP-Ab positive patients according to the indicated DLCO cut-off.
Binding of anti-p4.2 Ab expressed as a percentage of the binding obtained with the positive control sera from patient pt4. The horizontal bar marks the median and the box indicates the interquartile range; outlier values (more than 1.5 times the interquartile range) are marked with a circle.

Table III. Multivariate logistic regression analysis of anti-p4.2 antibody (Ab) levels and age in relation to DLCO and ILD.

Outcome variable	Independent variable retained in the model	B	SE	*p*	OR (95% CI)
DLCO<70	Anti-p4.2 Ab	0.99	0.38	0.011	2.69 (1.26-5.78)
DLCO≤60	Anti-p4.2 Ab	0.97	0.46	0.036	2.63 (1.07-6.51)
HRCT>1	Anti-p4.2 Ab	2.76	1.09	0.011	15.77 (1.86-134.012)
	Age	0.07	0.04	0.047	1.07 (1.00-1.16)

CI: confidence interval; DLCO: diffusing lung capacity for carbon monoxide; HRCT: high-resolution computed tomography; OR: odds ratio; SE: standard error.
Significance set at $p<0.05$.

Table IV. Multivariable logistic regression analysis of anti-p4.2 antibody levels in relation to DLCO and ILD.

Outcome variables	Patients, n	Multivariable	
		OR (95% CI)	*p*
DLCO<70	128	2.46 (1.09-5.52)	0.030
DLCO≤60	128	2.18 (0.84-5.66)	0.107
HRCT>1	121	16.67 (1.87-148.96)	0.012

DLCO: diffusing lung capacity for carbon monoxide; HRCT: high-resolution computed tomography.
Significance set at $p<0.05$.

the association with DLCO≤60 was also dependent on the presence of ILD.

Discussion

In the present investigation it has been shown that high levels of anti-p4.2 Ab are associated to impaired DLCO (DLCO<70%), and hence to pulmonary vasculopathy, independently from ILD. PAH is a devastating condition arising from progressive abnormal vascular remodelling starting long before clinical manifestations appear and the diagnosis is made (27). Thus, it is generally believed that patient outcomes would markedly improve if PAH is treated early and aggressively (27, 28). These considerations have prompted the search for reliable biomarkers to detect PAH at very early stages or even to predict it. For instance, a reduced DLCO has been found to be a very early sign of PVD and subsequently of PAH (14). Previous studies have defined two classes of auto-Ab, namely anti-ETaR and AT1R Ab, that can predict vasculopathy in SSc patients (15).

More recently, some of our group characterised a subset of anti-CENP-A Ab, defined by their reactivity with pc4.2; the levels of these Ab were associated to low DLCO (DLCO<70%) and, in a more limited number of patients, to PAH, suggesting their potential predictive value for PAH. As phage-based ELISA is not meant for routine laboratory use, in the present study the synthetic peptide p4.2, having the same primary amino acid sequence as the one displayed by pc4.2, was developed to be used for the first time in a cost-effective and easy-to-apply peptide-based ELISA.

Even so, it is noteworthy that peptide antigenic specificity can be influenced by the molecular environment in which the peptide is expressed (29). For instance, in the infection disease setting in humans, phage-derived peptides bearing the binding motif for the anti-dengue virus 1 mAb 15-F3 lost their reactivity with the corresponding Ab when tested by ELISA as synthetic peptides (outside the phage molecular environment) (30). Similarly, a phage-associated peptide specific for the anti-GCC receptor mAb GCC:B10 lost its antigenicity when chemically synthesised (31). Finally, peptides mimicking ice-binding proteins when presented by phage clones failed to exhibit an ice recrystallisation inhibition activity when used as synthetic peptides (32). All the above data are at variance with what was observed in our system, in which the p4.2 antigenicity was maintained outside the pc4.2 sequence, as indicated by *in vitro* assay and by clinical analysis, showing that anti-p4.2 Ab positive patients had an ILD-independent impaired DLCO (DLCO<70%).

Unlike anti-pc4.2 Ab, no association between anti-p4.2 Ab and PAH could

be established, nor could any solid significant statistical conclusion be drawn regarding this matter, due to either the inaccuracy of TTE-estimated sPAP as compared to PAH measured by RHC (33), or to the very low percentage of patients who developed PAH in our cohort. In fact, of 134 patients enrolled in the study and followed prospectively for 5 years, only 2 of them (1.49%) developed PAH, as assessed by RHC. This was quite surprising since 10-20% of PAH prevalence is expected in ACA positive SSc patients (11). The reason underlying the low percentage of PAH patients in our cohort may lie in the high percentage of patients (57.6%) taking vasodilator drugs, namely iloprost (46.15%) (28), bosentan (8.46%), or in combination (2.99%), both of which are also approved for PAH-SSc. Furthermore, bosentan has been shown to reduce the risk of PAH onset in SSc patients (34, 35). In addition, no comparison of anti-p4.2 Ab with the DETECT score could be done, as the latter has been validated only for a DLCO< 60, while in our cohort more than 81% of patients had a DLCO≥60, being anti-CENP-A positivity the only inclusion criterion. The reason(s) underlying the association of anti-p4.2 Ab with a reduced DLCO can only be speculated about at the moment. One possibility is that the peptide-associated antigenic determinant is also expressed on cell membranes (*e.g.* endothelial) involved in lung vascular remodelling, and that anti-p4.2 Ab can thus influence the initial phase of vascular remodelling. Alternatively, vascular remodelling may induce the expression of neoantigens expressing the p4.2 motif, favouring the clonal expansion of anti-p4.2 Ab already present in the serum, given that this is detected (albeit at lower levels) in serum of healthy donors. If this is the case, then it remains to be established whether anti-p4.2 Ab are natural or exogenous-antigen-induced Ab, and whether they can be the precursors of anti-CENP-A Ab, considering that anti-CENP-A Ab are not naturally occurring Ab (5). Experiments along this line are ongoing in our laboratory.

One limitation of this study is that no determination of anti-ETaR and AT1R Ab serum levels (15) to evaluate possible correlations with anti-p4.2 Ab levels was done. Further studies will establish whether anti-p4.2 Ab may replace or add value to anti-ETaR and AT1R Ab in predicting PVD.

Also, although a left heart dysfunction was excluded by TTE, left heart failure with preserved ejection fraction (LHFpEF) could not be completely ruled out as no left ventricular function analysis was carried on by cardiac magnetic resonance imaging (36). Indeed, the latter approach seems helpful in defining this particular condition even following RHC. In fact, pulmonary artery wedge pressure (PAWP) and pulmonary vascular resistance (PVR) might not be sensitive enough to exclude LHFpEH, hence in distinguishing precapillary PAH from mixed or postcapillary forms (37, 38).

This lack of data is regretful. However, we considered to firstly assess the feasibility of the anti-p4.2 Ab assay in relation to DLCO, a consolidated parameter of PVD, that can be done in any lab and may be available in any centre.

This study demonstrates that, by means of a simple peptide-based binding assay, it is possible to define a subset of anti-CENP-Ab positive patients at higher risk of developing PVD and that these patients should be strictly monitored for PAH onset.

Anti-p4.2 Ab may be an interesting biomarker expression of vascular remodelling before PAH takes place in SSc patients. The relationship between the functional significance and the specificity of these antibodies remains to be investigated.

Acknowledgements

The authors thank Giuseppina Dammacco for her excellent secretarial assistance.

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