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Anti-annexin V autoantibodies and vascular abnormalities in systemic sclerosis: a longitudinal study

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Abstract

Background: Annexins are a group of conserved proteins which exert several regulatory functions on various cellular activities. Increased frequency and levels of antibodies against annexin V have already been observed in several autoimmune diseases including systemic sclerosis (SSc), but their role as a vascular biomarker is unknown. The aim of this study was to determine the serum levels and the dynamical behavior of anti-annexin V antibodies over a 24 months follow-up in patients with SSc.

Methods: In this bicentric cross-sectional study, 70 patients with SSc were consecutively selected from March 2016 to April 2017. Demographic and clinical features, including the presence of active DUs, were collected. Serum anti-annexin V IgG and IgM antibodies were measured at baseline and after 6, 12 and 24 months of follow-up. Videocapillaroscopy was performed in all patients.

Results: Among the 70 SSc patients included anti-annexin V IgG was found in 11 patients (15.7%) (range of 15.88–39.48 U/mL) and anti-annexin V IgM in 10 patients (14.3%) (range of 14.16–22.69 U/mL) at baseline. During follow-up, the number of patients who were positive for anti-annexin V IgG and IgM remained stable over 24 months. Among the patients with positive anti-annexin V IgG at baseline the frequency of patients with necrosis or amputation of extremities, forced vital capacity less than 70% and pulmonary arterial hypertension (PAH) was significantly higher than in patients with negative anti-annexin V IgG antibodies. Patients with anti-annexin V IgG had also a higher Raynaud's Condition Score and a higher Health Assessment Questionnaire Disability Index (HAQ-DI) than patients without these antibodies at baseline. Patients with positive anti-annexin V IgM at baseline presented a higher frequency of PAH, compared to those with negative anti-annexin V IgM at baseline.

Conclusions: Anti-annexin V antibodies are stable and do not change their positivity during a 24 month follow-up in SSc patients. Anti-annexin V IgG was associated with more severe interstitial lung involvement and digital microangiopathy, and patients with anti-annexin V IgG or IgM had a higher occurrence of PAH indicating an association of these biomarker with more severe disease.

Keywords: Systemic sclerosis, Anti-annexin V, Digital ulcers, Vasculopathy, Biomarkers

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Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease, characterized by heterogeneous clinical presentation that affects the skin and several internal organs [1]. The pathogenesis of SSc includes interplay between vascular injury, abnormalities of the cellular and humoral immune systems and tissue fibrosis of the skin and internal organs such as lung, heart, and gastrointestinal tract [2, 3]. Circulating antibodies, alteration of immune mediators, and mononuclear cell infiltration into affected organs indicates that immune system dysfunction is important in the disease pathogenesis [2, 3]. Endothelial cell dysfunction is an early event in SSc and plays a role in the progression of vasculopathy and fibrosis [4].

In recent years, several efforts have been made on the identification of serum biomarkers associated with disease severity, and with specific clinical features such as peripheral vasculopathy in SSc [5–8].

Annexins are a group of conserved proteins which exert several regulatory functions on various cellular activities. Autoantibodies directed toward annexin I, II, V and XI have been reported in different autoimmune diseases, including autoimmune rheumatic diseases, but their role in immune response is controversial [9]. Annexin V belongs to a calcium-dependent phospholipid-binding protein family and exerts potent anticoagulant effects [9–11]. Annexin V is highly expressed by vascular endothelial cells and is also involved in the regulation of apoptosis and protection against both excessive coagulation and inflammatory activities [12–14]. Annexin V is also highly expressed by villous placental syncytiotrophoblast at the maternal-fetal interface and has been shown to play a thrombomodulatory role within the placental blood circulation [9].

Increased frequency and levels of antibodies against annexin V have already been observed in several autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), anti-phospholipid syndrome (APS), and SSc [9, 14, 15]. Anti-annexin V antibodies have also been associated with thrombotic and vessel occlusive events, recurrent miscarriages and preeclampsia, especially in patients with systemic lupus erythematosus and APS [9, 14].

In patients with SSc, it has been previously suggested that anti-annexin V antibodies could lead to a thrombogenic state due to interference with the effects of annexin V [9, 13]. Few studies have evaluated anti-annexin V antibodies in patients with SSc, most of them showing an association with digital ischemia, but the role of these antibodies in SSc is still unknown [10–12]. Thus, the aim of this study was to determine the serum levels and the dynamical behavior of anti-annexin V antibodies over a 24 months follow-up in patients with SSc. In addition, we evaluated the association of anti-annexin V antibodies with the severity of peripheral vasculopathy, digital ulcers occurrence and the microvascular changes observed in nailfold videocapillaroscopy during follow-up.

Patients and methods

Study design and patients

In this prospective observational cohort study, 70 patients with SSc attending the Rheumatology Division of the University Hospital Maria Aparecida Pedrossian of the Federal University of Mato Grosso do Sul (UFMS) and of the Federal University of São Paulo (UNIFESP) were consecutively selected from March 2016 to April 2017 (baseline data published in Advances in Rheumatology DOI: https://doi.org/10.1186/s42358-019-0057-9) [16]. All patients were evaluated in four visits: at baseline and after 6, 12 and 24 months of follow-up. Patients had to meet the ACR/EULAR 2013 classification criteria for SSc [17]. All subjects signed informed consent approved by the institutional ethical review board of both institutions (Federal University of São Paulo: CAAE: 53429216.5.1001.5505 and Federal University of Mato Grosso do Sul: CAAE: 49087115.6.0000.0021). Patients with overlapping rheumatic autoimmune diseases, malignancies and active infectious diseases were excluded.

Clinical assessment

At baseline, data regarding demographic and clinical features were collected, including information about age, gender, Raynaud's phenomenon (RP) duration before diagnosis, disease duration (defined as the onset of the first non-Raynaud's symptom), and modified Rodnan Skin Score (mRSS). The presence of calcinosis, telangiectasias, arthritis, renal crisis and esophageal dysmotility was also collected from all subjects. Interstitial lung involvement was evaluated by means of pulmonary function tests and computed tomography. The presence of pulmonary arterial hypertension (PAH) was assessed according to current definitions using Doppler echocardiography and right-sided heart catheterization [18]. The modified Rodnan Skin Score was evaluated in all patients by the same physician as previously described [19]. The SSc patients were also classified into diffuse cutaneous (dcSSc) or limited cutaneous (lcSSc) disease groups [20]. Drug therapy data were collected from all individuals at baseline.

Longitudinal evaluation

The presence and number of active digital ulcers (DUs) were recorded in each visit. Active DUs were defined as a loss of epithelialization and tissues involving, to different degrees, the epidermis, the dermis, the subcutaneous tissue and sometimes also involving the bone. The presence of necrosis and the amputation of the extremities

were also recorded [21]. Patients were also classified according to the presence of recurrent DUs defined by the presence of one or more DUs in at least two visits [22].

Patients were instructed to complete the Raynaud's Condition Score (RCS) during the week before each visit, in which the difficulty the patient experienced with RP in the prior 24 h was estimated on a 0–10 scale (0 = no difficulty; 10 = extreme difficulty) [23]. The Health Assessment Questionnaire Disability Index (HAQ-DI) (score from 0 to 3) was also recorded [24].

Nailfold videocapillaroscopy (NVC)

Videocapillaroscopy was performed using an optical videocapillaroscopic probe under a 200× magnification lens at the 3 visits (Optilia Medical OP-120020, Sweden). The images were captured and stored for further analysis. The following variables were assessed: the number of capillaries/mm, the number of enlarged capillaries (apical diameter > 20 μm), the number of giant capillaries (apical diameter > 50 μm), and the number of microhemorrhages. For the assessment of capillary loss (avascular score), the normal range of nine capillaries/mm was adopted [25, 26]. The average number for each capillaroscopic variable was calculated from the analysis of four consecutive fields (1 mm each) in eight digits, excluding the thumbs. The mean scores from the eight fingers were added, and the total value was divided by the number of fingers evaluated. For each parameter, a semi-quantitative rating scale was adopted as previously described [25].

Autoantibody measurements

Serum anti-annexin V IgG and IgM antibodies were measured at baseline and after 6, 12 and 24 months of follow-up. Peripheral venous blood (20 mL) was collected in dry tubes. Sera were frozen at − 20 °C until analysis. Anti-annexin V antibodies IgG and IgM levels were measured using enzyme-linked immunosorbent assay (ELISA) (ORGENTEC Diagnostika GmbH, Mainz, Germany), according to the manufacturers' instructions. Samples below the cut-off value of 8 units/mL were considered negative. Samples ≥ than 8 units/mL were considered positive, in compliance with the manufacturer's recommendation. Anticentromere (ACA), anti-Scl-70 and anti-RNA polymerase III (RNAP III) levels were measured at baseline using enzyme-linked immunosorbent assay (ELISA) (QUANTA Lite Centromere CENP-A & CENP-B, QUANTA Lite TM Scl-70 and QUANTA Lite RNA Pol III, respectively, INOVA Diagnostics, San Diego, CA, USA), according to the manufacturers' instructions. Samples above the cut-off value of 20 units/mL were considered positive.

ELISA was also used to measure anticardiolipin IgM/IgG (Sigma Laboratory, Darmstadt,Germany) and anti-beta 2 glycoprotein 1 (ORGENTEC Diagnostika GmbH, Mainz, Germany). Tests were considered positive if the titer was > 20.0 U/ml for IgG/IgM anticardiolipin and > 8.0 U/ml for IgG/IgM anti-beta 2 glycoprotein 1. Lupus anticoagulant was detected according to the recommendations of the International Society of Thrombosis and Hemostasis (ISTH) using tests to verify the prolongation of clotting assays, such as activated partial thromboplastin time (aPTT), kaolin clotting time, and dilute Russell viper venom time (DRVVT). Then, the presence of lupus anticoagulant was confirmed by mixing normal platelet-poor plasma with the patient's plasma.

Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate normality distribution. Differences between two groups were analyzed by t-test or the Mann-Whitney test for continuous variables. The chi-squared test was used to analyze categorical variables. The Kruskal-Wallis test was used to evaluate differences between three groups. The Dunn's post-test was used to pinpoint which specific means are significant from the others. Generalized linear models (GLMs) and Bonferroni post hoc tests were carried out to compare differences among different times of assessment (baseline, 6 months, 12 months and 24 months) and between groups. Pearson's correlation coefficients were used to evaluate the correlation between variables. Statistical analysis was performed using SPSS statistical software, version 23.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered significant [27].

Results

Among the 70 patients included, most were women (92.9%), with a mean age of 46.8 ± 12.5 years, a mean RP duration before diagnosis of 5.10 ± 6.15 years, and a mean disease duration of 9.41 ± 6.26 years. Twenty-five patients (35.7%) had dcSSc, and 45 patients (64.3%) had lcSSc. Active DUs were observed in 14 patients and active DUs or gangrene and amputation of the extremities in 15 patients at baseline. Eighteen patients (25.7%) were currently using intravenous cyclophosphamide, 9 patients (12.9%) were using methotrexate, and 7 patients (10.0%) were using mycophenolate. Corticosteroids (prednisone < 10 mg/day) were used by 17 patients (24.3%), calcium channel blockers by 45 patients (64.3%), phosphodiesterase-5 inhibitors by 18 patients (25.7%), antagonists of endothelin-1 (bosentan) by 3 patients (4.3%), and rituximab by 6 patients (8.6%). During the observation period, medication use was stable for the drug classes recorded except for cyclophosphamide. The evaluation of the scleroderma-specific autoantibodies showed the presence of ACA in 28 patients

(40%), anti-topo I in 21 patients (30%), and anti-RNAP III in 5 patients (7.1%) (Table 1).

The evaluation of the anti-annexin V autoantibodies showed the presence of anti-annexin V IgG in 11 patients (15.7%) (range of 15.88–39.48 U/mL) and anti-annexin V IgM in 10 patients (14.3%) (range of 14.16–22.69 U/mL) at baseline. During follow-up, the number of patients who were positive for anti-annexin V IgG and IgM remained stable over 24 months (Table 2).

The frequency of patients with active DUs remained stable during 24 months ($p = 0.638$). Among the 70 patients, 12 (17.1%) had recurrent DUs during longitudinal follow-up. The RCS was significantly lower at 6 months than at 12 or 24 months ($p = 0.015$), and no difference was observed between the 6-month analysis and baseline. The mean number of DU and the HAQ-DI remained stable during follow-up. The evaluation of NVC showed a significant decrease in the number of capillaries/mm and microhemorrhages and an increase in the number of enlarged capillaries and giant capillaries and in the avascular score throughout the 24-month follow-up (Table 2).

Table 1 Demographic and clinical features of the SSc patients

Variable	Patients with SSc (n 70)
Gender (F/M), (%)	65/5 (92.9/7.1)
RP duration (years), mean ± SD	5.10 ± 6.15
Disease duration (years), mean ± SD	9.41 ± 6.26
Cutaneous subset (Diffuse/Limited), n (%)	25/45 (35.7/64.3)
Modified Rodnan cutaneous score	13.11 ± 10.55
Calcinosis, n (%)	11 (15.7)
Telangiectasias, n (%)	38 (54.3)
Active digital ulcers, n (%)	14 (20.0)
Arthritis, n (%)	20 (28.6)
Esophageal involvement, n (%)	53 (75.7)
FVC < 70% predicted, n (%)	19 (27.1)
Interstitial lung involvement on CT scan, n (%)	34 (48.6)
PAH, n (%)	7 (10.0)
Renal crisis, n (%)	3 (4.3)
C-reactive protein (mg/L)	8.81 ± 8.97
ACA, n (%)	28 (40.0)
Anti-Scl-70, n (%)	21 (30.0)
Anti-RNA polymerase III, n (%)	5 (7.1)
IgG anti-cardiolipin	2 (2.9)
IgM anti-cardiolipin	3 (4.3)
IgG anti-beta 2 glycoprotein 1	0 (0.0)
IgM anti-beta 2 glycoprotein 1	1 (1.4)
Lupus anticoagulant	0 (0.0)

Results are presented as mean ± standard deviation or absolute frequency (relative frequency)
RP Raynaud's phenomenon, *FVC* forced vital capacity, *CT* computed tomography, *PAH* pulmonary arterial hypertension

The serum levels of anti-annexin V IgG and IgM antibodies were similar between patients with DUs and without DUs (Anti-annexin V IgG: 6.27 ± 6.05 U/mL versus 6.21 ± 6.78 U/mL, $p = 0.975$; Anti-annexin IgM: 4.43 ± 2.92 versus 6.25 ± 5.50 U/mL, $p = 0.097$, respectively) or in patients with active DUs or gangrene and amputation compared to those without these abnormalities at baseline (Anti-annexin V IgG: 6.04 ± 5.90 versus 6.27 ± 6.83 U/mL, $p = 0.904$; Anti-annexin V IgM: 4.38 ± 2.82 versus 6.29 ± 5.55 U/mL, $p = 0.073$, respectively).

Among the 11 patients with positive anti-annexin V IgG at baseline the frequency of patients with necrosis or amputation of extremities, forced vital capacity less than 70% and PAH was higher than in patients with negative anti-annexin V IgG antibodies. Patients with anti-annexin V IgG had also higher RCS and HAQ-DI scores than patients without these antibodies at baseline (Table 3). Patients with positive anti-annexin V IgM at baseline presented a higher frequency of PAH, compared to those with negative anti-annexin V IgM at baseline. The frequency of interstitial lung involvement on CT scan was of 72.7 and 70% in patients with anti-annexin V IgG and IgM antibodies compared to 47.5 and 48.3% in patients with negative anti-annexing V IgG and IgM antibodies, respectively. There was no significant difference in the frequency of active and recurrent digital ulcers, and other clinical variables between patients with positive or negative anti-annexin V IgG or IgM antibodies at baseline (Table 3).

Among the 13 patients with persistent positive anti-annexin V IgG measurement, 09 (69.2%) had persistent positive measurements for this autoantibody. Among the 11 patients with persistent positive anti-annexin V IgM measurement, 07 (63.6%) had persistent positive measurement for this autoantibody during longitudinal follow-up. Interestingly, 5 patients were positive for both antibodies (anti-annexin V IgG and IgM) in all measurements.

There was no correlation between the serum levels of anti-annexin V IgG or IgM at baseline and age, RP duration or disease duration, scleroderma renal crisis or telangiectasias or the number of active DUs evaluated at different time points (data not shown). However, the RCS and HAQ-DI score were statistically higher in patients with positive anti-annexin V IgG antibodies compared to those with negative anti-annexin V IgG at all evaluations (data not shown).

Discussion

This is the first study that evaluated the frequency of anti-annexin V antibodies during a longitudinal follow-up in patients with SSc. Vascular involvement and

Table 2 Longitudinal data of anti-annexin V serum levels, severity of Raynaud's phenomenon, HAQ-DI and nailfold capillaroscopy of the 70 SSc patients

Variable	Moment				*p* value
	Baseline	6 months	12 months	24 months	
Positive IgG anti-annexin V, n (%)	11 (15.7)	12 (17.1)	9 (12.9)	9 (12.9)	0.478
Positive IgM anti-annexin V, n (%)	10 (14.3)	11 (15.7)	7 (10.0)	8 (11.4)	0.396
Patients with active DUs, n (%)	14 (20.0)	9 (12.8)	7 (10.0)	10 (17.2)	0.638
Gangrene and amputation, n (%)	4 (5.7)	3 (4.3)	2 (2.9)	5 (7.1)	0.537
Raynaud Condition Score (RCS)	5.29 ± 2.27	4.67 ± 2.10	5.24 ± 2. 43	5.17 ± 2.41	0.015
Mean number of DUs	0.31 ± 0.78	0.21 ± 0.59	0.16 ± 0.52	0.28 ± 0.67	0.191
HAQ-DI	0.77 ± 0.05	0.71 ± 0.57	0.78 ± 0.58	0.81 ± 0.60	0.051
Nailfold videocapillaroscopy					
Number of capillaries/mm	7.31 ± 1.00	7.08 ± 0.97	6.63 ± 1.19	6.36 ± 1.21	< 0.001
Enlarged capillaries	1.14 ± 0.84	1.33 ± 0.87	1.60 ± 0.94	1.77 ± 0.99	< 0.001
Giant capillaries	0.21 ± 0.24	0.27 ± 0.30	0.43 ± 0.37	0.56 ± 0.47	< 0.001
Microhemorrhages	0.66 ± 0.62	0.59 ± 0.69	0.36 ± 0.42	0.43 ± 0.43	< 0.001
Avascular score	0.9 ± 0.69	1.05 ± 0.70	1.23 ± 0.74	1.34 ± 0.80	< 0.001

Results are presented as mean ± standard deviation or absolute number and frequency

endothelial dysfunction are primary events in SSc, resulting in vascular obliteration and decreased blood flow to the organs [28]. The clinical expression of vasculopathy is extremely heterogeneous and may manifest simply by RP, as well as by clinical manifestations such as digital ulcers or gangrene [29] or even life-threatening severe manifestations such as pulmonary arterial hypertension [30]. In this context, there is a need to identify biomarkers that are easily applicable in clinical practice, to identify patients at risk of developing more severe manifestations or certain phenotypes [31].

Thus, in the present study, the role of anti-annexin V IgM and IgG autoantibodies as a biomarker of peripheral microangiopathy or internal organ involvement was prospectively evaluated. In particular, the presence of active DUs, gangrene and amputation, and the microvascular changes evaluated by videocapillaroscopy, were evaluated during a 24 month follow-up.

In our study, anti-annexin V IgG antibodies were observed in 15.7% of patients and IgM class antibodies in 14.3% of patients at baseline. At baseline, patients with positive anti-annexin V IgG showed a higher frequency of gangrene and amputation of the extremities, a lower FCV and a higher frequency of PAH. Anti-annexin V IgG was also associated with a worse RCS and HAQ-DI.

Our results are in agreement with the results of Sugiura and Muro (1999), who found a frequency of anti-annexin V IgG positivity in 18.2% of SSc patients [11]. In addition, the authors found higher anti-annexin V titers in the group of patients with digital ischemia (ulcer of the fingertip or gangrene of the finger) compared to patients without digital ischemia (8.3 U/ml versus 2.7 U/ml, $p < 0.004$) [11]. Although an association with anti-annexin V and active digital ulcers was not observed in our study, an association with more severe peripheral microangiopathy evaluated by means of gangrene and amputation of the extremities was found, suggesting that anti-annexin V could be a biomarker of more severe vasculopathy. Moreover, the lower FCV and a higher frequency of PAH in patients with anti-annexin V suggest a possible association of anti-annexin V with more severe disease. In accordance with these results, anti-annexin V IgG-positive patients had worse quality of life indices, as measured by HAQ-DI.

Two other studies have evaluated anti-annexin V in patients with SSc. El Serougy et al. [12], observed significantly higher levels of anti-annexin V IgG antibodies in 40 Egyptian patients with SSc compared to healthy controls. They also found higher levels of anti-annexin V IgG in patients with digital ischemia (ulcers or gangrene) and, similar to our findings, higher levels in patients with pulmonary fibrosis. The latest study was performed by Habeeb et al. [10], who evaluated 20 SSc patients and found a prevalence of positivity of these antibodies in 75% of patients. This discrepant value might be related to the population studied or to the method used for the anti-annexin V measurement. The authors [10] used a French Zymutest anti-annexin V IgG ELISA kit, while in our and El Serougy's study [12] the same German Orgentec kit was used, which evaluated both IgG and IgM class anti-annexin V antibodies.

In accordance to previous studies [10–12], we did not find significant correlation between anti-annexin V serum antibodies and patients' age, gender, disease duration or clinical cutaneous involvement.

Table 3 Clinical variables according to the presence or absence of anti-annexin V at baseline

Variable	Anti-annexin V IgG		*p* value	Anti-annexin V IgM		*p* value
	Positive (*n* = 11)	Negative (*n* = 59)		Positive (*n* = 10)	Negative (*n* = 60)	
Age (years), mean ± SD	48.73 ± 11.30	46.42 ± 12.79	0.579	49.90 ± 15.55	46.27 ± 12.02	0.399
Gender (F/M), n (%)	10/1	55/4	0.586	9/1	56/4	0.549
RP duration before diagnosis (years), mean ± SD	3.09 ± 3.48	5.47 ± 6.48	0.241	3.80 ± 3.88	5.32 ± 6.45	0.475
Disease duration (years), mean ± SD	9.18 ± 4.29	9.46 ± 6.59	0.894	10.00 ± 7.38	9.32 ± 6.12	0.752
Cutaneous subset (Diffuse/Limited)	5/6	20/39	0.341	3/7	22/38	0.490
Modified Rodnan cutaneous score	17.91 ± 13.05	12.22 ± 9.89	0.101	11.80 ± 9.77	13.33 ± 10.74	0.674
Calcinosis, n (%)	3 (27.3)	8 (13.6)	0.232	2 (20.0)	9 (15.0)	0.493
Telangiectasia, n (%)	5 (45.5)	33 (55.9)	0.261	6 (60.0)	32 (53.3)	0.655
Puffy fingers, (%)	1 (9.1)	12 (20.3)	0.345	0 (0.0)	13 (21.7)	0.109
Active digital ulcers, n (%)	3 (27.3)	11 (18.6)	0.382	1 (10.0)	13 (21.7)	0.357
Recurrent digital ulcers, n (%)	4 (36.4)	11 (18.6)	0.177	1 (10.0)	14 (23.3)	0.314
Necrosis or amputation of extremities, n (%)	3 (27.3)	1 (1.7)	0.011	1 (10.0)	3 (5.0)	0.232
Arthritis, n (%)	4 (36.4)	16 (27.1)	0.385	3 (30.0)	17 (28.3)	0.591
Esophageal involvement, n (%)	10 (90.9)	43 (72.9)	0.188	9 (90.0)	44 (73.3)	0.239
FVC < 70% predicted, n (%)	6 (54.5)	13 (22.0)	0.036	3 (30.0)	16 (26.7)	0.548
Interstitial lung involvement on CT scan, n (%)	8 (72.7)	28 (47.5)	0.112	7 (70.0)	29 (48.3)	0.177
PAH, n (%)	3 (27.3)	4 (6.8)	0.048	3 (30.0)	4 (6.7)	0.036
Diastolic dysfunction, n (%)	5 (45.5)	12 (20.3)	0.085	3 (30.0)	14 (23.3)	0.457
Renal crisis, n (%)	0 (0.0)	3 (5.1)	0.594	0 (0.0)	3 (5.0)	0.625
Raynaud Condition Score	6.00 ± 2.12	5.07 ± 2.27	0.002	5.18 ± 2.44	5.09 ± 2.29	0.873
HAQ-DI (score 0–3)	1.17 ± 0.55	0.70 ± 0.49	0.006	0.73 ± 0.58	0.78 ± 0.52	0.777
ACA, n (%)	5 (45.5)	23 (38.9)	0.688	6 (60.0)	22 (36.7)	0.163
Anti-Scl-70, n (%)	5 (45.5)	16 (27.1)	0.223	3 (30.0)	18 (30.0)	1.000
Anti-RNA polymerase III, n (%)	0 (0.0)	5 (8.5)	0.316	0 (0.0)	5 (8.3)	0.343
Nailfold videocapillaroscopy						
Number of capillaries/mm	7.14 ± 1.03	7.35 ± 1.00	0.513	7.09 ± 0.89	7.36 ± 1.02	0.429
Enlarged capillaries	1.40 ± 0.73	1.0 ± 0.79	0.198	1.32 ± 0.57	1.08 ± 0.82	0.367
Giant capillaries	0.24 ± 0.23	0.21 ± 0.24	0.728	0.29 ± 0.21	0.21 ± 0.24	0.313
Microhemorrhages	0.56 ± 0.46	0.66 ± 0.63	0.624	1.0 ± 0.93	0.58 ± 0.51	0.192
Avascular score	1.16 ± 0.59	0.93 ± 0.74	0.330	1.23 ± 0.66	0.92 ± 0.72	0.218

Results are presented as mean ± standard deviation or absolute number and frequency

Recently there has been an increased interest in the study of anti-annexin V antibodies, since its inclusion has been considered as part of the diagnostic criteria of patients with antiphospholipid syndrome [32], particularly in those patients in which conventional autoantibodies are negative (anti cardiolipin, lupus anticoagulant and anti-beta 2 glycoprotein 1). Mekinian et al. [33] found that 68% of patients with clinical criteria for obstetrical antiphospholipid syndrome (APS) that were seronegative for conventional antiphospholipid antibodies (APL) have non-conventional APL, mostly represented by anti-annexin V IgG antibodies. As expected, in our study, the frequencies of anticardiolipin, anti-beta 2 glycoprotein 1 or lupus anticoagulant antibodies were low, not allowing an analysis between these antibodies and anti-annexin V. In other autoimmune diseases, anti-annexin V was found in 3.8% of patients with SLE with no clinical or serological features of APS, 28.0% of patients with SLE having only serological signs of APS, and 30.4% of patients with clinical symptoms and serological signs of APS [9].

During the 24 months follow-up, anti-annexin V IgG and IgM antibodies showed no statistically significant variations, indicating that longitudinal measurement of anti-annexin V are not useful in the clinical practice. In general, when the patient was positive for an anti-

annexin V antibody, he was always positive throughout the 24 months of clinical observation, without large variations in the titers of these autoantibodies. The number of patients with active DUs, gangrene and amputation and the mean number of DUs also remained stable during follow-up.

The study of microvascular abnormalities evaluated with videocapillaroscopy was also performed prospectively. In agreement with previous studies [34–36], a worsening of microvascular abnormalities was observed during follow-up. No worse videocapillaroscopy was observed in patients with positive anti-annexin V antibodies.

In relation to other vascular changes, patients with positive IgG and IgM anti-annexin V showed a higher frequency of PAH. Previous studies reinforce that anti-annexin V autoantibodies could play a role in pathogenesis of SSc. By binding to vascular endothelial cells, they would promote apoptosis and cytokine release that could contribute to pulmonary vasculopathy and fibrosis [9]. Indeed, in our study, a higher number of patients with anti-annexin V class IgG had FVC < 70% compared to patients without these antibodies. Although not statistical significant, interstitial lung involvement on CT scan was observed in higher frequency of patients with positive anti-annexin V antibodies. Thus, the present results suggest a possible association of anti-annexin V antibodies and the presence of PAH and pulmonary fibrosis.

Our study has some limitations including the lack of organ involvement evaluation during follow-up and the lack of a healthy control group.

In summary, we found that anti-annexin V antibodies remained stable during follow-up in patients with SSc. SSc patient's with positive anti-annexin V antibodies had worse digital microangiopathy and a higher frequency of interstitial lung involvement and PAH, suggesting that anti-annexin V antibodies could play a role in the pathogenesis of SSc patients as well as be associated with more severe disease.

Conclusion

In conclusion, in this 24-months prospective study, the presence of anti-annexin V IgG was associated with more severe interstitial lung involvement and digital microangiopathy, and the presence of anti-annexin V antibodies, either of the IgG or IgM class, with a higher occurrence of PAH. Prospective analyses are needed to confirm the value of these variables in predicting the occurrence of these manifestations in SSc patients.

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Authors' contributions
AMCH performed the nailfold videocapillaroscopy examination of all patients, and with CK was a major contributor in writing the manuscript. CK and ASS analyzed and interpreted the patient data regarding the statistical analysis. SHR performed with. LGJ all autoantibodies and serum biomarkers tests. All authors read and approved the final manuscript.

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