Systemic sclerosis: can we identify patients at risk?

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Chapter 8

Association between centromere and topoisomerase specific immune responses and the degree of microangiopathy in systemic sclerosis

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Published Journal of Rheumatology

Objectives

Autoreactive antibody responses, including the use of several isotypes of autoantibodies, have been shown to associate with clinical outcome in several rheumatic autoimmune diseases. The goal of this study was to evaluate whether 1) anti-centromere antibody (ACA) and anti-topoisomerase antibody (ATA) specific isotype expression and 2) organ involvement are associated with the degree of microangiopathy in Systemic Sclerosis (SSc).

Methods

ACA and ATA IgG, IgM and IgA levels were measured in baseline serum samples of ACA IgG+ and ATA IgG+ patients with SSc. The degree of microangiopathy was determined based on nailfold videocapillaroscopy (NVC) images collected at the same time point. Univariable and multivariable logistic regression analyses with autoantibodies, clinical characteristics, isotype expression and ACA and ATA IgG, IgM and IgA levels as independent variables, and NVC pattern as dependent variable were performed.

Results

In 164 patients, isotype levels and degree of microangiopathy were evaluated. Logistic regression confirmed the association of the degree of microangiopathy with the presence of digital ulcers (OR 3.07, 95% CI 1.43-6.60), interstitial lung disease (OR 3.41, 95% CI 1.11-10.61) and pulmonary arterial hypertension (OR 5.58, 95% CI 2.05-17.81). ATA positivity was associated with more severe microangiopathy (OR 2.09, 95% CI 1.05-4.13). Patients that solely expressed ACA IgG showed a trend towards less severe microangiopathy compared to patients also expressing ACA IgM and/or IgA. Levels of ACA IgG and ATA IgM were found to be associated with the severity of microangiopathy.

Conclusions

We observed an association between ACA and ATA responses and the degree of microangiopathy in SSc. These findings might indicate that the breadth of the autoimmune response, as reflected by autoantibody production and microvascular damage, interact in the pathophysiology of SSc.

INTRODUCTION

Systemic Sclerosis (SSc) is characterised by the triad of microvascular damage, dysregulation of innate and adaptive immunity and generalized fibrosis (1). The pathogenesis of SSc has still not been completely elucidated, and the primary cause of SSc remains to be determined (2). Approximately 95% of patients with SSc have antinuclear autoantibodies (ANA). These autoantibodies contribute to the disease classification and are associated with specific clinical manifestations, making them important tools for disease prognostication (3). Anti-centromere antibodies (ACA) and anti-topoisomerase antibodies (ATA) are the two most common ANA in patients with SSc (3). Of these, ACA is associated with a relatively mild disease course, while ATA is associated with more severe disease including diffuse skin and lung involvement. This clear association with a typical clinical phenotype suggests that the immune response, reflected by ATA or ACA production, is closely linked to disease pathophysiology. The exact pathogenicity of ATA or ACA, however, remains unclear (4).

A second important diagnostic and prognostic tool in SSc is nailfold videocapillaroscopy (NVC) which is an investigation that determines the degree of microangiopathy by using standardised magnification to visualize the capillaries in the nailfold. In SSc, specific patterns of capillary changes and the degree of these changes have been defined extensively (5). More severe microangiopathy is associated with worse disease in SSc patients, in recent studies an association between NVC pattern, organ involvement and disease progression was found (6-12). In addition, an association between NVC patterns and specific autoantibodies was also described (13). NVC can therefore be seen as an important biomarker that can be used to predict severe complications in SSc (14).

Some studies suggest that autoantibody production is secondary to vasculopathy, and thus specific autoantibodies can be viewed as bystanders in disease pathogenesis (2). However, other studies suggest that circulating autoantibodies may be directly implicated in the disease process. Higher levels of ATA have been shown to associate with the development of organ involvement (15). An association between autoantibody specific isotypes and disease severity has been found, with higher ATA IgM levels in ATA + SSc patients who showed disease progression, and higher ACA IgG and ACA IgM levels in ACA+ SSc patients who had a more severe disease (16, 17). A study performed by Ahmed et al. (18) demonstrated that SSc sera containing ACA or ATA can trigger fibrillin expression in human dermal endothelial cells and induce cell apoptosis, while Shen et al. (19) concluded that the pathognomonic ACA and ATA in SSc accelerate vascular endothelial cell senescence and functional impairment inducing Raynaud's Phenomenon (RP). Together, these studies implicate a possible association between ACA, ATA, and possibly specific isotype levels and vasculopathy. This association has not yet been evaluated in SSc.

As the presentation of SSc can be very heterogeneous and prediction of the disease course is still very difficult, a better understanding of the interaction between the specific auto-immune response and the degree of microangiopathy could not only improve our insights in disease pathogenesis, but could also contribute to more reliable disease prognostication, which is of utmost importance. In line with this, we hypothesized that an activated immune response, as reflected by higher ATA or ACA IgG levels associate with more severe microvascular damage.

METHODS

Study design and patients

SSc patients at the Leiden University Medical Center (LUMC) are included in an observational cohort study (Combined Care in Systemic Sclerosis; CCISS (12)), which was approved by the Ethics Committee (P09.003). The cohort study is designed in accordance with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent. This standardized annual care pathway comprises extensive screening, including autoantibody testing, electrocardiography (ECG), thoracic echocardiography, high resolution computed tomography (HRCT), pulmonary function test, and NVC. An exercise test, 24 hour Holter ECG or right heart catheterization (RHC) are performed if indicated. In our current study, patients who fulfilled the ACR/EULAR 2013 classification criteria for SSc (20), were positive for IgG either ATA or ACA, and had a clinical diagnosis of SSc were included. Use of current medication (calcium channel blockers, sildenafil, bosentan and Iloprost for vasoactive medication, and corticosteroids, cyclophosphamide, azathioprine, methotrexate, mycophenolate mofetil and azathioprine for immunosuppressive medication) at the time of blood sampling, baseline clinical characteristics and investigations were retracted from the database. Baseline characteristics were considered at time of inclusion in the cohort.

Nailfold videocapillaroscopy

NVC was performed at the same time point as the baseline characteristics and blood samples were collected. All images were obtained in the hospital in a comfortable room with a temperature of 22-25 degree Celsius. All fingers except for the thumbs from both hands were examined using a videocapillaroscope (LUMC: 2009-2015; Videocap 3.0, DS Medica; 2015 -2017, Inspectis pro; 2018 onwards) equipped with a probe with 200x magnification. NVC images were scored by trained observers and classified qualitatively as described previously; 'normal', 'non-specific' and 'scleroderma pattern' (21). A "normal pattern" is defined as a pattern of typical hairpin-like capillaries with a regular distribution. A "non-specific pattern" is defined as a pattern with abnormalities without fulfilling the definition of a 'scleroderma pattern' (22). A 'scleroderma pattern' was defined according to the standards set by Cutolo et al. and categorized in an 'early' 'active' or 'late' pattern (23). The presence of giant capillaries, hemorrhages and avascularity are the main denominators in the definition of a scleroderma pattern. A more severe degree of microangiopathy is defined as a late scleroderma pattern with capillary loss (< 4 capillaries per mm) as its main denominator. For our current evaluation, the images were re-examined by a trained investigator (NvL). The inter-observer agreement was high for qualitative pattern determination (ICC 0.97).

ACA and ATA assay and measurements

Total immunoglobulin ATA, IgG, IgM and IgA and total immunoglobulin ACA, IgG, IgM and IgA levels of all the collected samples were measured in baseline samples by fluorescence enzyme-linked immune sorbent assay (FEIA), using the Phadia250 system (ThermoFisher Scientific, Nieuwegein, the Netherlands). The cut-off levels for ATA and ACA IgG were set at 7 units/ml (U/mL) according to the manufacturer's instructions. Fifty serum samples of non-rheumatic age and sex matched subjects were measured to establish cut-off values (mean + 2SD) for IgM and IgA isotypes of ACA and ATA. A cut-off for ACA IgA was determined at 37 aU/ml, for ATA IgA at 77 aU/ml, for ACA IgM at 13 aU/ml and for ATA IgM at 432 aU/ml. To evaluate the specificity of the assay, 10 SSc patients who were negative for ATA IgG were tested and all had ATA-IgM and -IgA levels below the defined cut-off, in addition 10 SSc patients negative for ACA IgG were tested for ACA IgM and ACA IgA, and these levels were also below the defined cut-off. An 'expressed isotype' was defined as a level above the cut-off value. Outliers were checked and when necessary remeasured.

Organ involvement

Digital ulcers (DU) were present when there was clear visible tissue breakdown, and both ischemic and mechanical (results of microtrauma and increased skin tension) ulcers were included in this definition. Interstitial lung disease (ILD) was defined based on the combination of forced vital capacity (FVC) <70% and evidence for ILD on HRCT. An experienced radiologist evaluated the HRCT for ground glass opacifications, reticulations and honeycombing. We chose to use a combined value including both pulmonary function and HRCT to make sure that we only classify patients with clinically relevant pulmonary involvement as having ILD. Pulmonary arterial hypertension (PAH) was defined as an increase in mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest as assessed by RHC; including presence of pre-capillary PH, defined by a pulmonary capillary wedge pressure (PCWP) ≤15 mmHg and a PVR >3 Wood units (WU) on RHC, in the absence of other causes of precapillary PH such as PH due to lung diseases, chronic thromboembolic pulmonary hypertension, or other rare diseases. To evaluate myocardial involvement, we used different measurements. The Medsger subdomain reflecting myocardial involvement was evaluated, in which grade 0 represents a normal heart function, grade 1 denotes conduction abnormalities and a left ventricular ejection fraction (LVEF) between 44-49%, grade 2 signifies arrhythmias and a LVEF 40-45%, grade 3 indicates severe involvement with a LVEF < 40%. As the Medsger scale mainly relies on the LVEF for determination of myocardial involvement, using only this parameter could lead to underestimation of its presence, since in patients with SSc with myocardial involvement, LVEF is not always below the normal cut-off. Therefore, we additionally used a combined value where patients had to have at least two of the following: arrhythmias (> 2% ventricular or supraventricular arrhythmia, atrial fibrillation), conduction problems, decreased LVEF < 50%, diastolic or systolic dysfunction, pericarditis or pericardial effusion.

Statistical analysis

No sample size calculation was performed due to the explorative character of this study. Analyses were performed by IBM SPSS version 23. All analyses were performed cross-sectionally. NVC patterns and clinical features, at the time of blood sample collection for autoantibody determination, were compared between ACA+ and ATA+ SSc patients using descriptive statistics, and differences were tested for significance as appropriate. Disease duration was defined as duration since onset of Raynaud's Phenomenon (RP), as current SSc pathophysiology indicate that RP is a direct consequence of vasculopathy. We performed a Mann-Whitney U test to calculate the significance of the continuous variables. A Chi-square test was performed for the categorical variables. Fisher exact test was employed when appropriate. Binary logistic regression (univariable and multivariable) was performed, with autoantibodies, NVC pattern, isotype expression and ACA or ATA isotype levels as independent factor and organ involvement as dependent variables. Ordinal logistic regression analyses, with disease characteristics, autoantibodies and isotype expression and ACA or ATA IgG level as independent and NVC SSc pattern as dependent variables were also performed. Since age and disease duration can be a confounder for the association between organ involvement and degree of microangiopathy, we corrected for these variables in the multivariate analyses. In addition, variables with significant association in the univariate analysis were added as indicated. All isotype levels were transformed using log2. To adjust for multiple testing, Bonferroni correction was applied. P values < 0.05 were considered significant.

RESULTS

Study group

A total of 231 SSc patients (129 ACA+ and 102 ATA+) were included. The included patients had a mean age of 55 years (SD 14) and median disease duration from onset first non-RP of 4 years (IQR 1-11). As expected, females represented the majority of the study population (n= 186). ATA+ patients differed from ACA+ in sex (p<0.001), age (p= 0.01), disease duration (p<0.001), diffuse cutaneous subset (p<0.001), and ILD (p<0.001). The main demographic and clinical data of the patients are summarized in Table 1. An important difference between the ATA+ and the ACA+ group that could have an influence on the degree of microangiopathy is the disease duration since onset RP, which is longer in the ACA+ patients compared to the ATA+ patients (16 years vs 6 years). Complete data on NVC patterns were available for 164/231 patients (100 ACA+ and 64 ATA+). The missing NVC were all from patients with baseline visits before 2013, in 2013 the NVC became an annual standard examination.

Organ involvement and the degree of microangiopathy

In the univariable analysis (Table 2), a more severe degree of microangiopathy (late SSc pattern), as shown by NVC, was associated with ILD (OR 3.59, 95%CI 1.75-15.91), PAH (OR 5.85, 95% CI 1.90- 18.65), cardiac involvement (OR 2.95, 95%CI 1.20-7.23) and DU (OR 2.28, 95% CI1.17-4.47). Multivariable analysis showed that a more severe degree of microangiopathy was significantly associated with ILD (OR 3.41, 95%CI 1.11-10.61), PAH (OR 5.58, 95%CI 2.05-17.81) and DU (OR 3.07, 95%CI 1.43-6.60), with correction for age, disease duration and ATA positivity.

NVC patterns in autoantibody subgroups

A late SSc pattern was numerically seen more often in ATA+ patients vs ACA+ patients (31% vs 18%; p= 0.05* after Bonferroni correction; Table 1). The frequency of early (10% ATA, 18% ACA) and active (58% ATA, 68% ACA) SSc pattern were comparable between ATA+ and ACA+ patients. In the multivariable analysis (Table 5 supplementary file), after adjustment for vasoactive medication, age, and disease duration, ATA positivity was associated with more severe microangiopathy (OR 2.97, 95CI 1.41-6.24) compared to ACA positivity.

Baseline characteristics of included ACA+ and ATA+ SSc patients

	Total group	ACA+	ATA+
Demographic	n= 231	n= 129	n= 102
Female, n (%)	186 (81)	116 (90)	70 (69)
Age, mean (SD)	55 (14)	58 (13)	51 (14)
Smoking, ever, n (%)	138 (60)	76 (59)	62 (61)
Disease duration			
Since RP, median (IQR)	10 (4-20)	16 (6-26)	6 (2-13)
Since non RP, median (IQR)	4 (1-11)	5 (1-12)	3 (1-9)
Organ involvement			
DcSSc, n (%)	49 (21)	2 (2)	47 (46)
Puffy fingers, n (%)	74 (33)	38 (30)	36 (37)
Sclerodactylie, n (%)	151 (67)	71 (55)	80 (82)
mRSS, median (IQR)	4 (2-6)	3 (1-5)	6 (2-12)
Pitting Scars, n (%)	106 (46)	59 (46)	47 (46)
Teleangiectasia, n (%)	152 (66)	108 (84)	44 (44)
Digital ulcera, n (%)	43 (19)	30 (23)	13 (13)
DLCO % predicted mean (SD)	65 (17)	70 (17)	62 (17)
FVC % predicted, mean (SD)	92 (21)	92 (21)	91 (20)
ILD on HRCT, n (%)	55 (24)	11 (9)	44 (43)
PAH, n(%)	20 (13)	10 (8)	10 (10)
Nailfold videocapillaroscopy			
NVC early, n (%)	22 (15)	16 (18)	6 (10)
NVC active, n (%)	90 (63)	56 (68)	34 (58)
NVC late, n (%)*	33 (23)	15 (18)	18 (31)
NVC capillary loss < 7mm, n (%)	114 (70)	64 (65)	50 (78)
Medication			
CYC, n (%)	39 (17)	19 (15)	20 (20)
HSCT, n (%)	14 (7)	7 (7)	7 (7)
Iloprost/bosentan, n (%)	17 (12)	14 (11)	13 (13)
Methotrexate ever, n (%)	44 (19)	23 (18)	21 (20)
ACA or ATA characteristics			
IgA positivity, n (%)	-	95 (74)	100 (98)
IgA level (aU/mL), median (IQR)	-	73 (34-146)	2778 (933-8368)
IgM positivity, n (%)	-	95 (74)	66 (65)
IgM level (aU/mL), median (IQR)	-	65 (4-561)	822 (286-2162)
IgG level (U/mL), median (IQR)	-	478 (186-1031)	484 (170-934)

Table 1. Baseline characteristics. ACA= anti-centromere antibody, ATA= anti-topoisomerase antibody, CYC= cyclophosphamide, dcSSc= diffuse cutaneous SSc, DLCO= carbon monoxide diffusing capacity, FVC= forced vital capacity, HSCT= stem cell transplantation, ILD= interstitial lung disease, mRSS= Modified Rodnan Skin Score, NVC= nailfold video capillaroscopy, PAH= pulmonary arterial hypertension, RP= Raynaud phenomenon.

ATA and more severe microangiopathy is associated with organ involvement

	Univariable OR (95% CI)		
	Interstitial lung disease	Cardiac involvement	PAH
Male	1.71 (0.84-3.48**)**	0.59 (0.26-1.34)	1.04 (0.33-3.27)
Age	1.01 (0.99.1.04)	**1.07 (1.03-1.10)**	**1.08 (1.03-1.13)**
Disease duration since RP	0.99 (0.95-1.01)	1.04 (0.98-1.03)	1.00 (0.99-1.05)
Disease duration since NR	1.03 (0.99-1.06)	1.01 (0.97-1.05)	1.01 (097-1.07)
ATA	**6.68 (1.87-23.94)**	0.96 (0.47-1.95)	1.29 (0.52-3.24)
NVC SSc pattern	**3.59 (1.75-15.91)**	**2.95 (1.20-7.23)**	**5.85 (1.90-18.65)**
Immunosuppressiva	**4.64 (1.57-13.66)**	1.08 (0.50-2.31)	0.53 (0.26-1.99)

Table 2. In the multivariable logistic regression autoantibody, age, disease duration (since onset RP) and variables with significant association in univariate analysis were included. Interstitial lung disease (ILD) was defined as ILD on HRCT and a FVC < 70% of predicted. NVC pattern was entered as ordinal variable in the following order: early, active or late.

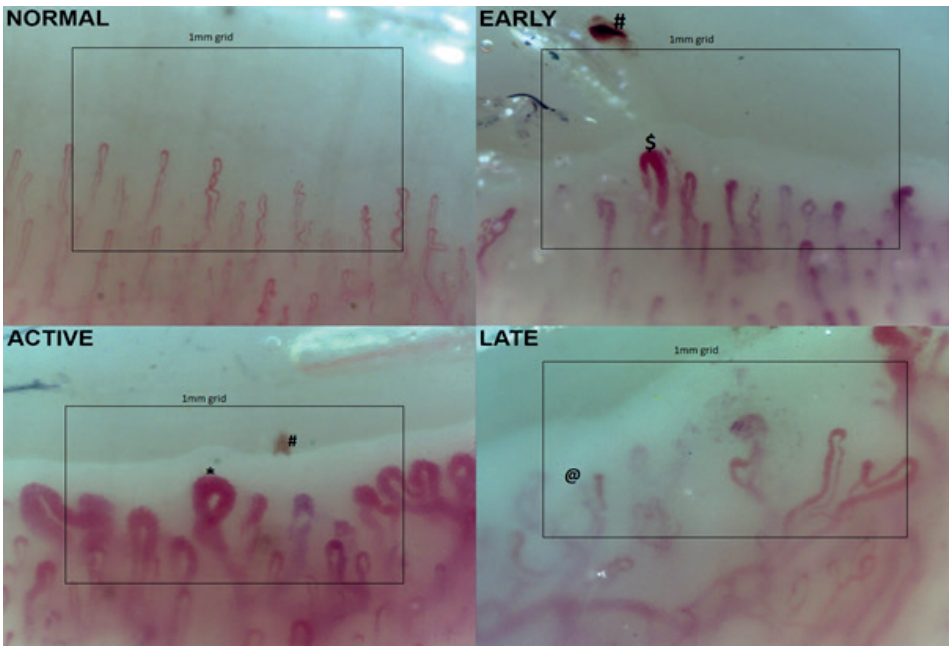

Figure 1. Nailfold videocapillaroscopy image made with INSPECTIS pro**.** Examples of NVC images with a 1 mm grid. # hemorrhages, $ dilation > 30uM, * giant (dilation > 50um), @ neoangiogeneses.

	Multivariable OR (95% CI)			
DU	Interstitial lung disease	Cardiac involvement	PAH	DU
1.32 (0.55-3.20)	-	-	-	-
0.99 (0.97-1.02)	1.01 (0.95-1.07)	**1.06 (1.01-1.11)**	**1.09 (1.02-1.18)**	0.98 (0.95-1.01)
1.01 (0.99-1.03)	0.98 (0.89-1.07)	1.01 (0.97-1.05)	1.02 (0.94-1.07)	0.99 (0.96-1.03)
1.04 (1.01-1.08)	-	-	-	-
2.07 (1.02-4.22)	**13.34 (2.87-52.61)**	1.28 (0.38-4.31)	3.92 (0.68-22.46)	0.36 (0.14-0.92)
2.28 (1.17-4.47)	**3.41 (1.11-10.61)**	2.17 (0.86-5.48)	**5.58 (2.05-17.81)**	**3.07 (1.43-6.60)**
0.46 (0.20-1.07)	1.79 (0.45-7.01)	-	-	-

ATA= anti-topoisomerase, CI= confidence interval, DU= digital ulcera, NR= non-Raynaud, NVC= nailfold videocapillaroscopy, OR= odds ratio, PAH= pulmonary arterial hypertension, RP= Raynaud Phenomenon, SSc= systemic sclerosis .

Isotype expression

In ACA IgG+ patients, 74% (n= 95) were ACA IgA+ and 74% (n= 95) ACA IgM+. Of the ACA + patients, 11% expressed solely ACA IgG, 16% were positive for IgG and IgM, 16% of the ACA patients were positive for IgG and IgA, and 58% were positive for ACA IgG, IgM and IgA. All ATA IgG + patients expressed more than one ATA specific isotype: ATA IgA+ was found in 98% (n= 100), ATA IgM+ was found in 65% (n= 66). Two percent of ATA IgG+ patients expressed also ATA IgM, in the absence of detectable IgA ATA, 33% of ATA IgG+ patients expressed also ATA IgA in the absence of detectable IgM ATA, and 65% expressed all three ATA isotypes.

Association ACA and ATA isotype expression with the degree of microangiopathy

As shown in Figure 2, the ACA IgG+ patients that expressed only ACA IgG, and no other ACA isotypes more frequently showed an early pattern and less frequently a late pattern when compared to ACA IgG+ patients expressing two or three ACA isotypes. The differences between the groups were not statistically significantly. The ATA IgG+ patients that concurrently expressed only ATA IgA showed a late pattern less often than ATA IgG+ patients expressing all three ATA isotypes. These differences were not statistically significant.

Numerically, ACA IgG levels were higher in ACA IgG+ patients with a late SSc pattern than in ACA IgG+ patients with an early SSc pattern (median: 630 U/mL vs 200 U/mL). ATA IgM levels were higher in ATA IgG+ patients with a late SSc pattern compared to ATA IgM in patients with an early pattern (median: 1515 aU/mL vs 691 aU/mL). These results were not statistically significant (supplementary file). In the multivariable analysis with adjustment for disease duration and use of vasoactive medication, antibody isotype levels were associated with degree of microangiopathy (Table 3 and 4). For ACA IgG levels (OR 2.49, 95%CI 1.04-5.83) and for ATA IgM levels (OR 2.70, 95%CI 1.06-4.22) the associations were significantly different.

ACA IgG levels are associated with more severe microangiopathy after adjustment for disease duration and vasoactive medication.

	Univariable OR (95% CI)	**Multivariable OR (95% CI)**
	NVC SSc pattern	NVC SSc pattern
Male	0.69 (0.17-2.77)	-
Age	1.02 (0.99-1.05)	-
Disease duration since RP	1.01 (0.97-1.04)	1.01 (0.98-1.05)
Disease duration since NR	1.01 (0.95-1.07)	
Autoantibody specific IgM positive	1.21 (0.42-3.52)	-
Autoantibody specific IgA positive	1.13 (0.45-2.81)	-
Autoantibody specific IgG levels, U/ml	2.24 (0.99-5.05)	**2.46 (1.04-5.83)**
Autoantibody specific IgM levels, aU/ml	0.99 (0.60-1.65)	-
Autoantibody specific IgA levels, aU/ml	1.51 (0.67-3.37)	-
Autoantibody specific isotype expression	0.80 (0.33-1.91)	-
Immunosuppresive medication	0.64 (0.22-1.88)	-
Vasoactive medication	**3.33 (1.03-12.11)**	**1.81 (1.07-2.87)**

Table 3. Univariable and multivariable logistic regression for **anti-centromere positive** patients. NVC pattern was entered as ordinal variable, in order: early, active or late. ACA= anti-centromere antibody, ATA= anti-topoisomerase antibody, CI= confidence interval, NR= non-Raynaud, NVC= nailfold videocapillaroscopy, OR= odds ratio, RP= Raynaud Phenomenon, SSc= systemic sclerosis.

ATA IgM levels are associated with more severe microangiopathy after adjustment for age and disease duration.

	Univariable OR (95% CI)	**Multivariable OR (95% CI)**
	NVC SSc pattern	NVC SSc pattern
Male	2.20 (0.64-7.53)	-
Age	**1.06 (1.02-1.10)**	1.05 (1.01-1.10)
Disease duration since RP	**1.06 (1.01-1.11)**	1.05 (1.0-1.11)
Disease duration since NP	**1.21 (0.88-1.67)**	-
Autoantibody specific IgM positive	1.29 (0.43-3.82)	-
Autoantibody specific IgA positive	1.00 (1.00-1.00)	-
Autoantibody specific IgG levels, U/ml	1.24 (0.41-3.77)	-
Autoantibody specific IgM levels, aU/ml	2.24 (0.97-5.18)	**2.70 (1.06-4.22)**
Autoantibody specific IgA levels, aU/ml	0.96 (0.42-2.18)	-
Autoantibody specific isotype expression	1.29 (0.43-3.82)	-
Immunosuppresive medication	1.65 (0.56-4.86)	-
Vasoactive medication	1.33 (0.30-5.96)	-

Table 4. Univariable and multivariable logistic regression for **anti-topoisomerase positive** patients. NVC pattern was entered as ordinal variable, in order: early, active or late. ACA= anti-centromere antibody, ATA= anti-topoisomerase antibody, CI= confidence interval, NR= non-Raynaud, NVC= nailfold videocapillaroscopy, OR= odds ratio, RP= Raynaud Phenomenon, SSc= systemic sclerosis.

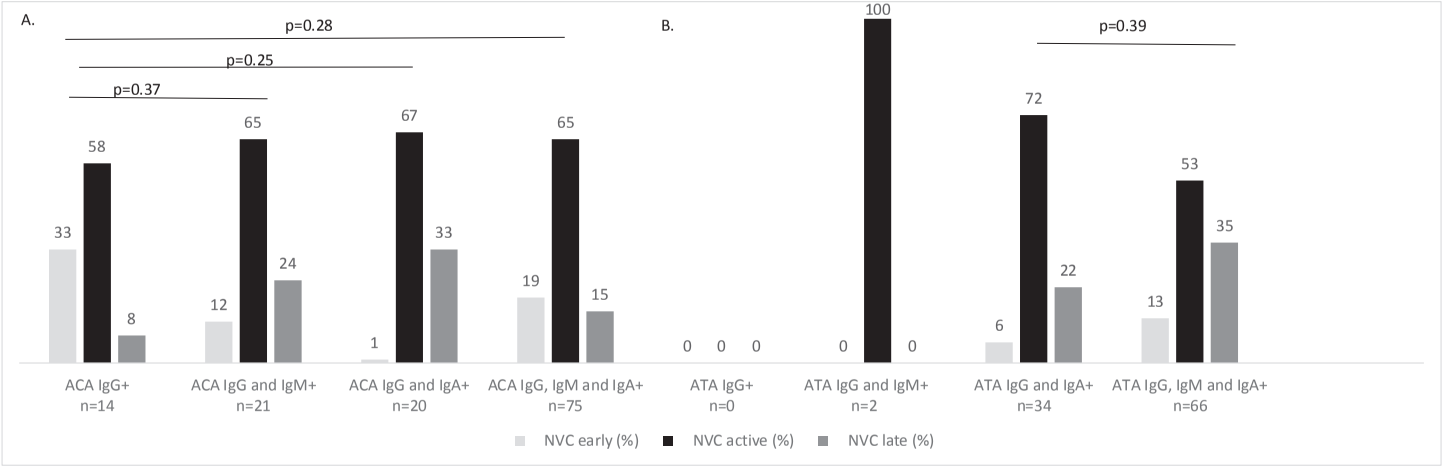

Figure 2. Presence of one, two or three autoantibody specific isotypes and the distribution of early, active or late SSc pattern. The significant values are on group level; 1A:group ACA IgG+ vs. ACA IgG+ and IgM+ p= 0.37 group ACA IgG + vs ACA IgG+ and IgM+ p= 0.25, group ACA IgG+ vs ACA IgG+, IgM+ and IgA+ p= 0.28 (no significant difference in NVC pattern prevalence). 1B; All ATA IgG+ patients expressed at least one additional ATA isotype. Two patients expressed ATA IgG+ and ATA IgM+ both with an active NVC pattern. Prevalence of NVC patterns was not significantly different between ATA IgG+ and IgA+ vs ATA IgG+, IgM+ and IgA+ patients (p= value 0.39). ACA= anti-centromere antibody, ATA= anti-topoisomerase antibody, NVC= nailfold videocapillaroscopy.

DISCUSSION

In our study we evaluated the association between specific ATA and ACA responses and the degree of microangiopathy in a Dutch SSc cohort. We first confirmed the association between more severe microangiopathy and organ involvement including ILD, PAH and DU in patients with SSc. Second, we showed that ATA+ SSc patients more often had severe microangiopathy compared to ACA+ patients. Finally, our results indicate a possible association between characteristics of specific antibody responses and the degree of microangiopathy. After adjustment for possible confounders, we observed a significant association between ACA IgG and ATA IgM levels and a more severe degree of microangiopathy.

In the association analysis we observed a trend for higher ATA IgM among ATA + patients with a late SSc pattern and higher ACA IgG among ACA+ patients with late SSc pattern on NVC. Only after correcting for possible confounders (including disease duration) did these associations become significant, with an association between a more severe degree of microangiopathy and levels of ACA IgG, and between a more severe degree of microangiopathy and levels of ATA IgM. The rationale behind these findings is not fully understood and we can only hypothesize about possible explanations. As ATA+ and ACA+ patients display clearly different clinical phenotypes, one might hypothesize that behaviour of ATA and ACA specific isotypes differs which might impact on their roles in pathophysiology. Further, ATA and ACA might bind to different cells or antigens which may be one reason for the differences between the two group. ATA bind to DNA topoisomerase I expressed by e.g. fibroblasts, and it may be that ATA is only pathogenic in case there is insufficient clearance of apoptotic bodies of endothelial cells containing DNA topoisomerase I. This would also fit with the observation that a more severe degree of microangiopathy is associated with organ involvement (24). ACA, can react against six different centromeric nucleoproteins, and could have a different ability in e.g. recruiting immune effector- or clearance mechanisms (25). Although ACA and ATA have been reported to react with endothelial cells, no data is published on differences between isotype binding and the effects on endothelial cells (18, 26). Another important factor in the pathogenesis of SSc including the endothelial cell damage, is the complement system. IgM and IgG have the ability to induce inflammation by activating complement, however, IgA is a weak activator for complement, this may be one of the explanations why no association between specific IgA and degree of microangiopathy was found (27).

In general, the production of IgM against protein-antigens is driven by short-lived plasmablasts derived from recently stimulated B cells and therefore presence of ATA and ACA- specific IgM suggests an ongoing active immune response. How IgM production is sustained in the presence of IgG against the same antigen is not fully understood, but

similar observations have been reported for anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis (28). Production of IgM could also result from a failure of class-switching resulting in prolonged survival of IgM- secreting plasmablasts (29). In both the ATA+ patients (65%) and the ACA+ patients (74%) continuous IgM expression next to IgG is observed, but proportionally IgM expression is more frequent in ACA+ patients.

Our results are partly in line with those reported by Markusse et al. and Caramaschi et al., who reported a relationship between organ involvement and more severe microangiopathy as assessed by NVC (12, 30). However, in these studies it was suggested that the presence of a specific IgG autoantibody is independent of the development of microangiopathy. Relations between ATA/ACA isotype profile and isotype levels and disease severity have not yet been evaluated in large SSc cohorts. ATA IgG and ATA IgA levels, and presence of ATA IgM have previously been described to correlate with skin scores and with disease severity in small cohorts (15, 31). We are currently working on verifying these results in a multicentre study.

Our study has some limitations that should be considered. As not all HRCTs were evaluated according to Goh, we were not able to discriminate between limited and extensive ILD. Therefore, we decided to apply a combined definition including presence of ILD on HRCT and FVC < 70% to make sure that only patients with clinically relevant ILD are classified as having ILD. Secondly, we only included patients positive for ATA IgG or ACA IgG at baseline we cannot fully exclude that there might be patients only positive for ACA IgM, ATA IgM, ACA IgA or ATA IgA that we did not include. This seems however unlikely as ATA IgA and IgM isotypes and ACA IgA and IgM isotype were absent in ANA+ SSc patients lacking a SSc specific antibody. To determine whether relevant ATA IgM or ATA IgA can be expected in SSc patients negative for ATA IgG, and vice versa for ACA IgM and ACA IgA, we have additionally measured expression of ATA IgM and ATA IgA in n= 38 samples of ACA IgG positive and ATA IgG negative patients, and measured expression of ACA IgM and ACA IgA in n= 46 samples of ATA IgG positive and ACA IgG negative patients. This showed that relevant expression of ATA/ACA IgM and ATA/ACA IgA is very rare in ATA/ACA IgG negative patients. Likewise, no conclusions can be drawn for the remaining antibody subgroups in SSc.

The current data were derived from a cohort study in a tertiary center where patients with a (preliminary) diagnosis of SSc were included at presentation at the out-patient clinic and therefore treatment prior to inclusion was uncontrolled. Furthermore, we do not know how (previous) immunosuppressive medication might have influenced our results. However, in our study, ACA and ATA-specific isotype levels were not different for SSc patients who used immunosuppressive medication compared to SSc patients who

did not use medication, and at baseline, the use of immunosuppressive medication was comparable between the ACA+ and ATA+ patients. Strikingly, in the multivariable analysis current use of vasoactive medication was associated with worse microangiopathy. Possibly, this association is reflecting confounding by indication, with patients with more severe microangiopathy more often suffering from DU. Finally, we decided not to include outcomes such as gastro-intestinal involvement and renal crisis in this analysis as endoscopies were not performed routinely, and the numbers for renal crisis was too low. In all patients, an HRCT and transthoracic echocardiography were performed routinely, regardless of risk factors for specific organ involvement.

In conclusion, we observed associations between specific ATA and ACA responses and the degree of microangiopathy in patients with SSc, indicating that dysregulated B cell responses and microvascular damage interact with each other in the pathophysiology of SSc. Further research is needed to confirm these observations, and to identify the possible mechanism behind this association.

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SUPPLEMENTARY FILE

ATA positivity is associated with degree of microangiopathy

	Univariable OR (95% CI)	Multivariable OR (95% CI)
	SSc pattern	SSc pattern
Male	1.54 (0.62-3.84)	-
Age	1.03 (1.00-1.05)	-
Disease duration since RP	1.01 (0.98-1.04)	1.01 (0.98-1.04)
Disease duration since NR	1.03 (0.99-1.08)	-
ATA antibody	**2.09 (1.05-4.13)**	**2.97 (1.41-6.24)**
Immunosuppresive medication	1.66 (0.81-3.39)	-
Vasoactive medication	**3.06 (1.09-8.60)**	**3.88 (1.31-11.54)**

Table 5. Logistic regression of all the patients together, not stratified for autoantibody. ATA= anti-topoisomerase antibody, SSc= systemic sclerosis, RP= Raynaud phenomenon, OR= odds ratio, CI= confidence interval.

Isotype levels compared between NVC SSc patterns in ACA+ and ATA+ SSc patients

NVC available baseline ACA+	n=99	IgG ACA level	IgM ACA level	IgA ACA level
Early SSc pattern, median (IQR)	n=12	200 (114-858)	49 (0-208)	63 (33-103)
Active SSc pattern, median (IQR)	n=56	430 (184-1067)	122 (22-602)	67 (17-152)
Late SSc pattern, median (IQR)	n=15	630 (181-1094)	50 (23-1210)	102 (23-178)
		NS	NS	NS
NVC available baseline ATA+	n=64	IgG ATA level	IgM ATA level	IgA ATA level
Early SSc pattern, median (IQR)	n=6	170 (77-969)	691 (432-871)	1848 (1285-4971)
Active SSc pattern, median (IQR)	n=34	578 (277-999)	802 (325-7288)	2782 (784-10254)
Late SSc pattern, median (IQR)	n=18	394 (164-641)	1515 (492-7718)	1810 (890-6868)
		NS	NS	NS

Table 6. ACA= anti-centromere antibody, ATA= anti-topoisomerase antibody, IgG levels U/mL, IgA and IgM aU/mL, IQR= inter quartile range, NS= non-significant, NVC= nailfold videocapillaroscopy, SSc= systemic sclerosis.