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Association Between Centromere- and Topoisomerase-specific Immune Responses and the Degree of Microangiopathy in Systemic Sclerosis

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ABSTRACT. ***Objective***. Autoreactive antibody responses, including the use of several isotypes of autoantibodies, have been shown to be associated with clinical outcome in several rheumatic autoimmune diseases. The goals of this study were to evaluate whether (1) anticentromere antibody (ACA)– and antitopoisomerase 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–positive (+) and ATA IgG+ patients with SSc. The degree of microangiopathy was determined based on nailfold videocapillaroscopy (NVC) images collected at the same point in time. 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 the 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 who expressed solely 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 microangiopathy severity.

Conclusion. 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, interacts in the pathophysiology of SSc.

Key Indexing Terms: autoantibodies, microangiopathy, pathophysiology, systemic sclerosis

Systemic sclerosis (SSc) is characterized 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 disease classification and are associated with specific clinical manifestations, making them important tools for disease prognostication[3]. Anticentromere antibodies (ACA) and antitopoisomerase antibodies (ATA) are the 2 most common ANA in patients with SSc[3]. Of these, ACA is associated with a relatively mild disease course, while ATA is associated with a 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 and 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 standardized magnification to visualize the capillaries in the nailfold. In SSc, specific patterns of capillary changes and the extent of these changes have been defined extensively[5]. More severe microangiopathy is associated with worse disease in patients with SSc, and in recent studies an association between NVC pattern, organ involvement, and disease progression was found[6,7,8,9,10,11,12]. 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].

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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+ patients with SSc who showed disease progression, and higher ACA IgG and ACA IgM levels in ACA+ patients with SSc 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 phenomenon (RP). Together, these studies implicate a possible association between ACA, ATA, and 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 autoimmune response and the degree of microangiopathy could not only improve our insights in disease pathogenesis, but could also contribute to a 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, associates with more severe microvascular damage.

MATERIALS AND METHODS

Study design and patients. Patients with SSc 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-h Holter ECG, and/or right heart catheterization (RHC) are performed if indicated. In our current study, patients who fulfilled the American College of Rheumatology/European League Against Rheumatism 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 [i.e., vasoactive (calcium channel blockers, sildenafil, bosentan, and iloprost) and immunosuppressive medication (corticosteroids, cyclophosphamide, azathioprine, methotrexate, mycophenolate mofetil, and azathioprine)] 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.

NVC. NVC was performed at the same time 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°C. All fingers, except for the thumbs on both hands, were examined using a videocapillaroscope (Videocap 3.0: 2009–2015; DS Medica: 2015–2017; Inspectis Pro: 2018 onwards) equipped with a probe with 200× magnification. NVC images were scored by trained observers and classified qualitatively as previously described: "normal pattern," "nonspecific pattern," and "scleroderma pattern"[21]. A normal pattern was defined as a pattern of typical hairpinlike capillaries with a regular distribution. A nonspecific pattern was defined as a pattern with abnormalities without fulfilling the definition of a scleroderma pattern[21,22]. A scleroderma pattern was defined according to the standards set by Cutolo, *et al* and categorized into an "early," "active," or "late" pattern[21,23]. 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/mm) as its main denominator. For our current evaluation, the images were reexamined by a trained investigator (NvL). The interobserver agreement was high for qualitative pattern determination (ICC 0.97).

ACA and ATA assay and measurements. Total Ig ATA, IgG, IgM, and IgA, and total Ig ACA, IgG, IgM, and IgA levels of all the collected samples were measured in baseline samples by fluorescence enzyme-linked immunosorbent assay (FEIA), using the Phadia 250 system (ThermoFisher Scientific). The cutoff levels for ATA and ACA IgG were set at 7 units/mL (U/mL), according to the manufacturer's instructions. Fifty serum samples of nonrheumatic age- and sex-matched subjects were measured to establish cutoff values (mean +2SD) for IgM and IgA isotypes of ACA and ATA. A cutoff 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 patients with SSc who were negative for ATA IgG were tested and all had ATA-IgM and IgA levels below the defined cutoff. In addition, 10 patients with SSc who were negative for ACA IgG were tested for ACA IgM and ACA IgA, and these levels were also below the defined cutoff. An "expressed isotype" was defined as a level above the cutoff value. Outliers were checked and remeasured when necessary.

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 of ILD on HRCT. An experienced radiologist evaluated the HRCT for ground-glass opacifications, reticulations, and honeycombing. We chose to use a combined value that included both pulmonary function and HRCT to make sure that we classified only patients with clinically relevant pulmonary involvement as having ILD. Pulmonary arterial hypertension (PAH) was defined as an increase in mean pulmonary arterial pressure ≥ 25 mmHg at rest, as assessed by RHC. This included the presence of precapillary pulmonary hypertension (PH), defined by a pulmonary capillary wedge pressure (PCWP) ≤ 15 mmHg and a pulmonary vascular resistance > 3 Wood units 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) of 44–49%, grade 2 signifies arrhythmias and an LVEF 40–45%, and grade 3 indicates severe involvement with an LVEF < 40%. As the Medsger severity scale relies mainly on the LVEF for determination of myocardial involvement, using only this variable could lead to underestimation of its presence, since in patients with SSc with myocardial involvement, LVEF is not always below the normal cutoff. Therefore, we additionally used a combined value for which patients had to have at least 2 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 SPSS v.23.0 (IBM Corp.). 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+ patients with

SSc using descriptive statistics, and differences were tested for significance as appropriate. Disease duration was defined as the duration since onset of RP, as current SSc pathophysiology indicates 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 (univariate and multivariate) was performed, with autoantibodies, NVC pattern, isotype expression, and ACA or ATA isotype levels as independent factors, and organ involvement as a dependent variable. Ordinal logistic regression analyses were also performed, with disease characteristics, autoantibodies, isotype expression, and ACA or ATA IgG, IgM, and IgA levels as independent variables, and NVC SSc patterns as dependent variables. Since age and disease duration can be confounders 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 patients with SSc (129 ACA+, 102 ATA+) were included. The included patients had a mean age of 55 years (SD 14) and a median disease duration from onset of 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+ patients 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+ groups 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 yrs vs 6 yrs). Complete data on NVC patterns were available for 164 patients (100 ACA+, 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 univariate 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% CI 1.17–4.47). Multivariate analysis showed that a more severe degree of microangiopathy was 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 seen more often numerically in ATA+ patients compared to ACA+ patients (31% vs 18%; P = 0.05, after Bonferroni correction; Table 1). The frequencies of early (10% ATA+, 18% ACA+) and active (58% ATA+, 68% ACA+; Table 1) SSc patterns were comparable between ATA+ and ACA+ patients. In the multivariate analysis (Supplementary Material, available from the authors upon request), after adjustment for vasoactive medication, age, and disease duration, ATA positivity was associated with more severe microangiopathy (OR 2.97, 95% CI 1.41–6.24) compared to ACA positivity.

Isotype expression. In ACA IgG+ patients, 74% (n = 95) were ACA IgA+ and 74% (n = 95) were ACA IgM+. Of the ACA+ patients, 11% expressed solely ACA IgG, 16% were positive for IgG and IgM, 16% were positive for IgG and IgA, and 58% were positive for ACA IgG, IgM, and IgA. All ATA IgG+ patients

Table 1. Baseline characteristics of included ACA+ and ATA+ SSc patients.

	Total Group	ACA+	ATA+
Demographics	n = 231	n = 129	n = 102
Sex, female	186 (81)	116 (90)	70 (69)
Age, yrs, mean (SD)	55 (14)	58 (13)	51 (14)
Smoking, ever	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	49 (21)	2 (2)	47 (46)
Puffy fingers	74 (33)	38 (30)	36 (37)
Sclerodactyly	151 (67)	71 (55)	80 (82)
mRSS, median (IQR)	4 (2–6)	3 (1–5)	6 (2–12)
Pitting scars	106 (46)	59 (46)	47 (46)
Telangiectasia	152 (66)	108 (84)	44 (44)
Digital ulcers	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	55 (24)	11 (9)	44 (43)
PAH	20 (13)	10 (8)	10 (10)
NVC			
Early	22 (15)	16 (18)	6 (10)
Active	90 (63)	56 (68)	34 (58)
Late	33 (23)	15 (18)	18 (31)
Capillary loss < 7 mm	114 (70)	64 (65)	50 (78)
Medication			
CYC	39 (17)	19 (15)	20 (20)
HSCT	14 (7)	7 (7)	7 (7)
Iloprost/bosentan	17 (12)	14 (11)	13 (13)
MTX, ever	44 (19)	23 (18)	21 (20)
ACA or ATA characteristics			
IgA positivity	–	95 (74)	100 (98)
IgA level (AU/mL), median (IQR)	–	73 (34–146)	2778 (933–8368)
IgM positivity	–	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)

Values are n (%) unless otherwise stated. ACA: anticentromere antibody; ATA: antitopoisomerase antibody; CYC: cyclophosphamide; dcSSc: diffuse cutaneous SSc; DLCO: carbon monoxide diffusing capacity; FVC: forced vital capacity; HRCT: high-resolution computed tomography; HSCT: stem cell transplantation; ILD: interstitial lung disease; mRSS: modified Rodnan skin score; MTX: methotrexate; NVC: nailfold video-capillaroscopy; PAH: pulmonary arterial hypertension; RP: Raynaud phenomenon.

Table 2. ATA and more severe microangiopathy is associated with organ involvement.

	Univariate, OR (95% CI)				Multivariate, OR (95% CI)			
	ILD	Cardiac Involvement	PAH	DU	ILD	Cardiac Involvement	PAH	DU
Sex, male	1.71 (0.84–3.48)	0.59 (0.26–1.34)	1.04 (0.33–3.27)	1.32 (0.55–3.20)	–	–	–	–
Age, yrs	1.01 (0.99–1.04)	**1.07 (1.03–1.10)**	**1.08 (1.03–1.13)**	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)
Disease duration since RP	0.99 (0.95–1.01)	1.04 (0.98–1.03)	1.00 (0.99–1.05)	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)
Disease duration since non-RP	1.03 (0.99–1.06)	1.01 (0.97–1.05)	1.01 (097–1.07)	**1.04 (1.01–1.08)**	–	–	–	–
ATA	**6.68 (1.87–23.94)**	0.96 (0.47–1.95)	1.29 (0.52–3.24)	**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)
NVC SSc pattern	**3.59 (1.75–15.91)**	**2.95 (1.20–7.23)**	**5.85 (1.90–18.65)**	**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)**
Immunosuppressives	**4.64 (1.57–13.66)**	1.08 (0.50–2.31)	0.53 (0.26–1.99)	0.46 (0.20–1.07)	1.79 (0.45–7.01)	–	–	–

In the multivariate logistic regression autoantibody, age, disease duration (since onset RP), and variables with significant association in univariate analysis were included. ILD was defined as ILD on HRCT and FVC < 70% of predicted. NVC pattern was entered as the ordinal variable in the following order: early, active, or late. Significant values are in bold. ATA: antitopoisomerase antibody; DU: digital ulcers; FVC: forced vital capacity; HRCT: high-resolution computed tomography; ILD: interstitial lung disease; NVC: nailfold videocapillaroscopy; PAH: pulmonary arterial hypertension; RP: Raynaud phenomenon; SSc: systemic sclerosis.

expressed more than 1 ATA-specific isotype: ATA IgA+ was found in 98% (n = 100) and ATA IgM+ was found in 65% (n = 66). Two percent of ATA IgG+ patients also expressed ATA IgM in the absence of detectable ATA IgA, 33% expressed ATA IgA in the absence of detectable ATA IgM, and 65% expressed all 3 ATA isotypes (data not shown).

Association of ACA and ATA isotype expression with the degree of microangiopathy. As shown in Figure 1, the ACA IgG+ patients that expressed only ACA IgG and no other ACA isotypes more frequently showed an early pattern and less frequently showed a late pattern when compared to ACA IgG+ patients expressing 2 or 3 ACA isotypes. The differences between the groups were not statistically significant. The ATA IgG+ patients that concurrently expressed only ATA IgA showed a late pattern less often than ATA IgG+ patients expressing all 3 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; Figure 2). These results were not statistically significant (Supplementary Material, available from the author upon request). In the multivariate analysis with adjustments for disease duration and use of vasoactive medication, antibody isotype levels were associated with degree of microangiopathy (Tables 3 and 4). For ACA IgG levels (OR 2.46, 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.

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+ patients with SSc more often had severe microangiopathy compared to ACA+ patients. Finally, our results indicated 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 the behavior of ATA- and ACA-specific isotypes differs,

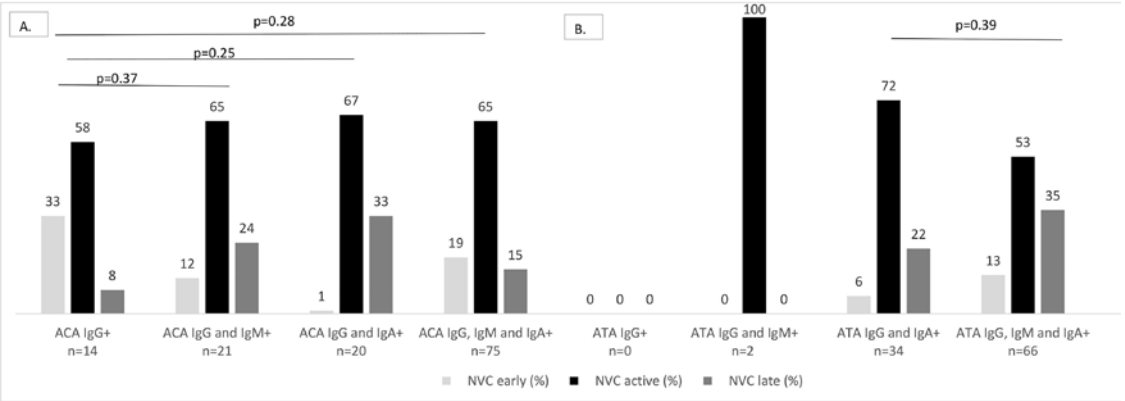

Figure 1. Presence of 1, 2, or 3 autoantibody-specific isotypes and the distribution of early, active, or late SSc pattern. The significant values are on group level. (A) ACA IgG+ vs ACA IgG+ and IgM+, $P = 0.37$; ACA IgG + vs ACA IgG+ and IgA+, $P = 0.25$; ACA IgG+ vs ACA IgG+, IgM+, and IgA+, $P = 0.28$ (no significant difference in NVC pattern prevalence). (B) All ATA IgG+ patients expressed at least 1 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+ patients vs ATA IgG+, IgM+, and IgA+ patients ($P = 0.39$). ACA: anticentromere antibody; ATA: antitopoisomerase antibody; NVC: nailfold videocapillaroscopy.

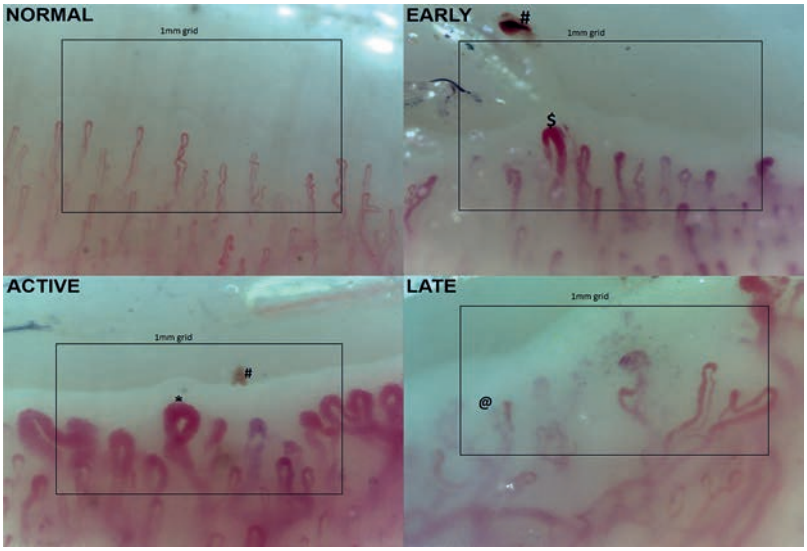

Figure 2. Nailfold videocapillaroscopy (NVC) images made with the INSPECTIS Pro. Examples of NVC images with a 1 mm grid. #: hemorrhages; $: dilation > 30 uM; *: giant (dilation > 50μm); @: abnormal shapes (neoangiogeneses)[33].

which may affect 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 2 groups. ATA bind to DNA topoisomerase I, expressed by fibroblasts, and it may be that ATA is pathogenic only when 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 6 different centromeric nucleoproteins and could have a different ability in recruiting immune effector or clearance mechanisms[25]. Although ACA and ATA have been reported to react with endothelial cells, no data have been published on differences between isotype binding and the effects on endothelial cells[18,26]. Another important factor in the pathogenesis of SSc, including 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, so this may be one of the reasons that 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 the 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 anticitrullinated protein antibodies in rheumatoid arthritis[28]. The production of IgM could also result from a failure of class-switching, thereby resulting in the prolonged survival of IgM-secreting plasmablasts[29]. In both the

Table 3. Univariate and multivariate logistic regression for ACA+ patients.

	Univariate, OR (95% CI) NVC SSc pattern	Multivariate, OR (95% CI) NVC SSc pattern
Sex, male	0.69 (0.17–2.77)	-
Age, yrs	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 non-RP	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)	-
Immunosuppressive medication	0.64 (0.22–1.88)	-
Vasoactive medication	**3.33 (1.03-12.11)**	**1.81 (1.07–2.87)**

Significant values are in bold. NVC pattern was entered as the ordinal variable in the following order: early, active, or late. ACA: anticentromere antibody; ATA: antitopoisomerase antibody; NVC: nailfold videocapillaroscopy; RP: Raynaud phenomenon; SSc: systemic sclerosis.

Table 4. Univariate and multivariate logistic regression for antitopoisomerase-positive patients.

	Univariate, OR (95% CI) NVC SSc pattern	Multivariate, OR (95% CI) NVC SSc pattern
Sex, male	2.20 (0.64–7.53)	-
Age, yrs	**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 non-RP	**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)	-
Immunosuppressive medication	1.65 (0.56–4.86)	-
Vasoactive medication	1.33 (0.30–5.96)	-

Significant values are in bold. NVC pattern was entered as the ordinal variable in the following order: early, active, or late. ACA: anticentromere antibody; ATA: antitopoisomerase antibody; NVC: nailfold videocapillaroscopy; RP: Raynaud phenomenon; SSc: systemic sclerosis.

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. Relationships between the ATA/ACA isotype profile, isotype levels, and disease severity have not yet been evaluated in large SSc cohorts. ATA IgG and IgA levels and the presence of ATA IgM have previously been described to correlate with skin scores and disease severity in small cohorts[15,31]. We are currently working on verifying these results in a multicenter study.

Our study has some limitations that should be considered. Since not all HRCT were evaluated according to Goh[32], 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 were classified as having ILD. Second, we included only patients positive for ATA IgG or ACA IgG at baseline, so we cannot fully exclude that there might have been patients who were positive only for ACA IgM, ATA IgM, ACA IgA, or ATA IgA that we did not include. However, this seems unlikely since ATA IgA and IgM isotypes

and ACA IgA and IgM isotypes were absent in ANA+ patients with SSc who were lacking an SSc-specific antibody. To determine whether relevant ATA IgM or ATA IgA can be expected in patients with SSc who were negative for ATA IgG, and similarly for ACA IgM and ACA IgA in patients who were negative for ACA IgG, we measured the expression of ATA IgM and ATA IgA in 38 samples of ACA IgG+ and ATA IgG– patients, and measured expression of ACA IgM and ACA IgA in 46 samples of ATA IgG+ and ACA IgG– patients. This showed that relevant expression of ATA/ACA IgM and ATA/ACA IgA is very rare in ATA/ACA IgG– 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 outpatient clinic; therefore, treatment prior to inclusion was uncontrolled. Further, 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 patients with SSc who used immunosuppressive medication compared to 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 multivariate analysis, current use of vasoactive medication was associated with worse microangiopathy. Possibly, this association reflects confounding by indication, with patients with more severe microangiopathy more often suffering from DU. Finally, we decided not to include outcomes such as gastrointestinal involvement and renal crisis in this analysis since endoscopies were not performed routinely and the numbers for renal crisis were too low. In all patients, an HRCT and transthoracic echocardiogram 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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