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Anticentromere Antibody Levels and Isotypes and the Development of Systemic Sclerosis

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Objective. Little is known on the disease course of very early systemic sclerosis (SSc). Among the information yet to be elucidated is whether anticentromere antibody (ACA) isotype levels can serve as biomarkers for future SSc development and for organ involvement. This study was undertaken to evaluate whether IgG, IgM, and IgA ACA levels in IgG ACA–positive patients are associated with disease severity and/or progression from very early SSc to definite SSc.

Methods. IgG ACA–positive patients from 5 different cohorts who had very early SSc or SSc fulfilling the American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) 2013 criteria were included. A diagnosis of very early SSc was based on the presence of IgG ACAs and Raynaud's phenomenon, and/or puffy fingers and/or abnormal nailfold capillaroscopy, but not fulfilling the ACR/EULAR 2013 criteria for SSc. Multivariable regression analyses were performed to determine the association between baseline ACA isotype levels and progression to definite SSc with organ involvement.

Results. Six hundred twenty-five IgG ACA–positive patients were included, of whom 138 (22%) fulfilled the criteria for very early SSc and 487 (78%) had definite SSc. Levels of IgG ACAs (odds ratio 2.5 [95% confidence interval 1.8–3.7]) and IgM ACAs (odds ratio 1.8 [95% confidence interval 1.3–2.3]) were significantly higher in patients with definite SSc. Of 115 patients with very early SSc with follow-up, progression to definite SSc occurred within 5 years in 48 (42%). Progression to definite SSc was associated with higher IgG ACA levels at baseline (odds ratio 4.3 [95% confidence interval 1.7–10.7]).

Conclusion. ACA isotype levels may serve as biomarkers to identify patients with very early SSc who are at risk for disease progression to definite SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a heterogeneous autoimmune disease with high mortality and morbidity (1,2). As early intervention has been shown to improve disease course and outcome, it is very important to detect SSc at an early stage, when therapeutic interventions can prevent progression of organ damage (3,4). The American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) 2013 criteria for SSc have a high sensitivity for accurately classifying patients as having SSc (5). However, there are still patients who do not fulfill these criteria, despite exhibiting early signs of SSc (6). Currently, there are no biomarkers to identify which patients with signs of very early SSc will progress to having definite SSc. Identification of this subgroup of patients with very early SSc, with more precise insights into

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their potential disease course, is crucial for early therapeutic interventions (7).

SSc-specific antinuclear autoantibodies (ANAs) are commonly used for disease and risk stratification. Anti–topoisomerase I antibodies and anticentromere antibodies (ACAs) are the most prevalent autoantibodies in SSc (8). The presence of ACAs is associated with limited skin involvement, higher prevalence of calcinosis, and gastrointestinal (GI) involvement (9–11). Compared to most other SSc-associated autoantibodies, the presence of ACAs generally carries a better prognosis with respect to survival (10,12). The major reactive antigen of ACAs has been identified as CENP-B, which has therefore been suggested as the primary target driving a selected B cell response characterized by IgG ACA production (13,14). Based on the observation that the generation of disease-specific ANAs is closely linked to disease development and clinical phenotype, it has been hypothesized that ANAs are implicated in disease pathogenesis (15–17). However, the exact role of these disease-specific ANAs and their underlying antigen triggers in SSc remains unclear.

In rheumatoid arthritis (RA), an autoimmune disease characterized by polyarthritis and the presence of rheumatoid factor and anti–citrullinated protein antibodies (ACPAs), an extended ACPA repertoire has been shown to be associated with disease development and disease severity, while the effector function of ACPAs is still not elucidated (18–20). At present, little information is available regarding ACA isotype levels in SSc. Detailed information on the ACA isotype distribution in ACA-positive patients with SSc can contribute to a better understanding of the characteristics and dynamics of the underlying autoreactive B cell response. Consistent with findings in RA, we hypothesize that in IgG ACA–positive SSc, the expansion of specific ACA isotype responses is associated with SSc development and severity, as reflected by organ involvement.

By taking advantage of the prospective and comprehensive clinical data available from 5 independent and well-described SSc cohorts (Leiden, Oslo, Zurich, Ghent, and Bordeaux), we aimed to evaluate whether individual ACA isotype levels are associated with disease severity in IgG ACA–positive patients with SSc. We also investigated whether these levels can identify subjects with very early SSc whose condition will progress to definite SSc.

PATIENTS AND METHODS

Patient population. The SSc cohorts in Leiden, Oslo, Zurich, Ghent, and Bordeaux are prospective cohorts including all consecutive patients with SSc (21–26). Patients in these cohorts undergo annual extensive screening, which includes a complete physical examination, laboratory testing, pulmonary function testing, transthoracic echocardiography, high-resolution computed tomography (HRCT), 24-hour electrocardiography, nailfold capillaroscopy (NFC) evaluation, and an optional cardiopulmonary exercise test. At every visit, blood samples are collected and stored in respective biobanks (27).

IgG ACA–positive patients who were included had to fulfill either the ACR/EULAR 2013 criteria for SSc (5) or criteria for very early SSc (28,29). Patients were classified as having very early SSc if they were IgG ACA positive and had Raynaud's phenomenon (RP) and/or puffy fingers and/or abnormal NFC, but did not fulfill the ACR/EULAR 2013 criteria for SSc (5,28,29). In this study, we used a prospectively collected data set from routine practices with post hoc analyses. Patients entering the cohorts before March 2019 were selected for the present study. Detailed information on all of the cohorts has been previously reported (21,23–25,30–32).

Ethics approval. Collection of biomaterials and analysis of their clinical associations was approved by the local ethics committees in Leiden (CME no. B16.037), Switzerland (no. PB 2016-02014 02014 and BASEC-Nr. 2018-01873), Norway (no. 2006/119), Ghent (no. 2008/385), and Bordeaux (no. 2012-A00081-42). All participants provided written informed consent.

Clinical characteristics. At the baseline visit, clinical data and blood samples (including samples obtained for autoantibody testing) were collected from all patients. Baseline was defined as the first visit in the SSc care pathway, which included screening for SSc. Patients were involved in the development of the SSc care pathway. Patients with SSc who fulfilled the ACR/EULAR 2013 criteria were categorized as having definite SSc either without organ involvement or with organ involvement, as described below.

For analyses, patients were categorized as having 1) very early SSc, 2) definite SSc without organ involvement, or 3) definite SSc with organ involvement. Follow-up data were collected only for the very early SSc group, as data from this group of patients were of particular interest due to the possibility of intervention early in their disease course. Follow-up consisted of an annual assessment in the SSc care pathway to monitor the course of the disease, including evaluations of the organ systems (skin, lung, heart, GI, renal, and musculoskeletal). Follow-up duration was defined as the time calculated from the baseline visit to the most recent visit. Disease duration was defined as the time from RP onset, since among patients with very early SSc, data on the time from onset of the first non-RP symptom were missing in those who did not have puffy fingers. We recorded the clinical characteristics required to evaluate the disease status of the patients (very early SSc, SSc with organ involvement, or SSc without organ involvement). The modified Rodnan skin thickness score (MRSS) (33), sclerodactyly, puffy fingers, peripheral vascular involvement including pitting scars, digital ulcers, and telangiectasia were evaluated and reported by the physician during evaluation. The NFC result was considered

abnormal if a scleroderma pattern was present, in accordance with the definitions approved by the EULAR Study Group on Microcirculation in Rheumatic Diseases and Scleroderma Clinical Trials Consortium (34,35). Use of immunosuppressive treatment at baseline, including hydroxychloroquine, mycophenolate mofetil, methotrexate, cyclophosphamide, azathioprine, and glucocorticoids, was recorded. Only ~0.5% of the patients were receiving biologic treatment at baseline; therefore, this was not considered.

Organ involvement. Digital ulcers were defined as areas with visually discernible depth and a loss of continuity of epithelial coverage and included both ischemic and traumatic ulcers. Interstitial lung disease (ILD) was considered to be present when evidenced on HRCT imaging. Myocardial involvement was assessed using a modified Medsger severity scale (36), which consists of at least 2 of the following: arrhythmias (>2% supraventricular and ventricular extrasystoles, or atrial fibrillation), conduction problems (bundle branch block), decreased left ventricular ejection fraction <54%, diastolic/systolic dysfunction, pericarditis, or pericardial effusion.

Pulmonary arterial hypertension (PAH) was defined as an increase in mean pulmonary arterial pressure of ≥25 mm Hg at rest as assessed by right heart catheterization, including the presence of precapillary pulmonary hypertension (PH), defined by a pulmonary capillary wedge pressure of ≤15 mm Hg and a pulmonary vascular resistance of >3 Wood units, in the absence of other causes of precapillary PH (i.e., PH due to lung diseases, chronic thromboembolic PH, or other rare diseases) (37). Renal crisis was defined based on clinical data (including an increase in blood pressure, increase in creatinine level, and oligo/anuric renal failure). GI involvement was defined based on a composite variable: presence of confirmed gastric antral vascular ectasia (GAVE) (data available for all patients), presence of fecal incontinence (data available for 413 of 625 patients), and/or malabsorption syndrome (data available for 317 of 625 patients), and/or weight loss >10% in 1 year (data available for 309 of 625 patients). Patients with very early SSc were considered to be progressors to definite SSc if they developed ILD, digital ulcers, PAH, renal crisis, myocardial involvement, or GI involvement and if they met the ACR/EULAR 2013 criteria during follow-up.

Anticentromere assay and measurement. Blood samples were stored, collected, and processed in accordance with the European Scleroderma Trials and Research biobank recommendations (27). All baseline samples were assessed in the clinical chemistry department of the Leiden University Medical Center. Total IgG, IgA, and IgM ACA levels (CENP-B) in all samples were measured by one of the authors (JAB) with an enzyme immunoassay (FEIA) using a Phadia 250 system (ThermoFisher Scientific). Immunofluorescence (IF) patterns were evaluated at baseline, and centromere ANA patterns (speckled) were found. IgG ACA levels (IgG CENP-B) were usually measured at commercial laboratories. The cutoff level for IgG ACA positivity was set at 7 units/ml, according to the manufacturer's instructions. IgM and IgA ACAs levels were defined as research-only parameters by the manufacturer. To define cutoff values for these parameters, levels in sera from 50 healthy subjects were measured, and the cutoff values for the presence of IgM and IgA were defined as 2 SD above the mean in these serum samples. The cutoff value for IgA ACAs was 37 arbitrary units (AU)/ml, and the cutoff value for IgM ACAs was 13 AU/ml.

Statistical analysis. Analyses were performed using IBM SPSS version 23, and graphs were created using GraphPad Prism 7 software. Descriptive statistics were used to summarize clinical and serologic features. To compare continuous independent variables, the Mann-Whitney U test was used for 2 groups, and the Kruskal-Wallis test with correction for multiple comparisons was used for >2 groups. Categorical variables were compared using the chi-square test. To evaluate cross-sectional associations between isotype levels and disease status, we used binary logistic regression with adjustment for age and disease duration (isotype levels [continuous; each isotype tested in a separate model] as predictor, and disease status [very early SSc, definite SSc without organ involvement, or definite SSc with organ involvement] as outcome measure).

Longitudinal analyses included the clinical evaluation of patients with very early SSc over time and progression of organ involvement in patients with definite SSc. To compare the clinical differences between progressors and nonprogressors, the Mann-Whitney U test and chi-square test were used. Multivariable logistic regression was used to assess the independent association between isotype levels and disease progression (isotype levels [continuous; each isotype tested in a separate model] as predictor, and disease progression [yes/no] as outcome measure), and receiver operating characteristic (ROC) curves were evaluated (Supplementary Figure 1 available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). The possibility of predicting disease progression to definite SSc based on IgG ACA level was evaluated. Data from the previous evaluation were carried forward and applied if follow-up data on ILD, PAH, and ejection fraction were missing (generally occurring if disease was clinically stable with stable pulmonary function test results, meaning additional testing was not performed). As data on the individual components of the composite GI involvement variable were not complete for all patients, the validity of this parameter was verified in a sensitivity analysis using the subgroup with complete data (Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract).

Data availability. All data relevant to this study are available in the manuscript, supplementary data files, and upon request from the corresponding author.

RESULTS

Clinical characteristics. In total, 625 IgG ACA–positive patients were included. Ninety percent (n = 558) were women, with a mean age of 58 years and a median disease duration since the first non-RP symptom of 6 years (interquartile range 2–9). Baseline characteristics of the 3 clinical groups are shown in Table 1. One hundred thirty-eight patients (22%) had very early SSc, 240 (38%) had SSc without organ involvement, and 247 (40%) had SSc with organ involvement.

IgG, IgM, and IgA ACA levels. Among all IgG ACA–positive subjects, 437 (76%) were also IgA ACA positive at baseline and 522 (89%) were also IgM ACA positive at baseline. A noncutaneous disease subset was more common in patients who were positive for both IgG and IgA ACAs compared to patients who were positive for IgG and IgM ACAs and patients who were positive for all 3 isotypes (47% versus 33% versus 27%, respectively). No other clinical differences were observed between subgroups defined by numbers of expressed isotypes (data not shown). Among patients in the very early SSc group, IgG and IgM ACA levels were significantly lower compared to patients in the definite SSc group (Figure 1 and Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). Using logistic regression with adjustment for age and disease duration since the first RP symptom, we found a significant association of IgG ACA levels (odds ratio 2.54 [95% confidence interval 1.75–3.69]) and IgM ACA levels (odds ratio 1.77 [95% confidence interval 1.34–2.34]) with disease status, with higher levels in patients with SSc (with and without organ involvement combined) compared to those with very early SSc. No significant associations were found between IgG, IgM, or IgA ACA isotype levels and definite SSc with or without organ involvement (Table 2). Findings confirming the same trend across all SSc centers are shown in Supplementary Table 2 and Supplementary Figures 3 and 4 (http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract).

To assess a possible effect of immunomodulatory treatment on ACA isotype levels, we performed a logistic regression analysis. No significant associations were found between use of immunosuppressive treatment and IgG ACA levels (odds ratio 1.4 [95% confidence interval 0.91–2.10]), IgM ACA levels (odds ratio 0.91

Table 1. Baseline characteristics and ACA isotype expression and levels in patients with very early SSc and patients with SSc without or with organ involvement*

Characteristic (n with data available)	Patients with very early SSc (n = 138)	Patients with SSc without organ involvement (n = 240)	Patients with SSc with organ involvement (n = 247)
Female (n = 625)	125 (91)	225 (91)	208 (87)
Age, median (IQR) years (n = 625)	52 (40–62)	57 (49–66)	62 (52–69)
Time since RP onset, median (IQR) years (n = 622)	5 (1–12)	10 (3–19)	8 (2–18)
Time since non-RP onset, median (IQR) years (n = 465)	NA	5 (2–11)	6 (2–12)
lcSSc (n = 482)	NA	202 (84)	187 (78)
dcSSc (n = 482)	NA	14 (6)	27 (11)
MRSS, median (IQR) (n = 589)	0 (0–0)	3 (0–5)	4 (0–6)
Digital ulcers (n = 616)	0	0	81 (33)
FVC % predicted, mean ± SD (n = 585)	107 (17)	107 (17)	107 (19)
DLco % predicted, mean ± SD (n = 596)	81 (15)	74 (14)	67 (18)
ILD on HRCT (n = 625)	0	0	86 (36)
PAH (n = 625)	0	0	52 (21)
Myocardial involvement (n = 563)	0	0	42 (22)
Renal crisis (n = 625)	0	0	3 (1)
GI involvement (n = 625)	0	0	120 (49)
Puffy fingers (n = 548)	21 (16)	71 (39)	36 (23)
Abnormal NFC (n = 488)	69 (55)	160 (84)	149 (86)
Immunosuppressive treatment (n = 625)	25 (18)†	48 (20)	112 (46)
ACA characteristics			
IgA positivity (n = 617)	88 (72)	177 (78)	172 (75)
IgM positivity (n = 617)	106 (86)	209 (91)	207 (90)
IgG level, median (IQR) units/ml (n = 617)	274 (93–662)	480 (197–990)	619 (263–1,077)
IgM level, median (IQR) AU/ml (n = 617)	101 (41–363)	183 (55–907)	251 (63–965)
IgA level, median (IQR) AU/ml (n = 617)	69 (35–103)	78 (39–166)	86 (37–187)

* Except where indicated otherwise, values are the number (%). ACA = anticentromere antibody; SSc = systemic sclerosis; IQR = interquartile range; RP = Raynaud's phenomenon; NA = not applicable; lcSSc = limited cutaneous SSc; dcSSc = diffuse cutaneous SSc; MRSS = modified Rodnan skin thickness score; FVC = forced vital capacity; DLco = diffusing capacity for carbon monoxide; ILD = interstitial lung disease; HRCT = high-resolution computed tomography; PAH = pulmonary arterial hypertension; GI = gastrointestinal; NFC = nailfold capillaroscopy.
† Eight patients were treated with glucocorticoids, 12 with methotrexate, and 5 with hydroxychloroquine.

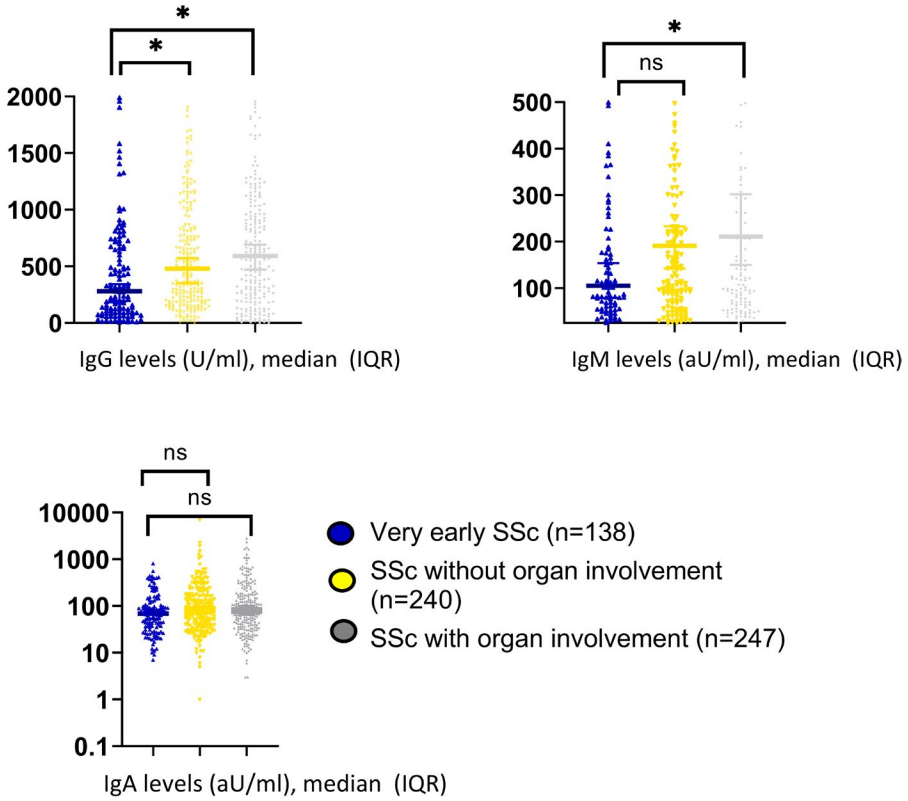

Figure 1. Anticentromere antibody (ACA) isotype levels in patients with very early systemic sclerosis (SSc), those with definite SSc without organ involvement, and those with definite SSc with organ involvement. Levels of IgG, IgM, and IgA ACAs in each group are shown. IgG and IgM ACA levels were significantly higher in patients with definite SSc with organ involvement compared to those with very early SSc; IgG ACA levels were also significantly higher in patients with definite SSc without organ involvement compared to those with very early SSc. Symbols represent individual patients; bars show the median and interquartile range (IQR). * = $P < 0.05$. NS = not significant.

[95% confidence interval 0.68–1.22]), or IgA ACA levels (odds ratio 0.74 [95% confidence interval 0.43–1.29]).

Evolution of very early SSc to definite SSc. Of the 138 patients classified as having very early SSc, 23 were lost to follow-up (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). In total, 48 (42%) experienced disease progression to definite SSc during a median follow-up period of 2 years (range 1–4). Of these progressors, 22 (46%) developed vital organ involvement, which consisted of ILD (n = 10; 21%), cardiac involvement (n = 5; 10%), or GI involvement (n = 7; 16%). Seventy-seven percent of progressors developed skin involvement, including an increase based on the minimum clinically important difference in MRSS (n = 11; 23%) (38), development of telangiectasia (n = 31; 65%), or sclerodactyly (n = 18; 38%). Both digital ulcers and pitting scars occurred in 17% of progressors. The remaining 67 patients did not develop organ involvement and their disease did not progress to fulfilling the ACR/EULAR 2013 criteria for SSc after a median follow-up of 2 years (range 1–5).

Compared to patients considered to be nonprogressors, those who were considered to be progressors were older and had a longer median follow-up duration (Table 3). IgG ACA levels were significantly higher in progressors compared to nonprogressors at baseline and also after adjustment for follow-up duration

Table 2. Association of IgG and IgM ACA levels with baseline disease status and progression to definite SSc*

	SSc vs. very early SSc, OR (95% CI)	SSc with organ involvement vs. SSc without organ involvement, OR (95% CI)	Very early SSc progression to definite SSc, OR (95% CI)
IgG ACA units/ml	2.54 (1.75–3.69)	1.09 (0.77–1.53)	4.27 (1.70–10.71)
IgM ACA AU/ml	1.77 (1.34–2.34)	1.11 (0.83–1.26)	1.75 (0.97–3.14)
IgA ACA AU/ml	1.40 (0.90–2.17)	0.96 (0.67–1.38)	1.36 (0.47–3.96)

* Odds ratios (ORs) were adjusted for age and disease duration. IgG, IgM, and IgA anticentromere antibody (ACA) levels were $\log_2$-transformed to overcome skewness in the data. Data on ACA isotype levels were available for 115 patients with very early systemic sclerosis (SSc). 95% CI = 95% confidence interval.

Table 3. Demographic and clinical characteristics of the patients with very early SSc whose disease progressed (progressors) and patients with very early SSc whose disease did not progress (nonprogressors) at follow-up*

	Progressor group (n = 48)	Nonprogressor group (n = 67)	*P*
Demographic characteristics			
Female	43 (90)	61 (91)	0.52
Age, mean ± SD years	53 (15)†	48 (13)	0.03
Disease duration since RP onset, median (IQR) years	5 (2–11)	6 (2–14)	0.69
Follow-up duration, median (IQR) years	5 (3–7)‡	2 (1–5)	<0.001
Clinical features			
Puffy fingers§	7 (15)	8 (12)	0.55
Abnormal NFC¶	26 (54)	34 (51)	0.45
ACA isotype#			
IgM positivity	36 (86)	51 (81)	0.36
IgA positivity	31 (76)	43 (68)	0.28

* Except where indicated otherwise, values are the number (%). No clinical follow-up data were available for 23 patients with very early systemic sclerosis (SSc). RP = Raynaud's phenomenon; IQR = interquartile range; NFC = nailfold capillaroscopy; ACA = anticentromere antibody.
† $P = 0.03$ versus nonprogressor group.
‡ $P < 0.001$ versus nonprogressor group.
§ Missing data for 10 patients.
¶ Missing data for 2 patients.
# Missing data for 9 patients (5 in the progressor group and 4 in the nonprogressor group).

(Figure 2). In logistic regression analyses with correction for age and follow-up duration, IgG ACA levels were significantly associated with disease progression to definite SSc (odds ratio 4.27 [95% confidence interval 1.70–10.71]) (Table 2). Puffy fingers were a significant predictor of progression to definite SSc in the univariable analysis (odds ratio 2.95 [95% confidence interval 1.31–6.62]),

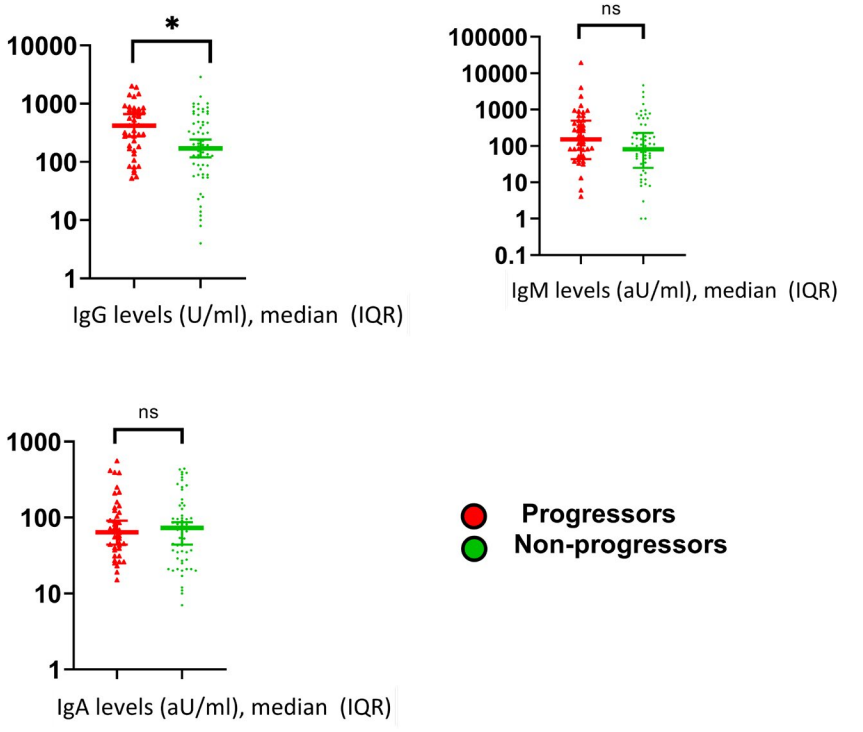

Figure 2. Anticentromere antibody (ACA) isotype levels in progressors and nonprogressors in the very early systemic sclerosis (SSc) group. IgG ACA levels were higher in patients with very early SSc whose condition progressed to definite SSc compared to nonprogressors. Symbols represent individual patients; bars show the median and interquartile range (IQR). * = $P < 0.05$. NS = not significant. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract.

whereas abnormal NFC did not show a significant association with progression to definite SSc (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). The ROC curves for progression to definite SSc in association with levels of IgG and IgM ACAs are shown in Supplementary Figures 1 and 5, with performance characteristics shown in Supplementary Tables 5 and 6 (http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). When we applied a threshold for the optimal sensitivity and negative predictive value for prediction of progression to definite SSc, we identified an IgG ACA level of 81 units/ml together with the presence of puffy fingers as having predictive capacity at baseline (Supplementary Figure 7). With this cutoff, 84% of progressors and 49% of nonprogressors were classified correctly at baseline. To further evaluate the predictive value of ACA isotype levels in SSc progression, we assessed their association with disease progression in patients with definite SSc at baseline and complete clinical data available at follow-up (n = 93) (Supplementary Table 5, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). In this subgroup, levels of IgG ACAs (odds ratio 2.79 [95% confidence interval 1.08–7.26]) and levels of IgM ACAs (odds ratio 2.06 [95% confidence interval 1.18–3.61]) were independently associated with disease progression.

DISCUSSION

In this study, we analyzed ACA isotype levels in patients with very early SSc and in patients with definite SSc to evaluate whether disease severity within ACA-positive patients is associated with characteristics of the ACA immune response. Secondly, we evaluated the clinical course of patients with very early SSc and assessed whether ACA isotype levels can identify subjects whose condition will progress to definite SSc. We demonstrated that patients with definite SSc have higher levels of IgG and IgM ACAs compared to patients with very early SSc. Moreover, we showed that in patients with very early SSc, higher levels of IgG ACAs are associated with disease progression to definite SSc within 2 years.

The lower IgG and IgM ACA levels in the very early SSc group might indicate a less pronounced immune response compared to the definite SSc group. We identified the highest levels of IgG, IgM, and IgA ACAs to be present in patients with SSc with organ involvement, and in patients with definite SSc, baseline IgG and IgM ACA levels were associated with future disease progression. These findings are consistent with our hypothesis that the immune response in patients with very early SSc is less pronounced compared to patients with definite SSc.

As shown by our data and by other study findings (30), although the classification might suggest short disease duration, some patients classified as having very early SSc showed similar disease duration as patients with definite SSc. This indicates that patients who fulfill the classification criteria for very early SSc are a heterogeneous group, in which the condition will eventually progress to definite SSc in some and will continue to only meet criteria for very early SSc in others (30). Our data show that the levels of ACA-specific immune response discriminate between those 2 subgroups (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). Similar findings have been observed in other rheumatic diseases, including RA (18,39). The observation that IgG ACA levels are numerically higher in patients with SSc who are positive for ACAs with organ involvement compared to patients with SSc who are positive for ACAs without organ involvement is consistent with our hypothesis. However, in this comparison, the differences observed were not statistically significant. We presume that the absence of broadly validated outcome measures for SSc might at least partially explain this lack of significance. Moreover, commonly accepted definitions of severe organ involvement such as ILD and diffuse skin involvement might be less sensitive in ACA-positive SSc, as severe fibrotic disease complications are less frequent in patients with ACAs than in anti–topoisomerase I–positive patients.

To date, the effects of disease duration on isotype levels are not fully understood. One could hypothesize that isotype levels decrease over time as antigen triggering diminishes (40). Whether ACA-specific immune response occurs before clinical disease development, and for how long, is unknown. One study showed that in patients with early SSc, the median duration from the time of RP onset to definite SSc was 4.6 years (41). There were no data reported regarding the first instance of specific antibody expression or different autoantibodies in that study. In our very early SSc group, 48 patients (42%) developed definite SSc over a median time period of 5 years.

We observed the strongest association between IgG ACA levels and disease subset (very early SSc versus definite SSc). Consistent with our hypothesis, it is tempting to speculate that either IgG ACAs and/or B cell responses underlying ACA production are involved in the disease pathogenesis. Since both microangiopathy (clinically shown by RP) and dysregulated immunity (reflected by the presence of specific ANAs) are among the earliest features of SSc, it could be speculated that specifically IgG and IgM ACAs contribute to endothelial cell damage, possibly by complement activation. Indeed, ACA-positive sera have been shown to affect endothelial cells (42). In the Leiden cohort, we recently demonstrated an association between ACA-specific immune response and degree of microangiopathy (43).

Finally, there are a number of possible implications of an association between both IgM and IgG ACAs and disease progression. In adaptive immune responses, IgM is the first isotype to appear after a vaccination or an infection. In normal adaptive immune responses, IgM disappears rapidly due to isotype switching with IgG taking over, and antibodies of the IgM isotype have a short lifespan (T½ = 8 days). The ongoing presence of IgM ACAs along with the association of levels of IgG ACAs with disease progression in the present study indicate ongoing immune activation accompanied by continuous production of IgM, which is most likely caused by recently activated B cells. Since there

is no evidence regarding the natural origin of IgA ACAs in SSc pathogenesis, we can only speculate about the implication of the high prevalence of IgA ACAs that was also observed. IgA is mostly found in mucous membranes, particularly the respiratory tract and the GI tract; as such, expression of disease-specific IgA ACAs might implicate involvement of these mucous membranes in SSc pathogenesis. Frequent pulmonary and GI involvement in patients with SSc supports this hypothesis, but how and where IgA ACAs are triggered is currently unknown.

Puffy fingers or abnormal NFC were found to be predictive of the diagnosis of very early SSc in a population with RP who had not yet received a diagnosis of very early SSc (28). Randone et al (44) identified SSc-specific autoantibodies, puffy fingers, and NFC abnormalities as predictors of disease progression in patients with RP and/or ANA positivity. We identified ACA characteristics to be predictive of disease progression; however, we were not able to confirm the association between abnormal NFC and disease progression. One explanation could be differences between the patient groups studied. In our study, the majority of the patients with very early SSc already had 8 points according to the ACR/EULAR 2013 classification criteria. In the study by Randone et al, the majority of the patients scored <6 points at baseline according to the ACR/EULAR 2013 classification criteria. Interestingly, disease progression rates between patients with 8 points and patients with <8 points were comparable. Secondly, we only included ACA-positive patients, whereas Randone and colleagues included patients with RP who could be negative for ANAs or positive for ANAs with different specificities. Strikingly, the number of patients who were considered to be progressors among those with very early SSc was comparable between the 2 studies (41% versus 42%), which highlights the necessity of biomarkers to adequately identify the patients at risk. Although not within the scope of the present study, evaluating the IgG ACA level as a possible predictive biomarker in clinical practice showed that, in combination with pulmonary fibrosis, 84% of progressors and 49% of nonprogressors could be accurately identified at baseline. However, this finding needs to be further evaluated and confirmed in independent cohorts.

Previous results on the association between disease severity and ACA-specific responses have been conflicting. Two longitudinal studies with a small sample size (n = 13 and n = 15) did not provide conclusive results on the associations between clinical characteristics and ACA isotypes; however, fluctuating levels of ACA isotypes over time were observed (45,46). These studies were limited by small sample sizes, the use of nonvalidated outcome measures, and older techniques to measure specific isotypes.

To our knowledge, our study is the first to perform complete evaluation of ACA isotype responses in patients with SSc, and specifically to evaluate ACA isotype response in association with clinical progression to SSc in the very early SSc group. Our results provide an answer to one of Witebsky's postulates (47), offering evidence that may be useful in further investigations into a possible pathogenetic role of ACAs in the SSc disease course. We believe that ACA isotypes can be seen as biomarkers for the underlying immune response, and the presence and levels of the different isotypes can be used as markers for the breadth of the immune response. In addition, we hypothesize that the breadth of the immune response is a proxy for the intensity of the immune response, i.e., continuous expression of more isotypes indicates more active triggering of the adaptive immune response, which is also supported by data in other autoimmune diseases (18,19,48).

This study has some limitations. We included patients who were positive for IgG ACAs at baseline, and cannot completely exclude the possibility that patients who were positive only for IgM or IgA ACAs were missed. However, as a sensitivity check, we additionally measured expression of IgA and IgM ACAs in 46 patients with SSc of various durations who were negative for IgG ACAs (negative both by Phadia FEIA and by IF assay), which confirmed that clear expression of IgM and IgA ACAs in patients who are negative for IgG ACAs is very rare (results not shown). Likewise, no conclusions can be drawn with regard to other antibody subgroups in SSc. Since samples were not analyzed longitudinally, the effect of starting or discontinuing immunosuppressive treatment remains unclear, although we did not find an association between immunosuppressive treatment and ACA isotype levels. Another limitation is the difference in follow-up duration among patients in the very early SSc group. However, we performed 2 additional sensitivity checks: 1) including patients with long follow-up duration and 2) including patients with short disease duration, both of which confirmed the significant association between levels of IgG ACAs and progression to definite SSc (Supplementary Tables 8 and 9, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). GI involvement was assessed based on available parameters including GAVE. This could have led to underestimating the prevalence of GI involvement, and therefore we performed a sensitivity check in a subgroup with additional data available (Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41814/abstract). Even with this broader definition of GI involvement, patients with organ involvement still showed the highest levels of IgG and IgM ACAs. To strengthen these results, the next step would be to evaluate ACA isotypes longitudinally and at the time of disease progression.

In conclusion, we have shown, to our knowledge for the first time and in a large multicenter ACA-positive SSc cohort, that IgG and IgM ACA levels are significantly higher in patients with definite SSc compared to patients with very early SSc. Moreover, we showed that in 42% of ACA-positive patients with very early SSc, disease progressed to definite SSc within 5 years and was associated with higher IgG ACA levels. Both observations indicate that CENP-B–specific IgG levels may be novel biomarkers in SSc and can potentially contribute to disease development.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version

to be published. Dr. van Leeuwen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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