Systemic sclerosis: can we identify patients at risk?

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Chapter 9

Analyses of anti-centromere antibody levels and isotypes in development of systemic sclerosis

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Objectives

Little is known on the disease course of very early systemic sclerosis (SSc). It is unknown whether anti-centromere antibody (ACA) isotype levels can serve as biomarkers for future SSc development and for organ involvement. We aim to evaluate whether ACA-IgG, -IgM and -IgA levels in ACA-IgG positive patients associate with disease severity and/or progression from very early SSc to definite SSc.

Methods

ACA-IgG positive patients with very early SSc and ACA-IgG positive patients fulfilling the 2013 ACR/EULAR criteria for SSc from five different cohorts were included. A diagnosis of very early SSc was based on the presence of ACA-IgG AND Raynaud and/or puffy fingers and/or abnormal nailfold capillaroscopy but not fulfilling the 2013 ACR/EULAR criteria. Multivariable regression analyses were performed to determine the association between baseline isotype levels and progression to SSc and organ involvement.

Results

Six hundred twenty-five ACA-IgG positive patients were included of whom 138 (22%) fulfilled very early SSc criteria and 487 (78%) had definite SSc. ACA-IgG (Odds Ratio (OR) 2.5, 95% CI 1.8-3.7) and ACA-IgM (OR 1.8, 95%CI 1.3 -2.3) levels were significantly higher in definite SSc patients. Of 115 very early SSc patients with follow-up, 48 (42%) progressed to definite SSc within five years. Progression to definite SSc was associated with higher ACA-IgG levels at baseline (OR 4.3, 95% CI 1.7-10.7).

Conclusion

ACA isotype levels might serve as a biomarker to identify very early SSc patients at risk for 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 2013 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) classification criteria for SSc have a high sensitivity for classifying patients correctly as SSc patients (5). However, there are still patients, who do not fulfil these criteria, despite showing early signs of SSc (6). Currently, no biomarkers to identify which patients with very early signs of SSc will progress to definite SSc exists. Identification of this subgroup of very early SSc patients, with more precise insights in their disease course, is crucial for early therapeutic interventions (7).

SSc specific anti-nuclear autoantibodies (ANA) are commonly used for disease and risk stratification. Anti-topoisomerase-1 antibodies (ATA) and anti-centromere antibodies (ACA) are the most prevalent autoantibodies in SSc (8). The presence of ACA is associated with limited skin involvement, higher prevalence of calcinoses, and gastro-intestinal involvement (GI) (9-11). Presence of ACA generally carries a better prognosis than most other SSc associated autoantibodies with respect to survival (10, 12). The major reactive antigen of ACA has been identified as CENP-B, which is therefore suggested as the primary target driving a selected B cell response characterized by ACA-IgG production (13, 14). Based on the observation that the generation of disease specific ANA antibodies is closely linked to disease development and clinical phenotype, we hypothesize ANA specific antibodies to be implicated in disease pathogeneses (15-17). However, the exact role of these disease specific ANA and their underlying antigenic triggers in SSc remain unclear.

In rheumatoid arthritis (RA), an autoimmune disease characterised by polyarthritis and by presence of rheumatoid factor (RF) and anti-cyclic citrullinated antibodies (ACPA), an extended ACPA repertoire has been shown to associate with disease development and disease severity, while the effector function of ACPA 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 of ACA positive SSc patients can contribute to a better understanding of the characteristics and dynamics of the underlying, auto-reactive B-cell response. In line with the observations in RA, we hypothesize that in ACA-IgG positive SSc, the expansion of specific ACA isotype responses associates with SSc development and severity, as reflected by organ involvement.

By taking advantage of five independent and well described SSc cohorts (Leiden, Oslo, Zurich, Ghent, Bordeaux) with prospective and comprehensive clinical data available, we aimed to evaluate whether the levels of individual ACA isotype levels associate with disease severity in ACA-IgG positive SSc patients, and whether these levels can identify very early SSc subjects that will progress to definite SSc.

METHOD

Patient population

The SSc cohorts in Leiden, Oslo, Ghent, Bordeaux and Zurich are prospective cohorts including all consecutive SSc patients (21-26). Patients in these cohorts undergo annual extensive screening which includes complete physical examination, laboratory testing, pulmonary function testing, thoracic echocardiography, high resolution computed tomography (HRCT), 24-hours electrocardiography (EKG), nailfold capillaroscopy (NC) evaluation, and, optional, cardiopulmonary exercise testing (CPET). At every visit, blood samples are collected and stored in respective Biobanks (27).

Included ACA-IgG positive patients had to fulfil either the ACR/EULAR 2013 SSc criteria or had to fulfil criteria for very early SSc (VEDOSS criteria) (5, 28, 29). Patients were classified as very early SSc, if additional to being ACA-IgG positive, had Raynaud Phenomenon (RP) and/or puffy fingers and/or abnormal NC, but did not fulfil ACR/EULAR 2013 criteria for SSc (28, 29). This study was performed with the use of a prospectively collected dataset from routine practice with post-hoc analyses. Patients entering the cohorts before March 2019 were selected for the present study. Details of all cohorts are described elsewhere (21, 23-25, 30-32).

Collection and analysis of biomaterial and their clinical associations have been approved by the local ethics committee (Leiden CME number B16.037, Switzerland: PB 2016-02014 02014 and BASEC-Nr. 2018-01873, Norway: No.2006/119, Ghent: 2008/385, Bordeaux: 2012-A00081-42). All participants provided written informed consent.

Clinical characteristics

At baseline visit clinical data and blood samples (including autoantibody testing) were collected for all included patients. Baseline was defined as the first visit at the SSc care pathway which includes screening for SSc. The SSc patients fulfilling the ACR/EULAR 2013 criteria were categorized as definite SSc without organ involvement and definite SSc with organ involvement (explained in the paragraph below). For analyses, patients were categorized as: 1.very early SSc, 2. definite SSc without organ involvement and 3. definite SSc with organ involvement. Follow-up data were only collected for the very early SSc group, as knowledge on this group of patients is of particular interest due to the possibilities to interfere 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, gastrointestinal, renal, musculoskeletal). Follow-up duration was the time duration calculated from the first baseline visit to the most recent visit. Disease duration was defined as time from onset of Raynaud Phenomenon (RP), since

in very early SSc time since onset non-RP was missing in patients without puffy fingers. We collected the required clinical characteristics to evaluate the disease status of the patients (very early SSc, SSc with or SSc without organ involvement). The modified Rodnan Skin Score (mRSS), sclerodactyly, puffy fingers, peripheral vascular involvement including pitting scars, digital ulcers (DU) and telangiectasia were evaluated and reported by the physician during evaluation. NC was considered abnormal if a scleroderma pattern was present, according to the definitions consented by the EULAR study Group on Microcirculation in Rheumatic Diseases and Scleroderma Clinical Trials Consortium (SCTC) (33, 34). Use of immunosuppressive treatment at baseline was recorded including: hydroxychloroquine, mycophenolate mofetil, methotrexate, cyclophosphamide, azathioprine and corticosteroids. Use of biologicals at baseline was only present in approximately 0.5% of the patients, therefore this was not taken into account.

Organ involvement

DU were defined as an area with a visually discernible depth and a loss of continuity of epithelial coverage and included both ischemic and traumatic ulcers. Interstitial lung disease (ILD) was present when there was evidence for ILD on HRCT. Myocardial involvement was assessed using a modified Medsger score,(35) which consists of at least two of the following: arrhythmias (> 2% (supra)ventricular extrasystoles, 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 ≥25 mmHg at rest as assessed by right heart catheterization (RHC); including presence of pre-capillary pulmonary hypertension (PH), defined by a pulmonary capillary wedge pressure ≤15 mmHg and a PVR >3 Wood units, in the absence of other causes of precapillary PH such as PH due to lung diseases, chronic thromboembolic PH or other rare diseases (36). Renal crisis was based on clinical expertise (including increase in blood pressure, increase in creatinine, oligo/anuric renal failure). GI involvement was defined based on a composite variable: as presence of confirmed gastric-antral vascular ectasia (GAVE, available for all patients), presence of fecal incontinence (data available in 413 patients), and/or malabsorption syndrome (data available in 317 patients), and or weight loss >10% in one year (data available in 309 patients). Very early SSc patients were considered progressors to definite SSc when they developed ILD, DU, PAH, renal crisis, myocardial involvement, or GI involvement and if they met the ACR/EULAR 2013 classification criteria during follow-up.

Anticentromere assay and measurement

Storage, collection and processing of blood samples was performed in line with the European League Against Rheumatism Scleroderma Trials and Research group (EUSTAR) biobank recommendations (27). All baseline samples were assessed in the clinical

chemistry department of the Leiden University Medical Centre (LUMC). Total ACA-IgG, ACA-IgA and ACA-IgM levels (CENP-B) of all the samples collected were measured by fluorescence enzyme-linked immune sorbent assay (FEIA), using Phadia250 system by J.B (Thermo Fisher Scientific, Nieuwegein, The Netherlands). Immunofluorescent patterns (IF) were evaluated at baseline and centromere anti-nuclear antibody patterns (speckled) were found. Commercial labs usually measure ACA-IgG (CENP-B IgG). The cut-off level for ACA-IgG positivity was set at 7 units/ml, according to the manufacturer's instructions. ACA-IgM and ACA-IgA were defined as research only parameters by the manufacturer. To define cut-off values for these parameters sera from fifty healthy subjects were measured and the cut-off values for the presence of IgM and IgA were defined as the mean plus 2SD for serum samples. Cut-off for ACA-IgA was determined at 37 AU/ml and for ACA-IgM at 13 AU/ml.

Statistical analysis

Analyses were performed by IBM SPSS version 23, and GraphPad Prism 7 was used for creating graphs. Descriptive statistics were used to summarize clinical and serological features and differences were tested as appropriate. For comparison of the continuous independent variables Mann- Whitney U test was used (for two groups), for more than two groups Kruskal –Wallis test with correction for multiple comparisons was used. Chi square test was used for categorical variables. To evaluate cross-sectional associations between isotype levels and disease status we used binary logistic regression with adjustment for age and disease duration (predictor: isotype levels [continuous, each isotype was tested in a separate model], outcome: disease status i.e. very early SSc, definite SSc without or definite SSc with organ involvement). Longitudinal analyses included the clinical evaluation of very early SSc patients over time, and progression of organ involvement in definite SSc. For clinical differences between progressors and non-progressors 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 (predictor: isotype levels [continuous, all isotypes tested in separate models], outcome: progression yes/no) and ROC-curves were evaluated (supplementary file). The possibility to predict progression to SSc based on ACA-IgG level was evaluated. Last observation carried forward was applied in case of missing data during follow-up for ILD, PAH, and ejection fraction in case of clinically stable disease with stable pulmonary function test results and no additional testing available. As the individual components of the composite GI involvement variable were not complete for all included patients, the validity of the parameter was checked in a sensitivity analysis using the subgroup with complete data (supplementary table S2).

RESULTS

Clinical characteristics

In total, 625 ACA-IgG positive patients were included. Ninety percent were female (n= 558) with a mean age of 58 years, a median disease duration since non-Raynaud of 6 years (IQR: 2-9). The baseline characteristics of the three clinical groups are shown in table 1. There were 138 patients with very early SSc (22%), 240 SSc patients without organ involvement (38%), and 247 SSc patients with organ involvement (40%).

ACA IgG, IgM and IgA levels

Of all the ACA-IgG positive subjects, 437 (76%) were ACA-IgA positive at baseline; and 522 (89%) were ACA-IgM positive at baseline. A non-cutaneous disease subset was more common in patients positive for both ACA-IgG and ACA-IgA compared to patients positive for ACA-IgG and ACA-IgM and patients positive for all three isotypes (respectively 47% vs 33%vs 27%). No other clinical differences were observed between subgroups defined by numbers of expressed isotypes (data not shown). In the very early SSc group, the ACA-IgG and ACA-IgM levels were significantly lower compared to patients in the definite SSc group (figure 1, figure S3). Using logistic regression, with adjustment for age and disease duration since RP, we found a significant association between ACA-IgG (OR 2.54 (1.75-3.69)) and ACA-IgM levels (OR 1.77 (1.34-2.34)) and disease status, with higher levels in the SSc patients (SSc with and without organ involvement combined) compared to the very early SSc patients (table 2). No significant associations were found between ACA-IgG, ACA-IgM or ACA-IgA isotypes and definite SSc with or without organ involvement (table 2). In the supplementary file results per included centre are shown, confirming the same trend across all SSc centres (supplementary table S1, figure S1 and figure S2).

To assess a possible effect of immunomodulatory treatment on ACA isotype levels, we performed a logistic regression. No significant associations were found for use of immunosuppressive treatment and ACA-IgG (OR 1.4 (0.91- 2.10)), ACA-IgM (OR 0.91 (0.68-1.22)) or ACA-IgA levels (OR 0.74 (0.43-1.29)).

Baseline characteristics and ACA isotype expression and levels in patients with very early SSc and SSc

	Very early SSc n=138	SSc without organ involvement n=240	SSc with organ involvement n=247	Data available in n. patients
Female, n(%)	125 (91)	225 (91)	208 (87)	625
Age, median (IQR)	52 (40-62)	57 (49-66)	62 (52-69)	625
Since RP, median(IQR) in years	5 (1-12)	10 (3-19)	8 (2-18)	622
Since non RP, median(IQR) in years ¥	NA	5 (2-11)	6 (2-12)	465
lcSSc, n(%)	NA	202 (84)	187 (78)	482Ω
dcSSc, n(%)	NA	14 (6)	27 (11)	482Ω
mRSS, median (IQR)	0 (0-0)	3 (0-5)	4 (0-6)	589
Digital ulcers, n(%)	0	0	81 (33)	616
FVC % predicted, mean (SD)	107 (17)	107 (17)	107 (19)	585
DLCO % predicted, mean (SD)	81 (15)	74 (14)	67 (18)	596
ILD on HRCT, n(%)	0	0	86 (36)	625
PAH, n(%)	0	0	52 (21)	625
Myocardial involvement, n(%)	0	0	42 (22)	563
Renal crisis, n(%)	0	0	3 (1)	625
GI involvement, n(%)	0	0	120 (49)	625
Puffy fingers, n(%)	21 (16)	71 (39)	36 (23)	548
Abnormal NC, n(%)€	69 (55)	160 (84)	149 (86)	488
Immunosuppressive treatment, n(%)	25 (18)	48 (20)	112 (46)	625
ACA characteristics				
IgA positivity, n(%)	88 (72)	177 (78)	172 (75)	617
IgM positivity, n(%)	106 (86)	209 (91)	207 (90)	617
IgG levels, U/ml, median (IQR)	274 (93-662)	480 (197-990)	619 (263-1077)	617
IgM levels, aU/ml, median (IQR)	101 (41-363)	183 (55-907)	251 (63-965)	617
IgA levels, aU/ml, median (IQR)	69 (35-103)	78 (39-166)	86 (37-187)	617

Table 1. Baseline characteristics of the three groups, SSc= systemic sclerosis, RP= Raynaud phenomenon, NA= not applicable; lcSSc= limited cutaneous, dcSSc= diffuse cutaneous SSc, FVC= forced vital capacity, DLCO= diffusing capacity for carbon monoxide, GAVE= gastric antral vascular ectasia, mRSS= modified Rodnan Skin Score, ILD= interstitial lung disease, IQR= interquartile range, HRCT= high resolution computed tomography, n= number, NC= nailfold capillaroscopy, PAH= pulmonary arterial hypertension, SD= standard deviation. ¥ n= 22 missing in definite SSc , € n= 137 missing (started with NC in 2013/2014 and not routinely performed in all cohorts). Medication in very early SSc group: n= 7 corticosteroids, n= 12 methotrexate, n= 5 hydroxychloroquine. Ω maximum available in n= 487 (since very early SSc cannot be categorized in a disease subset).

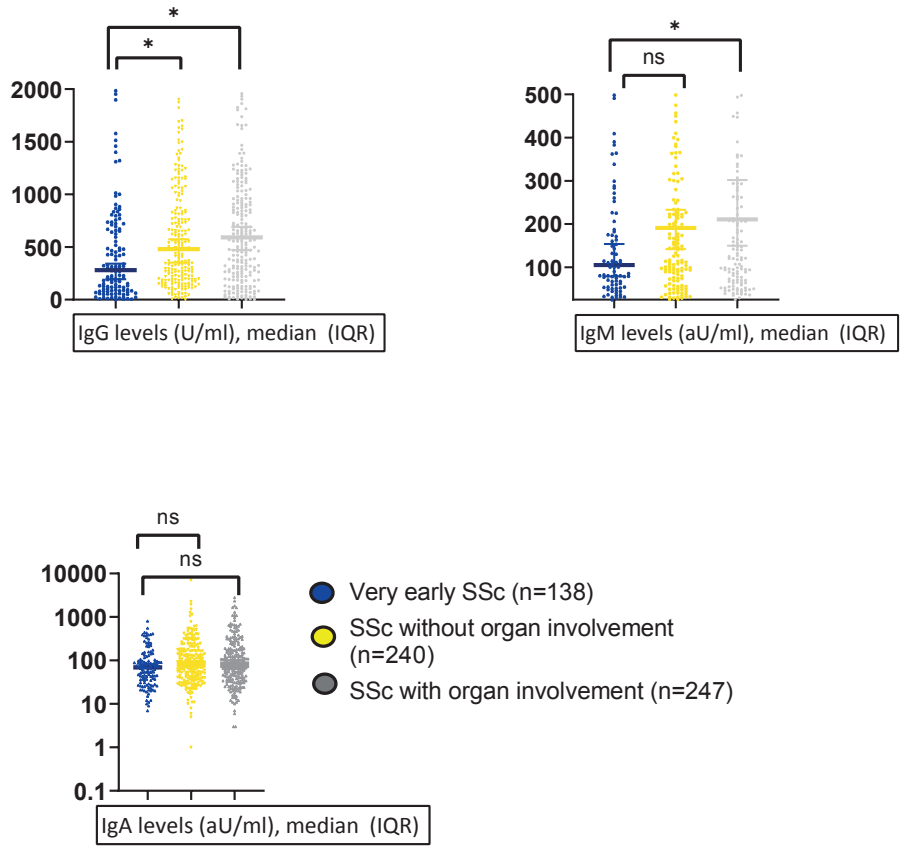

Figure 1. ACA isotype levels in very early SSc, definite SSc without and definite SSc with organ involvement**.** ACA-IgG, ACA-IgM and ACA-IgA levels between the three groups. ACA-IgG and ACA-IgM levels are significantly higher in definite SSc patients compared to very early SSc patients. * significant $p < 0.05$

ACA-IgG and ACA-IgM levels are associated with definite SSc, ACA-IgG levels are associated with disease progression towards definite SSc.

	SSc patients vs. very early SSc	SSc with organ involvement vs. SSc without organ involvement	Very early SSc Progression
	OR 95% CI	OR 95% CI	OR 95% CI
ACA IgG U/ml	**2.54 (1.75-3.69)**	1.09 (0.77-1.53)	**4.27 (1.70-10.71)**
ACA IgM aU/ml	**1.77 (1.34-2.34)**	1.11 (0.83-1.26)	1.75 (0.97-3.14)
ACA IgA aU/ml	1.40 (0.90-2.17)	0.96 (0.67-1.38)	1.36 (0.47-3.96)

Table 2. * adjusted for age and disease duration. ACA-IgG, -IgM and -IgA were log2 transformed to overcome skewness in the data. Very early SSc: ACA isotype levels available in n= 115 very early SSc patients.

Very early SSc evolving to definite SSc

Of the 138 patients classified as very early SSc, 23 were lost to FU (supplementary table S3). In total 48 (42%) progressed 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 minimal clinical important difference in mRSS (n= 11, 23%),(37) development of telangiectasia (n= 31, 65%) or sclerodactyly (18, n= 38%). Both DU and pitting scars occurred in 17% of the progressors. The remaining 67 patients did not develop organ involvement nor progressed to fulfilling ACR/EULAR 2013 criteria after median FU of 2 years (range 1-5).

Compared to non-progressors, very early SSc that progressed to definite SSc were older and had a longer follow-up duration (table 3). At baseline, ACA-IgG levels were significantly higher in progressors compared to non-progressors, also when adjusted for follow-up duration (figure 2). In logistic regression analyses with correction for age and follow-up duration ACA-IgG levels were significantly associated with progression to definite SSc (table 2 OR 4.27 (1.70-10.71)); puffy fingers were significant in the univariable analysis (OR 2.95 (1.31-6.62)), and abnormal NC did not show a significant association with progression to definite SSc (supplementary table S4). The ROC curves for ACA-IgG and ACA-IgM on progression can be found in the supplementary file figure S4, table S6-S7 and figure S5.When applying a threshold with optimal sensitivity and NPV, ACA IgG of 81 U/ml together with presence of puffy fingers can be applied (figure S5). With this cut of 84% of progressors and 49% non-progressors were classified correctly at baseline. To further evaluate the predictive value of ACA isotype levels for progression in SSc we evaluated association with disease progression in the patients with definite SSc at baseline and complete clinical follow-up data available (n= 93, table S5). In this subgroup ACA-IgG (OR 2.79 (1.08-7.26)) and ACA-IgM (OR 2.06 (1.18-3.61)) were independently associated with disease progression.

Very early SSc patients with follow-up data			
	Progressors n=48	Non progressors n=67	p value
Demographic			
Female, n(%)	43 (90)	61 (91)	0.52
Age, mean (SD)	**53 (15)**	**48 (13)**	**0.03**
Disease duration			
Since RP, median (IQR)	5 (2-11)	6 (2-14)	0.69
Follow-up duration in years, median (IQR)	**5 (3-7)**	**2 (1-5)**	**<0.001**
Clinical characteristics			
Puffy fingers, n(%)Ω	7 (15)	8 (12)	0.55
Abnormal NC, n(%)€	26 (54)	34 (51)	0.45
ACA characteristics			
IgM positivity, n(%)	36 (86)	51 (81)	0.36
IgA positivity, n(%)	31 (76)	43 (68)	0.28

Table 3. Differences between very early SSc patients that progressed during follow-up and very early SSc patients that did not show disease progression. No clinical follow-up data available n= 23 very early SSc patients. Missing data for ACA isotype levels in patients with follow-up; n= 8 very early SSc patients (5 in the progressor group, 4 in non progressors). Ω data available for n= 105 patients, € data available in n= 113 patients.

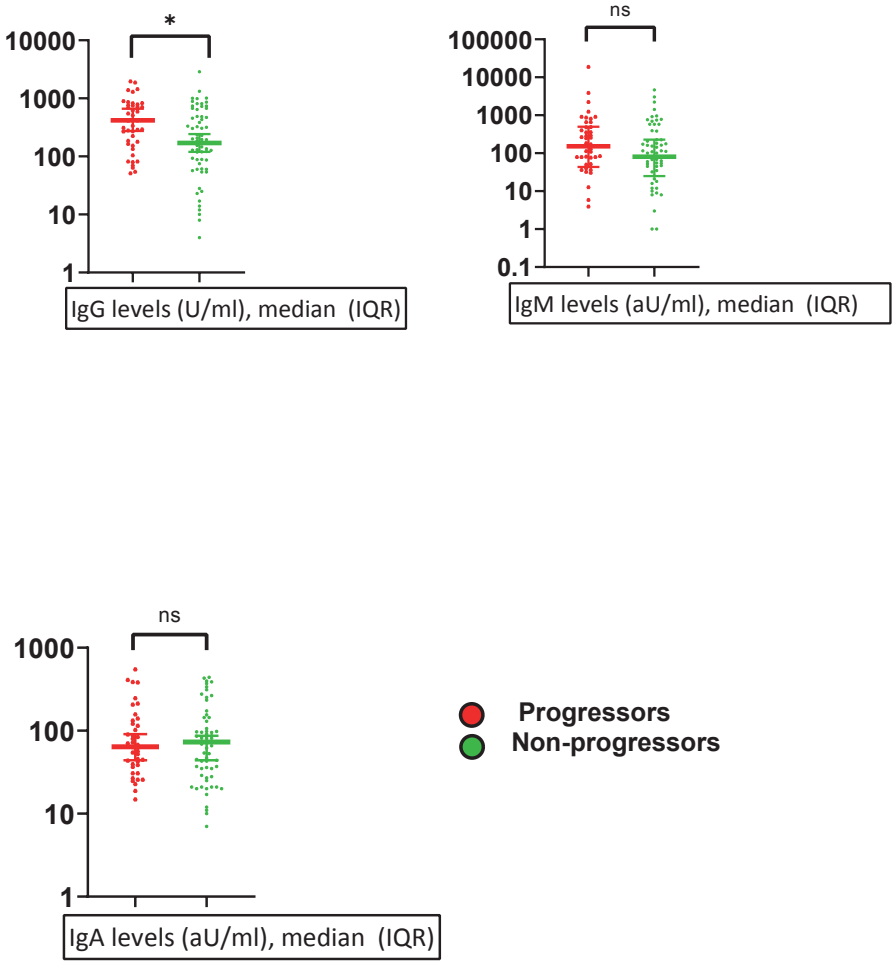

Figure 2. ACA-IgG, ACA-IgM and ACA-IgA levels between very early SSc patients that progress to definite SSc and patients that do not show progression. ACA-IgG levels are higher in patients that progress.

DISCUSSION

In this study, we analysed 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 that will progress to definite SSc. We show that definite SSc patients express higher ACA-IgG and higher ACA-IgM levels compared to very early SSc patients. Moreover, we show that in very early SSc, higher levels of ACA-IgG associate with progression to definite SSc within two years.

The lower ACA-IgG and ACA-IgM levels in the very early SSc group might indicate a less pronounced immune response compared to the patients with definite SSc. We found the highest ACA-IgG, ACA-IgM and ACA-IgA levels to be present in the SSc patients with organ involvement, and within the group of definite SSc patients baseline ACA-IgG levels and ACA-IgM levels associated with future disease progression. These findings are in line with our hypothesis that the immune response in very early SSc patients is less pronounced compared to patients with established disease. As shown by our data, and also by others,(30) although the classification might suggest short disease duration, some patients classified as 'very early SSc' in fact show similar disease duration as patients with definite SSc. This indicates that patients fulfilling classification criteria for very early SSc in fact consist of a heterogeneous group of patients: patients that will eventually progress to definite SSc and patients that will persist as 'very early' SSc and do not progress to more severe disease (30). Our data shows that the levels of the ACA specific immune response discriminates between those two subgroups (supplementary file). Similar observations have been made in other rheumatic diseases including RA (18, 38). The observation that ACA-IgG levels are numerically higher in ACA positive SSc patients with organ involvement compared to levels in ACA positive SSc patients without organ involvement is in line with our hypothesis. However, for this comparison observed differences 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 for severe organ involvement like ILD and diffuse skin involvement might be less sensitive in ACA positive SSc, as severe fibrotic disease complications are less frequent in ACA patients than in ATA positive patients.

Until now the effects of disease duration on isotype levels is not fully understood. One could hypothesize that isotypes levels decline over time as antigenic triggering diminishes (39). Whether and for how long ACA specific response occur before clinical disease development is unknown. In one study, a median duration since onset RP of 4.6

years was found from early SSc to definite SSc (40). No data about initiation of specific antibody expression nor data about different autoantibodies are available from this study. In our very early SSc group, 48 patients (42%) developed definite SSc over a median time of 5 years.

We observe the strongest associations between ACA-IgG and disease subset (very early SSc vs definite SSc). In line with our hypothesis, it is tempting to speculate that either ACA-IgG, and/or the B-cell responses underlying ACA production are involved in the disease pathogenesis. As both microangiopathy, clinically shown by RP, and dysregulated immunity reflected by presence of specific ANA, are among the earliest features of SSc one could speculate that specifically ACA-IgG and ACA-IgM contribute to endothelial cell damage, possibly by activating complement. Indeed, ACA positive sera have been shown to affect endothelial cells (41). In the Leiden cohort, we recently demonstrated an association between ACA-specific immune response and degree of microangiopathy (42). Finally, the implication of the association between both ACA-IgM and ACA-IgG with disease progression can be speculated about. 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 where IgG will take over, and, secondly, antibodies of the IgM isotype have a short life time ($T_{½}$= 8 days). The ongoing presence of ACA-IgM next to ACA-IgG and its association with disease progression points at ongoing immune activation accompanied by continuous production of IgM which is most likely caused by recently activated B cells. As there is no evidence regarding the nature origin of ACA-IgA in SSc pathogenesis we can only speculate about the implication of high prevalence of ACA-IgA. IgA is mostly found in mucous membranes, particularly the respiratory tract and the gastrointestinal tract; as such expression of disease specific ACA-IgA might implicate involvement of these mucous membranes in SSc pathogenesis. The frequent pulmonary and gastro-intestinal involvement in SSc patients supports this hypothesis, but how and where ACA-IgA is triggered is currently unknown.

Puffy fingers or abnormal NC were found to be predictive for the diagnosis of very early SSc in a population with RP and no diagnosis (yet) of very early SSc (28). Randone et al.(43) identified SSc specific autoantibodies, puffy fingers and NC abnormalities to be predictive for disease progression in patients with RP and/or ANA positivity. We identified ACA characteristics to be predictive for progression, however we were not able to confirm the association between abnormal NC with progression. One explanation could be the differences in included patients, in our study the majority of the very early SSc patients already had 8 points on the ACR/EULAR 2013 classification criteria; in the study performed by Randone et al. the majority of the patients scored <6 points on the ACR/EULAR 2013 classification criteria at baseline. Interestingly progression rates

between patients with 8 points at the ACR/EULAR criteria and patients with < 8 points were comparable. Secondly, we only included ACA positive patients while Randone and colleagues included RP patients that could be ANA negative, or ANA positive with different specificities. Strikingly, the amount of progressors among the very early SSc patients was comparable between the studies (41% vs 42%), which underlines the necessity of biomarkers to adequately identify the patients at risk. Although not the scope of the present study, evaluating ACA-IgG level as possible predictive biomarker in clinical practice showed that, in combination with PF 84% of progressors and 49% non-progressors could be identified correctly at baseline. However, this finding needs to be further evaluated and confirmed in independent cohorts

Previous results on 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 associations between clinical characteristics and ACA isotypes, they did observe fluctuating levels of ACA isotypes over time (44, 45). These studies were limited by small sample sizes, the use of invalidated outcome measurements and older techniques to measure specific isotypes. In conclusion, to our knowledge, our study is the first that performed complete evaluation of ACA isotype responses in patients with SSc, and specifically evaluated ACA isotype response in association with clinical progression to SSc in the very early SSc group. This study might be helpful to provide more evidence for evaluating a possible pathogenetic role of ACA in SSc disease course by answering one of the Witebsky's postulates (46). We believe that the ACA isotypes can be seen as biomarker for the underlying immune response, and the presence and levels of the different isotypes can be used as a marker 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 auto-immune diseases (18, 19, 47). This study has some limitations. We included patients with baseline positivity of ACA-IgG. We cannot completely exclude that SSc patients positive for ACA-IgM or -IgA solely have been missed. As a sensitivity check, we additionally measured expression of ACA-IgA and ACA-IgM in 46 ACA-IgG negative SSc patients (negative in both Phadia FEIA and in IF assay) with various disease durations, which confirmed that clear expression of ACA-IgM and/or IgA in ACA-IgG negative patients is not to be expected, since this was very rare (results not shown). Likewise, no conclusions can be drawn for the remaining antibody subgroups in SSc. As no longitudinal samples were analyzed the effect of starting or stopping immunosuppressive medication remains unclear, although we did not find an association between immunosuppressive medication and ACA isotype levels. Another limitation is the difference in follow-up duration in the very early SSc group,

however we performed two additional sensitivity checks, 1) including patients with a long follow-up duration and 2) including patients with a short disease duration, which both confirmed the significant association between ACA-IgG and progression to definite SSc (supplementary file table S8 and S9). GI involvement was assessed based on available parameters including GAVE; this could have led to underestimation of prevalence of GI involvement and therefore we performed a sensitivity check in a subgroup with additional data available (supplementary table S2). Even with this broader definition for GI involvement, patients with organ involvement still showed the highest ACA-IgG and ACA-IgM levels. To strengthen these results the next step would be to evaluate ACA isotypes longitudinal and at the time of progression.

In conclusion, we show for the first time and in a large multicentre ACA positive SSc cohort that ACA-IgG and ACA-IgM levels are significantly higher in definite SSc patients compared to very early SSc patients. Moreover, we show that 42% of ACA positive patients with very early SSc progresses to definite SSc within 5 years and that progression is associated with higher ACA-IgG levels. Both observations indicate CENPB-specific IgG levels as a novel biomarker in SSc and as potentially contributive to disease development.

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