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Antibodies recognizing sulfated carbohydrates are prevalent in systemic sclerosis and associated with pulmonary vascular disease

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Abstract

Background—Glycosylation represents an important modification that regulates biological processes in tissues relevant for disease pathogenesis in systemic sclerosis (SSc), including the endothelium and extracellular matrix. Whether patients with systemic sclerosis (SSc) develop antibodies to carbohydrates is not known.

Objectives—To determine the prevalence and clinical phenotype associated with serum IgG antibodies recognizing distinct glycans in patients with systemic sclerosis (SSc).

Methods—Pooled sera from patients with SSc and controls were screened for the presence of specific anti-carbohydrate antibodies using a novel array containing over 300 glycans. Antibody titers to 4-sulfated N-Acetyl-lactosamine (4S-LacNAc, [4OSO₃]Galβ1-4GlcNAc) were determined in 181 individual sera from SSc patients by ELISA and associated with disease phenotype.

Results—4S-LacNAc was identified as a target in pooled SSc serum. Anti-4SLacNAc antibodies were detected in 27/181 (14.9%) of SSc patients compared to 1/40 (2.5%) of healthy controls. Sulfation at position C-4 of galactose (4S-LacNAc) was found to be critical for immunogenicity. Anti-4SLacNAc antibody positive SSc patients had a higher prevalence of pulmonary hypertension by echocardiography (15/27; 55.7% versus anti-4S LacNAc negative patients 49/154; 31.8% p=0.02) with an odds ratio of 2.6 (CI 1.1, 6.3). Anti-4S-LacNAc positive patients accounted for 23.4% of all patients with pulmonary hypertension.

Conclusion—Sera from SSc patients contain IgG antibodies targeting distinct sulfated carbohydrates. The presence of anti-4S-LacNAc antibodies is associated with a high prevalence of pulmonary hypertension. These results suggest that specific posttranslational carbohydrate modifications may act as important immunogens in SSc and may contribute to disease pathogenesis.

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Keywords

Scleroderma; carbohydrates; antibodies; pulmonary hypertension

INTRODUCTION

Systemic Sclerosis (SSc) is an autoimmune disease that is characterized by endothelial cell damage, fibroblast activation, extracellular matrix accumulation and abnormal angiogenesis [1]. Autoantibodies that recognize membrane-bound antigens are thought to play an important pathogenic role, but direct evidence for this hypothesis remains limited [2]. Anti-endothelial cell antibodies (AECA) are found in 22–86 percent of SSc patients and have the capacity to modulate endothelial cell function as well as to induce apoptosis [3]. Similarly, anti-fibroblast antibodies from sera of patients with SSc have been shown to confer proinflammatory and proadhesive properties [4]. The targets of these antibodies remain largely unknown, but may include platelet-derived growth factor receptor (PDGF-R) [5, 6].

Cell surface structures in the target tissues in SSc, including the endothelium and extracellular matrix undergo extensive posttranslational modification by glycosylation. Changes in the glycosylation pattern can have a profound impact on a diversity of physiological functions, including fibroblast activation, wound healing, [7, 8], immune cell trafficking [9], and angiogenesis [10]. As a consequence, binding of antibodies to glycosylated structures *in vivo* may interfere with their function.

Whether patients with SSc develop specific antibodies that recognize distinct carbohydrate modifications is not known. Such antibodies would be prime candidates to interfere with glycosylation-dependent processes and thus may play an important role in the pathogenesis of the disease.

MATERIALS AND METHODS

Patients

One hundred eighty-one SSc patients were selected from the Johns Hopkins Scleroderma Center database. All patients met the American College of Rheumatology (ACR) criteria for SSc and were classified as having diffuse cutaneous SSc or limited cutaneous SSc depending on the extent of skin involvement. Sera from control groups included 40 consecutive patients with Systemic Lupus erythematosus (SLE), 40 patients with primary Sjogren's syndrome (SS), 16 SLE patients with secondary SS and 12 Rheumatoid arthritis (RA) patients with sicca complex, as well as 25 patients with idiopathic pulmonary arterial hypertension (IPAH) and 40 healthy controls. SLE patients met the 1997 revised ACR criteria for SLE, primary SS patients and secondary SS patients with SLE met the San Diego criteria for Sjogren's disease [11], patients with IPAH met the ACCF/AHA 2009 Expert Consensus criteria [12]. RA patients with sicca met the 1988 revised ARA criteria and fulfilled at least one subjective and objective criterion of the American-European consensus group criteria (AECC) [13]. Written informed consent was obtained from all patients prior to this study at the time of sample collection. The Johns Hopkins Institutional Review Board approved the present study.

Clinical phenotyping of Scleroderma patients

Demographic and clinical data, including age, sex, ethnicity, smoking status, disease duration (calculated from the date of onset of first non-Raynaud's phenomenon (RP) symptom), scleroderma subtype, specific organ involvement, and autoantibody status, were recorded for each patient at the time of clinical visit corresponding to serum collection.

Internal organ involvement was assessed using previously published criteria by Medsger et al. [14] and considered present when the relative Medsger severity score was ≥ 1 for the respective organ. Pulmonary involvement was determined based on abnormal findings on pulmonary function tests (PFTs) (forced vital capacity [FVC] and single-breath diffusing capacity for carbon monoxide [DLCO], measured as the absolute value as well as the percent predicted value for race, sex, and age, according to the American Thoracic Society recommendations [15]. For the purpose of this study, a patient was considered to have evidence of pulmonary arterial hypertension (PAH) if the estimated RVSP determined by Doppler echocardiography was > 40 mm Hg in separate tests and there was no overt clinical evidence of congestive heart failure, thromboembolic disease, or severe pulmonary interstitial fibrosis (FVC $< 50\%$). This assumption has been supported and confirmed in other studies [16]. Criteria for diagnosis of PAH by right heart catheterization were applied according to [12], and required the combination of a mean pulmonary artery pressure > 25 mm Hg; a pulmonary capillary wedge pressure ≥ 15 mm Hg; and a pulmonary vascular resistance > 3 Wood units. Skin involvement was scored according to the modified Rodnan skin thickness score (MRSS [range 0–51]) [17]. Sicca complex was considered present when patients fulfilled at least one subjective criterion of the AECC.

Glycan ELISA

Biotinylated glycans (Glycotect, Gaithersburg, USA) used for ELISA studies included the following structures: 3S-LacNAc [3OSO3]Gal β -[4GlcNAc], 4S-LacNAc [4OSO3]Gal β -[4GlcNAc], and 6S-LacAc [6OSO3]Gal β -[4GlcNAc]. 96 well microtiter Neutravidin plates preblocked with Superblock (Pierce, Rockford, USA) were coated with biotinylated glycans at 200 ng/well or PBS. Sera from disease patients and healthy controls were used at 1:250 dilution, unless further dilution was required to achieve an O.D. in the linear range of the assay. Antibody binding was detected using an HRP-labeled anti-human Fc γ -specific antibody (JacksonImmuno, West Grove, USA), followed by incubation with SureBlue (KPL, Gaithersburg, USA). Absorbance O.D. values at 450 nm and 690 nm for reference were transformed into Arbitrary Units (A.U.), based on a standard curve using serial dilutions of a high-titer positive SSc serum (FW-555).

Glycan microarray

The glycan microarrays from the CFG (<http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh11.shtml>) are prepared from amine functionalized glycan structures covalently coupled in microarrays to N-hydroxysuccinimide-derivatized microscope slides as previously described [18]. Pooled and individual sera from patients with disease and healthy controls were analyzed for binding to version 3.0 or 3.1 of the printed array (320 and 377 glycans, respectively) measured by net fluorescence units (FU) according to protocols described by the Protein-Glycan Interaction Core (Core H) of the CFG (http://www.functionalglycomics.org/glycomics/ProtocolServlet?view&operationType=view&pageType=view&protocolId=cfgPTC_245&sideMenu=no). The antibody-binding ratio (ABR) for individual glycans was calculated by division of net fluorescence units of samples from disease patients by FU from samples of healthy controls. Anti-glycan antibody levels in individual SSc patients were determined as follows: low positive ($>$ mean from healthy controls (HC) + 2 SD, medium positive ($>$ mean from HC + 5 SD), high positive ($>$ mean from HC + 10 SD).

Statistical analysis

Statistical significance for the results of the ELISA studies was calculated by Mann-Whitney test. Association of disease, sociodemographic characteristics and odds ratios were calculated by student's test and logistic regression.

RESULTS

Presence of distinct anti-carbohydrate antibodies in SSc

Pooled sera from 40 randomly selected patients with SSc and healthy controls were evaluated by glycan array CFG version 3.0 for specific binding to 320 distinct glycans (for a complete list see supplement 1). As demonstrated in Figure 1A, for the majority of glycans there was no significant difference in the antibody binding ratio (ABR) between pooled SSc and healthy control serum (mean ABR 1.57, SD 1.58). However, 10 distinct glycans were highly targeted by SSc sera with binding ratios > 2 SD above the mean with an ABR as high as 15 (Figure 1B). Two of the 10 glycans, 4S-LacNAc and 4,6S-LacNAc, demonstrated a high degree of structural similarity. Interestingly, pooled sera from SLE patients recognized a distinct set of glycans compared to patients with SSc (Figure 1C). Compared to pooled serum from patients with SLE, the ABR for 4S-LacNAc and 4,6S-LacNAc was several fold higher in SSc serum (13.7 fold versus 3.7 fold and 7.8 fold versus 3.0 fold, respectively).

Antibodies to 4-sulfated LacNAc are frequent and of high-titer in patients with SSc

For our subsequent studies we focused on 4S-LacNAc, since the associated ABR as determined by glycan array was higher compared to 4,6S-LacNAc. We developed an anti-4S-LacNAc-specific ELISA to screen a large number of patients and controls. Anti-4S-LacNAc antibodies were detected in 27/181 (14.9%) of SSc sera, compared to 1/40 (2.5%) of healthy controls (Figure 2A). Anti-4S-LacNAc antibodies were less frequent in SLE (4/40; 10.0%) and importantly, anti-4S-LacNAc antibody positive SLE patients exhibited relatively low titer antibodies compared to patients with SSc (mean A.U. 0.55 versus 1.25, respectively), although this was not statistically significant. These results were in agreement with our initial glycan array data using pooled sera from patients with SSc and SLE, which showed a relative specificity of SSc for 4S-LacNAc. Subsequently, we assayed 17 of our 181 SSc patient sera (nine anti-4-sulfated LacNAc positive and eight antibody-negative sera) individually in a second CFG glycan array. As demonstrated in Figure 2B, there was an excellent correlation for anti-4S-LacNAc antibody levels obtained by glycan array (fluorescence) and ELISA (A.U.) with an r^2 of 0.89.

Sulfation at position 4 of galactose plays a dominant role for immunoreactivity towards LacNAc

Sulfation of LacNAc may occur at various positions of the galactose and/or the N-Acetylglucosamine molecule. Further analysis of the binding of pooled SSc sera to 41 diverse sulfated glycans present CFG glycan array version 3.0 (Figure 3A) revealed that the mean ABR of sera from SSc and healthy controls was quite similar in the sulfate-containing subgroup (mean 1.77, SD 2.24) compared to the mean ABR of all glycans. This demonstrates that there was no non-specific reactivity towards sulfated structures in general. 4S-LacNAc as well as 4-6S-LacNAc were the only sulfated glycans that showed an ABR higher than 2 SD above the mean, e.g. an ABR above 6.25 (Figure 3A). In particular, LacNAc sulfated at position 3 or 6 of galactose, instead of position 4, led to a marked decrease in immunoreactivity, resulting in an ABR of 3.1 and 0.9, respectively. LacNAc by itself was not recognized as an SSc antigen (ABR 1.8). Surprisingly, additional sulfation of 4S-LacNAc at position 3 of galactose (3,4S-LacNAc) completely abolished antibody binding (ABR 1.3), whereas additional sulfation at position 6 of galactose (4,6S-LacNAc) retained some immunoreactivity (ABR 7.8). We confirmed these findings in a second CFG glycan array version 3.1 using 17 individual sera from SSc patients preselected according to their anti-4S-LacNAc status determined by ELISA (9 positive and 8 negative sera) along with 7 sera from healthy controls. As shown in Figure 4A, ABRs were highest for 4S- and 4,6S-LacNAc (up to 119 fold). In some patients, immunoreactivity extended to other sulfated glycans, although with a much lower ABR. Preferential recognition of 4S-LacNAc

over 3S- and 6S-LacNAc was confirmed by ELISA as well (Figure 4B). Pre-blocking of anti-4S-LacNAc positive IgG with biotinylated 4S-LacNAc completely abolished ELISA binding, whereas pre-blocking with biotinylated 3S-LacNAc/6S-LacNAc/NeuGc/GlcNAcGalGlc retained reactivity (see supplemental figure 1). In summary, this data strongly suggests that differential sulfation of LacNAc plays an important role for immunogenicity of the glycan in SSc patients.

Increased prevalence of pulmonary vascular disease and sicca in anti-4 sulfated LacNAc positive SSc patients

A detailed analysis of the clinical, serological and sociodemographic phenotype of anti-4S-LacNAc positive patients is shown in Table 1. There was no difference in disease type (diffuse versus limited), disease duration, age, gender, race, skin score, gastrointestinal and kidney involvement or mortality between anti-4S-LacNAc positive and negative patients. Anti-4S-LacNAc positive SSc patients had a higher prevalence of PAH defined by echocardiography (14/27; 56% compared to 49/154; 32%, $p=0.02$ as well as a higher lung severity score (1.9 versus 1.4, $p=0.037$). There was no significant difference between the two groups for % predicted FVC or DLCO. Data from right heart catheterization was available for 35 patients and confirmed the increased prevalence of PAH in the anti-4S-LacNAc positive group (6/27; 22.2% versus 11/154; 7.1%, $p=0.013$). The prevalence of sicca symptoms was increased significantly in the antibody-positive group (74% versus 44%, $p=0.006$). Interestingly, the mean Raynaud's severity score was significantly lower in anti-4S-LacNAc patients (1.5 versus 2.0, $p=0.016$). This was not due to a higher frequency of vasodilator use in 4S-LacNAc positive patients (19/26; 73.0% versus 80/132; 60.6%, $p=ns$). There was no association between anti-4S-LacNAc positivity and antibodies to Scl70 (topoisomerase I) or centromere protein. In multivariate analysis corrected for age, gender, race and disease type, all associations remained significant. For patients with anti-4S-LacNAc antibodies, the odds ratio for PAH was 2.6 (CI 1.1–6.3), for sicca it was 3.0 (CI 1.2–8.2). Raynaud's syndrome remained less severe with an odds ratio of 0.5 (CI 0.3–0.9).

We next determined whether anti-4S-LacNAc antibodies are associated with PAH or sicca in patient groups other than SSc; e.g. in 25 patients with IPAH, 40 patients with primary SS, 16 SLE patients with secondary SS and 12 RA patients with sicca complex. There was no increased frequency in anti-4S-LacNAc antibody positivity among IPAH patients (1/25; 4.0%). and similarly, patients with either primary SS, secondary SS associated with SLE, or RA and sicca complex did not demonstrate an increased frequency in anti-4S-LacNAc positivity compared to healthy controls (Figure 5).

DISCUSSION

To the best of our knowledge, this is the first study that provides a comprehensive analysis of the anti-glycan antibody repertoire in patients with SSc. Glycan arrays represent a powerful novel tool to efficiently probe human sera for the presence of antibodies to a large number of carbohydrates. The finding that patients with SSc develop a distinct pattern of IgG antibodies targeting specific carbohydrate modifications may potentially have important implications for disease pathogenesis, since glycosylated proteins and lipids are typically exposed at the cell membrane where autoantibodies can easily bind and interfere with their physiological function.

One of the key findings of our study is the fact that sulfation represents a critical carbohydrate component for the induction of immunogenicity in SSc patients. A striking observation was the specificity of the antibodies towards the 4-sulfated versus 3- or 6-sulfated galactose. These results suggest that production of immunogenic 4-sulfated galactose or related structures likely occurs *in vivo* at least in a subset of SSc patients. The

exact target of the antibodies remains unknown. Sulfation of LacNAc and polylectosamine (poly-LacNAc) is an important modification implicated in diverse cell-cell interaction, including selectin-mediated cell adhesion, cell-to-cell interaction through sialic acid binding Ig-like lectins (siglecs) and dendritic cell function [19] and occurs at position 6 of GlcNAc and galactose. Much less is known about the physiological role of sulfation of C-4 of galactose. Sulfation of C-4 galactose occurs in the carbohydrate-protein linkage region of glycosaminoglycans (GAGs) [20]. However, the sulfated galactose in this instance is β 1-3 linked to another galactose rather than β 1-4 linked to GlcNAc. Furthermore, C4-sulfation of a galactose-related carbohydrate, *N-acetylated* galactosamine (GalNAc), represents an important modification in several GAGs, including chondroitin sulfate A and Dermatan sulfate. GAGs are prominently expressed in the extracellular matrix and on endothelial cells, where they regulate processes that are thought to be dysregulated in SSc, including wound healing, angiogenesis and lymphocyte migration. Further studies are currently underway to determine whether anti-4S-LacNAc antibodies recognize specific GAGs.

Glycan arrays have been utilized successfully in other systemic autoimmune diseases for the identification of potential novel biomarkers for diagnosis and prognosis [21]. It has also recently become evident that a number of carbohydrate structures are targeted by serum antibodies of healthy individuals [22]. Anti-4S-LacNAc antibodies in our study showed remarkable specificity for the position of the sulfate within the LacNAc structure. The antibodies are of the IgG isotype, as demonstrated by using an anti-human Fc-gamma specific secondary antibody for detection, as well as retention of anti 4S-LacNAc reactivity in the isolated IgG fraction and affinity-purified anti-4S-LacNAc antibody from patient serum (see supplemental figure 1). The generation of these antibodies likely is a result of somatic hypermutation and affinity maturation. While antibodies to carbohydrate structures were previously thought to develop in a T-lymphocyte independent manner, this paradigm has recently been shifting [23].

The second important finding of our study is the association of anti-4S-LacNAc antibodies with pulmonary hypertension in SSc. Autoantibodies previously associated with pulmonary hypertension in SSc include anti-centromere [24] and anti-U3 RNP [25], with a frequency of about 22 and 17 percent of patients affected, respectively. In our study, over one half of anti-4S-LacNAc positive patients (55.6%) had echocardiographic evidence of pulmonary vascular disease and anti-4S-LacNAc antibody positivity identified almost a quarter (23.4 %) of all SSc patients with pulmonary vascular disease. Therefore the antibody may provide a valuable biomarker for pulmonary hypertension in SSc. We are currently extending our cross-sectional data by longitudinal studies that correlate the onset and progression of PAH in relation to anti-4S-LacNAc positivity and titer. Interestingly, we did not find an increased frequency of anti-4S-LacNAc antibody positivity in patients with IPH. Our finding is in agreement with the notion that IgG antibodies from patients with SSc-associated PAH versus IPAH may express distinct reactivity profiles with regards to endothelial cell antigens [26] and that autoimmune and inflammatory pathways likely play a more dominant role in SSc-related PAH versus IPAH [27].

Interestingly, anti-4S-LacNAc positivity was associated with pulmonary vascular disease, but a less severe Raynaud's phenotype. An association between pulmonary hypertension and severity of Raynaud's remains controversial [28–30]. It will be important to determine in longitudinal studies whether anti-4S-LacNAc positivity in individual patients fluctuates with the degree of Raynaud's activity.

Anti-4S-LacNAc positivity in our study was also strongly associated with sicca complex in SSc patients. However, sicca in this cohort was solely defined based on the subjective

criteria of the AECC. Further studies with objective testing will be needed to determine the phenotype of this subgroup of patients.

Finally, it is important to point out that while we adjusted for important variables in our multivariate analysis, including age, race, gender, and disease type, the associations we observed need to be confirmed in additional independent SSc patient populations.

In summary, using a novel glycan array approach, we identify 4S-LacNAc as an important carbohydrate antigen targeted by the immune response in SSc. Clinically, anti-4S-LacNAc positivity was associated with pulmonary hypertension and sicca. Our findings suggest that further studies are warranted to elucidate the role of carbohydrate modifications in SSc and other systemic autoimmune diseases. A better understanding of such processes may lead to novel opportunities for diagnosis and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Recognition of distinct carbohydrates by serum antibodies in SSc

(A) Antibody binding ratio (ABR) of pooled SSc serum to 320 glycans determined by glycan array CFG 3.0 as outlined in Materials and Methods. (B) List of glycan structures that are highly recognized by SSc serum with an ABR > 2 SD above the mean (mean 1.57; SD 1.58). Dotted line represents cut-off for 2SD above mean. (C) Comparison of ABR between pooled serum from SSc and SLE. SSc serum recognizes a distinct set of glycans (■ ABR > 2 SD from mean).

Figure 2. Antibodies to 4SLacNAc are frequent and of high titer in patients with SSc

(A) Antibodies to 4SLacNAc were determined by ELISA as outlined in Materials and Methods in individual sera from 181 patients with SSc, 40 disease patients (SLE) and 40 healthy controls. A.U. values are significantly higher in SSc patients compared to normal healthy controls ($p=0.008$). Dotted line represents cut-off for 2SD above mean (B) Correlation between fluorescence values obtained by glycan array and A.U. obtained by ELISA in 17 selected SSc patients, $r^2=0.89$

Figure 3. Differential recognition of sulfated glycans by individual sera from SSc patients
 Antibody binding to all sulfated glycans present in glycan array CFG version 3.1 from 17 preselected individual patient sera (9 anti-4S-LacNAc positive patients/8 antibody negative patients as determined by ELISA). (■) low positive, (■) medium positive, (■) high positive (as defined in Materials and Methods). (*) denotes anti-4S-LacNAc positive patients as determined by ELISA.

Figure 4. Preferential recognition of 4S-LacNAc by individual sera from SSc patients
(A) ABR to sulfated LacNAc glycans present in glycan array CFG version 3.1 in 17 preselected sera from patients with SSc (9 anti-4S-LacNAc positive/8 antibody negative patients as determined by ELISA). (*) denotes anti-4S-LacNAc positive patients as determined by ELISA. (B) Confirmation of preferential recognition of 4S-LacNAc compared to 3S- or 6S-LacNAc by ELISA. Results from 26/181 previously identified anti-4S-LacNAc positive patients (figure 2A) are shown. (□) 4S-LacNAc, (■) 3S-LacNAc, (▒) 6S-LacNAc). 1 of the 27 anti-4S-LacNAc positive sera was not available for further testing.

Figure 5. Antibodies to 4S-LacNAc are not increased in patients with sicca or pulmonary hypertension independent from a diagnosis of systemic sclerosis

Antibodies to 4-sulfated LacNAc were determined by ELISA as outlined in Materials and Methods in individual sera from 181 patients with SSc, 40 patients with primary Sjogren's, 16 patients with SLE and secondary Sjogren's, 12 patients with RA and sicca and 25 patients with IPAH (* $p=0.042$).

Sociodemographic and disease characteristics of 181 patients with systemic sclerosis according to the presence of anti-4SLacNAc antibodies.

Table 1

Variable(*)	Positive (n=27)	Negative (n=154)	P (‡)	Unadjusted OR (95% CI) (¶)	Adjusted OR (95% CI) (§)
Age, years (range 22–87)	59.3±13.6	52.4±12.8	NS	1.0 (0.9, 1.0)	1.0 (0.9, 1.0)
Female, %	85	82	NS	1.2 (0.4, 3.8)	1.2 (0.4, 4.0)
Race/Ethnicity, %					
White	81	72		1	1
Black	15	24	NS	0.5 (0.2, 1.7)	0.8 (0.2, 2.7)
Other	4	4		0.8 (0.1, 7.3)	1.2 (0.1, 11.5)
Smoking, %					
Never	59	53		1	1
Past	22	29	NS	0.7 (0.2, 1.8)	0.6 (0.2, 1.7)
Current	19	18		0.9 (0.3, 2.7)	1.1 (0.4, 3.5)
SSc type, %					
Limited	81	79	NS	0.9 (0.3, 2.5)	1.1 (0.4, 3.1)
Diffuse	19	21			
Disease duration, years (range 0.1–40.8) (†)	11.0±8.9	10.7±7.9	NS	1.0 (1.0, 1.1)	1.0 (0.9, 1.0)
Rodnan's skin score, modified (range 0–51)	4.9±5.8	6.9±8.4	NS	0.9 (0.9, 1.0)	0.9 (0.9, 1.0)
Gastrointestinal severity score (0–4)	1.4±0.6	1.2±0.9	NS	1.2 (0.8, 1.9)	1.3 (0.8, 2.0)
Musculoskeletal involvement, %	33	37	NS	0.8 (0.3, 2.0)	1.0 (0.4, 2.5)
Kidney involvement, %	11	8	NS	1.2 (0.3, 4.3)	0.9 (0.2, 3.6)
Sicca complex, %	74	44	0.006	3.6 (1.4, 9.0)	3.0 (1.2, 8.2)
Lung severity score (0–4)	1.9±1.4	1.3±1.2	0.037	1.4 (1.0, 1.9)	1.5 (1.1, 2.1)
Pulmonary function					
FVC, % predicted	85±18	85±19	NS	0.99 (0.97, 1.02)	0.99 (0.97, 1.02)
DLCO, % predicted	68±22	69±21		0.99 (0.98, 1.02)	0.99 (0.97, 1.02)
Elevated eRVSP (>40 mmHg), %	56	32	0.020	2.7 (1.2, 6.2)	2.6 (1.1, 6.3)
Raynaud's Phenomenon severity score (0–4)	1.5±0.7	2.0±0.9	0.016	0.5 (0.3, 0.9)	0.5 (0.3, 0.9)
Digital Loss (history of), %	33	39	NS	0.8 (0.3, 1.9)	0.8 (0.3, 1.9)
Mortality, % Deceased	19	26	NS	0.7 (0.2, 1.9)	0.5 (0.2, 1.7)
Autoantibodies, %					

Variable ^(*)	Positive (n=27)	Negative (n=154)	P ^(‡)	Unadjusted OR (95% CI) ^(¶)	Adjusted OR (95% CI) ^(§)
Anti-Scl70	7	17		0.4 (0.1, 1.8)	0.3 (0.1, 1.8)
Anti-centromere	52	44	NS	1.4 (0.6, 3.2)	1.0 (0.4, 2.5)

^(*)Values are the mean ± SD unless indicated otherwise. The range is given with each variable group, where applicable.

^(‡)Time from first non-Raynaud's symptom.

^(‡)Univariate analysis for positive anti-glycan testing as a function of the sociodemographic or disease characteristics, NS=not significant (p>0.05).

^(¶)Logistic regression model for positive anti-glycan testing as a function of the sociodemographic or disease characteristics.

^(§)ORs were estimated using logistic regression adjusting for age, gender, race, and disease type.