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Earliest Phase of Systemic Sclerosis Typified by Increased Levels of Inflammatory Proteins in the Serum

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Objective. **Patients with definite systemic sclerosis (SSc) who lack fibrotic features can be stratified into an intermediate stage of disease severity between preclinical/early SSc (EaSSc) and fibrotic subsets (limited cutaneous SSc [lcSSc] and diffuse cutaneous SSc [dcSSc]). The aim of the present study was to molecularly characterize nonfibrotic SSc and EaSSc on the basis of a broad panel of serum markers of inflammation and tissue damage, in order to increase the knowledge of the pathophysiologic mechanisms underlying SSc progression before the development of fibrosis.**

Methods. **An 88-plex immunoassay was performed in serum samples from a discovery cohort composed of 21 patients with EaSSc (meeting the LeRoy and Medsger criteria), 15 with nonfibrotic SSc (meeting the American College of Rheumatology/European League Against Rheumatism 2013 classification criteria, without skin or lung fibrosis), and 11 healthy controls. Analyte concentrations that were consistently significantly different at the exploratory *P* value threshold of 0.1 were selected for replication analysis in a larger group composed of 47 patients with EaSSc, 48 with nonfibrotic SSc, and 43 healthy controls, as well as 51 patients with lcSSc and 35 with dcSSc. The value of the replicated molecules in predicting SSc progression (at a family-wise error rate of 0.05) was tested.**

Results. **Based on the results of the explorative analysis, 16 molecules were selected for testing in the replication set. The results showed that CXCL10, CXCL11, tumor necrosis factor receptor type II (TNFRII), and chitinase 3–like protein 1 levels were significantly increased in patients with EaSSc and those with nonfibrotic SSc as compared to healthy controls. The disease in patients with high concentrations of CXCL10 and TNFRII was also characterized by a faster rate of progression from EaSSc and from nonfibrotic SSc to worse disease stages.**

Conclusion. **SSc patients with preclinical/early SSc and those with established, yet nonfibrotic, disease exhibit clear molecular alterations that are associated with faster rates of disease evolution. These data open novel avenues for disease interception in SSc.**

The hallmark of systemic sclerosis (SSc) consists of fibrosis involving the skin and internal organs, with pathologic development in the context of endothelial damage and immune system activation. The sequence of events leading to widespread collagen deposition in SSc is largely unknown, but microvascular injury and perivascular infiltration by mononuclear cells in genetically predisposed individuals are considered early events in the disease course (1,2). Raynaud's phenomenon (RP) is usually the first manifestation of the disease that may antedate by years the onset of definite SSc. The presence of RP, SSc-specific autoantibodies, and SSc-specific nailfold videocapillaroscopic (NVC) changes, even in the absence of any other sign of definite SSc, identifies individuals at higher risk of developing SSc, a subset referred to as those with early SSc (EaSSc) (3) or those with undifferentiated connective tissue disease at risk for SSc (4).

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For years, the occurrence of skin fibrosis has been considered the pivotal sign for identification and classification of patients with SSc (5). However, it has been widely recognized that a classification that relies so extensively on the presence of skin fibrosis lacks enough sensitivity to identify patients with limited or early disease (6). The American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2013 classification criteria for SSc (6) were specifically designed to circumvent this problem, and fibrotic features are no longer a prerequisite to formalize the diagnosis of SSc. The ACR/EULAR 2013 criteria thus allow the identification of SSc even in the absence of overt fibrosis. A consensus to properly define this patient subset—a subset formerly termed as "definite" SSc (7) or "noncutaneous" SSc (8)—does not yet exist, and it is doubtful whether this designation would represent a necessary intermediate phase of the disease between EaSSc and fibrotic SSc or whether such patients might cluster in a separate disease subset characterized by a smoldering, slowly progressing entity. From a clinical point of view, patients with nonfibrotic SSc could be stratified in an intermediate stage of disease severity between EaSSc (the least severe) and the fibrotic subsets of SSc (the most severe) (7).

This peculiar intermediate pathologic behavior can also be observed when a number of laboratory parameters are taken into account, including indices of inflammation or serum concentrations of circulating markers of vascular activation and dysfunction (7). Despite the existing evidence, a thorough molecular characterization of these patients and individuals with EaSSc is lacking. An increased knowledge about the biologic characteristics of these subsets and about the changes that occur early during evolution of the disease is indeed of paramount importance to understand the pathophysiologic mechanisms of SSc progression. Moreover, this increased knowledge would allow us to distinguish those patients whose disease is bound to progress from those whose disease will not progress and who will continue to express a milder clinical phenotype, thus paving the way for early intervention and/or disease interception.

Fueled by these considerations, we screened a broad panel of serum markers of inflammation, tissue damage, vascular dysfunction, metabolism, and remodeling in subsets of patients with definite SSc and those with EaSSc in comparison to healthy controls. With a 2-step research strategy, we first identified a number of analytes that were differently expressed in a discovery cohort of patients with EaSSc and those with definite SSc, and then replicated the results in a second, larger cohort. We next assessed the validated molecules for comparison to that in SSc patients with overt fibrosis. Finally, to define the prospective value of these molecules, we characterized the disease progression rates retrospectively in each subset of SSc patients in the replication cohort. To this end, the survival estimates for patients with high or low levels of the validated molecules were compared.

PATIENTS AND METHODS

Patients and healthy controls. Two different cohorts of patients were considered for the study and identified using a 2-step research design, with discovery and replication steps. All patients were recruited in Italy, at the Scleroderma Unit of the Fondazione IRCCS Ca' Granda Policlinico di Milano.

For the discovery step, 21 patients with EaSSc (3) and 15 patients with SSc who met the ACR/EULAR 2013 classification criteria (6) and who were without skin fibrosis (having a modified Rodnan skin thickness score of 0 on a scale of 0–3, where 3 indicates severe thickening [9]) and without any sign of interstitial lung disease (ILD) or pulmonary arterial hypertension (PAH) (nonfibrotic SSc) were enrolled. ILD was defined as a typical involvement of the lung parenchyma of >5% on high-resolution computed tomography (10) accompanied by reduction in the forced vital capacity (FVC) or diffusing capacity for carbon monoxide (DLCO) of <80% of predicted, as previously described (11). In accordance with standardized protocols, the presence of PAH is routinely screened in patients with EaSSc and those with SSc, and is confirmed by right-sided heart catheterization (12). None of the patients in the discovery cohort were suspected of having PAH. The presence of puffy fingers was ascertained by the referring physician and then independently verified, in a blinded manner, by one of the authors (LB), to correctly differentiate puffy fingers from sclerodactyly. When the 2 independent assessors were not in agreement, consensus was reached. The duration of disease was defined as the time from the occurrence of the first non-RP symptom. The NVC pattern was assessed by an experienced observer in our center and classified according to the criteria of Cutolo et al (13). Eleven healthy control subjects, matched to the patients by sex and age, were also included as a comparison group in the discovery step.

For the replication step, sera from 224 subjects, whose characteristics have previously been described elsewhere (7), were used. This group of patients included 47 patients with EaSSc, 48 with nonfibrotic SSc, and 43 age- and sex-matched healthy controls. In addition, 51 patients with limited cutaneous SSc (lcSSc) and 35 with diffuse cutaneous SSc (dcSSc) were enrolled.

Each patient underwent a complete evaluation to correctly allocate them to the different study groups, as previously described (7). The baseline clinical assessment was performed between the end of 2011 and mid-2012, allowing the follow-up data to be evaluated retrospectively for disease progression. Data were available for 143 patients (34 with EaSSc, 38 with nonfibrotic SSc, 41 with lcSSc, and 30 with dcSSc). Progression was defined as follows: 1) for those with dcSSc, death attributable to SSc, worsening of lung function (i.e., a reduction in the FVC of ≥10% from baseline or a reduction in the DLCO of ≥15% from baseline),

Table 1. Baseline demographic and clinical characteristics of the patients in the discovery cohort*

	EaSSc (n = 21)	Nonfibrotic SSc (n = 15)
Female, no. (%)	20 (95.2)	14 (93.3)
Age, mean ± SD years	53 ± 16	53 ± 14
RP duration, mean ± SD years	10 ± 8	13 ± 10
Disease duration, mean ± SD years	NA	8 ± 6
Autoantibodies, no. (%)		
ANA	18 (85.7)	15 (100)
Anti–RNAP III	0 (0)	0 (0)
ACA	14 (66.7)	11 (73.3)
Anti–topo I	2 (9.5)	2 (13.3)
NVC changes, no. (%)	17 (80.9)	14 (93.3)
Telangiectasias, no. (%)	0 (0)	4 (26.7)
Puffy fingers, no. (%)	0 (0)	13 (86.7)
Digital ulcers, no. (%)	0 (0)	2 (13.3)

* Patients were stratified according to disease subsets of early systemic sclerosis (EaSSc) and nonfibrotic SSc (definite SSc, without lung or skin fibrosis). RP = Raynaud's phenomenon; NA = not applicable; ANA = antinuclear antibody; anti–RNAP III = anti–RNA polymerase III antibody; ACA = anticentromere antibody; anti–topo I = anti–topoisomerase I antibody; NVC = nailfold videocapillaroscopic.

identification of PAH, or identification of digital ulcers in the absence of a history of digital ulceration; 2) for those with lcSSc, the same criteria as applied to dcSSc, as well as the progression of skin disease from limited to diffuse involvement; 3) for those with nonfibrotic SSc, the same criteria as applied to lcSSc, as well as the progression of skin or lung involvement and identification of fibrotic features; 4) for those with EaSSc, the same criteria as applied to nonfibrotic SSc, as well as the identification of telangiectasias or puffy fingers.

The baseline demographic and clinical characteristics of the patients in both the discovery cohort and the replication cohort are summarized in Tables 1 and 2. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee (Comitato Etico Area B).

Serum multiplex assay. A total of 88 markers of immune activation, inflammation, and tissue damage and additional markers of vascular remodeling and adhesion were selected for the analysis (a full list of the tested markers is available upon request from the corresponding author). Peripheral blood was obtained by venipuncture from all subjects at the time of enrollment. Serum was separated by centrifugation at 1,500*g* for 10 minutes and stored at −80°C until analyzed. Serum analyses were performed at the MultiPlex Core Facility of the Laboratory of Translational Immunology (University Medical Center Utrecht). An in house–developed multiplex immunoassay based on Luminex technology (xMAP; Luminex) was utilized, as previously described (14).

For statistical analysis, serum concentrations below the detection limit were converted to one-half of the lower limit of detection. When more than 5% of measurements were below the detection limit (out of range >5%), the analyte was excluded from further analysis. After performing this quality control step, 46 proteins passed the threshold to undergo statistical analysis in the discovery cohort (a complete list of the 42 molecules that did not meet this prerequisite is available upon request from the corresponding author).

Statistical analysis. Statistical analyses were conducted using SPSS software (version 22.0; IBM). Continuous variables are expressed as the mean ± SD; data were log-transformed before being analyzed. In the discovery phase, one-way analysis of variance (ANOVA) was performed, along with an ANOVA polynomial linear test for trend. In both tests, the exploratory significance threshold was $P = 0.1$.

Table 2. Baseline demographic and clinical characteristics of the patients in the replication cohort*

	EaSSc (n = 47)	Nonfibrotic SSc (n = 48)	lcSSc (n = 51)	dcSSc (n = 35)
Female, no. (%)	44 (93.6)	48 (100)	48 (94.1)	31 (88.6)
Age, mean ± SD years	53 ± 14	62 ± 13	62 ± 10	55 ± 13
RP duration, mean ± SD years	11 ± 10	17 ± 11	18 ± 11	15 ± 11
Disease duration, mean ± SD years	NA	9 ± 8	14 ± 8	12 ± 10
Autoantibodies, no. (%)				
None	6 (12.8)	1 (2.1)	3 (5.9)	1 (2.9)
ANA	41 (87.2)	47 (97.9)	48 (94.1)	34 (97.1)
Anti–RNAP III	3 (6.4)	0 (0)	3 (5.9)	3 (8.6)
ACA	22 (46.8)	42 (87.5)	22 (43.1)	1 (2.9)†
Anti–topo I	7 (14.9)	4 (8.3)	15 (29.4)	24 (68.6)†
NVC changes, no. (%)	42 (89.4)	48 (100)	NA	NA
Telangiectasias, no. (%)	0 (0)	29 (60.4)	34 (66.7)	30 (85.7)
Puffy fingers, no. (%)	0 (0)	40 (83.3)	NA	NA
Digital ulcers, no. (%)	0 (0)	3 (6.3)	12 (23.5)	7 (20)
Pitting scars, no. (%)	0 (0)	7 (14.6)	9 (17.6)	2 (5.7)
ILD, no. (%)	0 (0)	0 (0)	17 (33.3)	17 (48.6)
PAH, no. (%)	0 (0)	0 (0)	2 (4)	0 (0)
Use of immunosuppressants, no. (%)	0 (0)	1 (2.1)	7 (13.7)	12 (34.3)

* Patients were stratified according to disease subsets of early systemic sclerosis (EaSSc), nonfibrotic SSc (definite SSc, without lung or skin fibrosis), limited cutaneous SSc (lcSSc), and diffuse cutaneous SSc (dcSSc). RP = Raynaud's phenomenon; NA = not applicable; ANA = antinuclear antibody; anti–RNAP III = anti–RNA polymerase III antibody; NVC = nailfold videocapillaroscopic; ILD = interstitial lung disease; PAH = primary pulmonary arterial hypertension.
† One patient was double-positive for anticentromere antibodies (ACAs) and anti–topoisomerase I antibodies (anti–topo I).

Table 3. Summary of findings in the discovery cohort*

Analyte	Cohort			ANOVA *P*†	
	Healthy controls	EaSSc	Nonfibrotic SSc	F-test	Polynomial test for trend
IL-13, pg/ml	24.5 ± 21.4	258.5 ± 630.3	112.8 ± 273.5	NS	NS
IL-18, pg/ml	52.2 ± 20.0	86.6 ± 62.7	97.5 ± 33.8	0.039‡	0.012‡
G-CSF, ng/ml	0.439 ± 0.837	325.0 ± 1345.0	124.1 ± 454.7	0.019‡	NS
MIF, pg/ml	759.1 ± 631.5	916.9 ± 550.9	690.7 ± 429.4	NS	NS
CCL2, pg/ml	74.3 ± 29.0	96.2 ± 29.4	101.8 ± 39.7	0.076‡	0.036‡
CCL4, pg/ml	93.6 ± 59.2	129.5 ± 64.0	138.0 ± 55.6	0.051‡	0.020‡
CCL5, ng/ml	166.5 ± 49.5	142.1 ± 40.9	118.8 ± 58.8	0.033‡	0.013‡
CCL11, pg/ml	45.7 ± 25.6	118.3 ± 140.1	65.2 ± 31.4	0.041‡	NS
CCL17, pg/ml	210.8 ± 97.1	247.2 ± 123.3	264.9 ± 222.3	NS	NS
CCL18, ng/ml	565.7 ± 219.1	745.3 ± 647.3	896.6 ± 668.9	NS	NS
CCL19, pg/ml	28.3 ± 20.0	82.6 ± 86.3	182.2 ± 404.6	0.033‡	0.013‡
CCL22, pg/ml	494.7 ± 114.2	467.9 ± 185.7	505.9 ± 268.7	NS	NS
CCL25, pg/ml	729.2 ± 439.3	581.1 ± 311.2	562.1 ± 260.9	NS	NS
CXCL1, pg/ml	22.5 ± 76.1	188.0 ± 252.2	130.6 ± 110.4	NS	NS
CXCL5, pg/ml	549.3 ± 383.7	478.4 ± 294.3	462.6 ± 400.5	NS	NS
CXCL7, μg/ml	18.06 ± 4.12	15.43 ± 3.88	16.40 ± 3.78	NS	NS
CXCL9, pg/ml	76.6 ± 23.4	144.7 ± 93.5	93.7 ± 45.3	0.027‡	NS
CXCL10, pg/ml	193.5 ± 63.5	595.5 ± 409.2	583.0 ± 265.4	0.00005‡	0.00004‡
CXCL11, pg/ml	32.2 ± 24.7	61.8 ± 54.5	64.8 ± 49.9	NS	0.070‡
CXCL13, pg/ml	15.9 ± 8.6	52.4 ± 76.2	26.8 ± 30.4	0.066‡	NS
Osteopontin, ng/ml	9.0 ± 5.7	12.6 ± 5.0	13.0 ± 5.7	NS	NS
MMP-1, ng/ml	14.90 ± 7.12	16.86 ± 12.05	19.53 ± 16.72	NS	NS
MMP-3, ng/ml	16.39 ± 6.57	22.09 ± 14.52	20.37 ± 12.30	NS	NS
MMP-8, ng/ml	39.71 ± 18.56	51.80 ± 35.46	51.18 ± 28.10	NS	NS
MMP-9, ng/ml	1,860.9 ± 1,672.1	2,925.0 ± 1,718.3	3,072.7 ± 1,708.9	NS	NS
Cathepsin A, pg/ml	791.2 ± 347.0	1483.6 ± 1334.4	1483.6 ± 1334.4	NS	NS
Cathepsin B, ng/ml	12.66 ± 10.52	14.17 ± 7.39	12.83 ± 5.92	NS	NS
Cathepsin L, pg/ml	2,905.6 ± 360.5	2,884.0 ± 346.6	3,381.1 ± 992.0	0.051‡	0.062‡
Cathepsin S, ng/ml	9.99 ± 1.81	12.13 ± 2.78	11.45 ± 2.26	0.063‡	NS
TNFRII, pg/ml	1,041.6 ± 413.7	1,940.9 ± 912.1	1,660.1 ± 632.5	0.001‡	0.007‡
Galectin 1, ng/ml	19.68 ± 4.94	25.58 ± 6.40	28.38 ± 17.45	NS	NS
Galectin 3, ng/ml	43.12 ± 6.26	43.33 ± 8.18	38.27 ± 6.58	0.094‡	0.084‡
TIMP-1, ng/ml	225.1 ± 28.6	247.6 ± 38.3	253.5 ± 62.7	NS	NS
C5a, ng/ml	49.44 ± 10.46	58.91 ± 13.82	51.42 ± 18.15	NS	NS
S100A8, ng/ml	169.2 ± 55.2	145.0 ± 40.1	163.1 ± 76.1	NS	NS
CHI3L1, ng/ml	83.70 ± 74.27	95.45 ± 45.80	102.07 ± 35.71	NS	0.084‡
P-selectin, ng/ml	362.7 ± 226.4	448.9 ± 306.8	328.5 ± 140.1	NS	NS
Endoglin, pg/ml	686.9 ± 309.0	850.0 ± 297.2	793.3 ± 363.5	NS	NS
Angiopoietin 1, ng/ml	46.87 ± 10.13	45.40 ± 19.12	51.05 ± 18.74	NS	NS
PAI-1, μg/ml	11.72 ± 1.36	11.85 ± 1.47	11.81 ± 3.79	NS	NS
Adiponectin, μg/ml	234.5 ± 82.3	217.8 ± 86.0	206.7 ± 88.5	NS	NS
Adipsin, pg/ml	2,228.2 ± 173.0	2,171.9 ± 261.4	2,051.4 ± 342.4	NS	NS
Leptin, pg/ml	5,965.4 ± 3133.4	5,659.0 ± 3824.4	4,645.3 ± 952.2	NS	NS
Chemerin, ng/ml	331.5 ± 35.6	306.9 ± 43.0	311.5 ± 27.8	NS	NS
Apelin, ng/ml	24.86 ± 2.42	26.06 ± 4.35	25.04 ± 5.15	NS	NS
Resistin, ng/ml	41.67 ± 12.66	56.11 ± 38.94	58.30 ± 32.80	NS	NS

* Values are the mean ± SD. EaSSc = early systemic sclerosis; nonfibrotic SSc = definite SSc, without lung or skin fibrosis; IL-13 = interleukin-13; G-CSF = granulocyte colony-stimulating factor; MIF = macrophage migration inhibitory factor; MMP-1 = matrix metalloproteinase 1; TNFRII = tumor necrosis factor receptor type II; TIMP-1 = tissue inhibitor of metalloproteinases 1; CHI3L1 = chitinase 3–like protein 1; PAI-1 = plasminogen activator inhibitor 1.
† *P* values were determined by one-way analysis of variance (ANOVA) F-test or by one-way ANOVA polynomial test for trend among all 3 groups. Except where indicated otherwise, differences were not significant (NS).
‡ Significant difference at the *P* value threshold of <0.1.

In the replication step, the mean concentrations of the molecules selected in the discovery step were compared using an ANOVA test and an ANOVA polynomial linear test for trend. Results were declared significant at the level of $P = 0.05$, after adjustment for multiple comparisons according to the Bonferroni method (i.e., 0.05/13 = 0.0038). When the global ANOVA F score was significant at the adjusted threshold, pairwise comparisons with the Tukey's method were performed, with significant differences defined at the *P* value threshold of 0.05.

Heatmaps were used to visually explore the global behavior of the biomolecules that were replicated. To this end, data were normalized by scaling on an interval of 0–1. Normalization of the data and visualization were performed using Orange data mining software (15).

For analysis of evolution of the molecular concentrations, Turnbull's nonparametric estimator for interval-censored data (16) was used, since the collection of data was uneven, and statistical significance of the model was assessed using generalized log rank test statistics (17) after 10,000-fold exact permutation testing. Evolution of the concentration of each biomolecule was evaluated for those molecules that passed the replication step (as described above), choosing an optimal cutoff point that was within the 10th and 90th percentiles of the distribution of each molecule within each disease subset.

Data from patients in each disease subset were stacked to calculate the predicted survival and its associated test statistic in the overall SSc population. Survival analyses were performed using a custom Python code (written by LB; available upon request from the corresponding author). Matplotlib version 2.0.0 (18) was used to plot the survival curves.

RESULTS

Increased levels of CXCL10, CXCL11, tumor necrosis factor receptor type II (TNFRII), and chitinase 3–like protein 1 (CHI3L1) in EaSSc and nonfibrotic SSc. Of the 88 circulating proteins studied in the serum of subjects in the discovery cohort, comprising 36 SSc patients and 11 healthy control counterparts, 16 proteins were found to be altered in the patients at the exploratory *P* value threshold of 0.1. These 16 proteins were interleukin-18 (IL-18), granulocyte colony-stimulating factor (G-CSF), CCL2, CCL4, CCL5, CCL11, CCL19, CXCL9, CXCL10, CXCL11, CXCL13, cathepsin L, cathepsin S, TNFRII, galectin 3, and CHI3L1 (Table 3). All 16 analytes that passed the exploratory threshold for statistical significance in the discovery cohort were considered for replication analysis.

Quality testing was then independently performed in the replication cohort after the serum samples were assayed. In the replication cohort, the concentrations of G-CSF, cathepsin L, and galectin 3 were below the detection limit in >5% of samples tested, and therefore all 3 were excluded from further experiments. Thus, only 13 analytes, as listed in Table 4, underwent statistical analysis in the replication cohort, consisting of serum samples from 43 healthy donors and 181 patients with SSc, including the subsets of EaSSc, nonfibrotic SSc, lcSSc, and dcSSc.

In the replication analysis, 4 analytes, namely, CXCL10, CXCL11, TNFRII, and CHI3L1, were found to be increased both in patients with EaSSc and in patients with nonfibrotic SSc as compared to healthy controls (Table 4 and Figure 1A). Noteworthy, the levels of all 4 of these markers showed linearly increasing trends toward significant differences from healthy controls when compared to patients with EaSSc and to patients with nonfibrotic SSc. Importantly, in addition to their levels being increased in EaSSc and nonfibrotic SSc, these molecules were also up-regulated in patients with lcSSc and those with dcSSc (Figure 1B), suggesting that increased levels of CXCL10, CXCL11, TNFRII, and CHI3L1 in EaSSc and/or nonfibrotic SSc may mark disease progression.

This hypothesis was further substantiated by the fact that CXCL10, CXCL11, TNFRII, and CHI3L1 displayed an overall progressive increase in concentration when patients with EaSSc, patients with nonfibrotic SSc, and patients with fibrotic SSc (lcSSc and dcSSc considered as a whole group) were compared to healthy controls (Figure 1C). Some of the patients with lcSSc and those with dcSSc were receiving immunosuppressive medications at the time of study inclusion (Table 2); nevertheless, we found no difference in the concentrations of the replicated molecules when stratifying patients according to whether they were or were not receiving immunosuppressive therapy (data not shown). A large proportion of the patient population was positive for anticentromere antibodies (ACAs), but no association with the levels of the candidate proteins and the pattern of ACA expression could be detected (data not shown).

Association of CXCL10 and TNFRII concentrations with shortest disease progression times. Overall, 59 (41.2%) of 143 patients with available follow-up data within the replication cohort showed disease progression after a maximum follow-up time of 59.6 months. Nonexclusive causes of progression included death (n = 8), worsening of lung function (n = 38), new onset of PAH (n = 4), worsening of skin disease (n = 10), new onset of digital ulcers (n = 4), and identification of telangiectasias (n = 7). The estimated median time to evolution of disease (from all causes) was between 52.6 months and 52.9 months. Patients with higher levels of CXCL10 and TNFRII showed a shorter progression time from EaSSc and nonfibrotic SSc to other disease subsets. In particular, the most consistent results were observed in those with increased CXCL10 levels, both in the EaSSc (P = 0.01) and nonfibrotic SSc (P = 0.006) subsets

Table 4. Summary of findings in the replication cohort*

Variable	Analyte levels by cohort, pg/ml			ANOVA *P*†				
						Pairwise test		
	Healthy controls	EaSSc	Nonfibrotic SSc	Polynomial test for trend	F-test	EaSSc vs. healthy controls	Nonfibrotic SSc vs. healthy controls	EaSSc vs. nonfibrotic SSc
IL-18	77.16 ± 47.05	87.5 ± 58.88	75.58 ± 37.39	NS	NS	NA	NA	NA
CCL2	75.96 ± 33.29	79.79 ± 31.82	75.93 ± 35.69	NS	NS	NA	NA	NA
CCL4	54.11 ± 38.58	55.05 ± 28.56	62.13 ± 29.59	NS	NS	NA	NA	NA
CCL5	40,361.9 ± 40,387.4	32,650.89 ± 30,435.99	29,799.58 ± 31,104.92	NS	NS	NA	NA	NA
CCL11	53.98 ± 34.36	46.12 ± 22.25	41.47 ± 22.49	NS	0.027‡	NS	NS	NS
CCL19	7.18 ± 17.56	4.24 ± 9.95	7.87 ± 18.56	NS	NS	NA	NA	NA
CXCL9	3.12 ± 3.74	4.01 ± 4.95	5.98 ± 6.65	0.035‡	0.011‡	NS	0.029	NS
CXCL10	118.24 ± 70.81	163.7 ± 87.82	188.18 ± 115.77	0.001§	2.71×10^{-4}§	0.029	7.87×10^{-4}	NS
CXCL11	24.23 ± 25.8	47.39 ± 38.64	54.23 ± 42.76	7.98×10^{-5}§	4.01×10^{-5}§	0.002	1.18×10^{-4}	NS
CXCL13	4.29 ± 8.39	3.06 ± 5.5	5.62 ± 8.47	NS	NS	NA	NA	NA
Cathepsin S	4,099.37 ± 1,849.13	4,173.71 ± 1,636.27	5,080.06 ± 2,226.36	0.032‡	0.019‡	NS	NS	NS
TNFRII	1,656.96 ± 789.1	2,388.42 ± 1,137.45	3,034.52 ± 1,368.8	6.99×10^{-8}§	1.13×10^{-8}§	0.001	3.88×10^{-8}	0.042
CHI3L1	31,032.26 ± 21,818.7	45,230.43 ± 35,867.39	51,861.37 ± 31,904.37	0.003§	8.03×10^{-4}§	0.038	0.002	NS

* Analyte levels are the mean ± SD. EaSSc = early systemic sclerosis; nonfibrotic SSc = definite SSc, without lung or skin fibrosis; NA = not applicable; IL-18 = interleukin-18; TNFRII = tumor necrosis factor receptor type II; CHI3L1 = chitinase 3–like protein 1.

† *P* values were determined by one-way analysis of variance (ANOVA) polynomial test for trend among all 3 groups, one-way ANOVA F-test, or one-way ANOVA with Tukey's post hoc test for pairwise comparisons. Except where indicated otherwise, differences were not significant (NS).

‡ Significant difference at the uncorrected *P* value threshold of 0.05.

§ Significant difference at the *P* value threshold of 0.05 after Bonferroni correction for multiple comparisons.

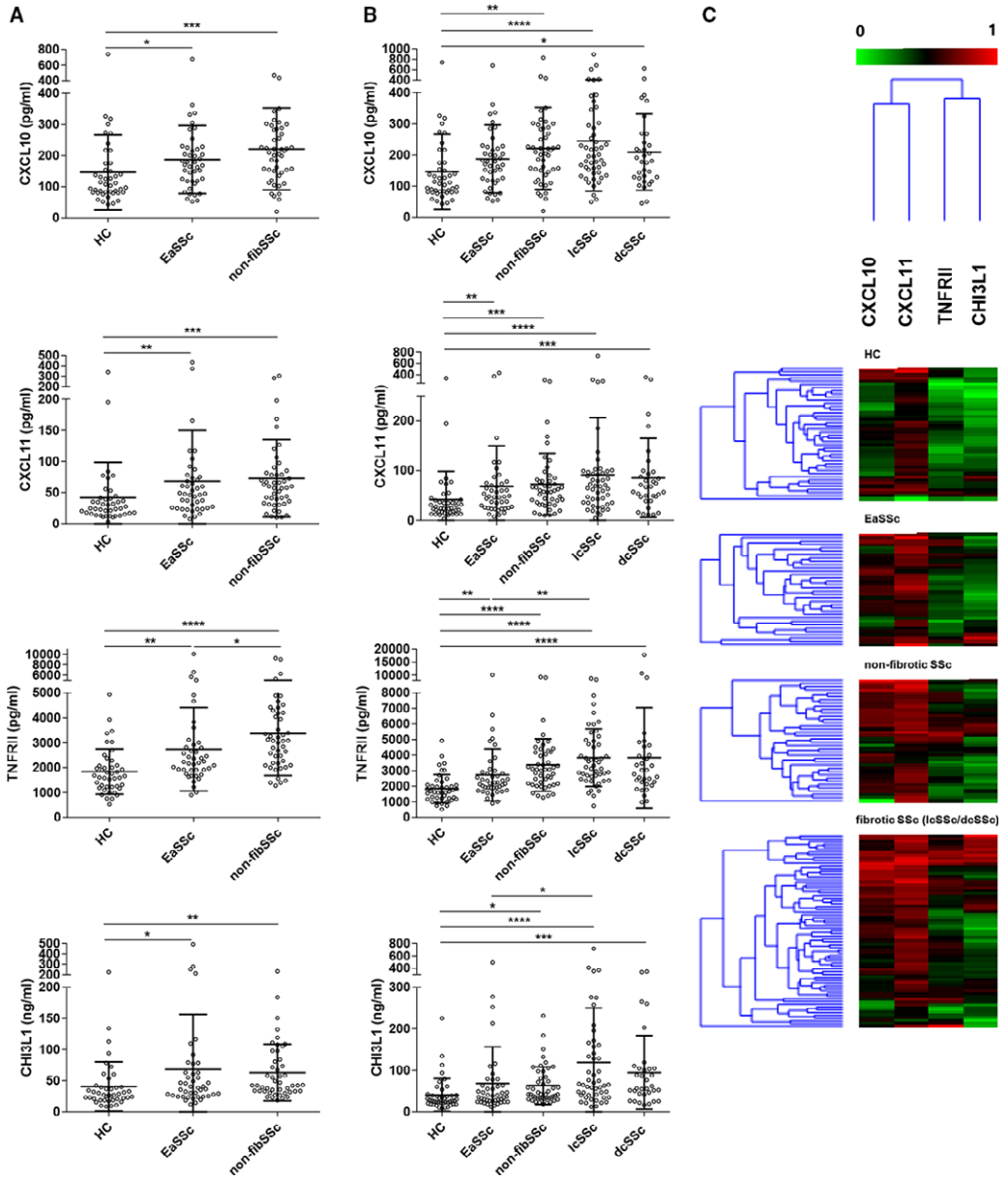

Figure 1. Replicated immune activation markers differently expressed in the circulation of patients with early systemic sclerosis (EaSSc), those with definite SSc in the absence of fibrosis of the skin and internal organs (nonfibrotic SSc [nonfibSSc]), or those with definite SSc in the presence of fibrosis of the skin and internal organs (limited cutaneous SSc [lcSSc] and diffuse cutaneous SSc [dcSSc]) compared to healthy controls (HC). **A** and **B,** Concentrations of the soluble analytes that were replicated as being significantly differently expressed (CXCL10, CXCL11, tumor necrosis factor receptor type II [TNFRII], and chitinase 3–like protein 1 [CHI3L1]) in comparisons of healthy controls to patients with EaSSc and those with nonfibrotic SSc **(A)** or to patients with EaSSc, those with nonfibrotic SSc, those with lcSSc, and those with dcSSc **(B)** in the replication cohort. Each symbol represents an individual patient; horizontal lines with bars show the mean and SD. Differences in the serum concentration levels were considered significant at the Bonferroni-adjusted threshold of $P < 0.0038$ by one-way analysis of variance (ANOVA) polynomial test for trend and/or one-way ANOVA test with Tukey's post hoc test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$. **C,** Heatmaps of analyte profiles in healthy controls and each SSc disease subset (patients with lcSSc and those with dcSSc considered jointly). Data were normalized for each analyte individually, and a color profile was made to show the cytokine pattern of each individual (represented by colored horizontal blocks: bright green = minimal expression; bright red = maximal expression).

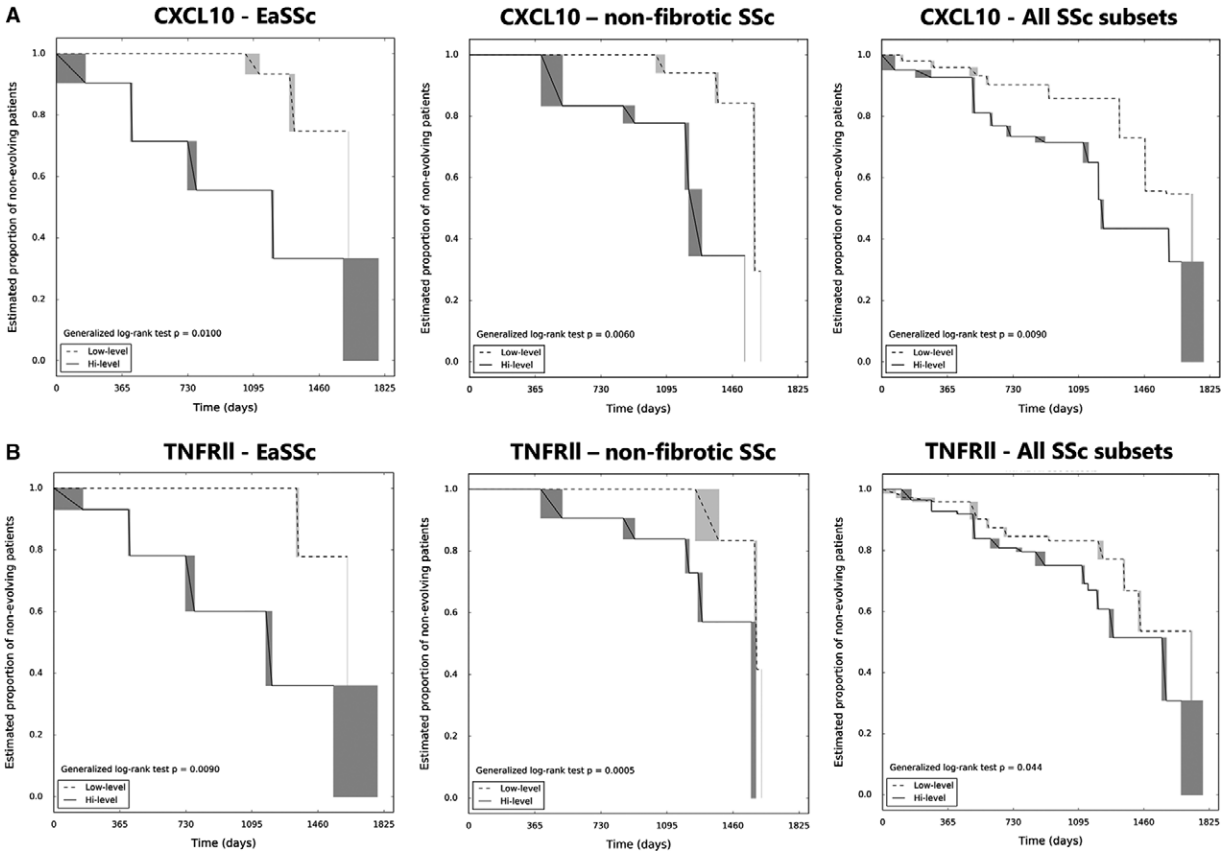

Figure 2. Survival curves of disease evolution times in patients with EaSSc, those with nonfibrotic SSc, and all SSc patients in relation to low serum levels versus high serum levels of CXCL10 **(A)** and TNFRII **(B)**. Survival estimates were determined using Turnbull's method for interval-censored data. Generalized log rank test statistic probabilities were calculated using 10,000-fold exact permutation testing. See Figure 1 for definitions.

(Figure 2A). These results, together with the different rates of progression observed in patients with dcSSc (P = 0.029), accounted for the overall different times of evolution to worse disease subsets between the high-expressing and low-expressing CXCL10 patient groups in the overall SSc population (P = 0.009) (Figure 2A).

Similarly, categorization of TNFRII levels could explain the different rates of disease progression in patients with EaSSc (P = 0.009) (Figure 2B), in those with nonfibrotic SSc (P = 0.0005) (Figure 2B), and in those with lcSSc (P = 0.0065) (data not shown). Furthermore, the concentrations of TNFRII were marginally associated with disease evolution times in the overall SSc population (P = 0.044) (Figure 2B).

Higher levels of CHI3L1 were associated with a shorter disease evolution time in patients with dcSSc (P = 0.004) (data not shown). However, levels of CHI3L1 were not associated with disease evolution times in patients in the early/nonfibrotic phase of SSc or in patients with lcSSc. Finally, the concentrations of CXCL11 were not associated with time to evolution in any of the disease subsets (data not shown).

DISCUSSION

SSc is a heterogeneous disease and patients can be clinically stratified into different clinical phenotypes. Although SSc with overt fibrosis has traditionally been the subject of extensive research, little is known about the pathophysiologic alterations that might characterize patients with preclinical features of scleroderma (EaSSc) or patients with a formal diagnosis of SSc who lack fibrotic features. Recent endeavors have clearly demonstrated that most patients with EaSSc are bound to progress toward definite SSc (11,19), and efforts have been made to formalize the criteria for recognition of SSc even in the absence of the prototypical hallmarks of fibrotic changes (6). Nonetheless, biomarkers that could be used to characterize the earliest stages of disease and its progression are still lacking. Thus, herein we have described for the first time the

identification and validation of 4 circulating biomarkers, CXCL10, CXCL11, TNFRII, and CHI3L1, that could enable the discrimination of EaSSc and nonfibrotic SSc subsets from healthy controls. Most importantly, the concentrations of CXCL10 and TNFRII also could identify those patients who show faster progression to definite disease or to lcSSc or dcSSc.

In previous studies, attempts have been made to categorize SSc based on the level of circulating inflammation markers. Liu and colleagues, for instance, based their composite score of plasma interferon (IFN)–inducible chemokines on the combination of CXCL10 and CXCL11, and described correlation of their levels with the IFN signature and with the severity of lung, muscle, and skin involvement in patients with fibrotic SSc (20). We have recently shown that patients with SSc without fibrotic features have the highest average IFN signature score and the highest prevalence of the IFN signature among the different disease subgroups (EaSSc, lcSSc, and dcSSc) when compared to healthy controls, with the EaSSc group closely following the nonfibrotic SSc group and surpassing patients with lcSSc and those with dcSSc in terms of the IFN score and prevalence (8). Consistent with these findings, we demonstrated herein that increased levels of CXCL10 and CXCL11 are present in the serum of patients with nonfibrotic SSc and, to a smaller extent, in the serum of patients with EaSSc. We herein also confirmed their increased levels in the circulation of patients with lcSSc and those with dcSSc, as has also been described previously (20,21), to levels similar to those observed in the nonfibrotic SSc group.

The antiangiogenic effects of CXCL10 (22), as well as its promoting role in the proliferation of vascular smooth muscle cells (23), support the notion of an early role of CXCL10 in amplifying endothelial damage and driving EaSSc and nonfibrotic SSc toward fibrotic modifications. This notion is corroborated by the findings observed in patients with localized scleroderma, in whom circulating CXCL10 levels are also increased, and whose increased levels correlate with disease activity (24). In this regard, the administration of anifrolumab, a monoclonal antibody directed against the IFNα receptor type 1, in patients with fibrotic SSc was found to be associated with a decrease in the blood and skin IFN signature (25) and with a drop in circulating CXCL10 levels as well (26); the down-regulation of the IFN signature correlated with the decrease in the levels of CXCL10. It is intriguing to speculate how the administration of anifrolumab would affect the rate of progression of EaSSc and nonfibrotic SSc, in particular in terms of halting the development of fibrotic features in those individuals with the highest CXCL10 levels, indicative of faster progression.

CXCL10 and CXCL11, as well as CXCL9—which also showed a trend toward increased levels in patients with nonfibrotic SSc in our cohort, but without reaching a statistically significant difference from healthy controls—all bind to CXCR3 on activated lymphocytes (T cells, natural killer [NK] cells, and NK T [NKT]–like cells) and endothelial cells (ECs). In spite of a well-documented increased expression of CXCL10 and CXCL9 in the serum and in the lesional skin of SSc patients, the expression of CXCR3 has been shown to be reduced in SSc skin and to be confined to ECs only (27). CXCR3-deficient mice challenged with bleomycin displayed a higher incidence of mortality attributable to increased lung fibrosis when compared to wild-type mice. A possible reason for this phenomenon is the decreased recruitment of NK and NKT-like cells in the lung and the consequent decline in levels of antifibrotic IFNγ in the tissue (28).

The decrease in CXCR3 levels in patients with SSc could reflect a mechanism of down-regulation upon overexposure to its ligands and to proinflammatory mediators and/or mirror the progressive decrease in leukocyte infiltration in late stages of fibrosis. In this regard, it would be of interest to investigate the expression of CXCR3 in patients with EaSSc and those with nonfibrotic SSc in whom fibrosis has not yet developed, and to follow up the pattern of expression over time in parallel with the progression of disease and lung involvement. These investigations would be particularly relevant in those individuals with higher circulating levels of CXCL10, who showed faster rates of disease progression in our cohort.

Circulating TNFRII has recently been described as a biomarker of disease activity in juvenile dermatomyositis (29), while in rheumatoid arthritis, not only is TNFRII typically associated with disease activity, but also its levels have been found to be increased in the circulation years before disease onset (30). Similarly, we found that the levels of TNFRII were increased in patients with preclinical EaSSc, and increased to a greater extent in those in whom SSc developed faster. In patients with nonfibrotic SSc, the observed increase in TNFRII was even higher than that in patients with EaSSc, and patients with the highest circulating concentrations had faster times of progression to fibrotic disease. It is indeed of interest to observe that TNFRII levels peaked in patients with lcSSc and those with dcSSc. Hügle et al also described an increase in TNFRII levels in the circulation of patients with fibrotic SSc, which marked the activation of the T cell compartment (31). In the same study, TNFRII was also

found to be overexpressed on T cells of the dermis of patients, and positively correlated with the extent of skin thickening. Furthermore, triggering of TNFRII on T cells in vitro led to the release of profibrotic cytokines, which further stimulated collagen production by fibroblasts (31). The shedding of soluble TNFRs reflects a status of broad immune activation and, in the case of TNFRII, predominantly mirrors the activation of T cells (32). Taken together, these data suggest that T cells might be implicated in the evolution process in SSc. Nevertheless, the biologic implications of increased TNFRII concentrations in the circulation of SSc patients need to be clarified in future studies, since the soluble receptor could either compete for TNFα binding with T cells—thereby inhibiting its effects—or act as a carrier of TNFα to different tissues and thus stabilize the TNFα serum concentration—thereby functioning as a reservoir.

A novel biomarker identified by our study is CHI3L1, a proinflammatory cytokine proposed as a biomarker of disease progression and severe prognosis in several chronic inflammatory diseases and cancer (33). Secreted by neutrophils, macrophages, vascular smooth muscle cells, synovial cells, chondrocytes from arthritic joints, hepatic stellate cells, and cancer cells, CHI3L1 binds to collagen and glycosaminoglycans in the extracellular matrix (34) and stimulates fibroblast growth, thus participating in tissue remodeling and the development of fibrosis. In SSc, its increased levels in the circulation has been associated with articular involvement and augmented levels of soluble IL-2 receptor α (35), a sign of T cell activation. CHI3L1 has also been linked to lung fibrosis and reduction in the DLco, with documented protein expression in macrophages and neutrophils in lung biopsy specimens from an SSc patient with lung inflammation and fibrosis (36). Its gradual increase in levels occurring linearly from healthy controls to patients with EaSSc to patients with nonfibrotic SSc to the highest levels in the lcSSc and dcSSc subsets, and its association with shortest time of disease progression in patients with dcSSc, could be a reflection of the different stages of disease severity. These findings offer grounds to study these molecules as prognostic biomarkers in SSc.

To our knowledge, this is the first study in a large cohort of patients with EaSSc and patients with nonfibrotic SSc conducted for validation of the biomarkers generated before the development of overt fibrosis. A limitation of our study is the fact that the serum proteins were not measured longitudinally and that the clinical data were collected retrospectively, and as a consequence, follow-up data were not available for each individual. In particular, it would have been of interest to assess how the levels of the validated molecules would be stratified after clinical progression. This second measurement, however, would have created problems with interpretation of the results, considering the high intermeasurement variability of the multiplex assays. Nevertheless, the results presented corroborate the concept of nonfibrotic SSc as a separate, intermediate entity linking the preclinical stage of SSc to the fibrotic, most severe subsets of the disease. We also for the first time provide evidence of molecules whose upregulation in the serum is associated with faster progression to definite disease in patients with EaSSc or faster development of fibrotic features in patients with nonfibrotic SSc. The present study thus offers new ground to understand the pathophysiologic mechanisms of SSc progression and opens new avenues for disease interception.

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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. Radstake 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.

Study conception and design. Cossu, Rossato, Beretta, Radstake.
Acquisition of data. Cossu, van Bon, Preti, Beretta.
Analysis and interpretation of data. Cossu, Beretta, Radstake.

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