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14. ABSTRACT
Systemic sclerosis (SSc-Scleroderma) is associated with substantial morbidity and mortality. Interstitial lung disease (ILD) is the leading cause of disease-related mortality. Response to immunosuppression is highly variable in patients with SSc-ILD. The currently available clinical markers are inadequate for identifying the likely responders. Our goal is to develop prediction tools using a combination of molecular biomarkers with potential clinical predictors. Serum based candidate biomarkers have been identified in the Scleroderma Lung Study II and replicated in an observational cohort. The predictive significance of these serum biomarkers was independent of clinical predictors. Peripheral blood gene expression modules predictive of ILD course were also identified. Moreover, gene expression changes ensuing from immunosuppressive treatment were characterized. The results of this project build the basis for prediction tools that can transform our current "one-size fits all" approach, enabling the timely initiation of the most effective treatment in SSc-ILD.

15. SUBJECT TERMS
Systemic sclerosis – scleroderma – interstitial lung disease - biomarker

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1. Introduction:

Systemic sclerosis (SSc-scleroderma) is associated with substantial morbidity and mortality. Interstitial lung disease (ILD) is the primary cause of disease related death. Immunosuppressive agents such as mycophenolate mofetil (MMF) and cyclophosphamide (CYC) are used for treatment of SSc-ILD. However, response to these treatments is highly variable and the clinical predictors cannot reliably identify the likely responders. In this project, we used the valuable samples collected in the Scleroderma Lung Study II (SLSII) clinical trial and an observational cohort of patients with early SSc to identify and verify molecular predictors of response to treatment. For this purpose, novel technologies and analytic approaches were used to determine key serum protein levels and transcript signatures. Moreover, the independent predictive significance of serum protein biomarkers beyond the information provided by clinical predictors (including high resolution chest CT markers) was verified in the Scleroderma Lung Study II.

2. Keywords:

Systemic sclerosis – scleroderma – interstitial lung disease – biomarker

3. Accomplishments:

What were the major goals of the project?

Major Task 1 : Institutional Review Board (IRB) and DOD Human Research Protection Office (HRPO) – 1 to 4 months

- Milestone Achieved #1: HRPO Approval was obtained on 1/9/2017

Major Task 2: Specific Aim 1: To determine the predictive significance of the peripheral blood type-I IFN signature – 5 to 24 months

- The Whole blood RNA samples have been extracted. Whole blood gene expression profiling in all baseline and 12-month SLSII samples has been completed using next generation RNA sequencing. The analysis of this large gene expression data set has been completed. For the first time, the peripheral blood cell (PBC) gene expression changes ensuing from oral CYC and MMF treatments have been characterized in SSc-ILD. This unbiased global approach has identified several gene expression modules that are predictive of ILD course in the MMF arm. A manuscript summarizing the results of this study has been prepared and will be submitted for publication this month. Furthermore, an abstract based on these results has been submitted to the upcoming American College of Rheumatology Convergence meeting.

Major Task 3: Specific Aim 2: To define the predictive significance of the skin immune dysregulation transcript signature for response to treatment – 5 to 24 months

- The skin biopsy samples were obtained from UCLA Pathology Repository and initial analysis on marker of fibrosis has been completed. Also, staining for two additional candidate biomarkers (interferon [IFN] inducible chemokines [CXCL9 and CXCL10]) identified in Task 4 has been completed in these skin biopsy samples. Moreover, a skin gene expression manuscript has been published in *Annals of Rheumatic Diseases*, examining predictive course of skin transcripts for the course of skin and lung involvement.

Major Task 4: Specific Aim 3: To characterize the predictive significance of key Th2 plasma cytokines for response to immunosuppression in SSc-ILD – 5 to 18 months

- The proposed serum protein profiling has been completed in the baseline SLSII samples. These studies indicated that KL-6, CCL18, c-reactive protein (CRP), and a serum IFN Protein Score have predictive significance for ILD-course. Moreover, the predictive significance of the serum IFN composite score has been replicated in an observational cohort of patients with early SSc. A manuscript summarizing the KL-6 and CCL18 results has been published in *Arthritis and Rheumatology in 2019*. Another manuscript summarizing the CRP and serum IFN Protein Score results was published in *Arthritis and Rheumatology* this year.

Major Task 5: Specific Aim 4: To develop and validate multivariable prediction tools for SSc-ILD with identified clinical and molecular predictors – 25 to 36 months

- The clinical factors predictive of ILD course have been identified in SLSII for both treatment arms. Moreover, it has been shown that two serum biomarkers (CRP and serum IFN Protein Score) identified in Task 4 were predictive of ILD course independent of these clinical predictors. These results have been published in *Arthritis and Rheumatology*.

What was accomplished under these goals?

Peripheral blood gene expression predictors of ILD course in SSc:

Deep whole genome RNA sequencing has been completed in 277 whole blood RNA samples, consisting of 134 baseline SLSII, 98 twelve-month SLSII, 45 matched healthy controls. Raw data were mapped and the initial quality control analysis was performed showing 100% concordance between the reported and transcript based gender information. Subsequently, transcript and modular analyses based on a previously described method (Chaussabel & Baldwin. Nat Rev Immunol 2014) were completed.

As listed in Table 1, the pairwise comparison of 12 month to baseline samples showed an upregulation of erythropoiesis, inflammation, and myeloid lineage related modules and a down-regulation of lymphoid lineage related modules in the CYC arm.

Table 1: QuSAGE analysis results for differentially expressed modules in pairwise comparison of 12-month to baseline samples in the CYC arm

Module	Annotation	Log Fold Change*	P_{FDR}
M2.3	Erythropoiesis	1.21	<0.0001
M6.18	Erythropoiesis	0.93	<0.0001
M3.1	Erythropoiesis	0.91	<0.0001
M4.4	Erythropoiesis	0.57	<0.0001
M5.15	Neutrophils / Granulocytes	0.53	<0.0001
M4.2	Inflammation	0.47	<0.0001
M5.3	Erythropoiesis	0.39	<0.0001
M1.1	Coagulation / Platelets	0.35	0.002
M3.3	Cell Cycle / Proliferation	0.34	<0.0001
M3.2	Myeloid Lineage	0.31	<0.0001
M6.11	Cell Cycle / Proliferation	0.28	0.0042
M4.14	Monocytes	0.27	<0.0001
M6.14	Coagulation / Platelets	0.26	0.0001
M6.6	Myeloid Lineage	0.26	<0.0001
M3.4	IFN Response	0.24	0.0103
M4.6	Myeloid Lineage	0.23	0.0001
M4.13	Inflammation	0.21	0.0055
M6.13	Inflammation	0.2	<0.0001
M3.6	Cytotoxic / NK Cell	-0.23	0.0095
M4.3	Protein Synthesis	-0.25	0.0021
M6.12	Lymphoid Lineage	-0.26	<0.0001
M4.7	Lymphoid Lineage	-0.3	<0.0001
M6.9	Lymphoid Lineage	-0.36	<0.0001
M4.15	Cytotoxic / NK Cell	-0.46	<0.0001
M6.15	T cells	-0.51	<0.0001
M6.19	T cells	-0.62	<0.0001
M4.1	T cells	-0.82	<0.0001
M4.11	Plasmablasts	-0.98	<0.0001
M4.10	B cells	-1.29	<0.0001

The modular changes ensuing from MMF treatment were more modest. As shown in Table 2, plasmablast and cell cycle modules were downregulated after MMF treatment.

Table 2: QuSAGE analysis results for differentially expressed modules in pairwise comparison of 12-month to baseline samples in the MMF arm

Module	Annotation	Log Fold Change*	P_{FDR}
M3.3	Cell cycle	-0.43	<0.0001
M6.11	Cell cycle/DNA repair	-0.39	0.0009
M4.11	Plasmablasts	-0.77	<0.0001

Next, predictive significance of transcript modules for the course of SSc-ILD based on the serially obtained forced vital capacity (FVC) 3 to 12 months after randomization was investigated. None of the baseline module scores significantly predicted the course of FVC% during this period in the CYC arm. In contrast, as shown in Figure 1, in the MMF arm, higher baseline Lymphoid Lineage (including T-cells and Cytotoxic/NK cells), as well as Mitochondrial predicted a better subsequent FVC% course while higher baseline Myeloid Lineage (including Neutrophils / Granulocytes) and Inflammation modules predicted a worse subsequent FVC% course. These results indicate, consistent with the primary mechanism of action of MMF on lymphocytes (Allison & Eugui. Immunopharmacology. 2000), patients with higher baseline lymphoid modules had a better response to MMF, while those with a higher myeloid cell lineage activation score had a poorer response.

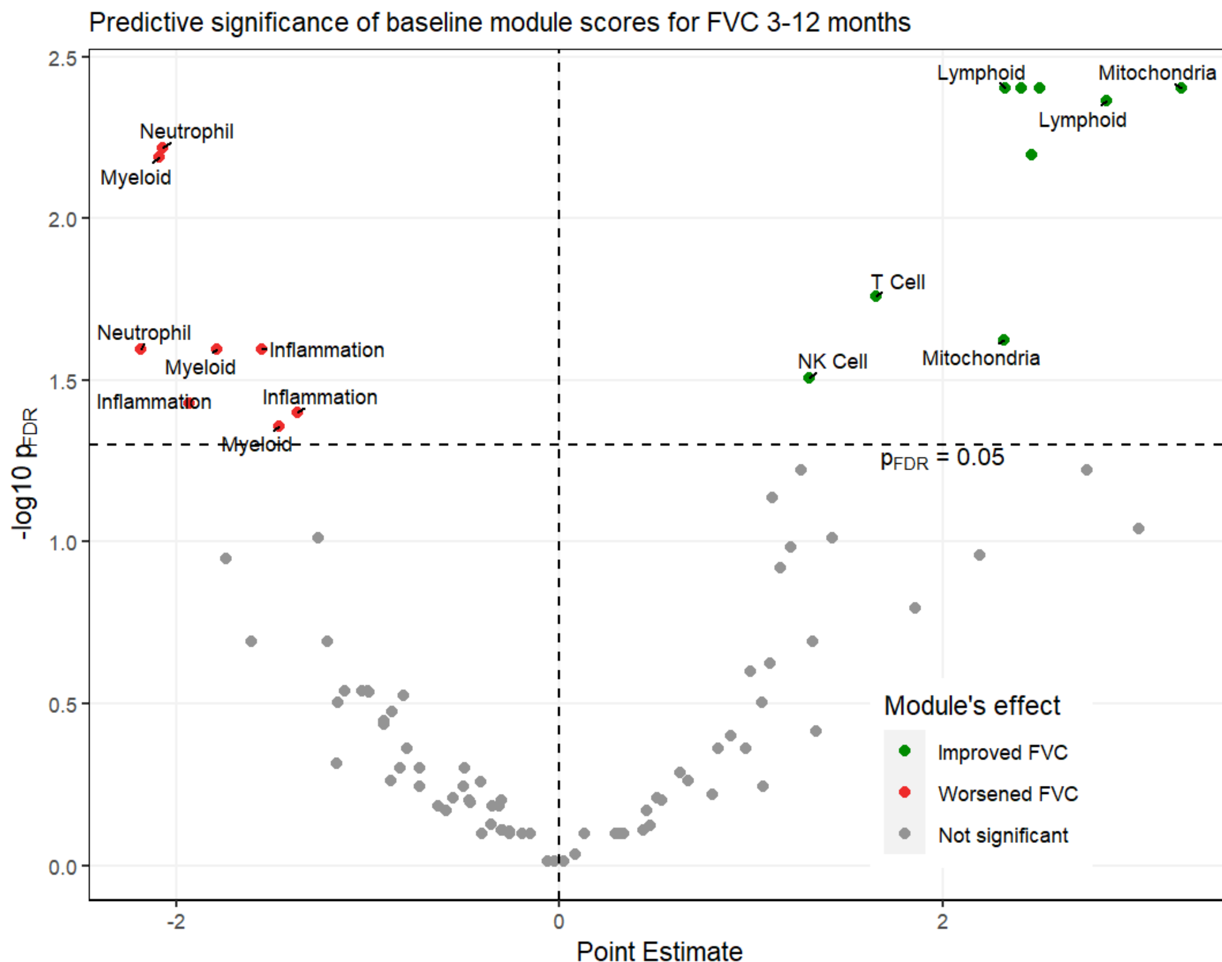

Figure 1: Predictive significance of baseline modular scores for FVC% during 3-12 month visits in the MMF arm. Higher T- and B-lymphocyte module scores predicted better ILD course while higher neutrophil/myeloid lineage module scores predicted worse ILD course.

Histological and gene expression studies in SSc skin:

Only a subgroup of SLSII patients (27.5%) had undergone a skin biopsy in SLSII study. The initial histological analysis indicated that histological dermal fibrosis decreased numerically between the baseline and 24-month visits but this difference did not reach statistical significance. Next, we investigated whether two prominent IFN inducible chemokines (CXL10 and CXCL9) are expressed in dermal inflammatory cells and fibroblasts in SSc skin and whether number of cells expressing these proteins decreased with treatment. As shown in Figure 2, CXCL9 positive inflammatory cells and fibroblasts decreased with immunosuppression. Similarly, CXL10 positive inflammatory cells decreased with treatment.

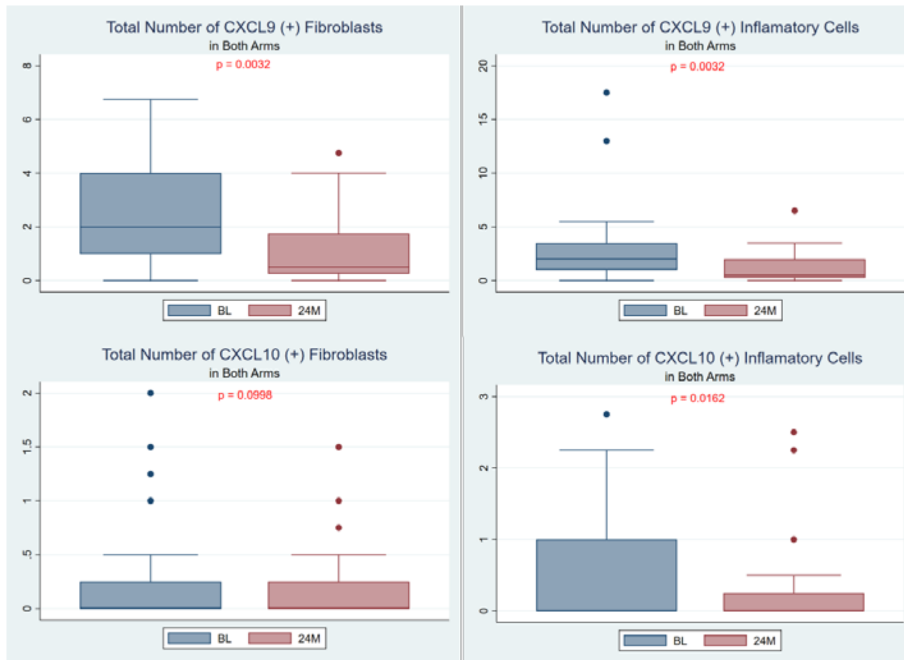

Figure 2: CXCL9 or CXCL10 positive cells in the dermal layer of patients with SSc in the baseline (BL) and 24 month (24M) visits of Scleroderma Lung Study II

Also, gene expression profiling was completed in skin samples in 48 patients with early SSc using valuable samples collected in the PRESS cohort. This study indicated that that SSc skin has a prominent inflammatory and fibrotic profile. In the multivariable model, adaptive immune cell signatures were significantly associated with shorter disease duration, while fibroblast and macrophage cell type signatures were associated with higher modified Rodnan Skin Score (mRSS). Immune cell signatures also correlated with skin thickness progression rate prior to biopsy, but did not predict subsequent mRSS progression. Moreover, the skin gene expression profile contrary to the above peripheral blood gene expression signatures were not predictive future FVC and ILD course. The results of this study have been recently published in *Annals of Rheumatic Diseases* (Skaug et al. Ann Rheum Dis. 2020 Mar;79(3):379-386 – see Appendix 2).

Serum protein predictors of ILD course in SSc:

First, we investigated whether two specific pneumoproteins, Krebs von den Lungen-6 [KL-6] and CC chemokine ligand 2 [CCL18]) predict response to immunosuppression with cyclophosphamide (CYC) and mycophenolate (MMF) in SSc-ILD. CCL18 was assayed by commercially available ELISA while KL-6 was measured using antibody coated latex microbeads and an automated analyzer.

Baseline serum KL-6 and CCL18 correlated with extent of ILD. After adjusting for baseline disease severity, higher baseline KL6 levels predicted progression of ILD as measured by the course of the FVC (CYC/MMF: Estimate -0.32/-0.72; *P*=0.024/0.005, respectively) and DLCO (CYC/MMF: Estimate -1.30/-1.28; *P*<0.001/<0.001, respectively) over 1 year for both treatment arms. Similarly, higher baseline CCL18 levels predicted progression of ILD as measured by the course of the FVC (CYC/MMF: Estimate -1.24/-0.35; *P*<0.001;0.007, respectively) and DLCO (CYC/MMF: Estimate -1.87/-1.25; *P*=0.001/<0.001, respectively) over 1 year for both treatment arms. These results indicate that Patients with higher baseline KL-6 and CCL18 levels were more likely to experience disease progression despite treatment with CYC and MMF. The results of this study were published in *Arthritis and Rheumatology* (Volkmann et al. Arthritis Rheumatol . 2019 Dec;71(12):2059-2067- see Appendix 1).

The second serum protein study focused on the main hypothesis of this DoD award, that SSc-ILD patients with a profibrotic immune profile will be more responsive to immunosuppression with a special focus on IFN inducible serum proteins.

Six IFN inducible serum proteins were measured: Monokine Induced by Gamma Interferon (MIG), Interferon Gamma Induced Protein 10 (IP-10), Monocyte Chemotactic Protein 2 (MCP-2), Beta 2 Microglobulin (B2M), Tumor Necrosis Factor Receptor 2 (TNFR-2), and Macrophage Inflammatory Protein 3 beta (MIP-3b). The corresponding gene names of these six proteins are CXCL9, CXCL10, CCL8, B2M, TNFRSF1B, and CCL19, respectively. This protein list was selected following a two-step process. In step one, 14 serum cytokines were identified that correlated significantly (r>0.3 and PFDR <0.05) with the IFN gene expression signature in the baseline samples collected in the Scleroderma: Cyclophosphamide or Transplantation (SCOT) study (see Assassi et al. Ann Rheum Dis. 2019). In Step 2, six of these proteins were also confirmed as inducible by type I IFN in human PBCs based on in-vitro studies, according to the information obtained from the Interferome V 2.0 database. A composite score of MIG, IP-10, MCP-2, B2M, TNFR-2, and MIP-3b was calculated.
As shown in Table 3, higher baseline IFN Protein Score predicted better ILD course based on higher serial FVC% over 3 to 12 months in both treatment arms after adjustment for baseline FVC% (MMF/CYC: Estimate=0.41/0.91; p=0.001/0.009).

Table 3: Predictive significance of IFN Protein Score for subsequent serial FVC%

MMF Arm*			
Predictive significance of baseline IFN Protein Score for serial FVC%s 3-12 months			
	Effect size	95% CI	p-value
Baseline IFN Protein Score	0.41	0.23 to 0.59	0.001
Baseline FVC%	0.84	0.82 to 0.86	<0.001
CYC Arm*			
Predictive significance of baseline IFN Protein Score for serial FVC%s 3-12 months			
	Effect size	95% CI	p-value
Baseline IFN Protein Score	0.91	0.56 to 1.13	0.009
Baseline FVC%	0.87	0.84 to 0.9	<0.001

* All models also included time as an independent variable

Our previous gene expression studies have shown that approximately half of patients with SSc have an IFN signature. Building on this finding, IFN Protein Score was dichotomized based on the median value in the baseline patient samples. As shown in Figure 3, patients with a positive baseline IFN Protein Score had a more favorable ILD course over 3 to 12 months in both treatment arms compared with patients with a negative IFN Protein Score (MMF/CYC: Estimate= 1.28/2.6; p=0.003/0.004).

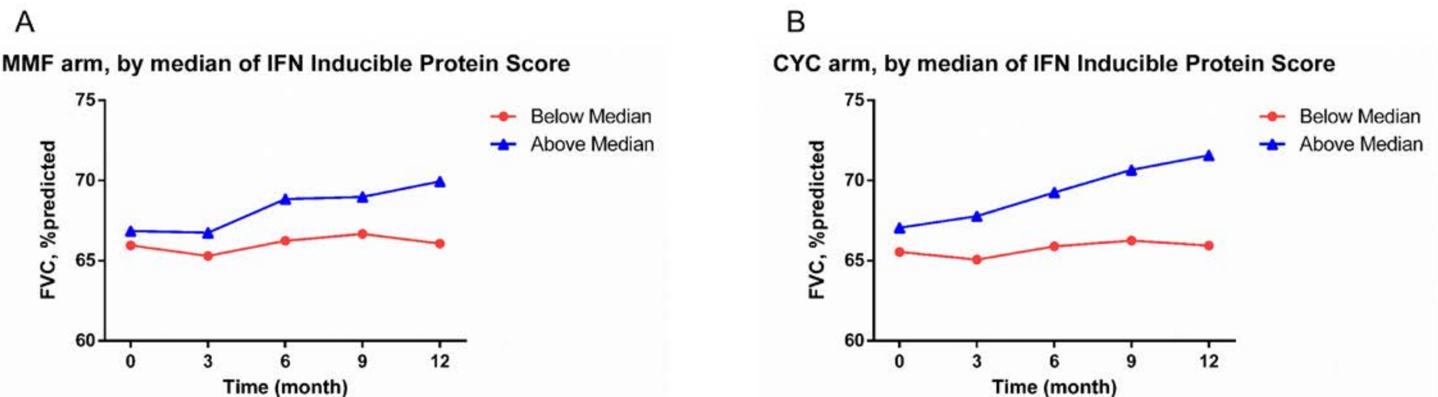

Figure 3: Course of FVC% 3-12 months after randomization based on the baseline IFN Inducible Protein Score, dichotomized using the median value. Patients with a positive IFN score had higher FVC% levels in the MMF (Panel A- p=0.003) and CYC (Panel B- p=0.004) arms. This analysis is adjusted for baseline FVC% and used the same analytic approach like Tables 3.

Independent validation of the predictive significance of IFN Protein Score:
The predictive significance of IFN Protein Score and CRP was investigated in an independent, observational cohort of patients with early diffuse SSc. In this cohort, 47 patients had a baseline serum sample and had FVC% measurements at the baseline and 12-month visits; of these, 31 (66%) had evidence of SSc-ILD on HRCT. Out of these 31 patients, 26 were treated with immunosuppressive agents (23 with MMF and 3 with methotrexate) during the first year follow-up period and were included in the present study. Confirming our findings in SLS II, higher baseline IFN Protein Score predicted increasing FVC levels; specifically, the baseline IFN Chemokine levels correlated positively with percent change in FVC% at 12 months (Spearman's correlation coefficient [r_s]=0.43; p=0.028). This correlation remained significant even after exclusion of the three patients treated with methotrexate (n=23; r_s=0.47; p=0.023).

Predictive significance of CRP in SLSII:

Previous studies in observational studies have indicated that CRP predicts worse ILD course and higher mortality in patients with SSc. However, these results had not been replicated in a clinical trial cohort. In order to address this question, the predictive significance of CRP was investigated in the SLSII. Contrary to the favorable (i.e. positive) predictive value of the IFN Protein Score, higher CRP levels predicted a worse ILD course reflected in lower serial FVC% over the 3 to 12-month visits in both treatment arms after adjustment for baseline FVC% (Table 4).

Table 4: Predictive significance of CRP for subsequent serial FVC%

MMF Arm*			
Predictive significance of baseline CRP for serial FVC%s 3-12 months			
	Effect size	95% CI	p-value
Baseline CRP	-0.15	-0.31 to -0.01	0.038
Baseline FVC%	0.83	0.78 to 0.86	<0.001
CYC Arm*			
Predictive significance of baseline CRP for serial FVC%s 3-12 months			
	Effect size	95% CI	p-value
Baseline CRP	-0.56	-0.72 to -0.45	<0.001
Baseline FVC%	0.90	0.86 to 0.92	<0.001

* All models also included time as an independent variable

Predictive significance of clinical factors for ILD course:

As shown in Table 5, the predictive significance of baseline demographic and clinical variables for serial FVC%s over the 3 to 12 month visits were first examined in separate models after adjustment for baseline FVC% for each treatment arm. Diffuse disease type, mRSS, African American race, and extent of radiographic involvement were predictive of ILD course in the MMF arm while African American race and diffuse cutaneous involvement were predictive of ILD course in the CYC arm.

Table 5: Separate analyses to examine the predictive significance of baseline demographic and clinical variables for serial FVC% 3 to 12 months*

	MMF Arm		**CYC Arm**	
Baseline variable	Point estimate (95% CI)	p-value	Point estimate (95% CI)	p-value
Age in year	-0.05 (-0.18 to 0.08)	0.462	0.04 (-0.06 to 0.15)	0.411
Female Sex	0.04 (-0.53 to 0.69)	0.891	1.17 (-0.09 to 2.26)	0.058
African American race	-0.68 (-1.26 to -0.13)	**0.032†**	-2.4 (-3.04 to -1.9)	**<0.001†**
Diffuse disease type	1.15 (0.43 to 2.06)	**0.005†**	-1.97 (-3.34 to -0.77)	**0.008†**
mRSS	0.07 (0.04 to 0.11)	**0.002†**	-0.04 (-0.14 to 0.06)	0.392
Anti-Topoisomerase	-0.14 (-1.12 to 0.81)	0.729	-0.35 (-2.23 to 1.62)	0.654
Anti-RNA polymerase	0.83 (-0.61 to 2.06)	0.175	1.08 (-2.08 to 4.17)	0.425
Extensive disease on HRCT #	-2.45 (-2.85 to -2.11)	**<0.001†**	0.09 (-2.18 to 2.36)	0.79

* Each row represents a separate model that included one baseline clinical variable, baseline FVC% and time as independent variables
† Baseline demographic and clinical variables showing predictive significance in separate models that were included in the subsequent extended multivariable model (Tables 6 and 7).
# QILD >20% on high resolution chest CT (HRCT)

Multivariable models with inclusion of clinical and serum predictors:

Next, the predictive significance of IFN Protein Score and CRP (both as continuous variables) was investigated in an extended multivariable model after adjustment for baseline FVC%, in addition to variables showing predictive significance in the above separate analyses in the MMF arm (Table 4) and in the CYC arm (Table 5). Similar to above findings, higher baseline IFN Protein Scores predicted better ILD course and higher baseline CRP levels predicted worse ILD course over the 3 to 12 month visits after adjustment for baseline demographic and clinical variables in both treatment arms. Results of these studies have been published in *Arthritis and Rheumatology* (Assassi et al. Arthritis Rheumatol. 2021 Jun;73(6):1005-1013- Appendix 3).

Table 6: Predictive significance of baseline IFN Inducible Protein Score and CRP after adjustment for baseline demographic and clinical variables for serial FVC% 3 to 12 months in the MMF arm*

Baseline variable	Point estimate	95% CI	p-value
IFN Inducible Protein Score	0.32	0.11 to 0.52	0.013
CRP†	-0.13	-0.24 to -0.01	0.041
African American race	0.95	0.43 to 1.41	0.004
Diffuse disease type	0.39	-0.19 to 1.05	0.139
mRSS	0.05	0.03 to 0.09	0.008
Baseline FVC%	0.81	0.78 to 0.83	<0.001
Extensive disease on HRCT	-2.27	-2.70 to -1.80	<0.001

* Time was also included as an independent variable
† Log2 transformed

Table 7: Predictive significance of baseline IFN Inducible Protein Score and CRP after adjustment for baseline demographic and clinical variables for serial FVC% 3 to 12 months in the CYC arm*†

Baseline variable	Point estimate	95% CI	p-value
IFN Inducible Protein Score	0.92	0.79 to 1.04	<0.001
CRP†	-0.46	-0.53 to -0.39	<0.001
African American race	-2.01	-2.31 to -1.71	<0.001
Diffuse disease type	-0.60	-0.91 to -0.33	0.005
Disease duration	0.19	0.12 to 0.26	0.002
Baseline FVC%	0.90	0.89 to 0.91	<0.001

* Time was also included as an independent variable
† Log2 transformed

Cumulatively, these results indicate that molecular biomarkers (such as serum IFN Inducible Protein Score and serum pneumoproteins) can contribute to identifying SSc-ILD patients who would benefit from immunosuppressive treatment. This can ultimately lead to timely initiation of the most effective treatment and prevention of the irreversible lung damage in patients with SSc-ILD.

What opportunities for training and professional development has the project provided?

Dr. Assassi attended the American College of Rheumatology (ACR) Annual Scientific Meetings during the funding period. Moreover, Dr. Assassi has mentored Dr. Skaug in analyzing the above-mentioned skin gene expression results and preparing the related manuscript published in *Annals of Rheumatic Diseases*. He also mentored Dr. Volkmann in analyzing the aforementioned pneumoprotein data which were published in *Arthritis and Rheumatology* in 2019. Dr. Assassi is also mentoring Ms. Nancy Wareing (MD/PhD Candidate) who is conducting a study on the predictive significance of neutrophil count for the FVC course in the SLS II study, the related manuscript will be submitted for publication this month.

How were the results disseminated to communities of interest?

The study results have been published in peer-reviewed scientific journals and presented at the ACR Annual Scientific Meetings, as well as Systemic Sclerosis World Congress (see Products for more details).

<u>Dissemination of study results to patients and their relatives:</u>

The study results were presented at the Biannual UTHealth – Scleroderma Foundation Texas Chapter Scleroderma Day in September 2019. This event is the largest scleroderma patient information venue in the state of Texas and is held every other year. Another presentation on SSc-ILD was given virtually in collaboration with American Thoracic Society and Scleroderma Foundation in March 2020. Finally, Dr. Assassi gave a virtual talk to Scleroderma Foundation Texas Chapter about manifestations and screening for SSc pulmonary involvement in 2021.

What do you plan to do during the next reporting period to accomplish the goals?

N/A

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The above reported biomarkers can contribute to more focused and individualized treatment options for SSc-ILD by identifying patients who would primarily benefit from MMF and CYC treatment. This is an important unmet clinical need in SSc-ILD as recently two additional medications (tocilizumab and nintedanib) have been approved for treatment of SSc-ILD but there are no reliable prediction models which can inform treatment choices in SSc-ILD. Considering that SSc-ILD is the primary cause of disease related mortality, more informed treatment decisions can represent a major progress in this field, by enabling the timely initiation of the most effective treatment and prevention of irreversible lung damage in patients with SSc-ILD.

What was the impact on other disciplines?

The discovered biomarkers might also have predictive significance in other autoimmune disease associated ILD conditions such as rheumatoid arthritis and dermatomyositis/polymyositis. This would also lead to more focused and individualized treatment options in patients suffering from those autoimmune lung diseases.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

SSc is associated with high morbidity and mortality. A more focused and effective therapeutic approach in these patients can substantially improve the lives of these patients and their relatives and ultimately decrease the societal burden of this disease. Moreover, SSc is a prototypic fibrotic disease. Fibrotic diseases affect millions of Americans and are one of the leading causes of morbidity and mortality in the USA. Understanding the predictors of response to treatment in SSc might have implications for other fibrotic diseases.

5. CHANGES/PROBLEMS:

Dr. Robert Elashoff, the PI at the UCLA site, retired during the funding period. Dr. Elizabeth Volkmann took over his role, this enabled the timely completion of above-mentioned analyses.

6. **PRODUCTS:**

Journal publications:

1. Salazar GA, Kuwana M, Wu M, Estrada-Y-Martin RM, Ying J, Charles J, Mayes MD, Assassi S. KL-6 But Not CCL-18 Is a Predictor of Early Progression in Systemic Sclerosis-related Interstitial Lung Disease. J Rheumatol. 2018 Aug;45(8):1153-1158
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16. Pending or under review manuscripts funded by this Award: Three original research articles based on data and samples from the GENISOS cohort are currently under review: 1) multiomic analysis of SSc molecular profile; 2) predictive significance of Scl-70 antibodies for the course of SSc-ILD; 3) predictors of perceived functional status in early systemic sclerosis. A manuscript summarizing the aforementioned results on the predictive significance of transcript modules for the course of SSc-ILD in SLSII will be submitted for publication this month. Another manuscript on the predictive significance of neutrophil count in the SLS II will be also submitted for publication this month.

Other publications, conference papers, and presentations

The following abstracts were presented at the last ACR Annual Scientific Meetings:

1. Volkmann ER, Tashkin DP, Kuwana M, Li N, Charles J, Hant FN, Bogatkevich GS, Akter T, Roth M, Kim HJG, Goldin J, Khanna D, Clements PJ, Furst DE, Elashoff R, Silver R, Assassi S. Specific Pneumoproteins Predict Progression of Interstitial Lung Disease in Systemic Sclerosis Patients Undergoing Treatment with Immunosuppression [abstract]. Arthritis Rheumatol. 2018; 70 (suppl 10).
2. Bellocchi C, Assassi S, Ying J, Mohan C, Santaniello A, Beretta L. Proteomic Aptamer Analysis Reveals a Distinct Profile of Very Early Systemic Sclerosis (SSc) Patients at Risk for Progression Toward Definite SSc [abstract]. Arthritis Rheumatol. 2018; 70 (suppl 10).
3. Assassi S, Li N, Volkmann E, Mayes M, Ying J, Roth M, Clements P, Furst D, Khanna D, Goldin J, Elashoff R, Tashkin D. Serum Interferon Chemokine Score Predicts Better Response to Immunosuppression in Systemic Sclerosis Related Interstitial Lung Disease. Arthritis Rheumatol. 2019; 71 (suppl 10).
4. Volkmann E, Tashkin D, Roth M, Li N, Kim G, Goldin J, Mayes M, Charles J, Clements P, Furst D, Khanna D, Elashoff R, Assassi S. The MUC5B Promoter Variant Does Not Predict Outcomes in Systemic Sclerosis-related Interstitial Lung Disease [abstract]. Arthritis Rheumatol. 2019; 71 (suppl 10).
5. Shanmugam V, Frech T, Steen V, Hummers L, Shah A, Bernstein E, Khanna D, Gordon J, Castelino F, Chung L, Hant F, Startup E, VanBuren J, Evnin L, Assassi S. The Collaborative National Quality and Efficacy Registry for Scleroderma: Data Completion Outcomes from a Multicenter United States Cohort Using Guideline-Based Registry Practices [abstract]. Arthritis Rheumatol. 2019; 71 (suppl 10).
6. Wareing N, Li N, Volkmann ER, Lyons M, Roth MD, Tashkin DO, Assassi S. Serum Neutrophil Count Predicts Progression of Interstitial Lung Disease and Mortality in Patients with Systemic Sclerosis Related Interstitial Lung Disease. [abstract]. Arthritis Rheumatol. 2020; 72 (suppl 10).
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8. Skaug B, Lyons M, Swindell W, Salazar G, Charles J, Vershel C, Mayes M, Assassi S. Large-scale Examination of Longitudinal Skin Gene Expression and Its Associations with Skin Thickness in Systemic Sclerosis [abstract]. Arthritis Rheumatol. 2020; 72 (suppl 10).

The following abstracts were presented as oral presentations at the Systemic Sclerosis World Congress in 2020:

1. Skaug B, Lyons M, Swindell WR, Salazar GA, Charles J, Theodore S, Mayes MD, Assassi S. Large-scale Examination of Longitudinal Skin Gene Expression and Its Associations with Skin Thickness in Systemic Sclerosis.
2. Assassi S, Ning L, Volkmann E, Mayes M, Ying Y, Roth M, Hinchcliff M, Khanna D, Frech T, Clements P, Furst D, Goldin J, Bernstein E, Castelino F, Domsic R, Gordon J, Hunt F, Shah A, Shanmugam V, Steen V, Elashoff R, Tashkin D. Interferon chemokine score predicts response to treatment in systemic sclerosis related interstitial lung disease.

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List of Appendices:

1. Volkmann ER, Tashkin DP, Kuwana M, Li N, Roth MD, Charles J, Hant FN, Bogatkevich GS, Akter T, Kim G, Goldin J, Khanna D, Clements PJ, Furst DE, Elashoff RM, Silver RM, Assassi S. Progression of Interstitial Lung Disease in Systemic Sclerosis: The Importance of Pneumoproteins Krebs von den Lungen 6 and CCL18. Arthritis Rheumatol. 2019 Dec;71(12):2059-2067.
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Pneumoproteins KL-6 and CCL-18 Predict Progression of Interstitial Lung Disease in Systemic Sclerosis

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Abstract

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Conflicts of Interest:
The authors report no financial conflicts of interest.

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Objective: To investigate the relationship between Krebs von den Lungen-6 [KL-6] and CC chemokine ligand 18 [CCL-18]) with severity and progression of systemic sclerosis-related interstitial lung disease (SSc-ILD).

Methods: Patients enrolled in Scleroderma Lung Study (SLS) II (cyclophosphamide [CYC] versus mycophenolate mofetil [MMF]) were included. Baseline and 12-month plasma samples were analyzed by ELISA to assess CCL-18 and KL-6 levels. The forced vital capacity (FVC) and the diffusing capacity for carbon monoxide (DL_{CO}) were measured every 3 months. Joint models were created to investigate the relationship between baseline CCL-18 and KL-6 and the course of the FVC and DL_{CO} over 1 year by treatment arm.

Results: Baseline KL-6 and CCL-18 levels each correlated with the extent of radiographic fibrosis. Levels of both CCL-18 and KL-6 declined significantly at one year. In both treatment arms, higher baseline KL-6 level predicted progression of ILD based on the course of the FVC (CYC/MMF: P=0.024/0.005) and DL_{CO} (CYC/MMF: P<0.001/0.004) over 1 year. Higher baseline CCL-18 level predicted progression of ILD based on the course of the FVC (CYC/MMF: P<0.001;0.007), DL_{CO} (CYC/MMF: P=0.001/<0.001) over 1 year, as well as mortality (CYC arm only P=0.0008).

Conclusion: In a rigorously-conducted clinical trial for SSc-ILD, KL-6 and CCL-18 levels correlated with ILD severity and declined with immunosuppression. Patients with higher baseline KL-6 and CCL-18 levels were more likely to experience disease progression despite treatment. KL-6 and CCL-18 could be used to identify patients with a progressive ILD phenotype who may benefit from a more aggressive initial treatment approach.

Keywords

Systemic sclerosis; Interstitial lung disease; Mycophenolate mofetil; Cyclophosphamide; Biomarkers

INTRODUCTION

Interstitial lung disease (ILD) occurs in the majority of patients with systemic sclerosis (SSc) [1]. While ILD is the leading cause of disease-related mortality among patients with SSc [2, 3], ILD progression rates vary considerably. Results of randomized controlled trials (RCTs) have demonstrated that some patients experience an improvement in lung function after treatment with immunosuppression, while other patients experience progression of ILD despite early and aggressive treatment [4, 5]. Furthermore, not all patients with ILD will develop symptoms or will have progressive disease even in the absence of treatment [1, 6–8].

Evidenced-based clinical tools to predict which patients with SSc-ILD are more likely to experience ILD progression do not exist. Specific clinical and biological factors have been associated with progression of ILD in observational studies (e.g., low forced vital capacity (FVC) [9], greater extent of ILD on high-resolution computed tomography (HRCT) imaging [10, 11], low diffusing capacity for carbon monoxide (DL_{CO}) [9, 12], and anti-topoisomerase I antibody positivity [9,12]). Moreover, several studies have identified serum/plasma protein candidate biomarkers that predict SSc-ILD progression, including interleukin

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(IL)-6 [13], C-reactive protein (CRP) [14], CC chemokine ligand 2 (CCL2) [15], CCL-18 [16, 17], CXCL4 [18], and KL-6 [19, 20].

Among these candidate biomarkers, KL-6 and CCL-18 have been found to predict outcomes in several different SSc-ILD populations [16, 17, 19, 20]. Because KL-6 and CCL-18 are pneumoproteins associated with lung parenchymal injury [21, 22], they may be more specific markers for monitoring and predicting the course of ILD in SSc. For example, in contrast to general inflammatory markers (e.g., IL-6 or C-reactive protein), the levels of KL-6 and CCL-18 may be less likely to be affected by extra-pulmonary fibrotic processes such as cutaneous sclerosis or infections.

Furthermore, KL-6 correlates with disease severity in different SSc-ILD populations [23–28]. Two observational studies [19, 20] have found that high KL-6 levels predict worse outcomes in SSc-ILD. A recent, small observational study demonstrated that high serum KL-6 level was associated with poor response to immunosuppression with cyclophosphamide (CYC) in SSc-ILD patients [29].

CCL-18 is a chemokine, which was previously known as pulmonary and activation-regulated chemokine (PARC), and studies have demonstrated higher levels of this chemokine in both serum and bronchoalveolar lavage fluid (BAL) samples of patients with ILD [30]. Observational studies have demonstrated that CCL-18 also predicts various ILD-related outcomes in SSc [16, 17, 31, 32].

Given the accumulating evidence that KL-6 and CCL-18 may be key markers of disease activity and progression in SSc-ILD, the present study sought to evaluate the predictive role of CCL-18 and KL-6 in the context of a RCT, in which all patients have equal access to care, uniform follow up and a standardized treatment approach. The present study aimed to determine whether KL-6 and CCL-18 are associated with the severity of ILD in a clinical trial cohort comprised of patients with well-characterized and active SSc-ILD. A secondary aim was to determine whether baseline levels of these peripherally-measured lung glycoproteins predict the progression of SSc-ILD in patients receiving immunosuppression with either mycophenolate mofetil (MMF) or CYC.

PATIENTS AND METHODS

Study participants

Data and plasma samples from participants enrolled in SLS II [5] were analyzed for this study. Eligibility criteria included the following key inclusion criteria: (1) adults, aged 18–75 years, (2) limited or diffuse cutaneous SSc [33], (3) active ILD as demonstrated by restrictive to borderline restrictive ventilatory impairment (FVC<80–85% but 45% predicted) AND the presence of any ground glass opacity (GGO; hazy opacity through which normal lung markings can be discerned) on high-resolution computed tomography (HRCT), (4) exertional dyspnea (Grade 2 on the Magnitude of Task component of the Mahler Baseline Dyspnea Index [BDI] [34]. Key exclusion criteria included pulmonary hypertension; clinically significant abnormalities on HRCT not attributable to SSc; smoking

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within the past 6 months; and evidence of significant airflow obstruction. Complete details of the SLS II study design have been previously reported [5].

Unaffected control participants were independently recruited at the University of Texas, Houston, and age-, ethnicity- and gender-matched to SLS II participants in an approximately 1 (control) to 3 (SLS II) ratio. The same healthy, unaffected controls were used for both the KL-6 and CCL-18 analyses. The Institutional Review Board of each site approved the studies; and only participants who provided informed consent were included in the present analyses.

Patient and Public Involvement

Patients and the public were not involved in the design or reporting of the results of this research study. Patients were involved in the conduct of the study because they served as participants.

SLS II Study Design

In SLS II, enrolled patients were randomized in a similar manner to either oral CYC for one year followed by one year of placebo or MMF for 2 years. For complete details of the SLS II protocol, please see the supplementary web appendix accompanying the main SLS II [5] manuscript. The FVC (primary SLS II endpoint) and DL_{CO} (secondary SLS II endpoint) were measured every 3 months, and the TLC was measured every 6 months during the trial. HRCT thoracic imaging was obtained at baseline in SLS II, and a Computer Aided Design (CAD) scoring system was employed to provide quantitative measures of different patterns of ILD as previously described [35]. Quantitative ILD (QILD) score was the sum of all abnormally classified scores, including scores for quantitative lung fibrosis (QLF, linear reticular markings with architectural distortion), GGO and honeycomb changes (clustered air-filled cysts with dense walls). Scores were calculated as percentage of total counted voxels for both the whole lung (WL), including both lungs, and for the zone (area-equivalent upper, middle or lower lung zone) of maximal involvement (ZM).

KL-6 and CCL-18 Assays

SLS II plasma samples were collected at the baseline and 12-month study visits in EDTA tubes and were immediately processed on-site on the day of collection, stored at -70^{0}C, and shipped on dry-ice to the central repository at the University of Texas - Houston. All SLS II patients with an available baseline plasma sample were included in the present study. Plasma samples from healthy controls collected at the University of Texas - Houston, were handled in the same manner except that no shipping was required. CCL-18 was assayed by commercially available ELISA kits (MIP-4/CCL-18 kit, Cell Sciences), while KL-6 was measured using latex-fixed anti-KL-6 monoclonal antibody with an automated analyzer (Nanopia KL-6; Sekisui Medical Co. Ltd.). All plasma assays were performed in duplicates and the coefficient of variance was <20%. Technicians performing the assays were blinded to the clinical diagnosis and outcome data.

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Statistical Analysis

Baseline characteristics—Summary statistics were generated for baseline characteristics. A two-sample t-test or Wilcoxon rank-sum test was used to compare continuous variables and a chi-square test was used to compare categorical variables. Kendall's tau correlations were performed to examine the relationship between KL-6/CCL-18 levels and baseline measures of extent of ILD, as measured by the FVC, DL_{CO}, QILD, and QLF.

Change of KL-6 and CCL-18 from baseline to 12 months—Summary statistics of KL-6/CCL-18 were calculated for baseline and 12 months. Wilcoxon signed-rank test was used to compare the data collected at the two time points.

Relationship between baseline KL-6 and CCL-18 with progression of SSc-ILD—A joint model analysis was used to determine whether baseline levels of KL-6 or CCL-18 predict progression of SSc-ILD. The joint model (used also in the main SLS II analysis [5]) adjusts for non-ignorable missing data due to treatment failure, death, and drop-outs [36]. The outcome for the primary outcome model was the course of FVC %-predicted measured in 3-month increments from 3 to 12 months. The longitudinal model of the joint analysis included the following covariates: baseline KL-6 or CCL-18, baseline FVC %-predicted, and a linear time trend. The outcome for the secondary outcome model was the course of DL_{CO} %-predicted measured in 3-month increments from 3 to 12 months. The longitudinal model of the joint analysis included the following covariates: baseline KL-6 or CCL-18, baseline DL_{CO} %-predicted, and a linear time trend. KL-6 and CCL-18 were log-transformed (with a base of 2) in these analyses to correct data skewness. We generated models for examining baseline KL-6 and CCL-18 as a continuous variable and also as a dichotomous variable (using the median as the cut point). The median was selected since there are no valid thresholds for defining high versus low KL-6 and CCL-18. In an exploratory analysis, we generated receiver operator curves (ROC) and logistic regression analysis to determine whether we could identify a threshold for KL-6 and CCL-18 that predicted disease progression. Since there is no universally accepted definition of disease progression in SSc-ILD we used the following two definitions: (1) FVC decline >= −5%; and (2) FVC decline >= −10% OR FVC decline between −5 and −9% accompanied by a DLCO decline >= −15%. The time course of 3–12 months was selected as this was the time period in which patients in both study arms (CYC and MMF) were receiving active treatment.

Relationship between baseline KL-6 and CCL-18 with long-term survival in SSc-ILD—Cox regression was used to assess the association between baseline KL-6/CCL-18 and long- term survival in SLS II. The model included baseline KL-6/CCL-18 (log-transformed) and baseline FVC %-predicted as covariates. The methods for obtaining long-term survival data in SLS II are described in detail in our recent publication [37].

All tests were 2-sided. The joint analyses were performed using the R package JMbayes, and all other analyses were conducted in SAS v9.4 (The SAS Institute, Cary, NC).

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RESULTS

Participant Characteristics

Baseline characteristics of SLS II participants who underwent KL-6 and CCL-18 analysis appear in Table 1. Among the 142 SLS II participants, 133 and 99 participants had KL-6 and CCL-18 measurements at baseline and 12 months, respectively. Compared with the SLS II cohort, unaffected controls (N=39) were similar in age (Mean 52.2 ± 9.5 years), sex (71.8% female), race (69.2% White, 23.1% Black, 7.7% Asian), and ethnicity (12.8% Hispanic/ Latino).

KL-6 Levels are Associated with Disease Severity

KL-6 levels were significantly higher in SSc patients (N=133) compared with unaffected controls (N=39) (1752.05 [1274.67] versus 330.70 [125.74] u/ml, P<0.0001). KL-6 levels correlated with SSc disease severity at baseline (Table 2). Specifically, increased KL-6 levels were associated with decreased DL_{CO}, decreased TLC, and increased radiographic extent of lung fibrosis as measured by the QILD-WL, QILD-ZM, QLF-WL, and QLF-ZM.

CCL-18 Levels are Associated with Disease Severity

CCL-18 levels were significantly higher in SSc patients (N=133) compared with healthy controls (N=39) (191.29 [111.08] versus 87.71 [28.28] ng/ml, P=0.0009). In addition, increased CCL-18 levels were associated with increased radiographic extent of lung fibrosis as measured by the QILD-WL and QLF-ZM (Table 2).

Relationship between KL-6 and CCL-18

CCL-18 levels correlated with KL-6 levels at baseline (r=0.18, P=0.036) and at 12 months (r=0.15, P=0.032). The change in CCL-18 levels from baseline to 12 months was not correlated with the change in KL-6 levels from baseline to 12 months in all participants (r=0.063, P=0.34), or in participants randomized to CYC (r=0.094, P=0.34) and to MMF (r=0.0068; P=0.95).

KL-6 and CCL-18 Levels Decrease after One Year of Immunosuppression

Among SLS II participants with baseline and 12-month KL-6 and CCL-18 measurements (N=99), treatment with CYC or MMF for one year led to significant reductions in these peripheral pneumoprotein levels (Figure 1). The average decline in KL-6 levels was 100.60 u/ml (N=99; p=0.045), while the average decline in CCL-18 levels was 61.24 ng/ml (N=98; P<0.0001). Among patients assigned to MMF, both KL-6 levels (N=49; P=0.016) and CCL-18 levels (N=51; P<0.0001) decreased significantly over 1 year (Supplementary Tables S1 and S2). Among patients assigned to CYC, CCL-18 levels (N=49; P=0.0008) decreased significantly over 1 year, although KL-6 levels did not (Supplementary Table S1 and S2). The average decline in KL-6 levels among patients assigned to CYC and MMF was 55.72 (819.44) and 146.40 (458.69) u/ml, respectively (Supplementary Table S2). The average decline in CCL-18 levels among patients assigned to CYC and MMF was 46.94 (87.10) and 75.55 (105.75) ng/ml, respectively (Supplementary Table S1).

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Baseline KL-6 Levels Predict SSc-ILD Progression

The predictive significance of KL-6 and CCL-18 was analyzed in each treatment arm separately. Among SLS II participants, higher baseline KL-6 levels predicted progression of ILD as measured by the course of the FVC%-predicted (CYC/MMF: Estimate −0.32/−0.72; P=0.024/0.005) and DL_{CO}%-predicted (CYC/MMF: Estimate −1.30/−1.28; P<0.001/0.004) over 1 year in the MMF, as well as the CYC arms, even after adjusting for baseline disease severity (Table 3).

After dichotomizing the KL-6 variable based on the median level in baseline SLS II samples (1448.2 u/ml), a high baseline KL-6 level was associated with increased progression of ILD as measured by the course of the FVC in the MMF arm (Estimate −1.19; P=0.018), but not in the CYC arm (Estimate −0.19; P=0.44) (Supplementary Table S3). High baseline KL-6 level was associated with increased progression of ILD as measured by the course of the DL_{CO} in the MMF arm (Estimate −0.46; P=0.030), but not in the CYC arm (Estimate −0.034; P=0.720) (Supplementary Table S4).

The results of the ROC analysis demonstrated that a KL-6 level greater than 1549 u/ml in the MMF arm was associated with an increased risk of progression using both definitions of ILD worsening. The sensitivity and specificity was 100% and 74%, respectively, when we used the definition of FVC decline >= −5%. The sensitivity and specificity was 100% and 71%, respectively, when we used the definition of FVC decline >= −10% OR FVC decline between −5 and −9% accompanied by a DLCO decline >= −15%). We were unable to identify a threshold for KL-6 with an adequate sensitivity and specificity in the CYC arm. Please see Supplementary Figure S1.

Baseline CCL-18 Levels Predict SSc-ILD Progression

Higher baseline CCL-18 levels predicted progression of ILD as measured by the course of the FVC (CYC/MMF: Estimate −1.24/−0.35; P<0.001;0.007) and DL_{CO} (CYC/MMF: Estimate −1.87/−1.26; P=0.001/<0.001) over 1 year for both treatment arms, even after adjusting for baseline disease severity (Table 4).

After dichotomizing the CCL-18 variable based on the median level in baseline SLS II samples (163.1 ng/ml), a high baseline CCL-18 level was associated with increased progression of ILD as measured by the course of the FVC both in the MMF arm (Estimate −0.61; P=0.039) and in the CYC arm (Estimate −0.01; P=0.010) (Supplementary Table S3). High baseline CCL-18 level was associated with increased progression of ILD as measured by the course of the DL_{CO} both in the MMF arm (Estimate −0.94; P<0.001) and in the CYC arm (Estimate −2.13; P<0.001) (Supplementary Table S4).

The ROC analysis failed to reveal a significant CCL-18 threshold for predicting ILD progression in either treatment with an adequate sensitivity and specificity (Supplementary Figure S2).

Baseline CCL-18, but Not KL-6, Predicts Long-term Survival in SSc-ILD

Data from the SLS II long-term follow up study [37] were used to explore whether baseline KL-6 or CCL-18 predicted long-term survival in patients with SSc-ILD. At the time of this

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analysis, 30 of 142 (21%) SLS II participants had died within 8 years after the first patient was randomized (CYC: 16; MMF: 14). The median follow-up time for all patients was 4 years. The majority of deaths in both cohorts were due to respiratory failure from underlying SSc (N=16) [37].

The Cox-proportional hazards model analysis demonstrated that SLS II participants with increased CCL-18 at baseline had an increased risk of mortality due to respiratory failure even after controlling for baseline disease severity in the CYC arm (HR: 3.09; P=0.018; Supplementary Table S5), but not in the MMF arm (Supplementary Table S6). Baseline KL-6 level was not associated with mortality due to respiratory failure in either treatment arm (Supplementary Tables S5, S6).

Similarly, baseline CCL-18 level was associated with mortality due to all causes (HR: 3.31; P=0.0008) in the CYC arm, but not the MMF arm. Patients with high CCL-18 based on the median had an increased risk of mortality in the CYC arm (P=0.006 by log-rank test; Supplementary Figure S3), but not in the MMF arm (Supplementary Figure S4). Baseline KL-6 level was not associated with all-cause mortality in either treatment arm (Supplementary Tables S7, S8). High KL-6 based on the median was not associated with an increased risk of mortality in the CYC arm or in the MMF arm (Supplementary Figures S5, S6).

DISCUSSION

To our knowledge, this is the first study to evaluate the relationship between plasma levels of KL-6 and CCL-18 with progression of ILD in the context of a relatively large RCT for SSc-ILD. Elevated levels of both KL-6 and CCL-18 at baseline predicted poor response to immunosuppressive therapy with either CYC or MMF.

At baseline, both KL-6 and CCL-18 levels each correlated with surrogate measures of ILD severity, including extent of radiographic fibrosis (KL-6, CCL-18), and % predicted TLC (KL-6) and DL_{CO} (KL-6). These findings are consistent with the findings of our previous publication of patients who participated in SLS I (CYC vs. placebo), in which baseline KL-6 levels correlated with the extent of radiographic fibrosis and with the DL_{CO} [23]. In contrast, neither KL-6 (SLS I and II) nor CCL-18 (SLS II) was associated with the baseline FVC % predicted. While the severity of SSc-ILD is often defined by the degree of ventilatory restriction (i.e. FVC % predicted), all pulmonary function test parameters are indirect and often variable measures of the extent of structural lung disease. This may explain why these peripheral pneumoproteins correlate more strongly with the extent of radiographic fibrosis as measured by quantitative computer-aided diagnostic techniques.

KL-6 and CCL-18 levels decreased in response to treatment with CYC and MMF for one year, although the magnitude of the decline was greater for CCL-18 than for KL-6. This may be due to the fact that CCL-18 is secreted by type 2 macrophages, whereas KL-6 is excreted by type II pneumocytes. Macrophages as inflammatory cells would be more likely to decrease their activity in response to immunosuppressive treatment than type II pneumocytes, which are epithelial in origin. For both pneumoproteins, patients assigned to

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MMF experienced the greatest decline in CCL-18 and KL-6 levels. This discrepancy could have been due to several factors. As reported previously [5], MMF was better tolerated than CYC in SLS II; thus, patients may have had better adherence to therapy with MMF than CYC and were more likely to achieve and maintain the target treatment dosage. Another possibility is that MMF targets pathways involving KL-6 and CCL-18 with greater potency than CYC. In SLS II, no difference was noted in the course of the FVC over two years between patients assigned to MMF versus CYC; however, there was a difference in the course of the DL_{CO}, favoring MMF [5]. More research is needed to further explore why KL-6 and CCL-18 levels declined to a greater degree in response to MMF than CYC treatment.

Even after adjusting for baseline disease severity, higher levels of KL-6 and CCL-18 predicted progression (worsening) of ILD in each of the two SLS II treatment arms. We opted to examine treatment arms separately since MMF and CYC have markedly different mechanisms of action; however, even in the combined cohort, both baseline KL-6 and CCL-18 predicted progression of ILD, as measured by the course of the DL_{CO} and FVC over one year (results available upon request).

The finding that high baseline KL-6 and CCL-18 predicted progression of ILD even after adjusting for baseline disease severity suggests that these two pneumoproteins could be used to identify patients with a more aggressive ILD phenotype. Despite treatment with MMF, patients with high baseline KL-6 and CCL-18 levels experienced a decline in their FVC and DL_{CO} over 12 months. Among patients assigned to CYC, those who had high baseline CCL-18 (but not KL-6) levels also experienced a decline in their FVC and DL_{CO} over 12 months, as well as increased risk of long-term mortality. In addition to helping to identify patients who may benefit from closer monitoring, KL-6 and CCL-18 measurements could also be used to select patients for combination ILD therapy (two immunosuppressants or an immunosuppressant plus and anti-fibrotic) or for cohort enrichment to identify patients who may be eligible for clinical trials investigating other novel therapies for progressive SSc-ILD.

We attempted to identify a threshold for KL-6 and CCL-18 for predicting worsening of ILD. We discovered that a KL-6 level greater than 1549 u/ml in the MMF arm was associated with an increased risk of progression using both definitions of ILD worsening with an excellent sensitivity and good specificity. However, we were unable to identify a threshold with adequate sensitivity and specificity in the CYC arm for KL-6, or for either treatment arm for CCL-18. This may have been due to loss of power due to dichotomization of the outcome. Moreover, while we used two different definitions of ILD progression, there is currently no consensus on a universally accepted definition of ILD progression in SSc.

This study has some limitations. We did not include an external validation cohort. We had planned to use the SLS I cohort as an external validation cohort, but the sample size of participants who underwent KL-6 measurement in SLS I and had complete follow up data was too small (N=40) to perform the joint model analysis. However, the baseline correlations between KL-6 and surrogate measures of ILD severity were similar between both SLS cohorts, suggesting that our findings are likely reproducible. Moreover, we

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demonstrated predictive potential of both KL-6 and CCL-18 in both treatment arms of SLS II, with each arm analyzed separately and one arm being CYC, as a means of semi-internal validation.

This study has important strengths. First, we evaluated ILD progression by using a joint model that included repeated measures of the FVC and DL_{CO}. Trends in the FVC and DL_{CO} determined from measurements at several time points may more accurately reflect true progression of ILD compared with changes in the FVC and DL_{CO} using measurements at only two time points. Indeed, our recent analysis of the long-term follow up data from SLS I and II revealed that the course of the FVC and DL_{CO} were better predictors of long-term mortality than the baseline FVC or DL_{CO} [37].

Using data from a rigorously-conducted clinical trial to study candidate biomarkers also limits potential confounding from variables, such as access to care and therapy and missing outcome data, that often occurs in the setting of observational studies in which patients receive varying medication regimens at baseline and subsequent visits (type, dose, duration) and varying follow up. Furthermore, in an exploratory analysis, we also found that high CCL-18 levels at baseline were associated with an increased risk of long-term mortality due to respiratory failure. These findings substantiate previously published work linking CCL-18 with progressive ILD and poor outcomes [16, 17, 31].

In summary, the present findings strongly suggest that KL-6 and CCL-18 are important peripheral markers of both disease severity and disease progression in patients with SSc-ILD. Measurement of these two pneumoproteins early in the course of SSc-ILD may help to identify those patients with a more aggressive SSc-ILD phenotype in both clinical practice and in research. Additional mechanistic studies are needed to determine precisely how KL-6 and CCL-18 contribute to the pathobiology of SSc-ILD. These additional studies may also reveal new therapeutic targets for intervention in SSc-ILD since currently available treatment options for this often fatal condition are still limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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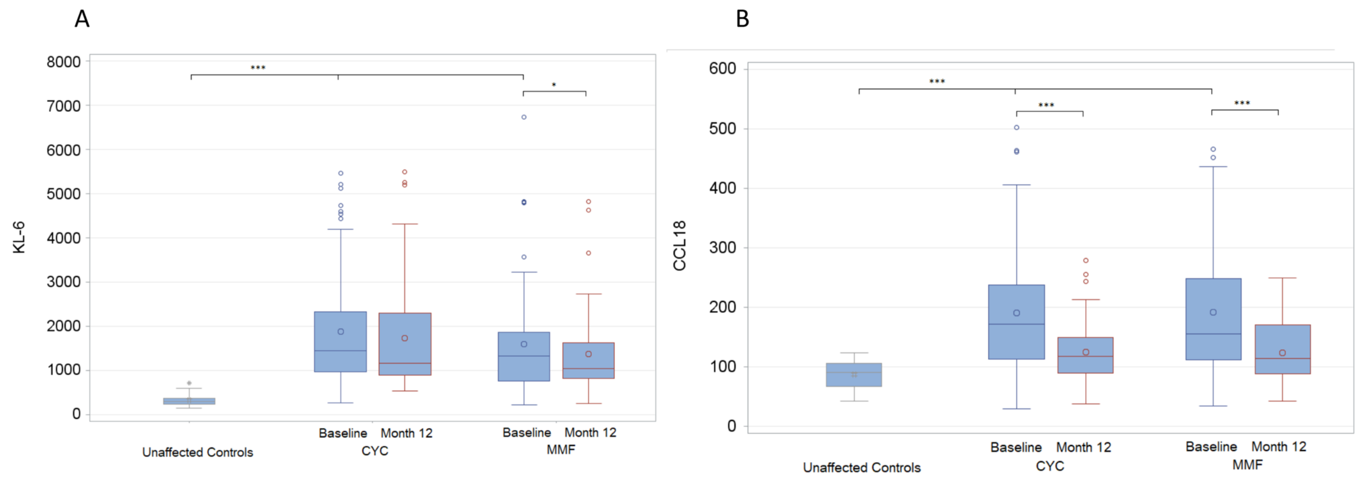

Figure 1. Change in KL-6 (A) and CCL-18 (B) from baseline to 12 months in SLS II.

The units for KL-6 are u/ml. The units for CCL-18 are ng/ml.

* p< 0.05, ** p< 0.01, *** p< 0.001.

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Table 1.

Baseline Characteristics of SLS II participants by study group and unaffected controls.

	SLS II		Controls
Measure	**CYC (N=71)**	**MMF (N=62)**	**(N=39)**
Age**, years	52.3 ± 9.5	52.9 ± 10.0	52.2 ± 9.5
Female	55 (77.5%)	44 (71.0%)	28 (71.8%)
Race***			
White	47 (66.2%)	46 (74.2%)	27 (69.2%)
African American	18 (25.4%)	10 (16.1%)	9 (23.1%)
Asian	3 (4.2%)	6 (9.7%)	3 (7.7%)
Other	3 (4.2%)	0 (0%)	0 (0%)
Diffuse cutaneous sclerosis	39 (54.9%)	38 (61.3%)	
Disease duration*, years	2.5 ± 1.8	2.7 ± 1.7	
FVC, % predicted	66.2 ± 9.9	66.5 ± 8.3	
FEV_1/FVC, %	83.5 ± 5.6	82.0 ± 5.7	
TLC, % predicted	65.4 ± 12.1	66.4 ± 10.2	
DL_{CO}***, % predicted	53.8 ± 14.2	54.9 ± 11.3	
BDI*** (focal score; 0–12)†	7.0 ± 2.3	7.3 ± 2.2	
HAQ-DI (score, 1–3)‡	0.7 ± 0.7	0.7 ± 0.6	
Modified Rodnan Skin Score (MRSS) (0–51)	14.1 ± 10.8	15.2 ± 10.3	
Lung fibrosis (QLF) score, whole lung (WL)*, %	9.1 ± 7.0	8.4 ± 7.1	
Lung fibrosis (QLF) score, worst zone (ZM)*, %	23.2 ± 19.2	22.8 ± 20.4	
Quantitative ILD (QILD) score***, % WL	32.1 ± 14.2	27.7 ± 13.8	
Quantitative ILD (QILD) score***, % ZM	53.2 ± 19.3	49.7 ± 21.2	

Data reported are mean ± SD or N (%)

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$

† High score denotes worse dyspnea

‡ High score denotes worse function

Definition of abbreviations: FVC = forced vital capacity; FEV_1 = forced expired volume in 1 sec; TLC = total lung capacity; DL_{CO} = single-breath diffusing capacity for carbon monoxide; % BDI = baseline dyspnea index; HAQ-DI = health assessment questionnaire for scleroderma-Disability Index; MRSS = Modified Rodnan Skin Score; QLF-WL, % = quantitative extent of lung fibrosis (reticulations) in whole lung on high-resolution computed tomography (HRCT); QLF-ZM, % = quantitative extent of lung fibrosis in the zone of maximal involvement on HRCT; QILD-WL, % = quantitative extent of interstitial lung disease (fibrosis + GGO + honeycombing) in whole lung on HRCT; QILD-ZM = quantitative extent of interstitial lung disease in the zone of maximal involvement on HRCT

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Table 2.

Baseline correlations between KL-6 and CCL-18 and SSc disease activity measures

Disease measure	KL-6-SLS II (N = 133)	CCL-18-SLS II (N = 133)
FVC%-predicted	−0.01	0.11
DL_{CO}%-predicted	−0.23***	−0.04
TLC%-predicted	−0.21***	0.01
QILD-WL	0.35***	0.14*
QILD-ZM	0.35***	0.18**
QLF-WL	0.36***	0.08
QLF-ZM	0.33***	0.10

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$

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Table 3.

High baseline KL-6 predicts progression of ILD based on the course of the FVC and DL_{CO} over 1 year in patients randomized to CYC and MMF.

Variable	Estimate	95% CI		P-Value
Outcome: Course of FVC over 12 months in CYC arm				
Intercept	9.99	7.02	12.26	0.001
KL-6	−0.32	−0.50	−0.11	0.024
Baseline FVC	0.88	0.87	0.90	<0.001
Time	0.10	0.028	0.17	0.004
Outcome: Course of FVC over 12 months in MMF arm				
Intercept	16.92	12.12	21.99	<0.001
KL-6	−0.72	−1.03	−0.32	0.005
Baseline FVC	0.85	0.80	0.89	<0.001
Time	0.054	−0.015	0.12	0.128
Outcome: Course of DL_{CO} over 12 months in CYC arm				
Intercept	18.11	14.63	20.60	<0.001
KL-6	−1.30	−1.51	−1.00	<0.001
Baseline DL_{CO}	0.85	0.84	0.87	<0.001
Time	−0.024	−0.12	0.076	0.634
Outcome: Course of DL_{CO} over 12 months in MMF arm				
Intercept	23.80	17.42	26.21	0.001
KL-6	−1.28	−1.46	−0.84	0.004
Baseline DL_{CO}	0.80	0.78	0.84	<0.001
Time	0.030	−0.051	0.11	0.428

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Table 4.

High baseline CCL-18 predicts progression of ILD based on the course of the FVC and DL_{CO} over 1 year in patients randomized to CYC and MMF.

Variable	Estimate	95% CI		P-Value
Outcome: Course of FVC over 12 months in CYC arm				
Intercept	13.80	11.92	15.65	<0.001
CCL-18	−1.24	−1.46	−1.03	<0.001
Baseline FVC	0.91	0.90	0.93	<0.001
Time	0.10	0.025	0.17	0.012
Outcome: Course of FVC over 12 months in MMF arm				
Intercept	10.21	8.34	11.89	<0.001
CCL-18	−0.35	−0.52	−0.16	0.007
Baseline FVC	0.88	0.86	0.90	<0.001
Time	0.057	−0.014	0.13	0.114
Outcome: Course of DL_{CO} over 12 months in CYC arm				
Intercept	16.91	12.16	19.61	<0.001
CCL-18	−1.87	−2.17	−1.19	0.001
Baseline DL_{CO}	0.87	0.84	0.89	<0.001
Time	−0.020	−0.11	0.065	0.642
Outcome: Course of DL_{CO} over 12 months in MMF arm				
Intercept	17.36	14.39	19.45	<0.001
CCL-18	−1.25	−1.49	−0.92	<0.001
Baseline DL_{CO}	0.85	0.82	0.87	<0.001
Time	0.040	−0.040	0.12	0.327

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Global skin gene expression analysis of early diffuse cutaneous systemic sclerosis shows a prominent innate and adaptive inflammatory profile

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Contributors SA, BS, DK, SV and TF designed the study. BS, DK, MEH, TF, VS, FH, JG, AAS, JB, AMSB, MW, SV, PB, NW, JC and SA were involved in acquisition of data. All authors were involved in analysis and interpretation of the data. All authors were involved in manuscript preparation and approved the final version of the manuscript.

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Abstract

Objectives—Determine global skin transcriptome patterns of early diffuse systemic sclerosis (SSc) and how they differ from later disease.

Methods—Skin biopsy RNA from 48 patients in the Prospective Registry for Early Systemic Sclerosis (PRESS) cohort (mean disease duration 1.3 years) and 33 matched healthy controls was examined by next-generation RNA sequencing. Data were analysed for cell type-specific signatures and compared with similarly obtained data from 55 previously biopsied patients in Genetics versus Environment in Scleroderma Outcomes Study cohort with longer disease duration (mean 7.4 years) and their matched controls. Correlations with histological features and clinical course were also evaluated.

Results—SSc patients in PRESS had a high prevalence of M2 (96%) and M1 (94%) macrophage and CD8 T cell (65%), CD4 T cell (60%) and B cell (69%) signatures. Immunohistochemical staining of immune cell markers correlated with the gene expression-based immune cell

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signatures. The prevalence of immune cell signatures in early diffuse SSc patients was higher than in patients with longer disease duration. In the multivariable model, adaptive immune cell signatures were significantly associated with shorter disease duration, while fibroblast and macrophage cell type signatures were associated with higher modified Rodnan Skin Score (mRSS). Immune cell signatures also correlated with skin thickness progression rate prior to biopsy, but did not predict subsequent mRSS progression.

Conclusions—Skin in early diffuse SSc has prominent innate and adaptive immune cell signatures. As a prominently affected end organ, these signatures reflect the preceding rate of disease progression. These findings could have implications in understanding SSc pathogenesis and clinical trial design.

INTRODUCTION

Systemic sclerosis (SSc) is a multi-system autoimmune and fibrotic disease associated with high morbidity and mortality.[12] Treatment options remain limited, and management is complicated by heterogeneity in clinical course and treatment response.

Whole transcriptome gene expression profiling can yield insights into disease pathogenesis and identify distinct subgroups of patients.[34] We and others have previously used microarray technology to measure global gene expression in skin biopsies from SSc patients in comparison to healthy controls (HCs),[5-12] revealing distinct gene expression patterns in SSc skin. Fibrotic and inflammatory gene expression signatures have been observed in a large percentage of patients, while a subset of patients has 'normal-like' gene expression profiles. These studies highlight heterogeneity in SSc skin gene expression. A large-scale study to characterise skin gene expression specifically in early, diffuse SSc in comparison to those with later stage disease has been lacking.

We investigated the transcript expression profiles of skin specimens from a large group of patients with early, diffuse SSc from the Prospective Registry for Early Systemic Sclerosis (PRESS) cohort using next generation RNA sequencing. These data were compared with HC skin and to patients in the Genetics versus Environment in Scleroderma Outcomes Study (GENISOS), in which patients had a longer average disease duration.

METHODS

Patients and control subjects

Patients were recruited from PRESS, an observational cohort of early diffuse SSc patients from 11 US academic medical centres.[13] Skin biopsies from 48 patients within 3 years of onset of first non-Raynaud's symptom were used for RNA sequencing, along with 33 biopsies from HCs matched to patients by age, sex and ethnicity. Ten repeat biopsies from eight SSc patients were also available. Skin biopsy was optional in PRESS, and all available biopsies in the PRESS cohort at the time of study were included. Patients fulfilled the 2013 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for SSc and had diffuse skin involvement.[14] Modified Rodnan Skin Score (mRSS) and local skin score at the biopsy site were recorded at the time of biopsy. Skin thickness progression rate (STPR) was calculated similarly to what was

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previously described,[15] using the equation mRSS at the time of biopsy/time from first puffy fingers or skin thickening. Participants provided informed and written consent.

Skin biopsy and RNA sequencing

Punch biopsies were obtained from the forearm skin. The methods for RNA sequencing and analysis are described in online supplementary methods. Data from the PRESS cohort were compared with similarly obtained data from the GENISOS cohort that included SSc patients with longer disease duration at the time of biopsy.[10] Although microarray technology was used for gene expression profiling in the previously published study, we performed RNA sequencing in these GENISOS samples (n=55) and matched HCs (n=33) for the present study in order to avoid heterogeneity resulting from methodological differences.

Analysis of cell type-specific expression

We performed cell type-specific gene expression analysis using the method we have used previously.[10,16] Details are provided in the online supplementary methods.

Assignment of patients to ‘intrinsic subsets’ based on skin gene expression

Fragments per kilobase million (FPKM) values were sent to JMF and MLW who were blinded to all clinical data and assigned each sample to one of four ‘intrinsic subsets’ using previously described methods.[17,18]

Immunohistochemistry

Immunohistochemical (IHC) analyses of skin biopsies are described in the online supplementary methods.

Statistical analysis

Associations between cell type signatures and clinical or histological features were analysed by Spearman’s rank order correlation. Cell type signature scores were log-transformed and compared between the PRESS and GENISOS cohorts by Student’s t-test. Multivariable regression analyses were performed with pooled data from both cohorts with adjustment for clinical variables noted in the text. mRSS and STPR within the intrinsic subsets were analysed by linear regression analyses, using the normal-like subset as a reference.

RESULTS

Demographics

Demographics and clinical characteristics of participants from the PRESS cohort and matched HCs, along with the GENISOS cohort and their matched HCs, are shown in table 1.

Transcript expression profile of early diffuse SSc skin

Three thousand eighty seven transcripts were differentially expressed between SSc patients and HCs using a false discovery rate cut-off of 0.05 and fold change cut-off of >1.5 or <0.67, including 927 long non-coding RNAs (lncRNAs). Unsupervised hierarchical clustering revealed nearly complete discrimination between differential transcript expression

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in HCs and SSc patients, with the exception of three SSc patients whose transcript expression profile largely resembled that of HCs (figure 1A). Lists of differentially expressed transcripts between SSc and HC and associations between transcripts and mRSS or forced vital capacity (FVC) in SSc patients at the time of skin biopsy are included in the supplementary data file on our webpage (https://www.uth.tmc.edu/scleroderma/).The most over-represented pathways in SSc skin based on Ingenuity Pathway Analysis were hepatic fibrosis, granulocyte and agranulocyte adhesion and diapedesis, and Th1 and Th2 activation pathways (figure 1B). Th1 and Th2 activation pathways had not been previously observed in the skin of SSc patients.[10] The top activated transcriptional regulators were predicted to be signal transducer and activator of transcription 1, interferon regulatory factor 7, and CCAAT enhancer binding protein beta, while the top activated cytokines/growth factors were interferon gamma, tumour necrosis factor and interleukin 1 beta (IL-1β) (figure 1C, D). Surprisingly, transforming growth factor beta (TGFβ) ranked 15th among upstream cytokines/growth factors (data not shown), in contrast to our prior study of patients with longer disease duration in which it had ranked first.[10]

Prominent innate and adaptive immune cell signatures in early diffuse SSc skin

Cell type-specific analysis revealed that most patients had increased innate and adaptive immune cell signatures compared with HCs (figure 2A). The most prevalent signatures upregulated in SSc compared with HC were those of M2 and M1 macrophages (96% and 94% of SSc patients, respectively). A fibroblast signature was present in 92% of patients. Most SSc patients also had CD4 T cell, CD8 T cell and B cell signatures (60%, 65% and 69%, respectively). No significant differences in cell type signatures were observed in male versus female patients or in RNA polymerase III antibody-positive versus topoisomerase I antibody-positive patients (online supplementary tables 1 and 2, respectively).

We compared the cell type signatures in PRESS patients to those of GENISOS patients for whom we had previously performed skin biopsies and analysed RNA expression by microarray.[10] To allow for comparison between the two cohorts, RNA sequencing was performed using the available GENISOS (n=55) and matched HC RNA samples (n=33) from that study. Differences in disease characteristics of the patients whose skin gene expression was analysed in GENISOS and PRESS are shown in table 1. On average, PRESS patients had a shorter disease duration at the time of biopsy than GENISOS patients (1.3 vs 7.4 years, respectively). Compared with GENISOS patients, PRESS patients had higher CD8 T cell, CD4 T cell, B cell and natural killer (NK) cell signatures in addition to M1 and M2 macrophage signatures (figure 2B). Fibroblast signatures were similar between the two cohorts, while hair outer root sheet and keratinocyte signatures were lower in PRESS compared with GENISOS. Restricting the analysis to GENISOS patients with diffuse SSc and >3 years disease duration (n=28), PRESS patients had higher immune cell signatures, although the differences were smaller in this subgroup analysis (online supplementary figure 1). The prevalence of upregulated CD8 T cell, CD4 T cell and B cell signatures was relatively low in GENISOS as a whole (22%, 20% and 22%, respectively), including among the 28 patients with diffuse cutaneous involvement and >3 years disease duration (21%, 18% and 21%, respectively) (online supplementary figure 2).

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To characterise clinical correlates of immune cell signatures within both cohorts, we pooled the data and performed multivariable regression analyses where the associations of disease duration, extent of skin involvement (as determined by mRSS), FVC % predicted and immunosuppression (comparing those on no immunosuppression to those on methotrexate, mycophenolate or cyclophosphamide at the time of biopsy) with cell type signatures (dependent variable) were examined. Adaptive immune cell signatures were inversely associated with disease duration after adjustment for mRSS, FVC % predicted and immunosuppression. By contrast, M1 and M2 macrophages and fibroblasts associated with mRSS but did not significantly associate with disease duration after adjustment for other clinical variables (table 2). These associations were similar after additional adjustment for PRESS versus GENISOS cohorts, suggesting that the observations were not driven by batch effects (online supplementary table 3). Of note, the investigated cell type signatures were not associated with immunosuppressive treatment in the univariable analysis (data not shown) or multivariable analysis (table 2).

Examination of available follow-up samples in the PRESS cohort

The majority of follow-up biopsies showed declines in immune cell signatures compared with their original biopsies (online supplementary figure 3A, B and online supplementary table 4). Fibroblast signatures were more variable at follow-up, with a small decline on average. Keratinocyte signatures were increased in most follow-up biopsies. Most of the patients with follow-up biopsies had a decline in mRSS from baseline to follow-up, and mRSS change correlated with changes in immune cell and fibroblast signatures numerically.

Histological associations with gene expression profiles

Paraffin-embedded skin biopsy samples concurrently collected from a subgroup of PRESS SSc patients were evaluated histologically using standard H&E staining and IHC staining for markers of macrophages (CD68, CD163, AIF1), endothelial cells (CD31) and myofibroblasts (α-smooth muscle actin(SMA)), as well as markers of adaptive immune cells CD3, CD4, CD8, CD20 and CD56 (it should be noted that CD4 is also expressed in monocytes/macrophages, although at a much lower intensity than in CD4 T cells,[19] and that CD56 is expressed in a subset but not all NK cells). Demographics for these samples are shown in online supplementary table 5, and representative slides are shown in online supplementary figure 4. As expected, SSc skin had increased collagen thickness, α-SMA expression and macrophage markers compared with HC skin (online supplementary table 6). Markers of adaptive immune cells were also increased in SSc compared with HC skin (online supplementary table 7). Clinical correlates of IHC staining are shown in online supplementary table 8.

Importantly, cell type signature scores for macrophages and adaptive immune cells based on RNA sequencing data correlated with IHC staining for markers of macrophages and adaptive immune cells, respectively (table 3). Histologically, CD68 and CD163 tracked roughly in parallel, consistent with the reported difficulty in discerning M1 from M2 subtypes with these markers in human cells.[20] Taken together, the correlations with IHC staining support the validity of the gene expression-based cell type signatures. Moreover, fibroblast gene expression signature scores correlated with α-SMA (Spearman's rank order correlation

coefficient 0.73, p<0.01) and collagen thickness (Spearman's rank order correlation coefficient 0.76, p<0.01).

Association of cell type signature with disease course

A summary of mRSS, FVC and immunosuppression use 12 months after initial skin biopsy is shown in online supplementary table 9. 78.6% of patients were taking immunosuppressive medication 12 months after initial biopsy, which is expected for a cohort of early diffuse SSc patients. Cell type signatures did not significantly predict change in mRSS 6 or 12 months after biopsy, or change in FVC 12 months after biopsy (online supplementary table 10). Similarly, transcripts recently described as predictive of mRSS progression[21] based on samples collected in a phase II study of tocilizumab did not significantly predict postbiopsy mRSS change in this cohort (online supplementary figure 5). Restricting the analysis to those treated with immunosuppressive medications during follow-up also did not show predictive significance for the immune cell signatures (data not shown).

We then looked for associations with the preceding STPR, which was found to be an independent predictor of mortality in patients with early diffuse SSc.[15] Significant correlations were seen between immune cell signatures and STPR preceding the biopsy (figure 3 and online supplementary table 10). Thus, immune cell signatures in this cohort were associated with STPR up to the time of biopsy, but did not predict subsequent progression.

Comparison to intrinsic subset analysis

The PRESS samples were also assigned to one of four intrinsic subsets (inflammatory, fibroproliferative, limited or normal-like) using previously described methodology by Dr Whitfield's group.[17,18] Thirty-two out of 33 HCs were classified as normal-like, with 1 out of 33 classified as limited (data not shown). Among SSc patients, 23 were classified as inflammatory, 19 as fibroproliferative and 6 as normal-like (figure 2A and online supplementary figure 6). As shown in the figures, there was an over-representation of adaptive immunity cell type signatures in the inflammatory subset of patient samples.

Examination of longitudinal samples revealed that among five samples classified as inflammatory, follow-up biopsies from three of these individuals were classified in non-inflammatory subsets (two fibroproliferative and one normal-like), whereas none of the individuals with biopsies in the fibroproliferative or normal-like subsets on the initial biopsy had a follow-up biopsy in the inflammatory subset ().

Regarding mRSS course, the intrinsic subsets did not significantly predict mRSS change 6 or 12 months postbiopsy in the overall cohort or in the subgroup of patients taking immunosuppressive agents during follow-up (online supplementary table 11). In agreement with the immune cell signature data, STPR preceding the biopsy was significantly higher in the inflammatory subset (online supplementary table 12).

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DISCUSSION

Histological and gene expression analyses have demonstrated variable degrees of innate and adaptive immune cells in affected SSc skin.[5-11 22-28] In the current study, we measured whole transcriptome expression and cell type signatures in skin specimens in a large cohort of patients specifically with early diffuse SSc and matched HCs. More than half of patients in this cohort had upregulation of CD8 T cell, CD4 T cell and B cell signatures, a higher prevalence than what was observed in patients with longer disease duration from the GENISOS cohort. We also observed a higher prevalence of M1 and M2 macrophage signatures in the skin of early diffuse SSc patients. In patients with longitudinally collected biopsies, immune cell signatures declined on average from initial to follow-up biopsies. These results parallel the clinical observation that early SSc has an edematous, inflammatory phase followed by a more fibrotic phase, and the histological findings in SSc showing an early 'cellular stage' characterised by cellular infiltrates in the dermis followed by a later 'fibrotic stage' characterised by increased collagen deposition.[22 23]

Multivariable regression analysis including all samples from the PRESS and GENISOS cohorts showed that adaptive immune cell signatures were significantly associated with shorter disease duration even after adjustment for immunosuppression, severity of skin disease (as assessed by mRSS) and lung disease (as assessed by FVC), whereas macrophage and fibroblast signatures associated predominately with mRSS. These results suggest that the determinants of adaptive versus innate immune cell infiltration in the skin may differ. This can also have implications for target population enrichment strategies in clinical trials, although the observation needs to be confirmed in future studies.

Ingenuity Pathway Analysis suggested that inflammatory cytokines had a more prominent role in driving the dysregulated gene expression in early diffuse SSc compared with later stage disease. Of note, the vast majority of early diffuse SSc patients with a fibroblast signature had a concomitant M1 and/or M2 macrophage signature, and many had concomitant adaptive immune cell signatures, suggesting co-occurrence of dysregulated fibroblast and immune cell function in a majority of early diffuse SSc patients. Our gene expression and IHC data add to the large body of evidence that macrophages are upregulated in SSc.[29 30] Macrophages are capable of detecting innate immune stimuli and producing both pro-inflammatory and pro-fibrotic cytokines, including some (eg, IL-6 and TGFβ) that are implicated in SSc pathogenesis. However, the effects of macrophages within the skin and other end organs in SSc require further study.

Taken together, our results indicate that innate and adaptive immune cell activity in the skin is a prominent feature of early diffuse SSc. TGFβ, a key pro-fibrotic cytokine implicated in SSc pathogenesis,[31] appears to have a less prominent role in driving the dysregulated gene expression observed during this early, inflammatory phase, in contrast to its prominent role in later-stage disease.

Histological scoring in concurrently collected skin samples supported the gene expression data, demonstrating upregulation of macrophage, adaptive immune cell and fibrotic markers. Immune cell markers correlated with their respective gene expression signatures, and

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fibrosis markers correlated with fibroblast gene expression signatures. These results support the validity of the gene expression-based cell type signatures.

The RNA processing method used here (ribosomal RNA reduction) enabled the provision of an unbiased comprehensive list of differentially expressed lncRNAs, because this method (unlike poly (A) enrichment) does not remove lncRNAs that do not have a poly(A) tail [32] We have provided a list of differentially expressed lncRNAs expressed in the skin of early diffuse SSc compared with HC, as well as their associations with mRSS. Although our currently available pathway and predicted upstream regulator analytic methods do not include analysis of lncRNAs, the list of disease-relevant lncRNAs represents a resource for follow-up mechanistic studies in this novel area of research.

The carefully collected clinical data in the well-phenotyped PRESS cohort enabled us to examine the correlation of the SSc gene expression profile with the progression rate of skin fibrosis prior to and following skin biopsy. Immune cell signatures were associated with preceding STPR, while they did not have predictive significance for postbiopsy mRSS change. Similarly, transcripts found to be predictive of mRSS progression in previous work[21] were not significantly associated with postbiopsy mRSS change in this study. Intrinsic subset classification (normal-like, inflammatory and fibroproliferative)[18] did not show predictive significance for mRSS change 6 or 12 months after biopsy. These findings suggest that the use of these previously described gene signatures and subsets for predicting changes in mRSS may not be generalisable to all cohorts. Further research will be needed to determine whether or not a model for prediction of disease progression based on skin gene expression can be universally applied across cohorts, particularly in patients on treatment with commonly used immunosuppressive medications typified in PRESS. The data in this study suggest that skin gene expression signatures in early diffuse SSc are more of a reflection of preceding skin thickness progression than predictors of subsequent progression, supporting the notion that skin is a prominent end organ in SSc rather than an effector organ that drives disease progression.

Our study has several strengths. We examined the transcript expression profile of a relatively large number of skin samples in a well-phenotyped early diffuse SSc cohort using a sensitive, comprehensive RNA sequencing method and compared the results to a later stage SSc transcript expression dataset generated using the same technology. The gene expression-based cell type signatures were validated by IHC staining in concurrently collected samples. There were some limitations to this study that merit discussion. Only a small subgroup of patients (n=8) had follow-up samples available, limiting the ability to analyse changes in gene expression during disease progression. Our future studies will focus on longitudinal collection of early diffuse SSc skin samples. As is common in observational studies and most previous SSc skin gene expression studies, patients enrolled in PRESS were treated according to the standard of care, with the majority being treated with mycophenolate mofetil or methotrexate, which might have affected skin transcript expression.

In conclusion, this large-scale analysis of whole transcriptome expression in the skin of early diffuse SSc patients revealed a high prevalence of both innate and adaptive immune cell activity. Immune cell signatures were associated with preceding STPR but were not

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predictive of subsequent mRSS progression. These results shed light on the early pathogenesis of diffuse SSc and could have implications for clinical trials targeting the immune system in SSc patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key messages

What is already known about this subject?

- Skin gene expression is altered in patients with systemic sclerosis (SSc) based on data from microarrays, but heterogeneity exists in skin gene expression profiles of SSc patients.

What does this study add?

- A large-scale analysis of skin transcript expression specifically in patients with early, diffuse cutaneous SSc and comparison to patients with later disease revealed that innate and adaptive immune cell gene expression is more prominent in early diffuse SSc compared with later disease. After adjustment for key clinical characteristics, adaptive immune cell signatures were associated with shorter disease duration.
- Immune cell signatures appeared to reflect preceding skin thickness progression rate but did not predict subsequent modified Rodnan Skin Score progression.

How might this impact on clinical practice or future developments?

- The prominence of innate and adaptive immune cell signatures in early diffuse SSc would seem to support the premise of using immune-modulatory therapies in this subgroup of patients.
- There appear to be limitations in the use of skin gene expression profiles to predict subsequent disease progression, perhaps related to heterogeneity among SSc patient cohorts.

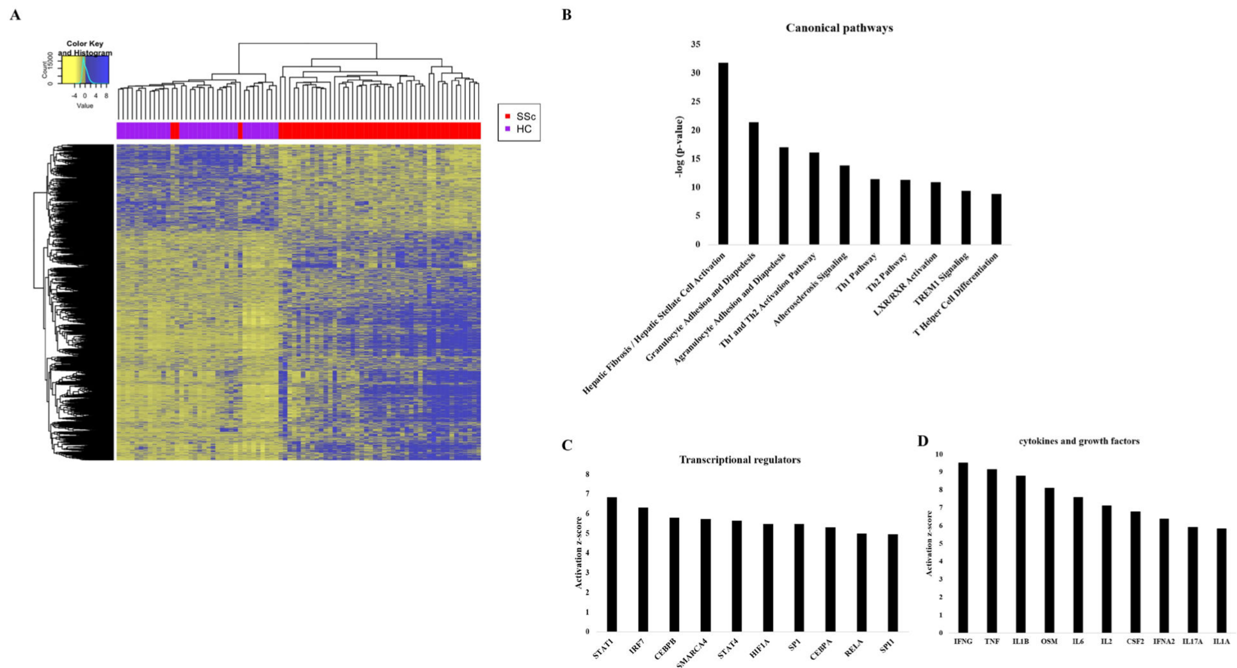

Figure 1.

Differentially expressed transcripts and pathways in Prospective Registry for Early Systemic Sclerosis systemic sclerosis (SSc) patients compared with healthy controls (HCs). (A) Heatmap of differentially expressed transcripts, represented by z-score normalised count values. Unsupervised hierarchical clustering is shown at the top, with HCs represented by purple squares and SSc patients represented by red squares. (B) Top 10 over-represented pathways in SSc compared with HC as determined by Ingenuity Pathway Analysis of differentially expressed transcripts (fold change >1.5 or <0.67 in SSc vs HC, with false discovery rate <0.05). (C) Top 10 predicted upstream transcriptional regulators in SSc compared with HC. (D) Top 10 predicted upstream cytokines/growth factors.

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Figure 2.

Cell type signatures in skin of PRESS SSc patients compared with healthy controls and compared with GENISOS SSc patients. (A) Cell type signature scores for each SSc sample (n=48). Scores represent the average fold-change (SSc/HC) for 125 cell type-specific signature genes (see online supplementary methods). Up-triangles indicate significantly higher scores for signature genes compared with non-signature genes (p<0.05, Wilcoxon rank-sum test). Down-triangles indicate significantly lower scores for signature genes compared with non-signature genes (p<0.05, Wilcoxon rank-sum test). Bottom margin values indicate the percentage of up-triangles (red) and down-triangles (blue), respectively. Patients were clustered based on signature scores (average linkage, Euclidean distance). The coloured boxes to the left of the cell type signature scores indicate the mRSS (left), local skin score at the site of the biopsy (middle) and the intrinsic subset classification, with legends at the right of the figure. White boxes (n=3) indicate no skin scores recorded at the time of the biopsy. (B) Signature scores for PRESS patients (n=48) were compared with those of GENISOS patients (n=55). The mean PRESS score is represented by round symbols with error bars spanning ±1 SD. The mean GENISOS score is represented by the midline for each grey box with boxes spanning ±1 SD. Right margin p values were obtained from a two-sample t-test of PRESS versus GENISOS scores (red: PRESS>GENISOS, FDR<0.05; blue: PRESS<GENISOS, FDR<0.05). Ctrl (HC), healthy control; DC, dendritic cell; GENISOS, Genetics versus Environment in Scleroderma Outcomes Study; hair ORS, hair outer root sheet; KC, keratinocyte; mRSS, modified Rodnan Skin Score; PRESS, Prospective Registry for Early Systemic Sclerosis; SSc, systemic sclerosis. FP, fibroproliferative subset; INF, inflammatory subset; NL, normal-like subset.

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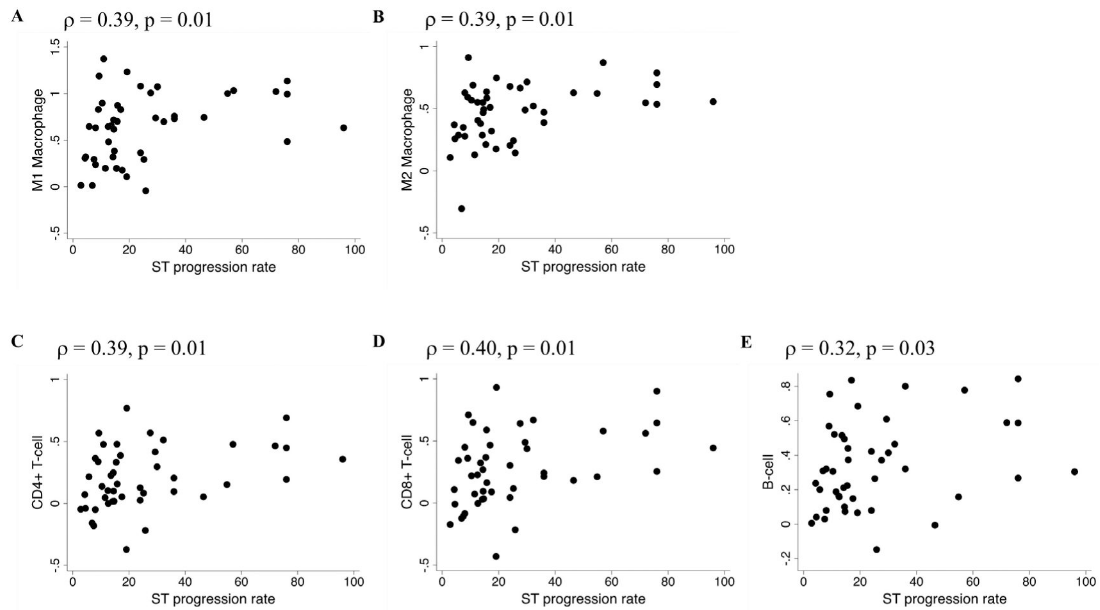

Figure 3.

Associations between preceding skin thickness progression rate and skin immune cell type signatures in PRESS SSc patients. Skin thickness progression rate (mRSS at the time of biopsy/years since first skin thickening or puffy fingers) preceding the skin biopsy is plotted on the x-axis. Cell type signature scores for (A) M1 macrophages, (B) M2 macrophages, (C) CD4 cells, (D) CD8 T cells or (E) B cells are plotted on the y-axis. mRSS, modified Rodnan Skin Score; PRESS, Prospective Registry for Early Systemic Sclerosis; SSc, systemic sclerosis; ST, skin thickness.

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Table 1

Demographics and clinical characteristics of SSc patients from PRESS and GENISOS cohorts and matched healthy controls at the time of skin biopsy

Characteristic	PRESS SSc (n=48)	HC matched to PRESS (n=33)	GENISOS SSc (n=55)	HC matched to GENISOS (n=33)
Age (years) at biopsy, mean (SD)	48.0 (15.0)	47.4 (13.4)	52.8 (12.6)	46.8 (11.7)
Race/ethnicity, n (%)				
White	30 (62.5)	24 (72.7)	38 (69.1)	19 (57.6)
Black	5 (10.4)	4 (12.1)	8 (14.5)	8 (24.2)
Hispanic	9 (18.8)	4 (12.1)	9 (16.4)	6 (18.2)
Other	4 (8.3)	1 (3.0)	0	0
Female, n (%)	30 (62.5)	22 (66.7)	40 (72.7)	27 (81.1)
Disease duration in years, mean (SD)	1.3 (0.9)		7.4 (5.2)	
Diffuse skin involvement, n (%)	48 (100)		37 (67.3)	
mRSS, mean (SD)	21.3 (8.7)		15.3 (10.4)	
Local skin score, mean (SD)	1.7 (0.8)		1.1 (0.9)	
FVC % predicted, mean (SD)	76.0 (19.8)		77.3 (19.8)	
SSc-associated autoantibody, n (%) *				
Antitopoisomerase I	12/41 (29.3)		15 (27.3)	
Anti-RNA polymerase III	17/38 (44.7)		17 (30.9)	
Anticentromere	1/36 (2.8)		7 (12.7)	
Mycophenolate, n (%) †	19 (39.6)		4 (7.3)	
Methotrexate, n (%) †	9 (18.8)		8 (14.5)	
Cyclophosphamide, n (%) †	1 (2.1)		1 (1.8)	

* indicates positive among those recorded.

† Indicates patients taking medication at the time of biopsy.

FVC, forced vital capacity; GENISOS, Genetics versus Environment in Scleroderma Outcomes Study; HC, healthy control; mRSS, modified Rodnan Skin Score;PRESS, Prospective Registry for Early Systemic Sclerosis; SSc, systemic sclerosis.

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Table 2

Multivariable regression analyses of key clinical variables with cell type-specific signatures in pooled PRESS and GENISOS datasets

	Coefficient	95% CI	P value
CD8 T cell *			
Disease duration	−0.026	−0.042 to −0.009	<0.01
mRSS	0.006	−0.002 to 0.014	0.12
FVC % pred	−0.001	−0.005 to 0.002	0.54
No immunosuppression	0.095	−0.058 to 0.249	0.22
CD4 T cell *			
Disease duration	−0.02	−0.034 to −0.006	<0.01
mRSS	0.004	−0.003 to 0.010	0.25
FVC % pred	−0.001	−0.004 to 0.002	0.49
No immunosuppression	0.06	−0.069 to 0.190	0.36
NK cell *			
Disease duration	−0.019	−0.031 to −0.007	<0.01
mRSS	0.004	−0.001 to 0.010	0.12
FVC % pred	−0.001	−0.004 to 0.001	0.39
No immunosuppression	0.086	−0.026 to 0.197	0.13
B cell *			
Disease duration	−0.023	−0.037 to −0.009	<0.01
mRSS	0.002	−0.005 to 0.009	0.56
FVC % pred	−0.001	−0.004 to 0.002	0.5
No immunosuppression	−0.014	−0.146 to 0.119	0.84
M1 macrophage *			
Disease duration	−0.013	−0.030 to 0.004	0.13
mRSS	0.013	0.005 to 0.021	<0.01
FVC % pred	−0.002	−0.005 to 0.002	0.36
No immunosuppression	0.04	−0.119 to 0.199	0.62
M2 macrophage *			
Disease duration	−0.001	−0.014 to 0.012	0.91
mRSS	0.014	0.007 to 0.020	<0.01
FVC % pred	−0.001	−0.003 to 0.002	0.61
No immunosuppression	0.005	−0.117 to 0.127	0.94
Fibroblast *			
Disease duration	0.001	−0.015 to 0.016	0.93
mRSS	0.016	0.008 to 0.023	<0.01
FVC % pred	0.001	−0.002 to 0.004	0.57
No immunosuppression	0.028	−0.119 to 0.174	0.71

[*] Cell type transcript signature used as the dependent variable in the multivariable model. FVC, forced vital capacity; GENISOS, Genetics versus Environment in Scleroderma Outcomes Study; mRSS, modified Rodnan Skin Score; NK, natural killer; PRESS, Prospective Registry for Early Systemic Sclerosis.

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Table 3

Correlation of immune cell gene expression signatures with immunohistochemical staining of immune cell markers

Cell abundance by IHC staining	Cell type signature score	Spearman's r (p value)
CD68	M1 macrophage	0.45 (0.02)
CD68	M2 macrophage	0.50 (0.01)
CD163	M1 macrophage	0.47 (0.02)
CD163	M2 macrophage	0.57 (<0.01)
AIF1	M1 macrophage	0.66 (<0.01)
AIF1	M2 macrophage	0.69 (<0.01)
CD3	CD4 T cell	0.61 (<0.01)
CD3	CD8 T cell	0.63 (<0.01)
CD4	CD4 T cell	0.49 (<0.01)
CD8	CD8 T cell	0.67 (<0.01)
CD20	B cell	0.54 (<0.01)
CD56	NK cell	0.24 (0.22)

IHC, immunohistochemical; NK, natural killer.

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Predictive Significance of Serum Interferon-Inducible Protein Score for Response to Treatment in Systemic Sclerosis–Related Interstitial Lung Disease

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Objective. Response to immunosuppression is highly variable in systemic sclerosis (SSc)–related interstitial lung disease (ILD). This study was undertaken to determine whether a composite serum interferon (IFN)–inducible protein score exhibits predictive significance for the response to immunosuppression in SSc-ILD.

Methods. Serum samples collected in the Scleroderma Lung Study II, a randomized controlled trial of mycophenolate mofetil (MMF) versus cyclophosphamide (CYC), were examined. Results were validated in an independent observational cohort receiving active treatment. A composite score of 6 IFN-inducible proteins IFNγ-inducible 10-kd protein, monokine induced by IFNγ, monocyte chemotactic protein 2, β_2-microglobulin, tumor necrosis factor receptor type II, and macrophage inflammatory protein 3β) was calculated, and its predictive significance for longitudinal forced vital capacity percent predicted measurements was evaluated.

Results. Higher baseline IFN-inducible protein score predicted better response over 3 to 12 months in the MMF arm (point estimate = 0.41, $P = 0.001$) and CYC arm (point estimate = 0.91, $P = 0.009$). In contrast, higher baseline C-reactive protein (CRP) levels were predictive of a worse ILD course in both treatment arms. The predictive significance of the IFN-inducible protein score and CRP levels remained after adjustment for baseline demographic and clinical predictors. During the second year of treatment, in which patients in the CYC arm were switched to placebo, a higher IFN-inducible protein score at 12 months showed a trend toward predicting a worse ILD course (point estimate = −0.61, $P = 0.068$), while it remained predictive of better response to active immunosuppression in the MMF arm (point estimate = 0.28, $P = 0.029$). The predictive significance of baseline IFN-inducible protein score was replicated in the independent cohort ($r_s = 0.43$, $P = 0.028$).

Conclusion. A higher IFN-inducible protein score in SSc-ILD is predictive of better response to immunosuppression and could potentially be used to identify patients who may derive the most benefit from MMF or CYC.

ClinicalTrials.gov identifier: NCT00883129.

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INTRODUCTION

Interstitial lung disease (ILD) is the leading cause of disease-related mortality in systemic sclerosis (SSc) (1,2). Scleroderma Lung Study I (SLS I) (3) and SLS II (4) showed that both cyclophosphamide (CYC) and mycophenolate mofetil (MMF) were effective in the treatment of SSc-related ILD (SSc-ILD) as measured by serially obtained forced vital capacity percent predicted (FVC%) values. Moreover, a follow-up study indicated that short-term improvement in FVC% was associated with improved long-term survival (5). However, response to immunosuppression was highly variable between patients in both clinical trials. In addition, CYC and MMF can be associated with serious side effects (3,4,6). Ideally, their use should be reserved for the subset of patients who are likely to respond to these medications. However, there are no widely accepted clinical or biologic parameters to predict response to immunosuppression in SSc-ILD. Moreover, the extent of lung fibrosis on high-resolution computed tomography (HRCT) of the chest did not predict change in FVC% from baseline in patients treated with CYC in SLS I (3). Thus, there is a substantial unmet clinical need for novel predictive biomarkers in SSc-ILD.

The interferon (IFN) signature is the most prominent and robustly replicated gene expression signature in peripheral blood cells from SSc patients. This signature was first described in whole blood samples (7,8) but has since been replicated in peripheral blood mononuclear cells (9), as well as in lymphocytes and monocytes (10). Those studies indicated that approximately half of SSc patients have a "lupus-like" IFN gene expression signature in their peripheral blood cells (7). However, serum samples are more accessible during routine clinical care and a more practical source for biomarker development than peripheral blood cell RNA samples. Recent studies have shown that certain serum proteins correlate with the IFN gene expression signature in SSc (11,12), enabling the utilization of these serum proteins as surrogate markers for IFN activation status. The predictive significance of the IFN transcript or serum protein signature for response to immunosuppression has not been investigated in SSc.

Capitalizing on the valuable, prospectively collected serum samples in the SLS II study (4), we determined whether a composite serum IFN-inducible protein score has predictive significance for response to immunosuppression in SSc-ILD. We hypothesized that SSc patients with higher serum IFN-inducible protein levels would be more responsive to immunosuppressive therapy with either MMF or CYC.

PATIENTS AND METHODS

Study participants. All SLS II patients with an available baseline serum sample were included in the present study. The eligibility criteria for SLS II have been published previously (4). Briefly, key inclusion criteria were as follows: adults ages 18–75 years with well-defined SSc with limited or diffuse cutaneous involvement (13); active ILD as demonstrated by restrictive-to–borderline restrictive ventilatory impairment (FVC% <80–85 but ≥45) AND the presence of any ground-glass opacity on HRCT; exertional dyspnea (grade 2 or worse on the Magnitude of Task component of the Mahler Baseline Dyspnea Index [14]); and disease duration of <7 years (based on the first non–Raynaud's phenomenon symptom due to SSc). Key exclusion criteria included clinically significant pulmonary hypertension, clinically significant abnormalities on HRCT not attributable to SSc, smoking within the past 6 months, evidence of significant airflow obstruction, prior use of oral CYC or MMF for longer than 8 weeks, or use of CYC and/or MMF in the 30 days prior to randomization. The SLS II protocol was approved by the institutional review board of participating sites, and written informed consent was obtained from all study participants.

SLS II study design. Patients were randomized to receive either MMF for 2 years or oral CYC for 1 year followed by placebo for 1 year. Based on this design, patients in both treatment arms were receiving active treatment during the first 12 months, while the participants in the MMF arm were continued on MMF therapy and those in the CYC arm were placed on placebo during the second year. The FVC% was the primary outcome and was measured every 3 months during the 24-month study period. Serum protein levels were also measured in sera collected from 39 healthy controls at the University of Texas Health Science Center at Houston (UTHSC-H) (see Supplementary Methods, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract). SSc-related autoantibodies were determined at the UTHSC-H divisional laboratories, and the extent of disease based on involvement >20% was measured on HRCT (15,16) (see Supplementary Methods for more details).

Serum protein assays and calculation of the IFN-inducible protein score. Serum samples were collected at the baseline, 12-month, and 24-month visits and were immediately processed on-site on the day of collection according to a standardized protocol, and were subsequently aliquoted, stored in –80°C freezers, and shipped on dry ice in batches to the central biorepository at the UTHSC-H. All 133 participants (63 in the MMF arm and 70 in the CYC arm) with an available serum sample were included in the present study. Serum samples from healthy controls were processed and stored in the same manner as those from SLS II, except that no shipment was required. Only unthawed serum aliquots from SLS II participants and healthy controls were used.

The primary focus of the present study was the measurement of 6 IFN-inducible proteins: monokine induced by IFNγ (MIG), IFNγ-inducible 10-kd protein (IP-10), monocyte chemotactic protein 2 (MCP-2), β_2-microglobulin (β_2m), tumor necrosis factor receptor type II (TNFRII), and macrophage inflammatory protein 3β (MIP-3β). The corresponding gene names of these 6 proteins are

CXCL9, CXCL10, CCL8, B2M, TNFRSF1B, and CCL19, respectively. This protein list was selected following a 2-step process. In step 1, 14 serum cytokines were identified that correlated significantly (r > 0.3 and false discovery rate–adjusted $P < 0.05$) with the IFN gene expression signature in the baseline samples collected in the Scleroderma: Cyclophosphamide or Transplantation (SCOT) study (see Supplementary Material in ref. 12). In step 2, 6 of these proteins were also confirmed as inducible by type I IFN in human peripheral blood cells based on in vitro studies, according to the information obtained from the Interferome V2.0 database (17).

Serum protein assays were performed at the Clinical Laboratory Improvement Amendments (CLIA)–certified laboratory of Myriad Rules-Based Medicine using multianalyte profiling (MAP) multiplexed immune assay. Although the primary focus of the present study was IFN-inducible proteins, these serum proteins could not be measured in isolation with predesigned multiplex panels. Therefore, 57 other serum proteins belonging to predesigned Myriad MAPs were also measured as part of the multiplex assay. For the analysis, proteins with levels below the lower limit of quantification in >50% of the baseline SLS II participants were excluded. For the remainder of the proteins, levels below the lower limit of quantification were replaced by the lower limit of quantification, while levels above the upper limit of quantitation were replaced by the upper limit of quantitation. The 6 IFN-inducible proteins listed above were within the dynamic range of their respective assays for all samples and no adjustments were necessary. Thirty-four of the other 57 proteins, including high-sensitivity C-reactive protein (CRP), were detectable in >50% of baseline SLS II samples and were further analyzed. In addition, Simoa assays (Quanterix) (18) were used for ultrasensitive detection of 2 low-abundant cytokines, B lymphocyte chemoattractant (CXCL13) and interleukin-6 (IL-6), which have previously been implicated as biomarkers in SSc-ILD (19,20).

A composite score of MIG, IP-10, MCP-2, β_2m, TNFRII, and MIP-3β was calculated using a previously described method (7,11,21–23). Specifically, the protein levels were divided by the top 95th percentile for each protein. Next, all values in the top 5% category were assigned a value of 1.0. Finally, the normalized values for the 6 proteins were summed to obtain the IFN-inducible protein score.

Confirmation cohort. For independent confirmation of the study results, patients with SSc enrolled in the Prospective Registry for Early Systemic Sclerosis (PRESS) cohort were evaluated. Briefly, PRESS is a multicenter, observational cohort of patients with early diffuse cutaneous SSc (disease duration <3 years from onset of the first non–Raynaud's phenomenon symptom of SSc) (24). All enrolled patients who fulfilled the following criteria were included in the present study: available serum sample at the baseline visit, no missing FVC% data at the baseline and 12-month visits, evidence consistent with SSc-ILD on HRCT, and treatment with immunosuppressive agents during the first year of the follow-up period. The serum samples in PRESS were processed and stored following the same procedures as in SLS II. Moreover, levels of IFN-inducible proteins and CRP were measured using the same assays in the Myriad Rules-Based Medicine laboratory.

Statistical analysis. Depending on the distribution, raw or $\log_2$-transformed cytokine data were analyzed. Similar to the primary clinical outcome analysis in SLS II (4), a joint model (25) combining a mixed-effects model for the longitudinally obtained FVC% values with a survival model to handle non-ignorable missing data due to study dropouts, treatment failure, or death was used. In the primary analysis, the outcome was the course of FVC% measured at 3-month increments from month 3 to month 12, which corresponds to the time period in which patients in both treatment arms were receiving active treatment. The longitudinal model in the primary analysis included the following covariates: baseline protein level, baseline FVC%, and a linear time trend. In addition, an extended multivariable analysis was performed that contained baseline protein levels (i.e., IFN-inducible protein score and CRP), in addition to baseline demographic and clinical variables that showed predictive significance in separate analyses ($P < 0.05$), baseline FVC%, and a linear time trend.

In a secondary analysis, we also investigated whether the serum protein levels at the 12-month visit had predictive significance for the course of FVC% over the 15-month to 24-month visits. The longitudinal model in this analysis included the following covariates: protein levels at the 12-month visit, FVC% at the 12-month visit, and linear splines with a knot at 21 months to characterize the time trend. The *P* value for the analysis of individual serum protein levels was adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (26).

In the confirmation cohort (PRESS), the majority of patients evaluated had only 2 FVC% measurements available during the first 12 months after enrollment (baseline and 12-month visit), thus a different, simplified approach for the analysis of data from these 2 time points was used. As previously described (27), the predictive significance of IFN-inducible protein score for percent change in FVC% ($[\text{FVC\%}_{\text{12-month visit}} - \text{FVC\%}_{\text{baseline}}]/\text{FVC\%}_{\text{baseline}}$) was analyzed by Spearman's correlation.

All tests were 2-sided. The joint analyses were performed using the R package JMbayes, and all other analyses were conducted in SAS version 9.4 (SAS Institute).

RESULTS

Baseline characteristics of the participants. Of the 142 patients enrolled, serum samples were available for 133 patients at baseline, 99 patients at the 12-month visit, and 84 patients at the 24-month visit. The healthy controls were similar to SLS II participants with regard to age, sex, and ethnic background (see Supplementary Table 1, available on the *Arthritis & Rheumatology* website

at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract for patient and control characteristics).

IFN-inducible protein score in patients and controls. The SLS II participants had a significantly higher IFN-inducible protein score at the baseline visit than healthy controls (fold difference 2.19; $P < 0.001$). As shown in Supplementary Figure 1 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract), the IFN-inducible protein score decreased significantly from the baseline visit to the 12-month visit (fold change 0.75; $P < 0.001$ for the MMF arm and fold change 0.76; $P < 0.001$ for CYC arm). In the subgroup of patients with serum samples available at both the 12-month and 24-month visits (n = 43 in the MMF arm and n = 41 in the CYC arm), the IFN-inducible protein score did not change significantly from the 12-month visit to the 24-month visit ($P = 0.994$ for MMF and $P = 0.529$ for CYC) (Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract). As shown in Supplementary Table 2 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract), the baseline demographic and clinical variables did not show a significant association/correlation with the concurrent IFN-inducible protein score.

Predictive significance of individual serum protein levels for ILD course. As described above, serum levels of 6 IFN-inducible proteins, as well as 36 serum proteins involved in other immune pathways, were measured in the baseline SLS II samples as part of the multiplex assay. We subsequently investigated whether any individual baseline protein levels had predictive significance for the course of FVC% from month 3 to month 12 of the follow-up period. As shown in Supplementary Table 3 (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract), only 2 serum proteins, MIG and IP-10 (both IFN-inducible proteins) showed predictive significance for FVC% in both treatment arms in the same direction after correction for multiple comparisons. Specifically, higher baseline MIG and IP-10 levels predicted higher serial FVC% levels. The point estimates for the other 4 IFN-inducible proteins were also toward higher serial FVC% levels, although their associations did not reach statistical significance. Of note, 2 other proteins (intercellular adhesion molecule 1 and eotaxin 1) also reached statistical significance in both treatment arms after correction for multiple comparisons, but the direction of prediction was not consistent between the 2 SLS II treatment arms for these 2 proteins.

Predictive significance of IFN-inducible protein score for ILD course. Next, the predictive significance of the IFN-inducible protein score was investigated. As shown in Table 1, a higher baseline IFN-inducible protein score predicted better ILD course based on higher serial FVC% values from month 3 to month 12 in both treatment arms after adjustment for baseline FVC% (point estimate 0.41, $P = 0.001$ for MMF and point estimate 0.91, $P = 0.009$ for CYC).

In the secondary analysis pertaining to the second year of SLS II, during which patients in the MMF arm continued to receive MMF and those in the CYC arm were switched to placebo (Table 1), higher IFN-inducible protein scores at 12 months continued to predict better response to immunosuppression in the MMF arm (point estimate 0.28, $P = 0.029$), while higher IFN-inducible protein scores at 12 months showed a trend toward predicting

Table 1. Predictive significance of IFN-inducible protein score for subsequent serial FVC% values in patients with SSc-ILD treated with MMF or CYC*

	Point estimate (95% CI)	*P*
MMF arm		
Predictive significance of baseline IFN-inducible protein score for serial FVC% values from month 3 to month 12		
Baseline IFN-inducible protein score	0.41 (0.23, 0.59)	0.001
Baseline FVC%	0.84 (0.82, 0.86)	<0.001
Predictive significance of 12-month IFN-inducible protein score for serial FVC% values from month 15 to month 24		
12-month IFN-inducible protein score	0.28 (0.11, 0.69)	0.029
12-month FVC%	0.96 (0.9, 0.98)	<0.001
CYC arm		
Predictive significance of baseline IFN-inducible protein score for serial FVC% values from month 3 to month 12		
Baseline IFN-inducible protein score	0.91 (0.56, 1.13)	0.009
Baseline FVC%	0.87 (0.84, 0.9)	<0.001
Predictive significance of 12-month IFN-inducible protein score for serial FVC% values from month 15 to month 24		
12-month IFN-inducible protein score	−0.61 (−1.5, 0.11)	0.068
12-month FVC%	1 (0.96, 1.08)	<0.001

* All models included time as an independent variable. IFN = interferon; FVC% = forced vital capacity percent predicted; SSc-ILD = systemic sclerosis–related interstitial lung disease; MMF = mycophenolate mofetil; CYC = cyclophosphamide; 95% CI = 95% confidence interval.

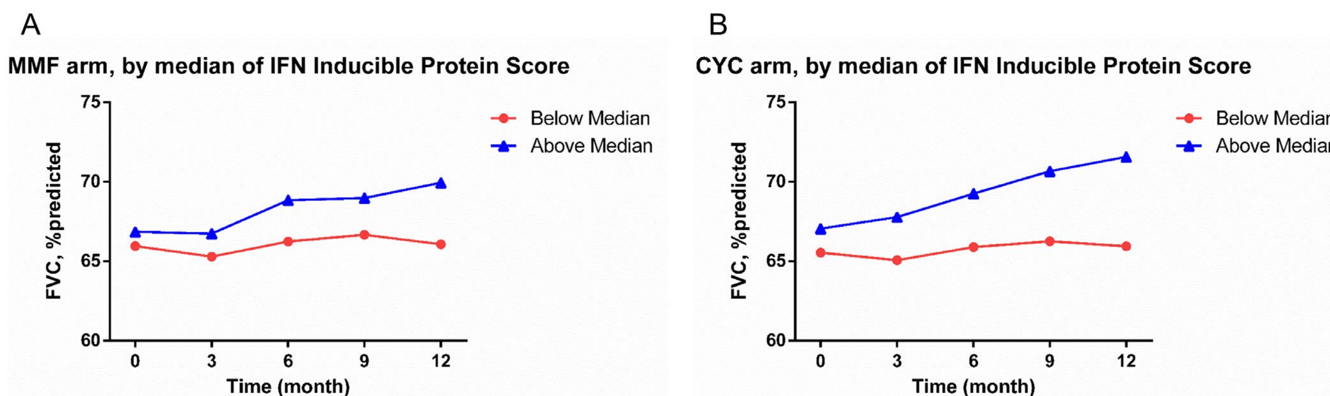

Figure 1. Course of forced vital capacity percent predicted (FVC%) from randomization to 12 months, according to interferon (IFN)–inducible protein score, in patients with systemic sclerosis (SSc)–related interstitial lung disease (ILD) treated with mycophenolate mofetil (MMF) (**A**) or cyclophosphamide (CYC) (**B**). IFN-inducible protein score was dichotomized using the median value. Patients with a positive IFN-inducible protein score (higher than the median value) had higher FVC% levels in the both the MMF arm ($P = 0.003$) and the CYC arm ($P = 0.004$). The analysis was adjusted for baseline FVC% and, similar to Tables 1–3, baseline FVC% and time were added as independent variables to the joint model.

lower serial FVC% values from month 15 to month 24 during the placebo treatment period in the CYC arm (point estimate = –0.61; $P = 0.068$).

Our previous gene expression studies have shown that approximately half of patients with SSc have an IFN signature (7,12). Building on this finding, the IFN-inducible protein score was dichotomized based on the median value in the baseline patient samples. As shown in Figure 1, patients with a positive baseline IFN-inducible protein score had a more favorable ILD course from month 3 to month 12 in both treatment arms compared with patients with a negative IFN-inducible protein score (point estimate 1.28, $P = 0.003$ for MMF and point estimate 2.6, $P = 0.004$ for CYC).

We also examined whether the baseline IFN-inducible protein score had predictive significance for the course of diffusing capacity for carbon monoxide percent predicted (DL_{CO}%) from month 3 to month 12 after randomization. Consistent with the FVC% findings, higher IFN-inducible protein score predicted higher serial DL_{CO}% in the CYC arm (point estimate 0.7 [95% confidence interval 0.47, 0.96]; $P < 0.001$). However, IFN-inducible protein score did not significantly predict DL_{CO}% course in the MMF arm (point estimate –0.15 [95% confidence interval –0.42, 0.15]; $P = 0.146$).

Table 2. Predictive significance of CRP for subsequent serial FVC% values in patients with SSc-ILD treated with MMF or CYC*

	Point estimate (95% CI)	*P*
MMF arm		
Predictive significance of baseline CRP for serial FVC% values from month 3 to month 12		
Baseline CRP†	−0.15 (−0.31, −0.01)	0.038
Baseline FVC%	0.83 (0.78, 0.86)	<0.001
Predictive significance of 12-month CRP for serial FVC% values from month 15 to month 24		
12-month CRP†	−0.61 (−0.7, −0.51)	<0.001
12-month FVC%	0.98 (0.96, 0.99)	<0.001
CYC arm		
Predictive significance of baseline CRP for serial FVC% values from month 3 to month 12		
Baseline CRP†	−0.56 (−0.72, −0.45)	<0.001
Baseline FVC%	0.90 (0.86, 0.92)	<0.001
Predictive significance of 12-month CRP for serial FVC% values from month 15 to month 24		
12-month CRP†	−0.3 (−0.93, −0.08)	0.027
12-month FVC%	1.01 (0.97, 1.12)	<0.001

* All models included time as an independent variable. CRP = C-reactive protein (see Table 1 for other definitions).
† Log_2 transformed.

Table 3. Separate analyses to examine the predictive significance of baseline demographic and clinical variables for serial FVC% values from month 3 to month 12 in patients with SSc-ILD treated with MMF or CYC*

	MMF arm		CYC arm	
Baseline variable	Point estimate (95% CI)	*P*	Point estimate (95% CI)	*P*
Age in years	−0.05 (−0.18, 0.08)	0.462	0.04 (−0.06, 0.15)	0.411
Female sex	0.04 (−0.53, 0.69)	0.891	1.17 (−0.09, 2.26)	0.058
African American race	−0.68 (−1.26, −0.13)	0.032†	−2.4 (−3.04, −1.9)	<0.001†
Diffuse disease type	1.15 (0.43, 2.06)	0.005†	−1.97 (−3.34, −0.77)	0.008†
Disease duration	0.04 (−0.06, 0.15)	0.314	0.12 (0.01, 0.25)	0.042†
MRSS	0.07 (0.04, 0.11)	0.002†	−0.04 (−0.14, 0.06)	0.392
Antitopoisomerase	−0.14 (−1.12, 0.81)	0.729	−0.35 (−2.23, 1.62)	0.654
Anti–RNA polymerase	0.83 (−0.61, 2.06)	0.175	1.08 (−2.08, 4.17)	0.425
Extensive disease on HRCT‡	−2.45 (−2.85, −2.11)	<0.001†	0.09 (−2.18, 2.36)	0.79

* Each row represents a separate model that included one baseline clinical variable, baseline FVC%, and time as independent variables. MRSS = modified Rodnan skin thickness score (see Table 1 for other definitions).
† Baseline demographic and clinical variables showing predictive significance in separate models that were included in the subsequent extended multivariable model (see Tables 4 and 5).
‡ Quantitative ILD >20% on high-resolution computed tomography (HRCT) of the chest.

Predictive significance of CRP level for ILD course. Contrary to the favorable (i.e., positive) predictive value of the IFN-inducible protein score, higher CRP levels predicted a worse ILD course reflected in lower serial FVC% values from month 3 to month 12 in both treatment arms after adjustment for baseline FVC% (Table 2). In the secondary analysis, higher CRP levels at 12 months again predicted a worse ILD course reflected by lower serial FVC% values from month 15 to month 24 in both treatment arms (Table 2).

IFN-inducible protein score and CRP level are independent predictors of ILD course. As shown in Table 3, the predictive significance of baseline demographic and clinical variables for serial FVC% values from month 3 to month 12 were first examined in separate models after adjustment for baseline FVC% for each treatment arm. Next, the predictive significance of the IFN-inducible protein score and CRP level (both as continuous variables) was investigated in an extended multivariable model after adjustment for baseline FVC%, in addition to variables showing predictive significance in the separate analyses described above, in the MMF arm (Table 4) and in the CYC arm (Table 5). Similar to the findings described above, higher baseline IFN-inducible protein scores predicted better ILD course, and higher baseline CRP levels predicted worse ILD course, from month 3 to month 12 after adjustment for baseline demographic and clinical variables in both treatment arms.

Table 4. Predictive significance of baseline IFN-inducible protein score and CRP level, after adjustment for baseline demographic and clinical variables, for serial FVC% values from month 3 to month 12 in patients with SSc-ILD treated with MMF*

Baseline variable	Point estimate (95% CI)	*P*
IFN-inducible protein score	0.32 (0.11, 0.52)	0.013
CRP†	−0.13 (−0.24, −0.01)	0.041
African American race	0.95 (0.43, 1.41)	0.004
Diffuse disease type	0.39 (−0.19, 1.05)	0.139
MRSS	0.05 (0.03, 0.09)	0.008
Baseline FVC%	0.81 (0.78, 0.83)	<0.001
Extensive disease on HRCT‡	−2.27 (−2.70, −1.80)	<0.001

* Time was included as an independent variable. CRP = C-reactive protein; MRSS = modified Rodnan skin thickness score (see Table 1 for other definitions).
† Log_2 transformed.
‡ Quantitative ILD >20% on high-resolution computed tomography (HRCT) of the chest.

Confirmation of the predictive significance of the IFN-inducible protein score in an independent cohort. The predictive significance of the IFN-inducible protein score and CRP level was investigated in the independent, observational PRESS cohort. In this cohort, 47 patients had a baseline serum sample and had FVC% measurements at the baseline and 12-month visits; of these, 31 (66%) had evidence of SSc-ILD on HRCT. Of these 31 patients, 26 were treated with immunosuppressive agents (23 with MMF and 3 with methotrexate) during the first year of the follow-up period and were included in the present study.

Table 5. Predictive significance of baseline IFN-inducible protein score and CRP level, after adjustment for baseline demographic and clinical variables, for serial FVC% values from month 3 to month 12 in patients with SSc-ILD treated with CYC*

Baseline variable	Point estimate (95% CI)	*P*
IFN-inducible protein score	0.92 (0.79, 1.04)	<0.001
CRP†	−0.46 (−0.53, −0.39)	<0.001
African American race	−2.01 (−2.31, −1.71)	<0.001
Diffuse disease type	−0.60 (−0.91, −0.33)	0.005
Disease duration	0.19 (0.12, 0.26)	0.002
Baseline FVC%	0.90 (0.89, 0.91)	<0.001

* Time was included as an independent variable. CRP = C-reactive protein (see Table 1 for other definitions).
† Log_2 transformed.

Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41627/abstract, shows their demographic and clinical characteristics. Confirming our findings in SLS II, higher baseline IFN-inducible protein score predicted increasing FVC% values; specifically, the baseline IFN-inducible protein levels correlated positively with percent change in FVC% at 12 months (Spearman's correlation coefficient [r_s] = 0.43, $P = 0.028$). This correlation remained significant even after exclusion of the 3 patients treated with methotrexate ($r_s = 0.47$, $P = 0.023$) (n = 23). Of note, baseline CRP level was not predictive of percent change in FVC% at 12 months in the PRESS cohort ($P = 0.828$).

DISCUSSION

In the well-characterized SLS II clinical trial cohort, a higher IFN-inducible protein score predicted better response to MMF, as well as CYC, while higher baseline CRP levels predicted a worse ILD course. Moreover, the predictive significance of the IFN-inducible protein score was independent of CRP level and clinical/demographic predictors. In the validation analysis, the predictive significance of the IFN-inducible protein score was confirmed in the PRESS cohort of patients with early diffuse cutaneous SSc.

In this study, a rigorous method was employed for calculation of the serum IFN-inducible protein score. Specifically, serum proteins included in the IFN-inducible protein score correlated with the peripheral blood cell IFN transcript signature in our previous study of untreated SSc patients using the same protein assays (12) and were induced by type I IFN in in vitro studies of human peripheral blood cells. Moreover, the method used for calculation of the composite score weighted each protein equally (21–23), ensuring that the overall IFN-inducible protein score is not skewed by a few outlier values of 1 or 2 proteins. Thus, the IFN-inducible protein score used in this study provides an accurate reflection of the type I IFN activation status in circulation in SSc-ILD. Of note, there is substantial overlap between type I and type II IFN-inducible genes/proteins. Based on the information in the Interferome database, the 6 serum proteins utilized can be induced by both type I and type II IFN. Therefore, we cannot exclude the possibility that the IFN composite score evaluated in this study is in part driven by type II IFN. However, in a pilot study of anifrolumab (a blocking antibody against IFNAR1) in 26 SSc patients, 2 of the proteins included in the composite score (β_2m and IP-10 [CXCL10]), decreased significantly after blocking the type I IFN receptor (28), providing direct human evidence that the IFN-inducible protein score is at least in part driven by type I IFN in patients with SSc.

In the present study, SSc-ILD patients with a higher IFN-inducible protein score were more responsive to immunosuppression with CYC or MMF. However, the results from the second year of the CYC arm (placebo phase) indicated that patients with an IFN excess profile at the 12-month visit had a worse ILD course without concurrent immunosuppressive treatment, while higher IFN-inducible protein score at the same visit continued to be predictive of better ILD course in patients assigned to the MMF arm, who continued to receive active immunosuppressive treatment during the second year of the study. This finding supports the notion that a high IFN score adversely affects SSc-ILD progression unless immunosuppressive treatment is administered. Thus, the IFN-inducible protein score in SSc acts as a predictive biomarker identifying likely responders to treatment rather than a prognostic biomarker that predicts the natural history of disease regardless of treatment status.

The deleterious effect of IFN excess in SSc is supported by previous murine model and human studies (for review, see ref. 29). In a previous study on the role of IFN regulatory factor 5 (IRF-5), bleomycin-induced dermal and lung fibrosis was attenuated in IRF-5–deficient mice. Moreover, there was in vitro evidence that profibrotic transcriptional activity of IRF-5 in fibroblasts was enhanced by transforming growth factor β (TGFβ) (30). In a more recent study on the role of IRF-7 in SSc pathogenesis, bleomycin-induced dermal fibrosis, as well as hypodermal fibrosis in tight skin mice, was attenuated in IRF-7–deficient mice. Moreover, IRF-7 blockade attenuated fibrotic response to TGFβ in SSc dermal fibroblasts (31). In terms of direct human data, a previous randomized controlled trial in which SSc patients were treated with recombinant IFNα or placebo had to be stopped prematurely because IFNα-treated patients demonstrated a significantly worse ILD course as measured by FVC% (32). More recently, in a phase I trial of the anti–type I IFN receptor antibody anifrolumab for the treatment of SSc, skin gene expression studies showed evidence of suppressed TGFβ signaling in the anifrolumab-treated group (28). Taken together, these data indicate that IFN excess is deleterious in SSc but also identifies patients who are more likely to benefit from immunosuppressive treatment.

In the present study, higher CRP level predicted worse ILD course in SSc patients receiving active immunosuppressive treatment as well as during the placebo phase in the CYC arm in the second year of the study, indicating that CRP, as a general marker of inflammation, is a prognostic biomarker that predicts worse FVC course regardless of treatment status. This finding is also supported by previous observational studies showing that higher baseline CRP levels are predictive of reduced survival (33) and faster FVC% decline in SSc (34). More recently, in a retrospective study of 24 SSc-ILD patients treated with 6 monthly infusions of CYC, a higher CRP level was significantly associated with poor response (35). Of note, higher CRP levels in the confirmation cohort did not predict the course of ILD in the present study. This might be due to the small sample size and/or the more heterogeneous patient population in the PRESS cohort, where a general marker of inflammation like CRP can be influenced by extrapulmonary factors. Moreover, patients in the PRESS cohort had different baseline characteristics than SLS II participants. Specifically, all PRESS patients had diffuse cutaneous involvement and had a disease duration of <3 years. Moreover, 30% of the

PRESS patients evaluated had baseline FVC% >85% and therefore would have not met one of the inclusion criteria for SLS II.

In addition to IFN-inducible proteins, levels of 36 immune pathway–related serum proteins, including IL-6, were measured in the present study. In a previous observational study, higher IL-6 levels were predictive of worse ILD course (19). In the present study, IL-6 levels were not predictive of FVC% course in either the MMF or the CYC arm (Supplementary Table 3). Similarly, anti–topoisomerase I was not predictive of ILD course (Table 3). This finding is not consistent with our previous finding in an observational cohort of early SSc patients with or without ILD, in which anti–topoisomerase I was predictive of a faster decline in FVC% (36), supporting the notion that antitopoisomerase loses its predictive significance in a study population that includes only patients with clinically significant ILD.

This study has several strengths. To our knowledge, this is the first study examining the predictive role of serum IFN-inducible proteins in a randomized controlled clinical trial of SSc-ILD. All serum protein assays were performed in the same CLIA-certified laboratory using rigorously standardized procedures. In SLS II, repeated FVC% measurements were available, allowing for a more accurate reflection of ILD progression. Patients were treated according to standardized, uniform treatment protocols, decreasing the potential confounding effect of treatment heterogeneity. Moreover, the predictive significance of the IFN-inducible protein score was shown in both the MMF and CYC arms separately, and confirmed in an independent observational study. Finally, SLS II was conducted in 14 centers across the US and included patients from a diverse ethnic background, increasing the generalizability of our findings.

There were several limitations to the present study. The sample size in the confirmation cohort was relatively small. Furthermore, SLS II did not include a placebo arm during the first year of the study period, although this limitation is partially mitigated by the fact that the IFN-inducible protein score showed prediction in opposite directions during the second year when patients in the CYC arm were switched to placebo while patients in the MMF arm continued receiving active immunosuppressive treatment. Furthermore, SLS II only included patients with a disease duration of <7 years; therefore, we could not investigate the predictive significance of the IFN-inducible protein score in patients with longstanding disease. Moreover, our findings should be further investigated in the recently completed study of nintedanib treatment for SSc-ILD (37) and future large clinical trials of antifibrotic agents in SSc-ILD, with the ultimate goal of developing prediction models for identifying patients who would primarily benefit from immunosuppressive versus antifibrotic treatment.

In conclusion, SSc-ILD patients with a higher serum IFN-inducible protein score are more likely to respond to MMF or CYC. The predictive significance of IFN-inducible protein score is independent of the general marker of inflammation CRP, which predicted worse ILD course regardless of the treatment regimen in SLS II. These serum proteins may be useful for more informed clinical decisions and clinical trial design and may ultimately lead to more personalized treatment regimens in SSc-ILD.

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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. Assassi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data. Assassi, Volkmann, Mayes, Roth, Hinchcliff, Khanna, Frech, Clements, Furst, Goldin, Bernstein, Castelino, Domsic, Gordon, Hant, Shah, Shanmugam, Steen, Tashkin.

Analysis and interpretation of data. Assassi, Li, Volkmann, Rünger, Ying, Elashoff, Tashkin.

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