ARTHRITIS & RHEUMATOLOGY
Vol. 70, No. 6, June 2018, pp 912–919
DOI 10.1002/art.40455

Skin Gene Expression Is Prognostic for the Trajectory of Skin Disease in Patients With Diffuse Cutaneous Systemic Sclerosis

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Objective. **At present, there are no clinical or laboratory measures that accurately forecast the progression of skin fibrosis and organ involvement in patients with systemic sclerosis (SSc). The goal of this study was to identify skin biomarkers that could be prognostic for the progression of skin fibrosis in patients with early diffuse cutaneous SSc (dcSSc).**

Methods. **We analyzed clinical data and gene expression in skin biopsy samples from 38 placebo-treated patients, part of the Roche Safety and Efficacy of Subcutaneous Tocilizumab in Adults with Systemic Sclerosis (FASSCINATE) phase II study of tocilizumab in SSc. RNA samples were analyzed using nCounter. A trajectory model based on a modified Rodnan skin thickness score was used to describe 3 skin disease trajectories over time. We examined the association of skin gene expression with skin score trajectory groups, by chi-square test. Logistic regression was used to examine the prognostic power of each gene identified.**

Results. **We found that placebo-treated patients with high expression of messenger RNA for CD14, SERPINE1, IL13RA1, CTGF, and OSMR at baseline were more likely to have progressive skin score trajectories. We also found that those genes were prognostic for the risk of skin progression and that IL13RA1, OSMR, and SERPINE1 performed the best.**

Conclusion. **Skin gene expression of biomarkers associated with macrophages (CD14, IL13RA1) and transforming growth factor β activation (SERPINE1, CTGF, OSMR) are prognostic for progressive skin disease in patients with dcSSc. These biomarkers may provide guidance in decision-making about which patients should be considered for aggressive therapies and/or for clinical trials.**

Currently there are no clinical or laboratory measures that accurately predict the progression of skin fibrosis and organ involvement in patients with systemic sclerosis (SSc). Several studies, including retrospective cohort analyses and randomized clinical trials, have shown that the severity of skin fibrosis, as assessed by the modified Rodnan skin thickness score (MRSS) (1), is predictive of disease mortality (2,3). In particular, Shand et al defined 3 distinct skin score trajectory subgroups, using latent variable modeling, and showed that patients with the worst skin score trajectories had significantly increased mortality (4).

Several clinical and serologic measures have been associated with progressive skin disease. It is generally accepted that the fastest rates of skin disease progression are recorded early in the disease (5). A recent observational study from the European League Against Rheumatism (EUSTAR) has shown that joint synovitis, short disease duration (<15 months), and low MRSS at baseline predict more progressive skin fibrosis (6). Anti–RNA polymerase III is associated with scleroderma renal crisis and more severe skin disease, though less associated with

ClinicalTrials.gov identifier: NCT01532869.

Supported by Boston University Medical Center (NIH Clinical and Translational Science Institute grant UL1-TR-000157) and the NIH (Research Project Grant Program grant 2R01-AR-051089). Dr. Lafyatis' work was supported by grant 5P30-AR-061271 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases Scleroderma Core Centers, and grants 1P50-AR-060780 and 2R01-AR-051089 from the Scleroderma Center of Research Translation.

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Drs. Sornasse, Chen-Harris, and Siegel own stock or stock options in Hoffmann-La Roche. Dr. Khanna has received consulting fees from Actelion, Bayer, Covis, Cytori, EMD Serono, Genentech/Roche, Gilead, GlaxoSmithKline, and Sanofi-Aventis (less than $10,000 each) and research support from Bayer, Bristol-Myers Squibb, Genentech/Roche, and Sanofi-Aventis. Dr. Jahreis owns stock or stock options in Hoffmann-La Roche and holds a patent for tocilizumab.

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Submitted for publication January 24, 2017; accepted in revised form February 8, 2018.

interstitial lung disease (5,7). Despite these findings, there remains no broadly accepted methodology for assessing the likelihood of progressive skin disease and no validated prognostic biomarkers of skin disease evolution, limiting patient risk–stratification and consequently the ability to select patients with progressive disease to receive innovative therapies.

We recently reported that CD14 expression correlates strongly with progressive skin disease (8). In this report, using data collected from the Safety and Efficacy of Subcutaneous Tocilizumab in Adults with Systemic Sclerosis (FASSCINATE) study, an international trial of tocilizumab (TCZ) in SSc (9), we describe trajectory patterns of skin score change over time, as determined using a group-based modeling approach in placebo-treated patients. We assessed several potential prognostic biomarkers associated with these changes. In addition, we examined the relationship of each biomarker to the change in skin score over time.

PATIENTS AND METHODS

Study design and participants. Samples and clinical data for the discovery cohort used in this study were from the FASSCINATE phase II study (10). Briefly, the FASSCINATE study was a randomized, double-blind, placebo-controlled, phase II study of TCZ (162 mg/week, administered subcutaneously) in early diffuse cutaneous SSc (dcSSc) patients age ≥18 years, with progressive disease of <5 years' duration since the first non–Raynaud's phenomenon sign or symptom. Investigators who enrolled at least one patient in the FASSCINATE study are listed in Appendix A. In the current analysis, we focused on the FASSCINATE patients treated with placebo. Of these patients (n = 44), we excluded 6 subjects. Two patients did not have a biopsy at baseline. One patient had only 2 MRSS values recorded (at baseline and at 8 weeks) and discontinued the study at week 16. The other 3 excluded patients had a decrease in MRSS of >12 units in 2 sequential assessments, suggesting that the measurement of the MRSS trajectory in these patients would be unreliable.

Skin biopsy gene expression analysis. RNA samples, used for assessment of gene expression, were analyzed using nCounter (NanoString Technologies). Expression of the genes was normalized to expression of 12 housekeeping genes. Of the 83 genes selected for confirmation and expression analysis, 62 transcripts were significantly overexpressed and 2 were significantly underexpressed in SSc patients compared with healthy controls (significance assessed by *t*-test with Bonferroni correction for multiple comparisons). Microarray data from the FASSCINATE trial, used for selecting prognostic genes, has been deposited at the GEO (accession no. GSE106358) at the NCBI.

Statistical analysis. We have described patterns of skin score change over time using a semiparametric mixture model (11). Specifically, the distinctive skin score trajectories were derived by modeling skin score as a function of time, i.e., the number of days in the study, using an SAS macro (more specifically, TRAJ, a procedure created by Jones et al [12] for estimating developmental trajectories). We assumed each skin score trajectory had a linear pattern of decline, and tested this assumption by including a quadratic term (i.e., testing for the possibility that change in skin score has a curved shape) and evaluated statistical significance of these terms for each trajectory group. Linear model terms were statistically significant ($P < 0.05$), but quadratic model terms were not; thus, we included only a linear term in our final models. The probability of each trajectory membership for a particular subject was estimated using the group-based model. Each subject was assigned to a specific trajectory group that had the highest estimated probability (i.e., posterior probability) compared with those of other trajectory groups. We used Bayesian information criterion (BIC) and entropy (i.e., amount of classification error indexed by average posterior probability) to assess the model fit. In general, models with lower BIC values provided a better fit to the data, and entropy statistics close to 1 (>0.8) convey a model with well-separated trajectories (11).

We divided expression of each gene into tertiles. The association of each gene with skin score trajectory groups was examined by chi-square test. Additionally, we examined the association between expression of each gene at baseline and change in skin score over time from baseline, using generalized estimating equations in SAS with the "exchange" option for the working correlation matrix. In the regression model, the lowest tertile of each gene expression measure was used as the referent group to test the difference in change in MRSS. Finally, we collapsed regressive and stable trajectories into one group and modeled the predictive ability of each gene by logistic regression. Using SAS, we then assessed the predictive ability of the model according to discrimination and calibration. Discrimination was evaluated using the area under the curve; the guidelines suggested that values of ≥0.70 are needed for adequate prediction. Calibration was determined using the Hosmer-Lemeshow test (13), with a significant result being indicative of poor calibration. Pearson's correlation coefficients were calculated using GraphPad Prism software. *P* values less than 0.05 were considered significant.

RESULTS

Study patients. All patients enrolled in the FASSCINATE study met the 1980 American College of

Table 1. Characteristics of placebo-treated patients from the FASSCINATE study*

Characteristic	Placebo-treated patients (n = 38)
Age	
Mean ± SD years	47.2 ± 13.0
Median (range)	49.5 (19–69)
Sex, no. (%)	
Female	30 (78.9)
Male	8 (21.1)
MRSS	
Mean ± SD	25.1 ± 5.2
Median (range)	25 (15–37)
Disease duration, mean ± SD months	19.8 ± 16.8

* Patients met the 1980 American College of Rheumatology criteria for systemic sclerosis. Disease duration was defined by the time since the first non–Raynaud's phenomenon symptom. FASSCINATE = Safety and Efficacy of Subcutaneous Tocilizumab in Adults with Systemic Sclerosis; MRSS = modified Rodnan skin thickness score.

Rheumatology criteria for SSc (14), had active disease of ≤5 years' duration since the first non–Raynaud's phenomenon symptom, and had an MRSS between 15 and 40. Additionally, at screening, active progressive disease of <1 year's duration was required for study inclusion, which was signified by any of the following: an increase in MRSS of ≥3 units, involvement of 1 new body area with an increase in MRSS of ≥2 units or involvement of >2 new body areas with an increase in MRSS of ≥1 unit, other documentation of worsening skin thickening in the previous 6 months, or ≥1 tendon friction rub accompanied by ≥1 laboratory criterion (C-reactive protein ≥10.0 mg/liter, erythrocyte sedimentation rate ≥28 mm/hour, or platelet count ≥330 × 1,000/μl) (9).

The discovery cohort, which was used to identify prognostic biomarkers, consisted of 38 patients from the placebo-treated patient group (Table 1). For validation, we studied microarray gene expression data from a second cohort of patients with dcSSc (20 patients total). The clinical features of the patients in this group were similar to those of the discovery cohort. All of the patients had early dcSSc (≤5 years from the first non–Raynaud's phenomenon symptom). However, patients in the validation group had some significant differences compared with those in the discovery group. First, patients in the validation group were treated with immunosuppressants, whereas the patients in the discovery group received only placebo during the study. Second, only 2 measurements of MRSS were obtained for patients in the validation group—1 at baseline and another at 24 weeks. Third, patients in the discovery cohort met certain additional criteria to define disease activity (15).

Gene expression and correlation with MRSS. Microarray data (from mid-forearm skin biopsies) that were generated as part of the clinical trial were analyzed for genes that were the most highly correlated with the

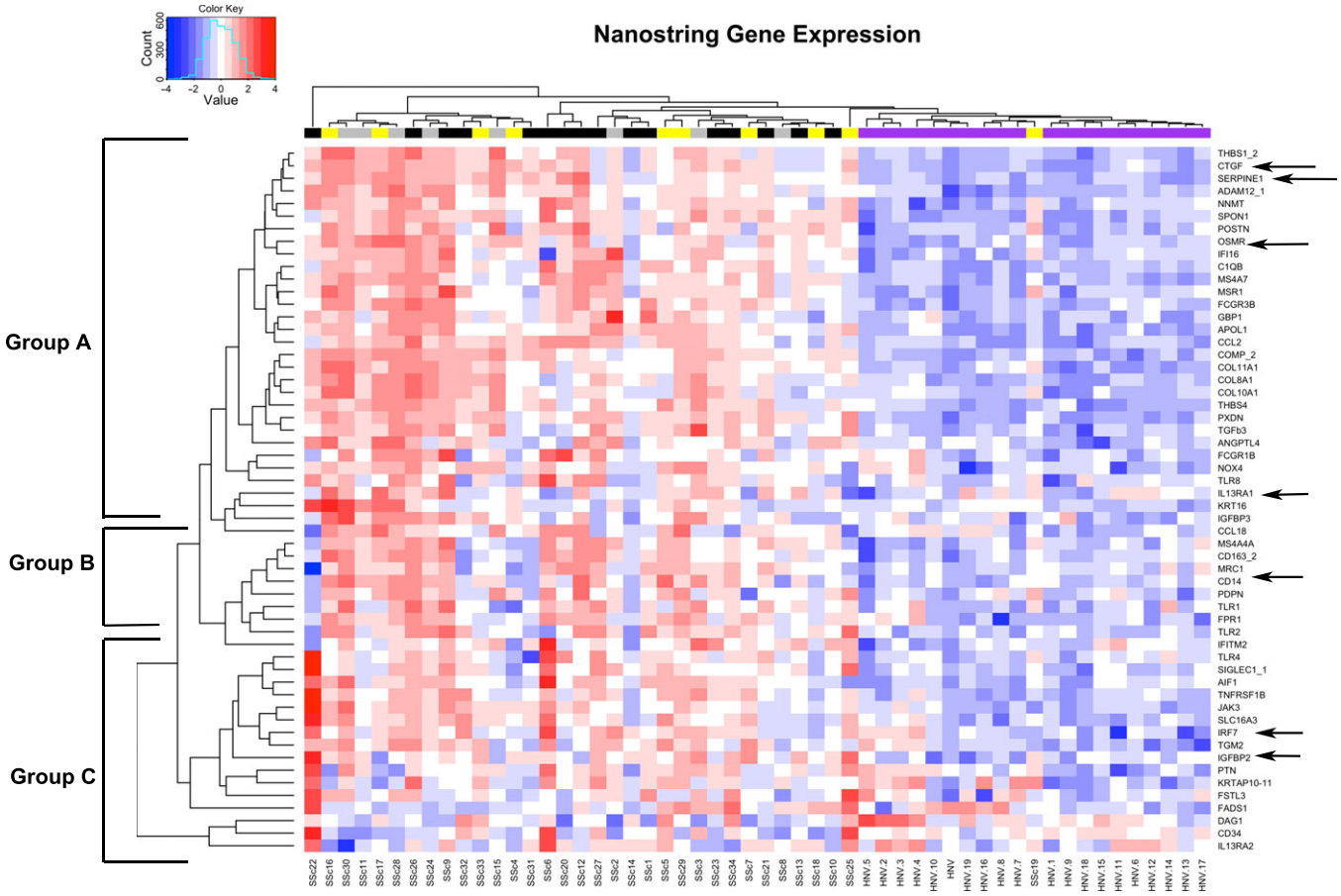

Figure 1. NanoString heatmap depicting gene expression in the skin of patients with diffuse cutaneous systemic sclerosis (dcSSc) compared to healthy controls, showing unsupervised hierarchical clustering in 20 healthy controls (violet bar) and 34 dcSSc patients (divided into regressive [yellow], stable [black], and progressive [gray] trajectories). Genes were grouped into 3 different clusters of transforming growth factor β/profibrotic genes (group A), macrophage-associated genes (group B), or genes without an evident biologic relationship (group C). Color code corresponds to Z scores of intensities. Red indicates higher expression and blue indicates lower expression. **Arrows** show genes selected for trajectory and tertiles analysis.

change in skin score from baseline to 6 months after treatment with placebo. From a microarray that was generated as part of the FASSCINATE study, we selected 83 genes that correlated highly with the changes in skin scores at 6 months (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40455/abstract). Sixty-two of these genes were overexpressed in dcSSc patients compared with healthy controls. Most of the genes that correlated most highly with the changes in MRSS were in gene clusters identifiable as part of transforming growth factor β (TGFβ)/profibrotic, interleukin-6 (IL-6)/STAT-3, or interferon pathways, or were associated with macrophages. Other genes of interest were also included in the nCounter panel, and gene expression from each of the patients was tested using nCounter technology, as described previously (9).

Using these gene expression data, we calculated the correlation coefficient (r) in 34 of the 38 placebo-treated patients between gene expression at baseline and the change in MRSS at week 16 (4 patients were not included because of missing values at 16 weeks and/or at baseline). Based on this correlation, we clustered all of the genes with an r value of >0.2 (Figure 1). By inspection, these genes were grouped into 3 different clusters. Two of the clusters (groups A and B) contained many recognizable genes based on known biologic relevance: TGFβ/profibrotic genes (group A) and macrophage-associated genes (group B). The third cluster contained genes without evident biologic relationships (group C).

We compared the correlation between baseline gene expression and the change in MRSS in this cohort of patients (the discovery cohort) with microarray data from a second group of dcSSc patients (the validation cohort). We found that the correlation coefficients had the same trend for many of the genes in both cohorts (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40455/abstract), even though the r values were different between the 2 groups. This might be due to differences in clinical features between the 2 groups, as well as the 2 different methods used for gene expression analyses (NanoString versus microarray).

From the group of the genes that we identified (see Supplementary Table 1), we chose 7 genes for further analysis based on the strength of correlation between baseline gene expression and the change in MRSS in both the discovery and validation cohorts ($r > 0.2$). Since expression levels of many of the genes correlated highly with each other, we limited our analysis of co-regulated genes to the genes showing the largest correlation coefficients. Notably, expression levels of the genes for CD14, CCL2, CD163, macrophage scavenger receptor 1 (MSR1), and membrane-spanning 4 domains, subfamily A, member 4A (MS4A4A) were highly correlated and therefore we chose to focus only on CD14. The following genes were analyzed further: CD14, IL13RA1, SERPINE1, OSMR, CTGF, IGFBP2, and IRF7 (Table 2). All of these genes were overexpressed in dcSSc patient skin compared with that of healthy controls (Figure 1).

Table 2. Comparison of correlations (r) between gene expression and changes in skin score in the discovery and validation groups

Gene	r	
	Discovery group	Validation group
IL13RA1	0.6	0.25
SERPINE1	0.54	0.31
OSMR	0.52	0.27
CTGF	0.45	0.23
CD14	0.59	0.36
IRF7	−0.2	−0.24
IGFBP2	−0.44	−0.32

Descriptive trajectory data. As shown in Figure 2, we identified 3 trajectory patterns of skin score change (y) over 48 weeks: 12 patients (30%) showed a regressive trajectory (y = 17.69773 − [0.12504 × weeks]), 18 (45%) showed a stable trajectory (y = 25.02055 − [0.8435 × weeks]), and 10 (25%) exhibited a progressive trajectory (y = 31.12353 + [0.11507 × weeks]). The progressive trajectory group started with a higher average MRSS (30.65) than the other 2 trajectory groups, and the average skin score had increased by 17.7% at the end of 48 weeks. The regressive trajectory group started with a lower average MRSS (19.93), which had decreased by 33.9% at the end of the follow-up period. The stable trajectory group

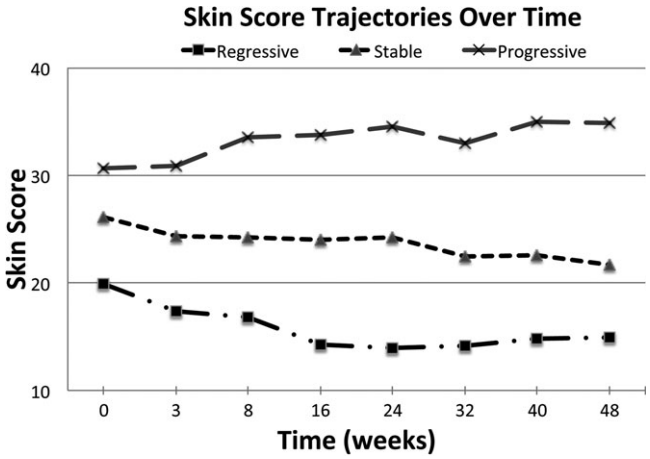

Figure 2. Skin score trajectory changes over time. Trajectories were defined according to baseline modified Rodnan skin score value and pattern. Symbols show the mean at each time point.

started with an intermediate average MRSS (26.12) that decreased slightly (16.2%) over the same time period. The average posterior probability of allocating study participants into trajectories (i.e., entropy) was ≥0.97, indicating an excellent precision that individuals were assigned to their most likely trajectories.

Association of gene expression and pattern of skin score progression. We examined the 7 genes selected from the cluster groups (CD14, IL13RA1, SERPINE1, OSMR, CTGF, IGFBP2, and IRF7) in relation to the skin score trajectory over time. Subjects expressing high levels of CD14, IL13RA1, SERPINE1, OSMR, and CTGF at baseline were more likely to exhibit a progressive skin score trajectory (Table 3). No association was found between levels of either IRF7 or IGFBP2 gene expression and skin score trajectories. We further examined the performance of each of these genes as prognostic biomarkers of progressive versus stable/regressive skin disease. Expression of 5 genes (CD14, IL13RA1, SERPINE1, OSMR, and CTGF) was prognostic for the risk of skin progression (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40455/abstract). IL13RA1 performed the best, followed by OSMR and SERPINE1.

Table 3. Association of gene expression and skin score trajectory over the follow-up period

	Skin score trajectory			
Gene expression	Regressive	Stable	Progressive	*P*
CD14				0.013
Low	5	6	1	
Medium	4	7	2	
High	0	5	8	
IL13RA1				0.026
Low	4	7	1	
Medium	5	6	2	
High	1	4	8	
SERPINE1				0.049
Low	5	6	1	
Medium	4	6	3	
High	1	4	8	
OSMR				0.058
Low	6	6	0	
Medium	2	6	5	
High	2	5	6	
CTGF				0.020
Low	5	5	2	
Medium	4	8	1	
High	1	4	8	
IRF7				0.345
Low	5	6	1	
Medium	2	6	5	
High	3	5	5	
IGFBP2				0.566
Low	3	4	5	
Medium	3	8	2	
High	4	5	4	

Table 4. Association of gene expression and skin score change over time

Gene expression tertile groups	Mean skin score change (95% confidence interval)	*P*
CD14		0.0793
Middle vs. lowest	−0.28 (−3.46, 2.90)	0.86
Highest vs. lowest	3.48 (0.31, 6.66)	0.03
IL13RA1		0.0532
Middle vs. lowest	1.63 (−1.51, 4.77)	0.31
Highest vs. lowest	4.08 (1.36, 6.80)	0.003
SERPINE1		0.0696
Middle vs. lowest	−0.74 (−3.99, 2.51)	0.65
Highest vs. lowest	3.16 (0.59, 5.72)	0.016
OSMR		0.0184
Middle vs. lowest	1.07 (−1.93, 4.07)	0.48
Highest vs. lowest	4.08 (1.87, 6.29)	0.0003
CTGF		0.0491
Middle vs. lowest	−0.71 (−3.86, 2.44)	0.66
Highest vs. lowest	3.32 (0.87, 5.76)	0.008
IRF7		0.4539
Middle vs. lowest	0.90 (−1.53, 3.32)	0.44
Highest vs. lowest	−1.23 (−4.39, 1.93)	0.47
IGFBP2		0.1241
Middle vs. lowest	0.37 (−1.94, 2.69)	0.75
Highest vs. lowest	−3.10 (−6.29, 0.10)	0.058

Association of gene expression and skin score change. Expression of several genes, i.e., CD14, IL13RA1, SERPINE1, OSMR, and CTGF, was also associated with skin score change over time from baseline. Compared with those in the lowest tertile, patients in the highest tertile of CD14, IL13RA1, SERPINE1, OSMR, and CTGF expression showed an increased MRSS. In contrast, those in the highest tertile of IGFBP2 expression appeared to show an improvement of MRSS over time (Table 4). Similar results were also observed in the validation group.

DISCUSSION

At present, it is difficult to predict the trajectory of skin disease in dcSSc patients on the basis of clinical criteria (16). After analyzing skin gene expression, our findings revealed that messenger RNA for CD14, SERPINE1, IL13RA1, CTGF, and OSMR expression is prognostic for the trajectory of skin disease in patients with active dcSSc for 1 year following skin biopsy. Thus, increased expression of these genes may serve as better markers for selecting patients with progressive skin disease for therapies/clinical trials than currently available methods.

In this study, we utilized skin biopsy samples from patients treated with placebo in the Roche FASSCINATE phase II study of TCZ in SSc (9). These samples provided a rare opportunity to examine prognostic biomarkers in a group of patients with active dcSSc who were not treated with any immunosuppressive drugs. However, the inclusion criteria for this study may have impacted the results.

All patients had active dcSSc of ≤5 years' duration since the first non–Raynaud's phenomenon symptom and, at screening, an MRSS between 15 and 40. Additionally at screening, active progressive disease of <1 year's duration was required, and was defined by an increase in MRSS of ≥3 units, involvement of 1 new body area with an increase in MRSS of ≥2 units or 2 new body areas with an increase in MRSS of ≥1 unit, other documentation of worsening skin thickening in the previous 6 months, or ≥1 tendon friction rub accompanied by ≥1 laboratory criterion (C-reactive protein ≥10.0 mg/liter, erythrocyte sedimentation rate ≥28 mm/hour, or platelet count ≥330 × 1,000/μl). Using these eligibility criteria to enrich the patient population for early active disease may have led to enrollment of patients with more progressive disease.

We found that the prognostic biomarkers identified using placebo-treated patients enrolled in the FASSCINATE study also showed trends in r values that were prognostic in a cohort of patients from Boston University Medical Center. The center's patients had received treatment with a variety of immunosuppressive medications (15). Thus, these prognostic biomarkers may have broader predictive value in other patients with early dcSSc. However, it is also possible that these biomarkers may act as predictors only in the preselected FASSCINATE cohort.

Defining progressive skin disease (by worsening MRSS) is important to enhance the patient population for clinical trials in which separation between the control and active treatment groups over a relatively short period of time (i.e., ≤1 year) is desirable. The 3 trajectory groups indicated that the patients whose disease symptoms were most likely to progress showed high baseline MRSS values. We found that only 25% of placebo-treated patients showed a progressive trajectory, despite an effort to enrich the patient population for active disease. Similar results were found previously in a larger study in which 192 patients with dcSSc were grouped using latent linear trajectory (4). In contrast, the trajectories we identified appear, at least on the surface, to be discrepant with the findings in a recent study of patients from the EUSTAR database (17). The most apparent possible reason for this difference would be a difference in patient selection between the 2 studies. The EUSTAR database is an observational study that recruits dcSSc patients with a broad range of disease durations, whereas FASSCINATE is a clinical trial in patients with early dcSSc and elevated acute-phase reactant levels.

In this study, we demonstrated that levels of the macrophage markers CD14, IL13RA1, MSR1, CD163, and MS4A4A correlate with progressive skin disease trajectories. Our current findings are in accordance with the results of previous studies, showing that levels of the macrophage markers SIGLEC1 and MRC1 are increased in lesional SSc skin (18,19). In addition, we recently showed that changes in skin gene expression of MS4A4A correlate highly with changes in the MRSS, helping define a 2-gene pharmacodynamic biomarker (20). Further, in peripheral blood mononuclear cells, IL13RA1 gene expression correlates highly with pulmonary arterial hypertension in patients with limited cutaneous SSc (21). Finally, we recently reported that treatment with TCZ results in the down-regulation of skin CD14 expression in SSc patients (9). Taken together, these observations indicate an important function of macrophages in dcSSc tissue inflammation and fibrosis. As these cells are found surrounding blood vessels, these data suggest that macrophages bridge the fibrotic and vascular features with the pathology seen in the skin of SSc patients.

Two of the prognostic biomarkers identified here, SERPINE1 and CTGF, are strongly induced by TGFβ (22,23). TGFβ has long been suspected to be an important mediator of fibrosis in SSc as well as a variety of other fibrotic diseases, including renal, pulmonary, cardiac, and liver fibrosis (24–26). These 2 genes were significantly decreased in patients treated with fresolimumab (anti-TGFβ antibody) (20), further supporting the notion that TGFβ has a role in the pathogenesis of this disease.

Finally, we identified OSMR, which forms the oncostatin M (OSM) receptor with the common signaling partner gp130, as a prognostic biomarker. OSM, an IL-6 family cytokine, is produced by a variety of immune cells, including macrophages, neutrophils, and activated T cells (27). It has been implicated in a number of biologic processes, including the induction of inflammation and the modulation of extracellular matrix. OSM is up-regulated in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and SSc (27) and is increased in the serum of dcSSc patients (28).

In conclusion, the present results indicate that dcSSc patients with elevated expression of CD14, CTGF, IL13RA1, OSMR, and SERPINE1 at baseline are more likely to have progressive skin score trajectories. The use of these biomarkers might help to guide decisions about which patients should be considered for therapies and/or for clinical trials. This observation will be further explored in the ongoing phase III study of TCZ in SSc patients (29).

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

Study conception and design. Stifano, Rice, Na, Jahreis, Zhang, Lafyatis. **Acquisition of data.** Stifano, Sornasse, Chen-Harris, Khanna, Jahreis. **Analysis and interpretation of data.** Stifano, Sornasse, Rice, Na, Chen-Harris, Zhang, Siegel, Lafyatis.

ADDITIONAL DISCLOSURES

Drs. Sornasse, Chen-Harris, Jahreis, and Siegel are employees of Genentech.

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APPENDIX A: FASSCINATE STUDY INVESTIGATORS

The study investigators who enrolled at least one patient in the FASSCINATE study are as follows: in Canada, Murray Baron (McGill University and Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec), Janet E. Pope (Saint Joseph's Health Care, London, Ontario); in France, Yannick Allanore (Hôpital Cochin, Paris), Joel Constans (Hôpital Saint André, Bordeaux), Thierry Martin (Hôpital Civil, Strasbourg), Carle Paul (Hôpital Larrey Université Paul Sabatier, Toulouse); in Germany, Frank Behrens (Centrum für innovative Diagnostik und Therapie Rheumatologie/Immunologie GmbH, Frankfurt am

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