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Scleroderma (S Bhattacharyya, Section Editor)

Insights Into Systemic Sclerosis from Gene Expression Profiling

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Abstract

Purpose of review The emergence of genomic data science stands poised to revolutionize our molecular understanding of the heterogeneity of complex diseases including systemic autoimmune diseases. In systemic sclerosis (SSc), bulk and single-cell transcriptomics have provided a new lens into the heterogeneity of this complex condition, both in terms of molecular heterogeneity, treatment response, and cell types important for the disease.
Recent findings Transcriptomics has revealed reproducible patterns of gene expression among SSc patients. These conserved patterns of gene expression provide insights into SSc etiology, and evidence suggests that these groups may have important implications for treatment decisions by targeting specific patients. Integration and analyses of publicly available data are providing new insights into the disease. Single-cell technologies are illuminating cell types that may be important in pathogenesis. The disease trajectory for SSc remains difficult to predict, but the interactions between adaptive and innate immune cells with tissue-resident stromal cells may play an important role.
Summary The heterogeneity in SSc can be broken down and quantified using molecular methods that range from bulk analysis to single cells. Further study of cellular and

molecular dynamics in end-target tissues is likely to result in better disease management through personalized, data-driven treatment decisions.

Introduction

Complex human diseases are genetically and clinically heterogeneous, arising from a combination of genetic and environmental factors. Despite the many clinical features, phenotypic variations, and molecular markers used to categorize and classify conditions in human health, complex diseases continue to challenge researchers. Patients with the same diagnosis exhibit unique disease trajectories, varying responses to therapy, and different clinical outcomes. This is particularly true for systemic sclerosis (SSc; scleroderma), a rare, multi-system fibrotic autoimmune disease with heterogeneous clinical presentation. Genome-wide gene expression profiling from patient tissues provides a rich source of data because it enables simultaneous quantification of thousands of molecular transcripts at a specific point in time. With the advent of high-throughput technologies to profile gene expression, scientists and clinicians are poised to incorporate detailed molecular and genomic information for more accurate diagnostic and prognostic purposes, leveraging genomic data science to better understand these conditions.

Clinical vs molecular heterogeneity

Clinical and demographic features associated with SSc severity include sex [1–3], age [4, 5], race [1, 6–8], anti-nuclear antibodies [9–12], specific organ involvement [13–16], and extent of skin fibrosis [17–19]. Each of these has been used to understand SSc etiology and improve treatment strategies, but these parameters do not fully capture the heterogeneity of the disease. For example, standard clinical and demographic features have proven insufficient for identifying patients that are more likely to benefit from a therapy in clinical trials. Molecular profiles are now showing promise to help break down the heterogeneity in SSc clinical trials [20].

Nearly 20 years ago, molecular subtypes based on gene expression were first characterized in diffuse large B cell lymphoma [21], a disease where grouping patients based on morphology failed to differentiate molecularly distinct patients. Since that time, molecular subtypes have been validated in multiple other cancers including breast invasive carcinoma [22–24], colorectal cancer [24, 25], lung squamous cell carcinoma [26, 27], serous ovarian carcinoma [28], and acute myeloid leukemia [29, 30]. Perou et al. characterized four molecular subtypes in breast cancer using unsupervised clustering of "intrinsic genes" identified from paired tumor samples [22], which have since been shown to correspond to histopathology and different clinical outcomes, such as response to therapy and survival time [31–33]. With robust precedents of molecular subtypes in many cancers, there is strong evidence to suggest the existence of clinically significant, transcriptionally unique subsets in systemic autoimmune diseases.

It is well established that there exist substantial gene expression differences between SSc and healthy controls, highlighted in studies of multiple tissues including skin [34, 35], lung [36], and peripheral blood cells [37–39]. Applying the rationale of Perou et al. [22], genome-wide gene expression of multiple affected tissues has revealed inherent heterogeneity, but also conserved patterns in subgroups of SSc patients [34, 35, 40, 41]. Four "intrinsic" gene expression

subsets, characterized by unique biological pathways, have been identified in SSc skin: inflammatory, fibroproliferative, limited, and normal-like [34, 42, 43]. The inflammatory subset is enriched by overrepresentation of immune system processes including inflammatory response, defense response, and wound healing. Cell cycle–related processes, including cell proliferation and mitosis, are upregulated in the fibroproliferative subset. The limited subset is composed solely of patients with limited cutaneous (lc) SSc patients. The normal-like subset contains SSc patients with gene expression that closely resembles healthy controls. The intrinsic subsets have subsequently been recapitulated in other affected organs from SSc patients [40, 44]. Other studies have suggested alternative gene expression groupings of SSc patients, but largely agree upon three distinct signals of inflammation, pro-fibrotic pathways, and normal-like, sometimes characterized by upregulation of keratinocyte-related signatures [45, 46•, 47••]. The pervasiveness and reproducibility of the SSc intrinsic subsets across tissues highlight the systemic nature of the disease and indicate that distinct molecular subsets of patients may need to be treated differently [44, 48].

Interpreting outcomes in SSc clinical trials using gene expression signatures

Though disease management has improved, prognosis for SSc continues to exhibit high mortality rates, primarily due to cardiac and pulmonary complications [49]. There have been many clinical trials in SSc, but the overwhelming majority have not met primary clinical endpoints and treatment effects are minimal or not significant [50, 51]. Despite this, in many cases, there are a few patients who do experience clinical benefit from treatment. For a handful of SSc clinical trials, gene expression analyses were performed in conjunction with the clinical studies (Table 1). Analyzing gene expression data from individual patients in clinical trials has helped to (1) elucidate the molecular mechanisms that contribute to clinical improvement in subsets of patients and (2) identify potential biomarkers for response to treatment. This phenomenon lends support to the need for precision medicine in SSc and an instance where intrinsic molecular subsets defined from gene expression may be informative.

Gene expression signatures have been linked to improvement in a variety of cases. For example, in clinical studies of nilotinib [52] and fresolimumab [44, 53], activation of the TGFβ pathway at baseline was an important factor related to improvement. Nilotinib is a tyrosine kinase inhibitor that targets BCR-ABL, c-kit, and PDGF, and fresolimumab is a monoclonal antibody that targets all isoforms of the protein TGFβ. Both treatments were shown to broadly decrease TGFβ pathway activity at the gene expression level in SSc patients that improved. In the faSScinate study of tocilizumab (anti-IL6), several immune-related genes were found to correlate with mRSS, including *SERPINE1* and *CTGF*, genes strongly induced by TGFβ [54]. Activation of the TGFβ pathway spans the inflammatory and fibroproliferative molecular subsets [55], although intrinsic molecular subset was not explicitly analyzed in the fresolimumab or faSScinate studies. However, other clinical trials have more directly linked intrinsic subsets to clinical improvement.

Table 1. Summary of clinical trials with genomic data analyzed to date

Clinical trials		Major findings
Nilotinib	Gordon et al. 2015	Inflammatory patients and patients with high TGFβ expression were more likely to improve.
Fresolimumab	Rice et al. 2015 Taroni et al. 2016	Activation of the TGFβ pathway at baseline was important for improvement.
Tocilizumab	Stifano et al. 2018	The expression of genes induced by TGFβ was correlated to improvement.
Abatacept	Chakravarty et al. 2015	Four out of five SSc patients who improved were inflammatory.
	Khanna et al. 2020	Inflammatory patients are more likely to see decreases in skin severity; fibroproliferative patients are more likely to see increases in pulmonary function.
Mycophenolate mofetil (MMF)	Hinchcliff et al. 2013	Four out of seven improvers belonged to the inflammatory subset.
	Hinchcliff et al. 2018	Inflammatory signature decreases with MMF therapy and rebounds upon treatment cessation.
Belimumab (with background MMF)	Gordon et al. 2017	Decreases in the inflammatory signature and movement to the normal-like subset coincide with improvement.
Stem cell transplantation	Franks et al. 2020	The fibroproliferative subset is the most likely, and normal-like the least likely, to see increased event-free survival with stem cell transplant.
Lenabasum	Spiera et al. 2020	Inflammatory and fibrosis gene expression signatures decrease with treatment.

In Hinchcliff et al., an investigator-initiated study of mycophenolate mofetil (MMF), four out of seven patients treated with MMF improved, and all four were classified as inflammatory at baseline [43]. These results suggested that the inflammatory patients were the most likely to respond to MMF treatment. In a continued analysis of an expanded cohort, Hinchcliff and Toledo et al. [56] used a gene expression signature to quantify inflammatory normalized enrichment scores (NES) from serial longitudinal biopsies. After 24 months of MMF treatment, the inflammatory signature notably decreased over time for many subjects, and this was coupled with stable or decreasing mRSS. Some subjects ceased MMF treatment at 24 months, and in these patients, the inflammatory score sharply rebounded. The rebound in inflammatory score was reflected clinically by an increase in mRSS score and increased relative numbers of inflammatory cells. These findings indicate that the inflammatory signature may be an important regulator of skin fibrosis.

Gordon et al. [52] was an investigator-initiated, industry-supported, single-center, randomized, double-blind, placebo-controlled, pilot study to assess the

safety and tolerability of belimumab in patients with dcSSc receiving background MMF therapy. Belimumab (anti-BLyS) is a targeted biological treatment that decreases B cell survival and production of autoantibodies [57]. The authors used gene expression data from baseline and post-treatment biopsy samples to assign samples to intrinsic subsets and quantify an inflammatory subset score. Several patients initially classified as inflammatory or proliferative at baseline changed subsets following treatment to the normal-like subset. This change was consistent with decreased mRSS and clinical improvement for many subjects. The authors quantified an inflammatory subset score and found that it correlated with the change in mRSS, most strongly for patients assigned to the inflammatory subset at baseline. These findings suggest that an overall reduction in inflammatory gene expression and movement toward the normal-like subset is associated with reduction of skin fibrosis.

In Speira et al. [58••], an industry-supported clinical trial of lenabasum, an agonist of cannabinoid receptor 2 (*CB2*), the authors performed gene expression profiling at baseline and post-treatment of study subjects. An inflammatory score and an extracellular matrix organization score were calculated for each sample based on gene set centroids. In the paired analysis, both the inflammatory scores and extracellular matrix organization scores decreased in treated subjects, indicating that lenabasum may modulate both inflammatory and fibrotic disease pathways [58••].

Chakravarty et al. [59] was an investigator-initiated placebo-controlled clinical trial of abatacept (CTLA4-Ig), a modified antibody designed to prevent activation of T cells by blocking the co-stimulating signal from antigen-presenting cells. Here, four of five patients who improved on abatacept, as determined by change in mRSS, were in the inflammatory intrinsic gene expression subset. Improvement was accompanied by a decrease in gene expression for immune pathways, including the CD28 and CTLA4 receptors—the target of abatacept [59].

Khanna et al. [60••] tested the a priori hypothesis, developed from the pilot study of abatacept, that the inflammatory subset is most likely to experience a significant decrease of mRSS during abatacept therapy in a large, randomized, placebo-controlled clinical trial of abatacept. In ASSET (Abatacept Systemic SclErosis Trial), a phase 2 study, the authors performed RNA-sequencing (RNA-seq) analysis of skin biopsies and classified patients into intrinsic gene expression subsets prior to study unblinding. Significant reduction in mRSS occurred in the inflammatory and normal-like subset, but not in fibroproliferative. However, the fibroproliferative subset showed a clinically relevant numerical increase in forced vital capacity (FVC%) predicted with abatacept treatment, while all other groups showed decreases. Skin improvement with abatacept was noted for the normal-like subset, but these results were not significantly different between active agent and placebo-controlled arms. These patients may be experiencing spontaneous improvement, a phenomenon that has been previously recognized in the field [51, 61, 62]. These data show, for the first time in a placebo-controlled trial, that intrinsic skin gene expression subsets may predict differential response to a targeted biological therapy, and that therapy may impact different facets of SSc pathogenesis in skin or lung. This suggests that stratification of cases according to intrinsic gene expression subsets may maximize the number of informative SSc cases in clinical trials and potentially improve future clinical practice.

The Scleroderma: Cyclophosphamide or Transplantation (SCOT) trial demonstrated a significant long-term benefit of myeloablative autologous stem cell transplantation for SSc patients [63]. Due to the high potential risk and cost of these procedures for patients, Franks et al. [64••] performed a follow-up computational analysis to identify how stem cell transplantation and cyclophosphamide treatments impacted the long-term survival of patients in the inflammatory, fibroproliferative, and normal-like intrinsic subsets. Using baseline gene expression from peripheral blood cells and machine learning techniques, the authors were able to classify patients based on previously validated gene expression signatures of the intrinsic subsets. While there were no significant differences in long-term survival between the treatment arms for the inflammatory and normal-like subsets, the fibroproliferative subset saw markedly different results. SSc patients assigned to the fibroproliferative subset who underwent transplant experienced longer event-free survival compared to those who received cyclophosphamide, indicating that it may be beneficial to prioritize these patients for transplant as opposed to immunosuppressive treatments, which they are less likely to benefit from.

The clinical trials described here are proof of principle that precision medicine in SSc is not only possible, but within reach. Immunosuppressive treatments are more likely to target the inflammatory patients, while stem cell transplantation may be more likely to benefit the fibroproliferative subset. Some findings have indicated that reduction in skin fibrosis coincides with decreases in the inflammatory signature and movement toward the normal-like subset [56, 60••, 65]. Depending on the baseline intrinsic subset, improvement may look different for different patients.

For future clinical trials, it may be advantageous to consider intrinsic subset in study inclusion criteria to reduce molecular heterogeneity in the cohort. One limitation of the prior work is that analyses were performed using a single tissue (e.g., skin biopsy or blood sample) from SSc patients. These analyses only reflect the disease biology of a single tissue and likely do not represent the full molecular picture of SSc as a multi-system disease. As suggested in Khanna et al. [60••], treatment may impact organs differently and careful consideration must be given to the tissue analyzed.

Advances in genomic data science: data integration, network analyses, and machine learning

In analyzing transcriptomic data, scientists are presented with several data science challenges including interpretation of high dimensional data [66–68], reproducibility of results [69], and platform compatibility issues in comparing different studies [70]. Despite these difficulties being amplified in small studies with limited sample sizes, sophisticated computational approaches and meta-analyses continue to improve the understanding of SSc etiology. Several studies have applied network analyses [47••, 48, 71, 72, 73•, 74••], machine learning [39, 64••, 75, 76], integration of different data types [77•], or a combination of these approaches [44] to characterize subsets of SSc patients, identify markers of disease risk, and implicate possible pathogenic cell types.

The gene expression intrinsic subsets discussed were first identified in multiple distinct cohorts, and subsequent studies have focused on identifying

robust biological signatures using data-driven approaches and cross-study validation methods. Mahoney et al. [48] first used weighted gene co-expression network analysis (WGCNA) [78] to identify conserved gene expression modules between three different data sets. In this study, the authors were able to demonstrate that gene sets derived from skin gene expression could be identified across multiple cohorts that were biologically meaningful, corresponded to the previously characterized intrinsic subsets (inflammatory and fibroproliferative), and may be related to genetic risk in SSc [48]. In order to assign single samples to intrinsic subsets, Franks et al. [75] curated datasets to train a machine learning classifier and obtained ~85% accuracy in validation metrics. This classifier has been used in multiple pilot clinical trials, significantly aiding in gene expression analyses by quickly assigning intrinsic subset using defined and validated criteria [60••, 64••, 65].

Signatures that differentiate SSc patients from healthy controls or between subsets of SSc patients may be useful beyond classification purposes. For example, Lofgren et al. [76] developed a 415-gene signature that successfully distinguishes SSc patients from controls. Moreover, the authors described the SSc skin severity score (4S) as a sample-specific numeric measure of this 415-gene signature that is correlated with current mRSS and predictive of future mRSS with MMF treatment. Wang et al. [72] calculated "regulator scores" as an aggregate measure of target genes' expression values for 836 regulators, such as transcription factors and miRNAs. In addition to stratifying the intrinsic subsets, many regulator scores correlated strongly with clinical measures and disease severity. For example, NMYC and CART1, two cell cycle and metabolism regulators, activity scores differentiated higher mRSS in fibroproliferative patients, while SMAD4 and NFAT activity scores identified inflammatory SSc patients with higher mRSS [72].

With the identification of disease activity signatures and regulators, potential therapeutics can be selected that are expected to target these genes and pathways. Taroni et al. [44] described the common phenomenon that improvement in clinical trials is often accompanied by the reduction of highly expressed immune-related genes and inflammatory pathways. By employing networks and machine learning approaches, the authors compared clinical trials and identified patients who did not improve on one treatment who may have improved on another [44]. Furthermore still, Kim et al. [74••] developed a network-based analysis to identify novel drugs likely to impact SSc-associated genes.

Other studies have identified disease signatures that implicate pathogenic cell types and polarization states. Skaug et al. [79•] recently demonstrated the existence of innate and adaptive immune cell signatures in early dcSSc. The authors reported that adaptive immune cell signatures positively correlated with longer disease duration, and macrophage and fibroblast signatures correlated with increased mRSS [79•]. Building upon the framework presented in Mahoney et al. [48], Taroni et al. [71] extended this approach to consider different tissues affected by SSc. In addition to finding tissue-specific signatures, the authors identified a common pathogenic gene signature of an immune-fibrotic axis indicating a prominent role for pro-fibrotic macrophages [71]. Fibroblast polarization is also likely a driving factor in SSc severity. Showalter et al. [77•] combined histological analysis with machine learning approaches for gene expression analysis to identify distinct dermal fibroblast polarizations. αSMA positively correlated with high skin score severity, while CD34 negatively correlated with skin score severity. Using these markers to binarize scores, the authors identified a 47-gene signature of an

inflammatory fibroblast polarization state that decreases over time in clinical improvers treated with nilotinib or belimumab [77•].

Of note, the current literature focuses primarily on linear data projections which are typically faster, more robust, and more interpretable than nonlinear methods. However, nonlinear methods (e.g., neural networks [80, 81], and variational autoencoders [82]) are gaining traction in biomedical data science and may represent a logical next step to elucidate more complicated data structure in SSc gene expression. Importantly, high-level transcriptomic analyses and clinical studies must go hand in hand. Nuances in data collection, analysis, and interpretation abound, and expertise from bioinformaticians and clinicians will be necessary for translational and impactful results.

Toward single-cell resolution

There is strong evidence that immunosuppressive therapies target immune cells and related biological processes important in inflammatory SSc patients and lead to clinical improvement [43, 59, 65]. The findings from multiple computational studies of gene expression in SSc support the concept of an immune-fibrotic axis, where alternatively activated macrophages contribute to adaptive immune cell processes and fibroblast activation [56, 71]. The recent advancements in high-throughput methods to study transcriptomes of single cells enable the study of this complex interplay between adaptive immune cells, innate immune cells, and stromal cells.

There have been efforts to characterize cell subtypes in SSc skin and lung with single-cell transcriptomic resolution, and this provides greater context for the molecular kinetics of disease progression compared to bulk tissue RNA-sequencing. Figure 1 summarizes the major findings from bulk and single-cell sequencing in SSc skin and lung. Apostolidis et al. [83] applied single-cell sequencing methods to characterize endothelial cells in SSc and healthy control skin. SSc endothelial cells displayed markers of extracellular matrix generation and negative regulation of epithelial-to-mesenchymal transition associated with vascular injury and activation. In a single-cell study of SSc skin tissue focused on lymphocytes, Gaydosik et al. [84] identified a distinct CXCL13+ T-cell subset that may promote autoantibody generation by enabling B cell responses. Valenzi et al. [85] performed single-cell sequencing to describe the heterogeneity of fibroblast populations in healthy control lung samples and SSc patients with interstitial lung disease (ILD). Importantly, a new population of myofibroblasts displayed active proliferation signals, which could provide an explanation for the apparent proliferation signature in fibroproliferative patients. Reyfman et al. [86•] performed a single-cell sequencing study aimed to describe cell types in pulmonary fibrosis and included several samples from SSc patients with ILD. Though this study did not report a broad range of fibroblast heterogeneity, significant heterogeneity prevailed in macrophages and epithelial cells. Several SSc macrophage states have been reported in prior bulk-sequencing studies of monocytes in peripheral blood including upregulation of glycolysis, hypoxia, and mTOR signaling coupled with downregulation of INFγ response pathways [87], pro-fibrotic activation profiles [88], and inflammatory signatures [89••]. Indeed, Valenzi et al. [90] confirmed decreased INFγ signatures in SSc-ILD compared to IPF patients, and increased type I interferon signaling especially in $SPP1^{hi}$ macrophages.

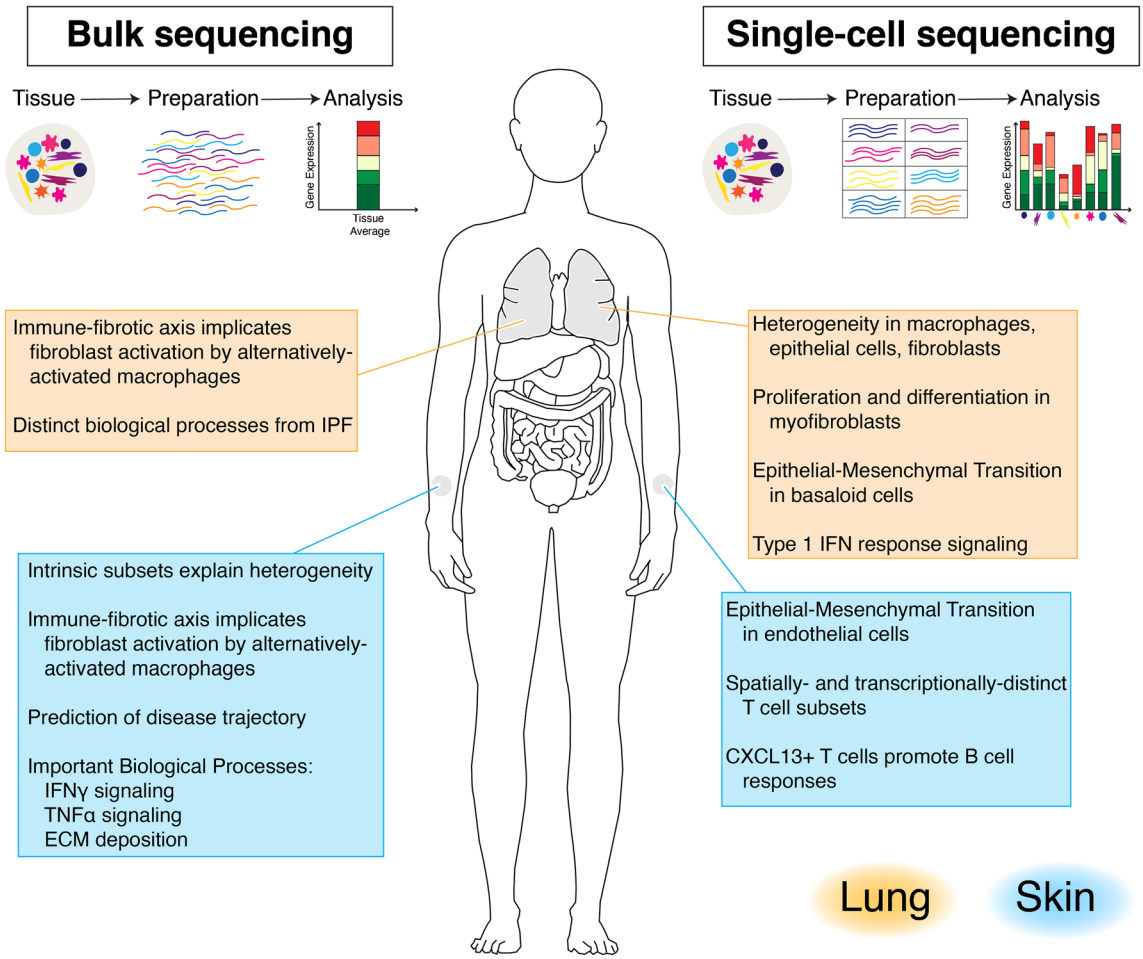

Figure 1. Summary of major findings from bulk (left) and single-cell (right) transcriptomics of skin and lung in SSc

These findings warrant further study in an expanded cohort of SSc patients with and without ILD to identify the polarization states specific to SSc and identify precursors of pathogenic cell states.

The field of single-cell sequencing has only begun to take stride in SSc and is sure to see great advancement in the coming years. It will be increasingly important for future studies to include diverse patients to represent the spectrum of disease heterogeneity and effectively leverage the large sample sizes of cells profiled to identify biologically meaningful results. Additionally, cross-platform analyses that relate the intrinsic subsets (identified using whole tissue profiling) to their corresponding single-cell compositions will connect historical data sets and interpretations with new data.

Conclusions

The studies described here have demonstrated the predictive potential of gene expression and genomic data science for clinical outcomes, give insight into the

personalized nature of SSc, and have important implications in treatment strategies. Additionally, these studies highlight the intricate coupling of immunologic and genomic signatures, and results indicate that a reset of both may be necessary for disease resolution. Although SSc remains a difficult disease to understand and treat, there has been substantial progress made with the addition of gene expression data. It is our hope that precision medicine in SSc flourishes and effectively utilizes genomic information to target treatment responses in SSc. If we can leverage gene expression and genomic data to identify important molecular processes early in the disease stage, then more effective treatment strategies can be designed and prescribed for individuals, ultimately leading to improved clinical outcomes in SSc.

Compliance with Ethical Standards

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest
Jennifer Franks declares that she has no conflict of interest. Michael Whitfield reports grants, personal fees, and other from Celdara Medical LLC, grants and personal fees from Bristol Myers Squibb, grants and personal fees from Corbus Pharmaceuticals, personal fees from Abbvie, and personal fees from Acceleron, outside the submitted work.

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• Of importance
•• Of major importance

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