Biomarkers as an opportunity to stratify for outcome in systemic sclerosis

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Abstract

Systemic sclerosis (SSc) is a highly complex disease whose heterogeneity includes multiple aspects of the condition, such as clinical presentation, progression, extent and type of organ involvement, and clinical outcomes. Thus far, these features remain not easily predictable both at the patient group level and in a given patient with regard to age at onset and clinical course. The unpredictable clinical course represents an obstacle to focusing potentially effective treatment in patients that need it the most. At the time of organ involvement and clinical diagnosis, most of the clinical manifestations are irreversible; therefore, predicting outcomes becomes crucial. This can explain the multiple attempts to identify prognostic, predictive, and monitoring—both soluble and imaging—biomarkers over the past years. They range from the currently most used biomarkers, the autoantibodies associated with disease-specific clinical features and course, to the single recently proposed skin, lung, cardiac involvement biomarkers and to the composite scores capturing multiple aspects of the disease. This review will focus on soluble and imaging biomarkers that recently showed promising evidence for outcome stratification in patients with SSc.

Keywords: Systemic sclerosis, scleroderma, biomarkers, outcome measures, cytokines, imaging

Introduction

Systemic sclerosis (SSc) is a highly heterogeneous complex disease characterized by early microvascular abnormalities, immune dysregulation, and chronic inflammation, and subsequent fibrosis of the skin and internal organs (1). The disease heterogeneity includes multiple aspects of the condition such as clinical presentation, progression, extent and type of organ involvement, and clinical outcomes. Thus far, these features remain not easily predictable both at the patient group level and in a given patient with regard to age at onset and clinical course (2). The unpredictable clinical course represents an obstacle to focusing potentially effective treatment in patients that need it the most. At the time of organ involvement and clinical diagnosis, most of the clinical manifestations are irreversible; therefore, predicting outcomes becomes crucial. This can explain the multiple attempts to identify disease biomarkers over the past years (2-4).

“Biomarker” definition has been recently established by the U.S. Food and Drug Administration and the National Institutes of Health as part of their joint Biomarkers, EndpointS, and other Tools resource. A biomarker is “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions.” Definition of biomarkers includes not only factors measured in biological samples, but also imaging data or other measurable factors, such as biomechanical properties (5).

One of the purposes of biomarkers is to link a measurement to a prediction of clinical outcome assessments (COAs). COAs are direct measures of “how a person feels, functions, or survives” (6).

Therefore, the identification of biomarkers for patient stratification in SSc is useful both for cohort enrichment in designing clinical trials and for selection of more intensive diagnostic screening and follow-up evaluation and early intervention in clinical management. Biomarkers are of crucial importance to stratify for outcome in SSc when assessing likely disease outcomes with treatment. Although prognostic biomarkers serve as indicators of differential disease outcomes (clinical event, disease recurrence, or progression) and can be useful to stratify the risk for negative clinical outcomes, and identify patients who could benefit from more intensive evaluation while allowing others to avoid unnecessary addition-
al diagnostic tests or medical interventions, in clinical trials, predictive biomarkers are those markers able to discriminate those who will respond or not respond to therapy (6).

A monitoring biomarker is a biomarker that can be measured serially to assess the status of a disease or to detect a treatment effect. “Monitoring” is a broad concept, so there is overlap with other categories of biomarkers (6).

Although referring the reader to the extensive overviews on the application of biomarkers in SSc provided by several authors (2-4, 7, 8), this review will instead focus on the recently proposed soluble and imaging biomarkers potentially useful to stratify for outcome patients with SSc.

Soluble biomarkers

Autoantibodies

Risk stratification in SSc is currently based on demographics and clinical features including disease subsets and autoantibodies. To date, the most frequently used prognostic factors for disease course are autoantibodies, each associated with different SSc subsets and predictive of the occurrence of specific organ involvement (reviewed in 9-11). Three SSc specific antibodies—anticentromere, anti-topoisomerase I (anti-Scl-70), and anti-RNA polymerase III (anti-RNAP III)—were included in the 2013 American College of Rheumatology/European League Against Rheumatism classification criteria for SSc (12). Table 1 summarizes the most significant clinical associations with autoantibodies in patients with SSc.

In particular, significant evidence on the prognostic role of anti-RNAP III antibody (Ab) recently emerged. Anti-RNAP III Ab is detected with high specificity in patients with SSc (98%-100%). Association of anti-RNAP III Ab with malignancies was recognized by several studies, regardless of the method of Ab detection. A percentage of 17.7%-43.8% of patients with SSc with anti-RNAP III Ab manifest internal malignancies. Genetic alterations of the POLR3A gene, encoding for RNA polymerases III polypeptide A, were found in tumor cells of patients with SSc with anti-RNA polymerases III, but not in the cases without the Ab suggesting that mutation in the autoantigen in the cancer cells may initiate an autoimmune response resulting in the onset of SSc as a paraneoplastic disease in these patients (13-17).

A recent case-control study conducted on the large multicenter cohort of EUSTAR registry confirmed that scleroderma renal crisis (SRC), gastric antral vascular ectasias, rapid progression of skin thickness, and malignancies concomitant to SSc onset as independent characteristics, associated with anti-RNAP III Ab (18). Association with pulmonary hypertension (PH) was not confirmed. In addition, the study highlighted that the majority of cancer was diagnosed within an interval between 6 months before and 12 months after SSc onset; the most prevalent cancer type was breast cancer; older patients or those with diffuse cutaneous SSc (dcSSc) were particularly at risk; malignancies other than breast cancer were much more frequent in males (19). Malignancy screening at the time of diagnosis and tight screening in the first 2-5 years were recommended for patients with anti-RNAP III Ab. Thus, anti-RNAP III Ab serves as a predictive biomarker of malignancy (18).

Pneumoproteins and cytokines

KL-6 and CCL-18

Lung involvement is the leading cause of disease-related death in SSc (21). Currently, lung involvement is the leading cause of disease-related death in SSc (21).

Table 1. Main clinical associations of autoantibodies in patients with SSc.

| Autoantibodies | Associated clinical features |
|---------------|-----------------------------|
| Anti-topoisomerase I (anti-Scl-70) | dcSSc, ILD, severe digital vasculopathy |
| Anticentromere | lcSSc, PAH, more favorable prognosis, lower mortality rate |
| Anti-RNA polymerase III | dcSSc, rapid skin thickening progression, SRC, GAVE, malignancy at diagnosis |
| Anti-U3 RNP | ILD, PAH, SRC, lower GI involvement in early disease, myopathy |
| Anti-Th/To | lcSSc, ILD, PAH |
| Anti-U11/U12 RNP | ILD |
| Anti-PM-Scl | PM/DM overlap, ILD |
| Anti-Ku | Muscle and joint involvement |
| Anti-Ro52/TRIM21 | ILD, poor survival |
| Anti-U1 RNP | MCTD, Inflammatory arthritis, Myositis, PAH |
| Anti-RuvBL1/2 | dcSSc, muscle involvement |
| Anti-eIF2B | ILD |

SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc; ILD: interstitial lung disease; lcSSc: limited cutaneous systemic sclerosis; PAH: pulmonary arterial hypertension; SRC: scleroderma renal crisis; GAVE: gastric antral vascular ectasias; GI: gastrointestinal; PM/DM: polymyositis/dermatomyositis; MCTD: mixed connective tissue disease.
function tests and chest imaging help to predict who has SSC-associated interstitial lung disease (ILD) and whether it will progress (22). In the absence of standardized validated prognostic and predictive biomarkers of ILD, a strategy that combines both lung function tests and chest imaging is currently recommended (22). However, several recent studies showed promising results on the potential of soluble biomarkers to predict development and course of ILD that will require further validation.

The most studied soluble prognostic biomarkers of ILD in SSC include Krebs von den Lungen-6 (KL-6) and CC chemokine ligand (CCL)-18. They are associated with lung parenchymal injury (23); thus, unlike the other soluble biomarkers, less likely to be influenced by involvement of other organs.

KL-6 is a glycoprotein expressed mainly by alveolar type II pneumocytes respiratory bronchiolar epithelial cells (24), whereas CCL-18 [also known as pulmonary activation-regulated chemokine] is a chemokine mainly produced by alveolar macrophages (25).

An observational follow-up study in 50 patients with untreated early stage SSC-ILD indicated that baseline KL-6 levels were correlated with the forced vital capacity (FVC) decline rate. In addition, high KL-6 levels were predictive of long-term development of end-stage lung disease (% predicted FVC<50%, requiring oxygen, or ILD-related death). A KL-6 value higher than 1,273 U/mL was proposed as a reliable predictor of end-stage lung disease development (26).

In a subsequent follow-up study, baseline KL-6 and CCL-18 levels were found to be higher in patients than in healthy controls (HC) (27). However, in this cohort of 82 patients with early SSC-ILD, including those on immunosuppressive therapy, only higher KL-6 levels were predictive of faster FVC% decline at the 1-year follow-up. The authors suggested that KL-6 is a promising pneumoprotein that can contribute to SSC-ILD clinical trial enrichment.

By contrast, earlier studies found CCL18 to be predictive of ILD event (28) and of short-term FVC decline (29).

In a large multicenter observational study, on 427 patients with SSC, Elhai et al. (30) showed that KL-6 levels correlated at baseline with FVC, diffusing capacity for carbon monoxide and extent of lung fibrosis and could, therefore, be used to assess the severity of lung fibrosis. CCL18 appeared to be a potential predictive marker for progression of ILD in SSC as serum levels were an independent predictor of a >10% decrease in the FVC and de novo development of extensive disease (30). By contrast, KL-6, unlike other studies, did not show predictive significance. In the same study, SP-D level was analyzed and found to correlate with presence of lung fibrosis, thus, suggesting its role as relevant diagnostic biomarker for SSC-associated ILD (30).

Baseline and 12-month plasma KL-6 and CCL18 levels were also analyzed in patients enrolled in the Scleroderma Lung Study II (SLSII) (cyclophosphamide [CYC] versus mycophenolate mofetil [MMF]) (31). For both markers, levels correlated with the extent of radiographic fibrosis and significantly declined with immunosuppression at 1 year. In both CYC and MMF arms, higher baseline KL-6 and CCL18 levels predicted progression of ILD on the basis of the course of FVC and diffusing capacity of the lung for carbon monoxide (DLCO) over 1 year, despite treatment.

These data cumulatively support the notion that both KL-6 and CCL18 levels might serve as prognostic biomarkers of progressive ILD, useful to identify patients requiring an early aggressive treatment.

**Interleukin-6 and C-reactive protein**

Interleukin-6 (IL-6) is a pleiotropic cytokine known to be involved in immune regulation and inflammatory responses. Recently, significant evidence emerged on potential role of IL-6 and of its associated family members in SSC pathogenesis (32-34).

Increased circulating IL-6 levels have been shown in other studies to be markers of pulmonary arterial hypertension (PAH) (37, 38), cardiac involvement (39), and cardiopulmonary severity (40).

In this context, serum IL-6 levels may help to stratify patient subgroups for disease activity and survival outcome. In addition, longitudinal evaluations of IL-6 levels could be useful markers of changes in skin and internal organs and in monitoring response in the context of interventional trials as observed in studies including patients with high baseline levels who showed a reduction with treatment (41-43).

The association between IL-6 expression and pathogenic potential in specific organ manifestations, particularly skin and lung fibrosis, and in PAH, may inform future therapeutic strategies and elucidate potential targeted therapies (33).

C-reactive protein (CRP) is an acute-phase molecule directly controlled by IL-6. The predictive significance of CRP level for long-term ILD progression was investigated by Liu et al. (44) in a large early SSC [Genetics versus Environment in Scleroderma Outcome Study (GENISO)] cohort. Baseline CRP levels were found to be higher in patients with SSC than in controls. More importantly, higher baseline CRP levels were associated with shorter survival and predicted the long-term decline in FVC. Confirming these results, in a longitudinal study of the Australian Scleroderma Cohort, a 2-fold increase of CRP associated with significant deterioration in FVC (45). High CRP levels were recently utilized as an enrichment criterion for a placebo-controlled tocilizumab clinical trial (41).

These data collectively suggest that CRP might serve as a prognostic biomarker for worse ILD course and for identification of patients requiring more intensive monitoring and treatment.

**CCL2**

The role of CCL2/monocyte chemotactic protein (MCP)-1 as a potential biomarker in SSC had been extensively studied over the past years. CCL2 has been shown to be released by fibroblasts in later fibrotic stages, to prompt the migration of fibrocytes to tissues, to stimulate production of large amounts of collagen, and to be overexpressed in non-fibrotic skin similar to involved fibrotic skin areas (46-48). Collectively, these data suggested a critical role in profibrotic and fibrotic stages in SSC.

In a recent study carried out in 2 independent cohorts [GENISO discovery cohort and Canadian Scleroderma Research Group replication cohort], higher baseline plasma CCL2 levels were found to be predictive of ILD progression with faster decline in FVC% values over time and poorer survival in patients with early SSC. The study supported the notion that CCL2 has a role as a prognostic biomarker and is a potential therapeutic target (49).
Earlier studies on CCL2/MCP-1 showed correlation of elevated serum levels of CCL2/MCP-1 with dcSSc, early disease, anti-topoisomerase I/III Ab, PF, greater frequency of major organ-based complications and disease activity score (50, 51). Taken together, the studies suggested CCL2/MCP-1 measurement to be a useful marker of risk stratification in early stage disease.

A longitudinal study with serial evaluations of serum levels of chemokines/cytokines showed that CCL2/MCP-1 levels were increased at baseline and decreased each following year, accompanied with an improvement in skin fibrosis. Variations of CCL2/MCP-1 were significantly associated with the variations of skin thickness score and vital capacity during 3 years. These results suggested that CCL2/MCP-1 is a serological indicator of skin fibrosis activity and lung involvement in patients with SSc (52).

**CXCL4**

A recent proteome-wide analysis and validation study showed that chemokine (C-X-C motif) ligand 4 (CXCL4; Platelet Factor 4) is the predominant protein secreted by plasmacytoid dendritic cells (pDCs) in SSc, both in circulation and in skin (53). In that observational study, CXCL4 plasma levels were found to be significantly higher in patients with SSc than in healthy and other disease controls. CXCL4 levels correlated highly with disease phenotype and disease progression in large, independent, and clinically well-characterized SSc patient cohorts, whereas levels of CCL2, CXCL10, CCL5, von Willebrand factor, and CCL18 did not show such correlation. CXCL4 levels correlated with skin and lung fibrosis and with PAH in SSc indicating that plasma CXCL4 levels could be useful to predict the risk and progression in SSc (53).

In another recent study that analyzed patients receiving immunosuppressive therapy within the SLSII, the authors observed that levels of CXCL4 did not correlate with extent of ILD at baseline and decreased in all patients treated with immunosuppressive therapy. In particular, changes in CXCL4 levels at 12 months predicted future progression of SSc-ILD from 12 to 24 months suggesting that intermediate-term changes in CXCL4 may have predictive significance for long-term progression of SSc-ILD in patients receiving immunosuppressive therapy (54).

Lande et al. (55) recently found that CXCL4 forms liquid-crystalline complexes with human and bacterial DNA that amplify toll-like receptor 9 (TLR-9)-mediated interferon (IFN)-α production in SSc pDCs. In the same study, it was also shown that CXCL4-DNA complexes activate pDCs in a TLR-9-dependent manner but independent of canonical signaling function via CXCR3, a known CXCL4 receptor. Interestingly, CXCL4-DNA complexes were found to be present in vivo and to correlate with IFN type I (IFN-I) signature in the blood, and this correlation increased in early SSc. The findings suggested that disrupting CXCL4 adjuvant activity could represent a therapeutic opportunity in SSc (55).

**Composite biomarkers**

The heterogeneity and complexity of the disease warrant the development of complex and composite biomarkers that are able to capture multiple organ involvement and provide a better prediction because each plays a role in the summative outcome of interest.

**Enhanced liver fibrosis test**

One of the most recently studied composite biomarkers including extracellular matrix (ECM) constituents is the enhanced liver fibrosis (ELF) score, originally developed on chronic liver diseases and subsequently investigated in patients with SSc (56, 57). It is an algorithm including the serum concentrations of amino-terminal propeptide of procollagen type III (PIIINP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), and hyaluronic acid (HA) using specific immune assays developed for the test, performed on an automated platform. PIIINP is the amino-terminal peptide cleaved when collagen type III is formed. TIMP-1 is an inhibitor of matrix metalloproteinases such as interstitial collagenase, gelatinase, and stromelysin, involved in degrading components of the ECM. HA is a glycosaminoglycan of the ECM. In a first single-center study of 210 patients with SSc, ELF was found above normal value in 83% of patients with SSc (56). ELF score correlated with several measures of fibrosis and with overall disease activity, severity, and health assessment questionnaire disability index. In the multivariate analysis, skin and lung involvement were independently associated with the ELF score (56). These results were recently validated in an independent cross-sectional multicenter study including 247 patients with SSc from 6 rheumatology centers (57). Collectively, in a total of 457 patients with SSc enrolled in 7 different centers, ELF score and the single markers were significantly higher in patients with distinct fibrotic phenotype, i.e., dcSSc subset, severe skin involvement, fibrosis on chest high-resolution computed tomography (HRCT) scan, abnormal pulmonary function and DLCO, and they independently associated with skin and lung involvement. To date, ELF score has been validated as a biomarker of fibrosis in SSc only in cross-sectional studies. A longitudinal multicenter study is on-going to identify an SSc specific algorithm with predictive value for skin and lung progression.

**IFN-inducible chemokines**

Recently, research interest in biomarkers for patients stratification also focused on IFN-I pathway whose activation is associated with severe clinical manifestations in SSc. The IFN signature is the most prominent gene expression profile in the peripheral blood cells of patients with SSc and is observed from the earliest phases of the disease, even before overt skin fibrosis (58). IFN excess in SSc has been confirmed by IFN-inducible chemokines evaluation in serum and plasma samples.

Liu et al. (59) observed that patients with SSc had higher plasma levels of IFN-inducible chemokines than matched HC. IFN-inducible chemokine levels of IP-10/CXCL10 and I-TAC/ CXCL11 correlated with the IFN gene expression signature and the calculated score with severity of skin, lung, and skeletal muscle involvement. The IFN-inducible chemokine score was also associated with the absence of anti-RNAP III antibodies and presence of anti-U1 RNP antibodies, but not with disease duration, disease type, or other autoantibodies. The proposed IFN-inducible chemokine score, marking a more severe form of SSc, regardless of disease duration and clinical subtype, was, therefore, suggested as a useful marker for risk stratification of patients with SSc (59).

The serum levels of 6 IFN-inducible chemokines (IP-10/CXCL10, monokine induced by gamma interferon, MCP-2, beta-2-microglobulin, macrophage inflammatory protein-3 beta/CCL19, tumor necrosis factor receptor-2) were recently measured in 135 participants of SLSli and 45 unaffected controls matched for age, gender, and race in a ratio of 1:3 to SLS II participants. A serum IFN composite score was calculated on the basis of levels of these 6 chemokines. Higher serum IFN chemokine score in SSc-ILD predicted better response to immunosuppression with MMF and CYC and could be potentially useful to identify patients who may derive the most benefit from these 2 treatments (60).

Furthermore, baseline IFN transcript signature normalized with myeloblation followed by autologous stem cell transplantation in patients with SSc enrolled in the Short Course Oncology Therapy trial (61). Immunosuppression with CYC did not have similar effect. Similar trajecto-
ries were observed in serum protein composite scores. In addition, changes in the transcript signatures were correlated with improvement in the lung volumes and skin fibrosis (61).

Another recent study from our group aimed to determine whether the serum concentration of the IFN-inducible chemokines CCL2, CCL8, CCL19, CXCL9, CXCL10, and CXCL11 combined in an IFN score could be used to stratify patients with dcSSc for severe clinical outcome at 12 months (62). Our preliminary results showed that FN score was higher in 143 patients with SSC than in 35 HC, however, within the SSC group, there was no association with disease subset or duration. High serum IFN score predicted worse clinical outcome at 12 months in dcSSc as measured by the global rank composite score (63) and composite response index in dcSSc (64). We suggested that the proposed IFN score could aid stratification both in clinical trials design and clinical management (62).

**NT-proBNP and high-sensitivity cardiac troponin**

N-Terminal pro-hormone Brain Natriuretic Peptide is a useful biomarker for early diagnosis of PAH and for cardiac involvement in SSC as shown by several studies over the past decade (65-68), whereas more recent is the evidence that high-sensitivity troponin (hs-Tn) may play a role as a marker of cardiac disease in patients with SSC (69-71).

A prospective study identified as predictors of PAH in SSC decreased DLCO/VA ratio (<70%) and increased NT-proBNP suggesting that use of these markers should result in improved PAH risk stratification and allow earlier initiation of therapy (72).

In a large cohort of patients with SSC, NT-proBNP was shown to be an independent predictor of 3-years mortality in a multivariate analysis. Using 125-ng/L concentration as a threshold value, NT-proBNP reliably and independently predicted 3-year mortality, with a sensitivity of 78.1% and a negative predictive value of 97.6%, respectively (73).

More recently, another study showed that hs-TnT and NT-proBNP plasma concentrations were increased in patients with SSC, even in those free of cardiovascular risk factors. The authors found that combination of hs-TnT and NT-proBNP had high positive and negative predictive values for the diagnosis of precapillary PH (74).

Levels of hs-TnT and NT-proBNP were also assessed in a prospective cohort study aiming to define the role of 24h-ECG-Holter as an additional risk-stratification technique in the identification of patients with SSC at high risk of life-threatening arrhythmias and sudden cardiac death (69). The authors found that the number of ventricular ectopic beats (VEBs) correlated with hs-TnT and NT-proBNP levels. The study highlighted the prognostic importance of high hs-TnT levels and right bundle branch block on ECG as independent predictors of high number of VEBs. It was suggested that a careful clinical evaluation with assessment of hs-TnT plasma levels should represent the first step in the risk stratification for sudden cardiac death, considering the ability to detect patients with SSC at higher arrhythmic risk and, therefore, eligible for a comprehensive cardiac evaluations including 24h-ECG Holter (69).

It was also reported that hs-Tn shows a good correlation with echocardiography abnormalities (70) and might be a potential biomarker of subclinical cardiac involvement in patients with SSC (71).

Our group recently investigated cardiovascular biomarkers in the context of an implantable loop recorder (ILR) and contrast-enhanced cardiac magnetic resonance (CMR) study in patients with SSC and now known cardiovascular disease (75). Patients with SSC, who developed incidental significant arrhythmias over a 3-year follow-up had higher baseline hs-TnT and N-terminal pro-brain natriuretic peptide and CMR-extracellular volume (indicating diffuse fibrosis). The study showed an association of hs-Tn and NT-proBNP with the CMR measures of fibrosis and myocardial perfusion reserve. On the basis of the study results, we suggested that hs-Tn and NT-proBNP together with disease phenotype and CMR measures of fibrosis are useful biomarkers to identify at-risk patients that would benefit from ILR significant arrhythmia screening (75). Consistently with our preliminary analysis, we also showed that abnormal electrophysiological (EP) testing is associated with future incidental significant arrhythmia in patients with SSC and those with abnormal EP tests at baseline tended to have notably higher serum NT-proBNP and hs-Tn levels (76). Although indicating a subclinical cardiomyopathy, these biomarkers might have predictive utility in the identification of future clinical subclinical heart disease.

A recent follow-up study also investigated predictive significance of plasma hs-TnT and NT-proBNP levels in identifying patients with SSC with cardiac involvement and at higher risk of cardiac death (77). The authors found that patients with SSC with increased cardiac markers presented a lower left-ventricular ejec-

Collectively these results suggest that evaluation of hs-Tn and NT-proBNP levels may represent a useful screening test to detect patients with SSC non-invasively for early heart involvement and/or PAH, thus improving risk stratification.

**Imaging biomarkers**

More recently, researchers’ interest focused toward imaging techniques that can directly measure the organs and outcome of interest, particularly skin and lung. These include magnetic resonance imaging (MRI), ultrasound (US) of lung and skin, including elastasonography (ES) and, more recently, optical coherence tomography (OCT) (78).

**Magnetic resonance angiography**

In the recent years, the progress in MRI has delivered the implementation of the magnetic resonance angiography (MRA) both with and without the use of contrast material. A few studies have used MRA to evaluate vascular abnormalities in the hands of patients with SSC (79-83).

Allanore et al. (79) evaluated vascular abnormalities in the hands of patients with SSC, using MRA with gadolinium injection and, as primary criteria, distality and quality of arterial opacification, avascular areas, and venous return. The study showed diffuse lesions involving both arterial and venous vessels of small caliber as well as the microcirculation of the hands of patients with SSC.

Wang et al. (80) used the 2-dimensional (2D) time-of-flight (TOF) micro-MRA on a 3Tesla clinical MRI scanner and evaluated the data for the proper palmar digital artery lumen area, the number of visible dorsal digital veins, and a semi-quantitative vascular score, which evaluated the overall integrity of digital vessels. The SSC subjects had a significantly decreased digital artery lumen area, reduced number of digital veins, and lowered overall vascular score compared with healthy volunteers.

Zhang et al. (81) used 3D TOF MRA and studied the source images and maximum intensity projection reconstruction; they also measured the digital artery count and lumen area of the selec-
tive section of the vessel (from the native TOF images) and compared them with measurements of the control group. They observed a decrease in the lumen area in the SSc group compared with the control group. A 4-level grading system was made according to the severity.

Our group first developed a digital arterial volume index (DAVIX) to obtain a quantification of the arterial blood volume using no contrast 2D TOF MRA of digital arteries on a 3Tesla scanner (82). We showed that the index was significantly lower in patients with SSc than in HC and that fingers of patients with digital ulcers (DU) had a mean DAVIX lower than those without DU (82). We later determined its value in predicting the onset of DUs in 91 consecutive patients with SSc clinical diagnosis, 63 of them classified as SSc (12, 83). In patients without DUs at baseline, who developed new DUs within 12 months of follow-up, the DAVIX was 3-fold lower than in patients who did not develop DU. Most importantly in patients with no current DU, a DAVIX <0.47 gave a 35% risk of developing DUs. In this context, the predictive value of DAVIX for the future onset of DU could be usefully employed as a stratification tool in clinical trials and, if validated, in clinical practice (83).

Lung ultrasonography

A novel and recent application of imaging biomarkers for the assessment of ILD in SSc is the lung US (LUS). It includes detection and quantification of B-lines, which consist of "comet tails"—artifacts fanning out from the lung surface—generated by the reflection of the US beam from thickened sub-pleural interlobar septa detectable in between the lung intercostal spaces (84). As pointed out by the systematic literature review of the OMERACT US Group, papers published on this topic included observational, cross-sectional, and/or descriptive studies (84). The primary aim of all the studies was to determine the correlation between LUS and HRCT findings in detecting PF. All reported results demonstrated a positive correlation between LUS B-lines and HRCT in the assessment of ILD that, however, was not confirmed by a multivariate analysis. The OMERACT US Group also highlighted that reported US B-lines scoring systems, acquisition, used transducer, and protocols were different across the studies and a consensus should be reached to standardize the procedure (84). Despite the promising and growing evidence on the utility of LUS in ILD, validity of LUS in detecting ILD in the early stages, its accuracy to assess the response to the therapy, the correct timing of LUS for diagnosis and follow-up and its potential in monitoring the progression of ILD-SSc will need to be addressed in future studies.

Skin ultrasonography

Skin US offers a potential for objective and reliable assessment of skin involvement in SSc. A comprehensive systematic literature review on US application to the assessment of skin in SSc has been recently reported by Santiago et al. (85). The authors highlighted the limitations and heterogeneity of the published reports and the need of standardization of the technique with the modern US tools. Specifically, they reported that, in the selected studies on this topic, the most frequently used measurement was skin thickness followed by evaluation of echogenicity and/or stiffness and/or vascularity. The main comparator was the total and site-specific modified Rodnan skin score. They also highlighted that a few studies reported inter-rater and intra-rater reproducibility, which, however, appeared to be excellent (85).

Use of ultra-high frequency US (50 MHz) has been preliminarily reported in the evaluation of skin involvement in 21 patients with SSc and 6 HC by Naredo et al. (86). The authors showed that ultra-high frequency US allows a very detailed imaging of skin layers, a reliable measurement of dermal thickness, and a discriminative capacity between dermis and hypodermis texture features in SSc and healthy subjects (86). The promising results warrant certainly further investigation.

Within the US techniques, elastography (ES) deserves a separate mention. Strain ES allows the examination of the elastic properties of skin with a color scale superimposed on the gray scale image produced by the conventional US. The principle is that the excessive dermal deposition of collagenous and non-collagenous ECM causing fibrosis reduces skin elasticity (3). Thus, it examines the "deformability" of the tissue during its controlled compression with an ultrasonographic transducer. Specific color patterns of dermis have been identified in patients with SSc compared with healthy subjects (87).

A very promising elastographic technique, which may provide a quantitative and more operator independent assessment of dermal properties, is the shear-wave elastography (SWE). Specifically, SWE evaluates skin stiffness measuring the speed at which the transducer-generated wave is propagated across the examined tissue. The resulting shear-wave velocity was shown to be more accurate and reproducible than results obtained during strain ES (88). Recent cross-sectional studies demonstrated that SWE can discriminate between SSc and HC skin and, additionally, between clinically unaffected SSc skin and the skin of HC (89-92). The sensitivity to change over time of SWE has been demonstrated by a recent single-center longitudinal study in 21 SSc and 15 HC (93). If confirmed by independent and larger studies, SWE could be a useful monitoring biomarker of skin involvement in SSc.

Optical coherence tomography

OCT is an emerging non-invasive imaging technique in dermatology (94) and rheumatology, recently applied to nail psoriasis (95, 96) and SSc skin fibrosis (97, 98). It is similar in principle to US; however, it measures the intensity of backscattered near-infrared light rather than acoustic waves (3, 78). In a limited timeframe (few seconds), OCT can provide "virtual" biopsies of the examined sample with high-resolution images similar to the tissue architecture observed in routine histology (scan depth of 2 mm, axial resolution of 5-10 μm, and lateral resolution of 7.5 μm) (99). It enables visualization of the stratum corneum, epidermis (ED), upper dermis, appendages, and blood vessels (100).

OCT studies on various skin diseases have been conducted, though the technology has yet to be implemented as a standard procedure in clinical practice (101). Use of OCT technology for quantification of skin fibrosis is in the formative stages and a tremendous growth potential has been foreseen, similar to the US development paradigm that has evolved over the past 30 years (102).

Our group first used a Swept-Source OCT system to detect and quantify skin fibrosis in patients with SSc (97). In that cross-sectional study, 21 patients with SSc with different severities of skin involvement, 1 patient with morphea, and 22 HC were included in the study and evaluated at the forearm skin. In healthy skin, the ED appeared as a hypo-reflective layer compared with the underlying papillary dermic (PD). The different reflective properties allowed the easy identification of dermal-epidermal junction (DEJ). The reticular dermis (RD) presented as a hypo-reflective region, below the PD. Blood vessels were visible in PD and RD as signal-poor cavities. In severely involved SSc skin, the ED appeared less hypo-reflective than the normal skin, visualization of the DEJ was difficult, and there was no clear distinction of PD and RD. Only rare vessels were visualized in comparison with normal skin. Comparison of OCT images with corresponding skin histology indicated a progressive loss of visualization of the DEJ associated with dermal fibrosis. Furthermore, SSc-affected skin showed a consistent decrease of optical density (OD) in the PD, progressively worse in patients with more severe modified Rodnan skin score (mRSS). In addition, clinically unaffected skin
was also distinguishable from healthy skin for its specific pattern of OD decrease in the RD. OCT analysis of affected and unaffected skin in a patient with plaque morphea showed a similar pattern of severe SSc and HC, respectively. The study also evaluated intra- and inter-observer reliability of the technique, which was excellent (97). These preliminary findings were confirmed by Pires et al. (98) who examined proximal third finger and dorsal forearm skin of 33 patients with SSc and 35 HC using a Swept-Source OCT of different brand. Stratifying patients according to the mRSS, they showed a difference across groups with different severities of skin involvement, more significant between HC or mild skin fibrosis and severe skin fibrosis groups. They concluded that skin appearance of patients with SSc in OCT images is clearly different from HC, and OCT can provide a unique perspective for objectively assessing skin fibrosis.

In a multicenter study on 87 subjects including 43 SSc and 44 matched HC, our group recently found that OCT measures demonstrated discriminative ability in the detection of SSc skin regardless of the severity of skin involvement and, more importantly, of clinically unaffected SSc skin as compared with HC. These results are consistent with gene array data showing scleroderma specific signatures in affected and clinically unaffected skin. These preliminary data, if validated, will inform future studies on at-risk patients with clinically unaffected skin, which may define a role for OCT in detecting subclinical SSc (103).

Because of the recent and still limited application of OCT in a few studies in SSc, there are currently several limitations to its applicability, ranging from the unavailability of the machine in most centers to the lack of standardization of the sites to assess. Evidence of the sensitivity to change over time is emerging from preliminary results of observational follow-up studies showing that, over a 24-month follow-up period, the changes of OD assessed by OCT are consistent with changes of mRSS (104). However, the technique, despite promising preliminary evidences, is still awaiting definitive validation as a tool to monitor skin changes and/or to stratify patients at risk of developing severe fibrotic disease and/or to assess response to therapy. If validated in future longitudinal and multicenter studies, OCT would be an ideal biomarker in clinical trials to assess response to therapy, as images stored in each center could undergo centralized analysis allowing a quantification of skin involvement with an excellent reliability (97).

**Conclusion**

SSc is one of the rheumatologic conditions with highest morbidity and still orphan of validated prognostic and predictive biomarkers. One of the major difficulties in the design of intervention trials in SSc is the lack of a validated, reliable, and feasible measure of response to therapy and the poor ability of identifying patients who will develop a severe clinical outcome. Although fibrotic involvement of the skin carries the major prognostic value and it is often the primary outcome of intervention trials, the implementation of a specific algorithm and/or composite score able to reflect disease as a whole is one of the major unmet need in the field and, to this aim, serum and imaging biomarkers are invaluable tools. International collaborations carry the potential of identifying key strategies for stratification useful both for clinical management and design of clinical trials.

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