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## Biomarkers in systemic sclerosis.

Susan V. Castro  
*Thomas Jefferson University*

Sergio A. Jimenez  
*Thomas Jefferson University*

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Susan V. Castro, Ph.D. and Sergio A. Jimenez, M.D.  
Jefferson Institute of Molecular Medicine  
Thomas Jefferson University  
Philadelphia, PA 19107, USA

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**Address all correspondence to:**

Sergio A. Jimenez, M.D.  
Jefferson Institute of Molecular Medicine  
Thomas Jefferson University  
233 S. 10th Street, Room 509 BLSB  
Philadelphia, PA 19107-5541  
Phone: 215-503-5042 / Fax: 215-923-4649  
E-mail: sergio.jimenez@jefferson.edu

**Summary**

Systemic Sclerosis (SSc) is an autoimmune inflammatory disorder of unknown etiology characterized by severe and often progressive cutaneous and visceral fibrosis, pronounced alterations in the microvasculature and frequent cellular and humoral immunity abnormalities, culminating in a severe and progressive fibrotic process. Numerous biomarkers reflecting the three main pathogenetic mechanisms in SSc have been described, however, aside from several disease-specific autoantibodies other biomarkers have not been thoroughly validated and they would need further study. Thus, there is an unmet need for validated biomarkers for diagnosis, disease classification, and evaluation of organ involvement and therapeutic response in SSc.

## Introduction

Systemic Sclerosis (SSc) is an autoimmune inflammatory disorder of unknown etiology characterized by severe and often progressive cutaneous and visceral fibrosis, pronounced alterations in the microvasculature and frequent cellular and humoral immunity abnormalities [1-4]. Clinically, SSc is heterogeneous, ranging from skin sclerosis confined to the fingers, face and/or distal parts of the extremities (sclerodactyly or acrosclerosis) with limited internal organ involvement, to diffuse skin involvement and severe fibrosis of multiple internal organs, and occasionally a fulminant course with rapid development of vital organ failure and a lethal outcome (fulminant SSc) [5]. The most apparent and almost universal clinical features of SSc are related to the progressive fibrosis of the skin, the microvasculature, and numerous internal organs. Morbidity and mortality in SSc are high and are related to the extent of the fibrotic and microvascular alterations. The extent and rate of progression of tissue fibrosis is of paramount importance in determining the clinical features and the prognosis of SSc. Indeed, fibrosis of the skin correlates with both survival and functional limitations [5-7].

The etiology of SSc is not known, however, it is currently accepted that the disease results from complex interactions between one or more environmental factors and a genetic predisposition in the host [1-4]. These genetics-environmental interactions eventually result in the development of generalized and often progressive skin and tissue fibrosis accompanied by a severe fibroproliferative/occlusive vasculopathy and by prominent abnormalities in cellular and humoral immunity with the occurrence of chronic inflammatory cell infiltration, derangement of cytokine and growth factor functional balance and development of numerous autoantibodies as illustrated in **Figure 1** [3,4,8]. At present, it is not clear which of these components of SSc pathogenesis is of primary importance or how they interrelate to cause the progressive fibrotic process. However, numerous recent studies have suggested that there is a sequence of pathogenetic events initiated by unknown etiologic factors that trigger microvascular injury with prominent structural and functional endothelial cell abnormalities which result in progressive fibroproliferative vasculopathy and vessel rarefaction [9-11]. The endothelial dysfunction also leads to the attraction of specific cellular elements from the bloodstream and bone marrow and their transmigration into the surrounding tissue, leading to the establishment of a chronic inflammatory process with participation of macrophages and T- and B- lymphocytes and the secretion and release of a variety of cytokines and growth factors from these cells. This sequence of events, diagrammatically illustrated in **Figure 2**, culminates in the development of a severe and progressive fibrotic process and in the production of disease-specific autoantibodies. The remarkable progress in the understanding of numerous basic mechanisms involved in the complex pathogenesis of SSc has opened new avenues for the development of novel and effective therapeutic approaches. At the same time, it has become apparent that there is an unmet need for validated biomarkers that can be used for diagnosis, disease classification, identification of organ involvement, and evaluation of therapeutic response in SSc.

## Biomarkers in Systemic Sclerosis

The NIH Biomarkers Definitions Working Group was convened by the NIH Director's initiative on Biomarkers and Surrogate Endpoints. The expert working group provided definitions and identified the characteristics and requirements of biological measurements to be employed for the development and assessment of human therapeutics. A "Biological Marker" or "Biomarker" was defined as a characteristic that is *OBJECTIVELY* measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a

therapeutic intervention. Other important characteristics of a biomarker include the following: it should reflect the underlying biologic process being evaluated; should allow the prediction of clinical course or prognosis of a disease process; should be sensitive to therapeutic effects; should be easily obtainable, preferably by non-invasive means; and should eventually be validated in clinical studies.

Although there has been extensive interest in the development of outcome measures for SSc [12-16], biomarkers that allow early diagnosis and assessment of disease activity or that carry a predictive prognostic value are not available for SSc. The clinical semi-quantitative assessment of skin thickness (modified Rodnan skin score or mRSS) is currently the gold standard and the only outcome measure used in clinical trials of SSc disease modifying agents. The original description of the method showed that the score correlated with skin biopsy sample weight and, thus, it was assumed to be a reflection of the fibrotic process causing skin induration and thickening. Although non-invasive and cost effective, the mRSS entails several shortcomings, ranging from the subjectivity of skin palpation assessments to the difficulty of scoring borderline changes in skin involvement. Furthermore, it is not possible to differentiate fibrotic skin thickening from that resulting from tissue edema, inflammation, vascular bed engorgement or skin tethering. It is, therefore, generally accepted that the development of objective and reliable markers reflecting the severity of tissue fibrosis would be of invaluable help in determining the efficacy of a given treatment in clinical trials, both by allowing a reduction in the number of patients needed for the studies to achieve statistical power and by offering an objective and quantitative method independent of the subjective assessment of the investigators involved in the study.

In contrast to the remarkable progress with biomarkers for cancer and cardiovascular disease [17-20], there are very few validated biomarkers for the assessment of SSc disease activity and for clinical subset disease classification, and their utility has not been extensively tested or validated in clinical trials [21]. Most importantly, there is an important need to develop, test, and validate accurate and objective measures of tissue fibrosis and vasculopathy in SSc and markers that may reflect a therapeutic response of the disease process for use in clinical trials.

Biomarkers for SSc can be grouped based on their ability to assist in SSc diagnosis (“diagnostic biomarkers”), to determine distinct clinical subsets which may have specific patterns of organ involvement or evolution (“clinical subset biomarkers”), to predict specific organ involvement or specific clinical manifestation such as for example tissue fibrosis (“fibrosis biomarker”) or vascular alterations (“vascular biomarker”), and to assess disease activity which may allow prediction of the clinical course or mortality (“prognostic biomarker”) or determination of the effectiveness of a therapeutic intervention (“therapeutic response biomarker”). This latter group of biomarkers are also often utilized as endpoints in clinical trials of potential treatments or interventions.

### **Autoantibodies as Systemic Sclerosis Diagnostic Biomarkers**

At present there are no specific diagnostic tests for SSc and the disorder is diagnosed primarily based on the collective appearance of a cluster of clinical symptoms such as, for example, Raynaud’s phenomenon, telangiectasias, esophageal dysfunction with gastro-esophageal reflux, characteristic pigmentary changes, presence of digital ulcers, or calcinotic lesions accompanying clinically detectable skin induration. Indeed, the diagnostic criteria commonly employed for classification of SSc is entirely based on clinical manifestations and

does not include any measurable serologic or laboratory parameters. However, it is well recognized that the presence of specific autoantibodies is one of the most common manifestations of SSc and greater than 90% of SSc patients harbor antinuclear antibodies in their serum [22-25]. Numerous autoantibodies have been described in SSc patients (**Table 1**). Some of these are highly specific for SSc including anti-Scl-70 and anti-centromere antibodies and have been, therefore, used as Diagnostic Biomarkers to support or confirm the clinical diagnosis of SSc. Anti-Scl-70 antibodies are directed against DNA topoisomerase I and are almost exclusively present in sera from patients with the diffuse form of SSc [26,27], and they correlate with the development of severe interstitial lung disease. Anti-centromere antibodies recognize several protein components of the tri-laminar kinetochore [28]. These antibodies are usually present in patients with the limited form of SSc and are found in 45-50% of these patients. In contrast to anti-Scl-70 antibodies, anti-centromere antibodies are only found in about 10% of patients with diffuse SSc. These two autoantibodies are mutually exclusive, co-existing in the same patient only in rare instances.

There are numerous other autoantibodies less commonly present in SSc patients, including anti-RNA polymerases I and III antibodies in patients with rapidly progressive diffuse disease and high frequency of SSc renal crisis, anti-fibrillarin antibodies commonly found in diffuse SSc, and anti-PM-Scl antibodies that are often present in patients with a polymyositis/SSc overlap syndrome [29-31].

### **Biomarkers for Clinical Disease Subset Classification**

It has long been recognized that there are at least two distinct clinical subsets of SSc differing in their clinical presentation and evolution, but most importantly, with clearly different outcomes regarding frequency and severity of organ involvement as well as overall mortality [5,32-34]. The extent of cutaneous sclerotic involvement has been found to accurately distinguish the two clinical subsets in the majority of cases. The first subset is characterized by diffuse cutaneous involvement frequently including the thighs, abdomen and chest, associated with a progressive course, frequent and severe visceral organ involvement occurring in the early stages of disease evolution including development of SSc renal crisis and pulmonary fibrosis and high SSc-related mortality. In contrast, in the second subset there is limited cutaneous involvement confined to the acral parts of the extremities and the face, and usually displays a more prolonged and protracted evolution, lesser severity of visceral organ involvement except for the relatively common occurrence of Pulmonary Arterial Hypertension (PAH) at a late stage of the disease, and a more benign prognosis with prolonged overall survival. When fully established the two clinical SSc subsets display clearly distinguishable patterns of cutaneous involvement, however, in early stages of presentation their manifestations often overlap. Thus, there is a substantial need for biomarkers that may allow the accurate identification of the clinical SSc subset at early stages of disease. Although currently there are no specific biomarkers to separate these two clinical subsets, the pattern of antinuclear autoantibodies present in the sera of affected individuals can be considered as biomarkers of the pattern of disease subset; anticentromere antibodies are almost exclusively present in the limited SSc subset, whereas Scl-70, anti RNA polymerase I and III, and anti-fibrillarin antibodies are almost exclusively associated with the diffuse form of SSc [22-31].

### **Biomarkers of Endothelial Cell Dysfunction**

Vascular dysfunction is considered to be one of the earliest clinical manifestations of SSc and it has been suggested to be a crucial initiating event in SSc pathogenesis [9-11,35-38] as illustrated in **Figure 2**. Endothelial injury leads to pronounced vascular fibroproliferative lesions in multiple organs, however, the effects of vascular dysfunction are most dramatic when they involve the pulmonary and renal arterioles, causing renal crisis and PAH, respectively, the two most prevalent causes of morbidity and mortality in patients with SSc.

Since the pioneering studies of Kahaleh and LeRoy, focusing attention on the important role of endothelial cells in SSc pathogenesis and their original demonstration that specific endothelial cell proteins such as the von Willebrand factor (vWf) are abnormally elevated in the sera of patients with SSc [36], there has been intense investigation and numerous studies have described potentially important biomarkers that may provide information about the functional status of endothelial cells and their dysfunction in SSc [39-43]. In the original study of Kahaleh [36], vWf was found elevated in the plasma of patients with SSc and patients with Raynaud's phenomenon, in comparison with normal controls. These studies have been confirmed subsequently, and it has been suggested that this biomarker correlates with the severity of SSc [39] and with the presence of pulmonary involvement [44] and the extent of radiologically demonstrated interstitial lung disease [45]. Of interest was the observation that ADAMTS-13, an enzyme that is involved in the cleavage and processing of vWf, was found to be reduced in patients with SSc, suggesting that measurements of the activity of this enzyme may represent a biomarker of vascular involvement or endothelial cell dysfunction in patients with the disorder [46].

Numerous other molecules involved in different aspects of the pathogenesis of endothelial dysfunction in SSc have also been suggested as potential biomarkers for endothelial perturbations in the disorder. Among these are circulating levels of adhesion molecules, thrombospondin, thrombomodulin, endothelin-1, the N-terminal pro-peptide of the brain natriuretic peptide (NT-pro-BNP), vascular endothelial growth factor (VEGF), endostatin, plasminogen activator, and metabolites of the arachidonic acid cascade such as prostacyclin and thromboxane or nitrous oxide circulating metabolites.

Endothelin-1 (ET-1), is a 21-amino acid polypeptide produced by endothelial cells capable of potent vasoconstrictive activity and the ability to stimulate proliferation of smooth muscle cells. Numerous studies have conclusively demonstrated that ET-1 and its specific cellular receptors play a crucial role in the proliferative vasculopathy of SSc, in particular, in the vascular alterations of SSc-associated PAH [47-50]. Thus, there has been intense interest in ET-1 measurements as a biomarker of SSc vasculopathy. Serum ET-1 levels have been found to be elevated in plasma of SSc patients and to increase following cold exposure and triggering of Raynaud's phenomenon. Elevated ET-1 levels correlated with other indicators of endothelial cell activation such as increased vWf, as well as with the levels of other endothelial cell proteins such as thrombomodulin and adhesion molecules including soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1). Furthermore, immunohistochemistry studies demonstrated the presence of elevated expression of endothelin-1 and endothelin receptors in pulmonary parenchyma at early stages of development of interstitial lung disease and fibrosing alveolitis of SSc [51], suggesting that ET-1 measurements may not only reflect crucial alterations in endothelial cell function involved in the pathogenesis of PAH

but may also be indicators of the profibrotic activity responsible for the exaggerated production of connective tissue macromolecules characteristic of the disease.

Adhesion molecules involved in cell-cell interactions and cell-extracellular matrix interactions are also important in the pathogenesis of the earlier stages of vascular alterations in SSc and have been suggested as potential biomarkers for SSc vasculopathy. Increased expression of endothelial leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), e-selectin and p-selectin has been found in affected skin from SSc patients with higher levels present in samples from the diffuse form of the disease, indicating that these proteins may participate in the early stages of tissue fibrosis as well. Elevated serum levels of these adhesion molecules have been found in SSc patients compared to normal individuals [52-54] and other studies demonstrated that these levels correlated with increased severity and extent of visceral organ involvement in the disease [55].

Numerous recent studies have also shown that in addition to functional abnormalities in endothelial cells in SSc, there might be abnormalities in angiogenesis and endothelial repair. The rarefaction of small capillaries with a reduction in capillary density in affected SSc tissues is consistent with abnormal and disordered angiogenesis. Therefore, markers that may reflect the angiogenesis process have been suggested as important in the evaluation of vascular alterations in SSc [56]. One of the key mediators of angiogenesis, VEGF, has been studied extensively as a potential biomarker for the vascular abnormalities in SSc [41,42,56-58]. Indeed, high VEGF levels have been found in patients with early SSc and these levels correlated with the presence of pulmonary fibrosis and abnormalities in pulmonary function including reductions in vital capacity and DLCO. High levels of VEGF were also found to correlate with shorter disease duration as well as with aggressive and rapidly progressive diffuse cutaneous SSc, although other studies failed to show such a correlation [59].

### **Biomarkers of Pulmonary Hypertension**

Pulmonary artery hypertension (PAH) has recently emerged as one of the most important and serious clinical problems in patients with SSc [60,61]. Although PAH is not the most common pulmonary involvement in SSc patients, it frequently leads to severe respiratory disability and often to a fatal outcome with a high mortality. In most instances the clinical course of untreated PAH is one of rapid progression leading to respiratory failure or to death within two to three years after it becomes clinically detectable. Currently, owing to the remarkable reduction in mortality from SSc renal crisis, it is apparent that PAH has become one of the leading causes of mortality in this disease [60,62]. PAH in patients with SSc can occur as a sequelae to interstitial lung disease, although often it develops as a late manifestation in patients with the limited cutaneous form of SSc in the absence of pulmonary fibrosis. There are no currently validated laboratory tests or serologic markers that can provide a specific diagnostic for PAH. However, given the important role that ET-1 plays in the pathogenesis of PAH and the remarkable clinical effects and survival improvement resulting from the therapeutic use of endothelin-1 receptor blockade, measurements of circulating ET-1 levels were examined as possible biomarkers for SSc-related PAH [50,51] and a more recent study showed that ET-1 plasma levels were significantly higher in SSc patients with PAH and with positive anticentromere antibodies. Furthermore, there was a positive linear correlation between these levels and systolic pulmonary artery pressure [63]. Thus, it was suggested that ET-1 plasma levels may be a biomarker for detection and monitoring of PAH in SSc. Recent interest has also



been focused on the measurement of plasma levels of NT-pro-BNP. Although the plasma levels of this peptide reflect myocardial responses to various stimuli such as mechanical stretch or hypoxia and are not specific for PAH, recent studies showed that plasma NT-pro-BNP determinations may predict prospectively the development of clinical PAH, may also be indicative of survival, and may represent an accurate surrogate marker to follow the response and evaluate the effects of therapeutic agents for SSc related PAH [64-68].

### **Biomarkers of Pulmonary Fibrosis**

In recent years, interstitial lung disease associated with SSc has become the leading cause of morbidity and mortality in the disease [62]. Thus, the search for biomarkers that may predict the development of pulmonary fibrosis and/or correlate with the clinical course and clinical response to potential therapeutic agents has become an important goal and numerous studies have been performed to identify such biomarkers [70,71]. Substantial interest has been placed on proteins that are synthesized, produced and secreted by type II alveolar epithelial cells. Some of these proteins appear to predict the progression of interstitial lung involvement and may also represent early markers indicative of the development of this complication. Three proteins that appear to be specific for pulmonary involvement are the Krebs von den Lungen 6 antigen (KL-6) and pulmonary surfactants A and D (PS-A and -D). Several studies have demonstrated that serum levels of KL-6 as measured by ELISA are substantially higher in SSc patients than in normal individuals [72-74]. Furthermore, these levels were substantially higher in patients with pulmonary fibrosis compared to patients without lung involvement. An important study performed a longitudinal evaluation of KL-6 levels in sera of a large cohort of SSc patients. The results showed a marked elevation of KL-6 levels, which occurred in close temporal association with the clinical diagnosis of pulmonary fibrosis, particularly in patients with positive anti-topoisomerase-1 antibodies [75]. Measurements of PS-A and -D were also performed in a cohort of Japanese patients with SSc and it was found that both surfactant-related proteins were significantly elevated in patients with a diagnosis of interstitial lung disease [76]. The same study showed that the sensitivity for the diagnosis and identification of pulmonary fibrosis was higher for the PS-D isoform than for the PS-A isoform. In contrast, the specificity for PS-A was higher (100% compared to 83%) than the specificity for PS-D. A correlation with functional abnormalities was also established and an important study showed that the levels of PS-D displayed a negative correlation with vital capacity and diffusion capacity for carbon monoxide [71]. Comparative studies of KL-6 and PS-D demonstrated that there was a positive correlation between the levels of both proteins and there was similarity as well in their sensitivity and specificity for the diagnosis of interstitial lung disease [77]. A recent study of the cohort included in the Scleroderma Lung Study examined the baseline levels of PS-D and KL-6 in patients with or without alveolitis as defined by high resolution computerized tomography and bronchioalveolar lavage and found that SSc patients overall had higher values for both proteins than normal individuals. Furthermore, significant differences were found in these levels in SSc patients with alveolitis compared to those without alveolitis [78].

Another potential marker of pulmonary involvement is the pulmonary and activation regulated chemokine (PARC). This chemokine is also known as CCL-18 and it has been found to be elevated in patients with SSc was associated with the development of pulmonary fibrosis as well as with reductions in vital capacity and diffusion capacity and correlated closely with the activity of inflammatory changes in the lungs [79]. Furthermore, PARC levels in bronchioalveolar lavage from SSc patients with active alveolitis correlated with the presence of

an inflammatory process in the lungs as well as with the number and type of inflammatory cells [80].

A recently identified glycoprotein belonging to the chitinase family, YKL-40, has also been suggested as a potential marker for pulmonary involvement in SSc [81,82]. Recent studies suggested that levels of YKL-40 may correlate with tissue remodeling and, therefore, may be of value in the assessment of the pulmonary fibrotic process associated with SSc. Although earlier results were encouraging, elevated levels of the protein have been found in a variety of clinical conditions including liver fibrosis and numerous malignancies, therefore, the utility of YKL-40 measurements for SSc associated pulmonary fibrosis needs further evaluation.

### **Biomarkers of cellular immune system and cytokine alterations**

The presence of mononuclear cell infiltrates in affected skin and visceral organs from SSc patients has long been recognized. Early in the presentation of SSc, biopsies from affected skin show prominent infiltration with activated macrophages and T- and B-cell lymphocytes [83]. Further expansion of T-cells within the affected tissues appears to be oligoclonal as shown in studies of T-cell receptor transcripts in SSc skin [84]. The expanded T-cell populations in affected SSc tissues release numerous cytokines, chemokines and growth factors which initiate and/or perpetuate the fibrotic process as well as the endothelial and vascular alterations. Important effects of these released soluble products include modulation of fibroblast proliferation and induction of expression of a myofibroblast phenotype with the acquisition of motile cell features, expression of  $\alpha$  smooth muscle actin, and marked increase in their levels of collagen production. These cytokines and growth factors also exert potent effects on vascular wall cells which result in the development of the typical fibroproliferative/occlusive vasculopathy including stimulation of proliferation of smooth muscle cells in the media and modification of numerous endothelial cell functions.

Given the crucial role that inflammatory cells and the chronic inflammatory process play in the pathogenesis of various aspects of SSc, extensive efforts have been devoted to identify biomarkers that may reflect the cytokine, chemokine, and growth factor alterations in the disease. The soluble receptor for interleukin 2 (srIL-2) was one of the earliest identified biomarkers reflecting inflammatory and immunologic activation in SSc. Several studies demonstrated a close correlation of serum levels of srIL-2 with clinical and disease activity in patients with SSc [85-87]. In one of these studies the levels of srIL-2 correlated inversely with the duration of SSc, being more elevated in patients with recent onset and rapidly progressive forms of the disorder [85]. The values also correlated with the severity and extent of skin sclerosis, as assessed by the modified Rodnan skin score. The serum levels of the soluble receptor for TNF- $\alpha$  (srTNF- $\alpha$ ) have also been proposed as indicators of activity of the immunologic process in SSc and significant correlations with the severity of SSc and the presence of pulmonary involvement have been described [88,89]. However, subsequent studies have not confirmed that the serum levels of srTNF- $\alpha$  are useful indicators of the severity or of the rate of progression of the disease and additional studies are required to further evaluate the validity of srTNF- $\alpha$  serum levels as indicators of the ongoing inflammatory and immune dysfunction in SSc.

A recent study measured the levels of the chemokine CCL-2 in a large cohort of SSc patients and found higher levels in both diffuse and limited cutaneous SSc clinical subsets, although marked CCL-2 elevations were associated with anti-topoisomerase or anti-RNA polymerase I/III antibodies and with greater frequency of pulmonary and cardiac involvement [90]. Another study examined CCL-2 and CXCL-10 longitudinally and found that CXCL-10

levels were substantially elevated in newly diagnosed SSc and the highest values were associated with more severe clinical manifestations including pulmonary and kidney involvement [91]. The longitudinal study demonstrated a reduction in CXCL-10 with stable levels of CCL-2, suggesting a temporal switch from a Th1 to a Th2 stage [91]. The value of these two chemokines as biomarkers for SSc, however, must be tempered by recent studies which showed elevated levels in other disorders including autoimmune thyroiditis, hepatitis C infection and psoriatic arthritis. Numerous other cytokines, chemokines and regulatory proteins that have been considered to be important participants in the immune activation in SSc have been suggested as potential biomarkers including CD-40, CCL-2, IL-15, IL-23, BAFF, FAS, and others [92-97]. However, further studies to validate their sensitivity and specificity and to confirm their potential usefulness as biomarkers of this process will be required.

### **Specific Biomarkers for Fibrosis**

Tissue fibrosis is the hallmark of SSc and is responsible for most of its clinical manifestations. The extent and severity of tissue fibrosis correlate with prognosis and mortality in SSc. Thus, there is an unmet need for reliable and accurate biomarkers that reflect the fibrotic process in SSc. Since the discovery of the potent profibrotic and immunomodulatory activities of TGF- $\beta$ , this pleotropic growth factor has been considered a crucial participant in the pathogenesis of the fibrotic process in SSc and other fibroproliferative diseases [98-100]. One of the most important effects of TGF- $\beta$  is the stimulation of synthesis and production of numerous extracellular matrix (ECM) molecules involved in tissue fibrosis. TGF- $\beta$  also decreases the synthesis of collagen-degrading metalloproteinases and stimulates the production of protease inhibitors such as tissue inhibitors of metalloproteinases-1 (TIMP-1). TGF- $\beta$  also induces tissue resident fibroblasts to change their differentiated phenotype and become myofibroblasts, activated cells capable of producing elevated levels of ECM macromolecules and expressing  $\alpha$ -smooth muscle actin. In addition to TGF- $\beta$ , numerous studies have shown that connective tissue growth factor (CTGF) also plays a crucial role in tissue fibrosis owing to its potent profibrotic effects [101]. TGF- $\beta$  stimulates CTGF synthesis in fibroblasts, vascular smooth muscle cells and endothelial cells. CTGF also appears to have an autocrine function stimulating its own production and, thus, maintaining a continuous or prolonged cycle of excessive scarring and fibrosis.

The crucial role of TGF- $\beta$  and CTGF in tissue fibrosis suggests that measurements of their serum levels may reflect the activity of the fibrotic process. However, a recent study measuring TGF- $\beta$  levels in sera from patients with diffuse SSc in comparison with sera from patients with limited SSc and normal controls failed to show a correlation with the mRSS, although the values were lower in patients with diffuse SSc than in patients with limited SSc, and were even lower than in normal individuals [102]. Few studies have performed measurements of CTGF although it appears that a circulating peptide containing the N-terminal region of CTGF may be of value as a biomarker of tissue fibrosis [103]. Thus, extensive studies will be required to validate whether measurements of serum levels of these growth factors may be useful biomarkers for the process of fibrosis in the disease.

The increased expression of the genes encoding interstitial collagens types I and III and the marked elevation of the production of the corresponding proteins in SSc led to numerous studies investigating circulating or urinary levels of collagen molecules or collagen fragments as biomarkers that may reflect the activity of the ongoing fibrotic process [104-109]. The serum levels of the telopeptide corresponding to the crosslinked carboxy-terminal end of type I collagen

as well as the amino-terminal propeptide of type I procollagen have been given substantial attention since they reflect the degradation and synthesis of type I collagen, respectively. In one study the levels of the crosslinked carboxy-terminal telopeptide were elevated in greater than 80% of patients with SSc and displayed a positive correlation with the extent of skin involvement [104]. The measurements of the type I procollagen peptide, however, did not discriminate between normal individuals and patients with SSc in this study. A more recent study [105] showed that approximately 50% of SSc patients had increased serum levels of the carboxy-terminal telopeptide of type I collagen and that these levels correlated with the mRSS and were higher in patients with diffuse cutaneous involvement. The measurements of the amino-terminal type III collagen propeptides appear to be more reflective of the activity of the fibrotic process and substantially elevated levels are found in patients with SSc compared to controls and in patients with diffuse cutaneous SSc and increasing clinical activity [106,107]. Elevated levels of type III procollagen peptides also correlated with pulmonary involvement, reduction in vital capacity and diffusion capacity, as well as with the extent of cutaneous involvement [107,108], and were an independent predictor of a poor prognosis and an unfavorable survival [109]. Although the measurements of metabolites derived from the biosynthesis and degradation of types I and III collagens are very likely a reflection of the fibrotic process, owing to the fact that most of the type I collagen in the body is present in bone, metabolites derived from this molecule would reflect, to a large extent, the remodeling and degradation of type I collagen in the skeletal system. In contrast, the levels of type III collagen metabolites may be more reflective of a fibrotic process, particularly at the earlier stages of the disease since it has been generally accepted that type III collagen synthesis is disproportionately increased at the initiation of tissue fibrosis.

Another protein that has been suggested as a potential biomarker to reflect the fibrotic process in SSc is the cartilage oligomeric matrix protein (COMP), which is also an important fibroblast product. Indeed, some studies have measured serum levels of COMP and described significant correlations with the extent of skin involvement and with the severity of SSc [110]. Increased expression of COMP was also demonstrated in skin samples from SSc patients as well as in fibroblasts cultured from these samples [111,112]. However, more extensive studies would be required to confirm the validity of COMP as a marker of tissue fibrosis in SSc.

### **Analysis of Gene Expression Employing Microarrays**

The recent development of high throughput gene expression profiling technologies such as cDNA microarrays, combined with advanced computational approaches, have provided basic and clinical investigators with the ability to identify and characterize high-resolution expression profiles of numerous disease states and to dissect molecular networks that underlie specific disease phenotypes. Within a few years following their introduction, microarrays are now routinely used in almost every line of biomedical research with the most impressive examples of the successful utilization of this technology in cancer research. In the field of SSc research, the application of microarray technology holds the promise that it may allow the identification of molecular signatures specific for SSc, which could provide clues to the elucidation of the pathogenetic mechanisms involved or responsible for the disease as well as valuable molecular signatures that can be used as biomarkers of utility as diagnostic or prognostic tools and as markers of the effectiveness of disease modifying therapies. Indeed, recent microarray studies of intact skin, peripheral blood mononuclear cells or cultured dermal fibroblasts disclosed distinct patterns of gene expression capable of distinguishing patients with limited SSc from those with

diffuse SSc and allowing the identification of separate subsets within these two groups that correlate with various clinical parameters and internal organ involvement [113-118]. Microarray studies have also been employed to identify specific patterns of gene expression in SSc associated pulmonary fibrosis [119] and pulmonary hypertension [120] and have identified a subset of SSc patients who display a TGF- $\beta$  signature in their skin [121]. Thus, global gene expression studies promise to provide molecular signatures which will be useful as molecular biomarkers for the diagnosis of SSc, identification of its clinical subsets, evaluation of effectiveness of disease-modifying therapies, and to stratify patients who may respond and benefit from specific therapies, as shown recently for imatinib mesylate [121,122].

### **Use of Proteomics to Identify Biomarkers**

The field of proteomics is defined as the study of the entire complement of proteins (proteome) present or produced by a cell or organism employing large scale separation and identification. Proteomic studies related to human diseases attempt to assess and identify qualitative and quantitative protein differences between healthy and diseased cells. The remarkable technical advances and instrumentation development achieved in the last decade have been successfully applied to numerous biological fields including the discovery of biomarkers. However, proteomics studies in SSc have been very limited and the few studies available have mainly focused on the understanding of pathophysiologic events rather than in the identification of disease biomarkers [123,124].

### **Future Perspective**

The diagnosis of SSc at an early stage prior to the occurrence of obvious cutaneous fibrosis is a challenging task. The assessment and unequivocal assignment of SSc clinical subset (i.e.: diffuse cutaneous versus limited cutaneous) and the evaluation of clinical effectiveness of therapeutic interventions is of great relevance to patient management. The ability to accurately separate SSc patients with rapid progression from those with slow progression and to estimate the prognosis of the disease would also be of remarkable clinical and therapeutic value. Although there has been substantial effort devoted to the development and identification of useful biomarkers for SSc we are still very far from reaching the full potential of biomarker research for SSc. The application of novel genomic, global gene expression, and proteomic approaches opens up new and promising opportunities to approach this lofty goal.

## **Executive Summary**

- Despite extensive studies to develop outcome measures for SSc, fully validated biomarkers that allow early diagnosis and assessment of disease activity or that carry a predictive prognostic value are not available.

### **Autoantibodies as Systemic Sclerosis Diagnostic Biomarkers**

- Numerous circulating autoantibodies highly specific for SSc, e.g., anti-Scl-70 and anti-centromere antibodies are used as diagnostic biomarkers to support or confirm the clinical diagnosis of SSc.

### **Biomarkers for Clinical Disease Subset Classification**

- Some autoantibodies are biomarkers of the clinical disease subset, namely anticentromere antibodies for limited SSc subset, and Scl-70, anti RNA polymerase I and III, and anti-fibrillarin antibodies for the diffuse form of SSc.

### **Biomarkers of Endothelial Cell Dysfunction**

- Numerous molecules involved in various aspects of the pathogenesis of endothelial dysfunction (e.g., von Willebrand factor, adhesion molecules, vascular endothelial growth factor) are potential biomarkers for endothelial perturbations in SSc.

### **Biomarkers of Pulmonary Hypertension**

- Endothelin-1 and N-terminal pro-brain natriuretic peptide plasma levels are biomarkers for detection and monitoring of pulmonary artery hypertension (PAH) and may be surrogate markers to evaluate the effects of therapeutic agents for SSc related PAH.

### **Biomarkers of Pulmonary Fibrosis**

- Serum levels of KL-6, pulmonary surfactants A and D, and PARC are indicators of the development of pulmonary fibrosis in SSc.

### **Biomarkers of cellular immune system and cytokine alterations**

- The levels of numerous cytokines and chemokines that reflect the participation of immune and inflammatory processes have been suggested as potential biomarkers.

### **Specific Biomarkers for Fibrosis**

- Circulating or urinary levels of molecules or fragments of interstitial type I and III collagens are potential biomarkers for the activity of the ongoing fibrotic process.
- Elevated levels of type III procollagen peptides correlate with pulmonary involvement and predict a poor prognosis and an unfavorable survival.

### **Analysis of Gene Expression Employing Microarrays and Use of Proteomics to Identify Biomarkers**

- The application of novel genomic, global gene expression, and proteomic approaches opens up new and promising opportunities for the development of useful SSc biomarkers and has already allowed the identification of patient subsets with specific signatures that may indicate different pathophysiological events or differential therapeutic responses.

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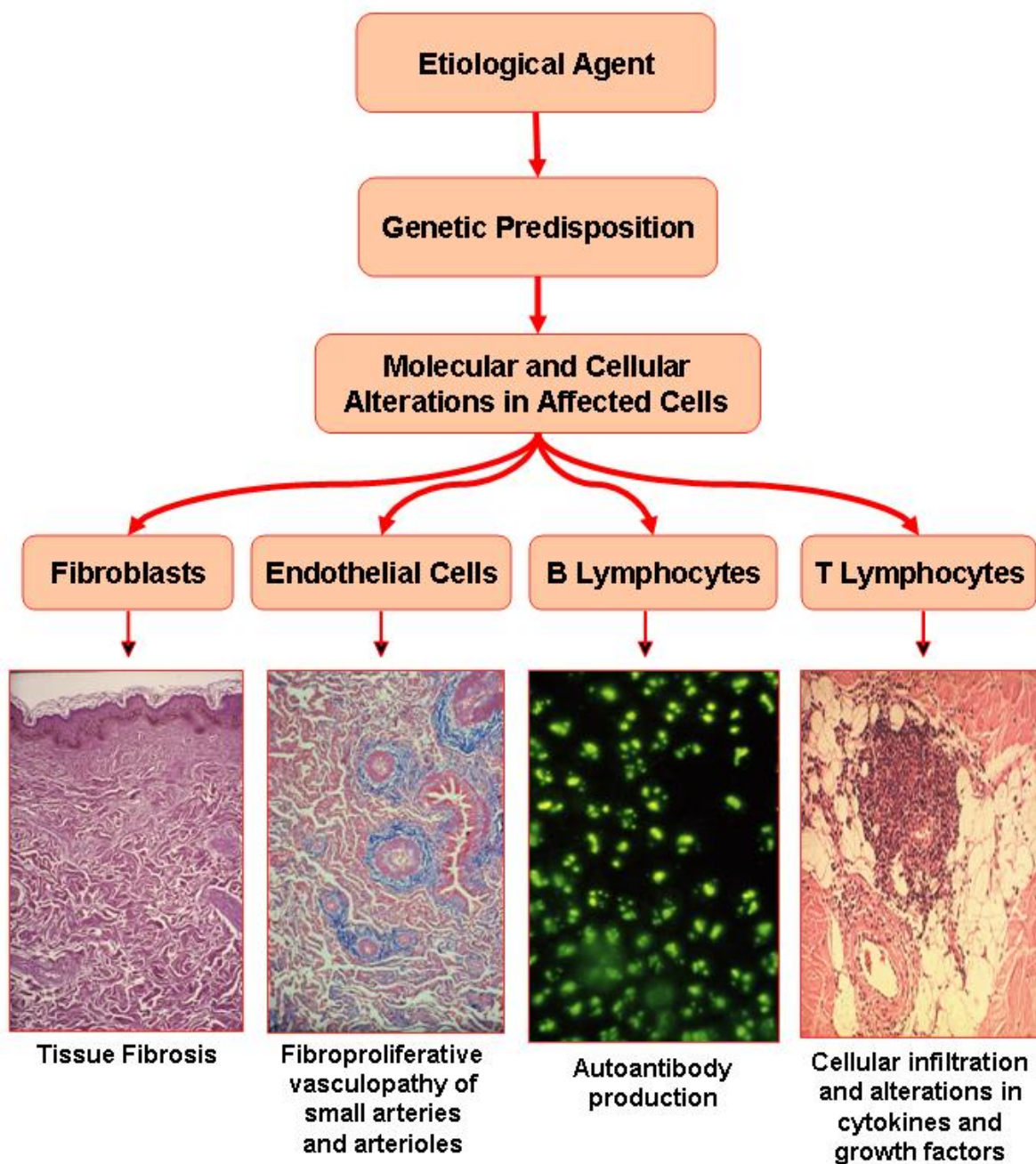
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**Table 1.** Potential biomarkers for SSc diagnosis, clinical subset classification, and process/organ involvement.

Biomarker	Class/Function	Clinical Association	Response to Treatment
<b>Diagnostic and Clinical Subset Classification</b>			
anti-Scl-70	Anti-DNA topoisomerase I antibody	Diffuse SSc, Pulmonary fibrosis	No
anti-centromere	Anti-Kinetochore protein antibody	Limited SSc, Pulmonary hypertension	No
anti-RNA polymerase I anti-RNA polymerase III	Antibodies to RNA polymerases	Diffuse SSc, renal involvement	No
anti-fibrillarin	Antibody to 34 kDa nucleolar protein component of U3-RNP	Diffuse SSc	No
anti-PM-Scl	Antibody to complex of 110-20 kDa nucleolar and nuclear proteins	Polymyositis/SSc overlap	No
Anti-Th/To	Antibody to RNase P ribonucleoprotein complexes	Limited SSc	No
<b>Vascular</b>			
von Willebrand factor (vWf)	Hemostasis	Endothelial cell dysfunction, SSc severity, ILD extent	Unknown
Adhesion molecules	Cell-cell interactions	Endothelial cell dysfunction	Unknown
Vascular endothelial growth factor (VEGF)	Growth factor	Endothelial cell dysfunction	Unknown
<b>Fibrotic</b>			
Type III procollagen peptides	Fibroblast and endothelial cell extracellular matrix protein	Pulmonary involvement, poor prognosis/survival	Yes
Transforming growth factor- (TGF- )	Growth factor	Fibrosis	Unknown
Connective tissue growth factor (CTGF)	Growth factor	Fibrosis	Unknown
Cartilage oligomeric matrix protein (COMP)	Cartilage and fibroblast extracellular matrix protein	Fibrosis	Unknown
<b>Immunologic (Cytokines and Chemokines)</b>			
soluble receptor for interleukin 2 (sIL-2)	Inflammatory and Immune system alterations	Chronic inflammatory process	Unknown
soluble receptor for tissue necrosis factor- (sTNF- )	Inflammatory and Immune system alterations	Chronic inflammatory process	Unknown
<b>Pulmonary Hypertension</b>			
Endothelin-1 (ET-1)	Vasoconstrictor Endothelial cell product	Pulmonary artery hypertension	Yes
N-terminal pro-brain natriuretic peptide (NT-pro-BNP)	Myocardial protein induced by mechanical stretch	Pulmonary artery hypertension	Yes
<b>Pulmonary Fibrosis</b>			
Krebs von den Lungen 6 antigen (KL-6)	Mucinous glycoprotein from type II pneumocytes	Pulmonary fibrosis	Yes
Pulmonary surfactant A	Product of type II pneumocytes	Pulmonary fibrosis (sensitive)	Yes
Pulmonary surfactant D	Product of type II pneumocytes	Pulmonary fibrosis (specific)	Unknown
Pulmonary and activation regulated chemokine (PARC)	Chemokine	Pulmonary fibrosis	Unknown
YKL-40 (human cartilage glycoprotein-39 (HC gp-39))	Tissue remodeling	Pulmonary fibrosis	Unknown

**Figure 1. General overview of the pathogenesis of SSc.** The illustrations on the bottom row show examples of, from left to right, the fibrotic process (biopsy of skin), microvascular alterations in pulmonary arterioles, autoantibodies detected by immunofluorescence, and mononuclear inflammatory cell infiltrates in affected skin. Adapted from Reference 3.





**Figure 2. Postulated sequence of events in the pathogenesis of SSc.** Sequence of pathogenic processes leading to tissue fibrosis and autoantibody production. The process is initiated by microvascular injury which induces chronic inflammation with participation of macrophages and T lymphocytes, as well as B lymphocyte activation leading to autoantibody production. The secreted products from the inflammatory cells result in fibroblast activation and phenotypic conversion into myofibroblasts, key events in the development of fibrosis.

