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**Evaluation of possible correlation between skin ultrasound and  
circulating fibrocytes in limited cutaneous systemic sclerosis patients**

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*To my parents with all my love*

I close my eyes

And I see you everywhere

I step outside

It's like I'm breathing you in the  
air

I can feel you're there

(A. & M. Bocelli)

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## **Chapter I**

### **Systemic Sclerosis**

#### **1.1 Definition**

Systemic sclerosis or scleroderma (SSc) is a connective tissue disease with multifactorial aetiology and autoimmune pathogenesis, characterized by early endothelial damage and progressive fibrosis, affecting the skin and internal organs (lungs, heart, digestive tract and kidneys) (1.2).

Another characteristic of the disease is the vascular impairment which includes morphological and functional changes, that lead to Raynaud's phenomenon (RP) and ulcers, frequently at periungual level (3).

SSc is classified according to the extent of skin fibrosis into two main forms: limited cutaneous and diffuse cutaneous involvement (2).

The limited subtype (lcSSc) is characterized by the association of Raynaud's phenomenon with fibrosis of the skin limited to the hands, face, feet and forearms level. Although this form is only sometimes characterized by serious organ involvement, affected individuals run a higher risk of pulmonary hypertension (2).

In the diffuse form (dcSSc) the clinical course is quick and cutaneous sclerosis, usually preceded a few months before by Raynaud's phenomenon, which rapidly extends to the entire body surface, including the trunk. This form is characterized by early organ involvement of lung, kidney, gastro-intestinal tract and myocardium (2).

A subgroup of patients with lcSSc has the CREST syndrome (calcinosis of the skin, Raynaud's phenomenon, oesophageal dysfunction, sclerodactyly, and telangiectasia).

Another subtype is scleroderma sine scleroderma, that as no skin involvement (2).

#### **1.2 Classification**

A new more sensitive and specific set of criteria for systemic sclerosis has been proposed to identify individuals with SSc for inclusion in clinical study by the American College of Rheumatology (ACR) and the European League Against

Rheumatism (EULAR). Diagnostic measures, such as anti-nuclear antibodies and nailfold capillaroscopy have been included in these criteria for the first time (4,5).

The criteria are based on various parameters, which are commonly used for the diagnosis of scleroderma. Noteworthy is the fact that these criteria are not diagnostic criteria as such are not applicable to patients with scleroderma-like disorders or patients who do not have sclerodactyly. The revised criteria for the classification of the SSc are presented in Table I (5).

### **1.3 Epidemiology**

SSc is a rare disease, diagnosed each year in 67 males and 265 females/100,000 persons (6-8). It is estimated to occur in 2,3-3,0 persons/1 million (6-8). However, as there are numerous oligosymptomatic forms that remains undiagnosed its prevalence is underestimated.

Although there is no racial prevalence, systemic sclerosis is rare in Japanese and Chinese populations. There is a substantial prevalence of women over men (female:male ratio 9:1). The dcSSc form occurs equally in both males and females, conversely the lcSSc form has a strong female predominance (female:male ratio 10:1). SSc usually appears in women in the 30-40 age range and about 85% of cases develop in persons 20-60 years old (6,7). There is no clear family predisposition (the correlation between monozygotic twins is 4%) or of any external known agent (6,7).

Generally, renal and pulmonary complications are responsible for the death of scleroderma patients (pulmonary hypertension 12%, lung fibrosis and heart disease 9%).

### **1.4 The most serious clinical manifestations**

SSc has complex pathogenesis. The clinical manifestations arise from three distinct processes: severe vascular fibro-proliferative lesions of the small arteries and arterioles; dysregulation of collagen and extracellular matrix production and deposition at the level of the skin and internal organs; alterations in both humoral and

cellular immunity. Clinically manifestation of SSc is localized at level of skin, vascular system and internal organs (2.8).

### *Cutaneous manifestations*

Skin damage is characteristic of the disease and is absent in a small percentage of patients. It sometimes includes sclerosis, discoloration and calcinosis.

Cutaneous sclerosis evolves in three stages: 1) the skin appears oedematous, 2) it is sclerotic and 3) it becomes atrophic.

The deposition of compact collagen fibres at the level of the dermis precedes epidermal thinning and skin appendage atrophy. The T cells are retrieved and a wide and progressive fibrosis of the skin and subcutaneous layers develops, favouring the onset of additional morphological alterations in the capillaries at a nail-bed level. The cutaneous fibrosis is usually symmetrical and the skin involvement may be limited to the fingers (sclerodactyly) or involve the entire body surface. The hands undergo a progressive retraction, with irreversible flexion deformity (claw hands).

A beak-shaped nose and a reduced aperture of the mouth ("facies scleroderma"), are typical facial features associated with SSc teleangiectases. The fibrous process also affects the sweat glands, reducing their secretory function.

Skin lesions in SSc are often discoloured with hyper/hypo pigmented areas, which may be very extensive. Another very common finding is calcinosis, i.e. coarse phosphate and calcium deposits which may also ulcerate the skin surface. The subcutaneous calcifications are localized more frequently at a finger tip level, bone eminences and the ear (8).

### *Vascular events*

Raynaud's phenomenon (RP), ischemic ulcers and telangiectasia, are all expressions of the alterations in the microvascular morphology (9-11). The best method to assess and classify microvascular damage is nailfold videocapillaroscopy (NVC) (3-5,9-11). NVC is the safest, most non-invasive, less expensive, reliable and reproducible method allowing for the evaluation of structural changes in the peripheral microcirculation in SSc. NVC is able to recognise the structural damage at an early stage and is a useful monitoring tool (9-11).

NVC is a well-established and validated technique for the identification of nailfold capillary changes to distinguish secondary RP from both primary RP and healthy subjects. It also identifies morphological patterns that are specific to various SSc stages (“Early”, “Active” and “Late” patterns of microvascular damage, according to the classification by Cutolo et al) and calculates the microangiopathy evolution score (MES) used to follow disease evolution (9-11).

Recently, several studies have demonstrated that SSc microangiopathy correlates with disease subsets and their severity (12-25). Furthermore, some authors have shown cross-sectional and predictive associations between progressive capillaroscopic changes and the impairment of internal organ function, i.e. lung disease, skin fibrosis and digital ulcers (12-25). It has now become clear that SSc patients with a “Late” NVC pattern have an increased risk of a complex disease and moderate to severe skin or visceral involvement, compared to patients with an “Early” or “Active” NVC pattern (12,16-20).

The diagnostic, prognostic and therapeutic implications of NVC microvessel morphological analysis allows for the best clinical management. Therefore, NVC abnormalities have been included in the parameters established by the 2013 ACR/EULAR classification criteria, the 2001 LeRoy criteria for the classification of early systemic sclerosis and the 2013 VEDOSS criteria for very early diagnosis of SSc (4,5,26).

#### *Visceral manifestations*

Visceral manifestations are very frequent in SSc and mainly affect the lungs, heart, oesophagus, intestines and kidneys (27-38).

#### *Pulmonary and cardiac manifestations*

The lungs are affected in 70-80% of scleroderma patients. The pulmonary manifestations of systemic sclerosis include interstitial lung disease, pulmonary arterial hypertension (PAH), pleurisy and pleural effusion (27-31). Interstitial fibrosis is frequent in patients with dcSSc, where the characteristic lesion is interstitial fibrosis, with dyspnoea and characteristic “velcro crackles” on auscultation (8,27-31). Pulmonary function testing may reveal a restrictive ventilatory defect, characterized by a reduction in forced vital capacity, lung compliance and diffusing capacity. High-

resolution computed tomography (HRCT) is the most sensitive diagnostic radiological instrument and is able to detect the disease at an early stage, even in the presence of a negative X-ray. A “ground-glass” appearance is a typical feature of early fibrosis. (28-31).

Pulmonary hypertension (PAH) is a common finding and may be regarded as one of the manifestations of the widespread small vessel arteriopathy that characterizes this disease: it can be the consequence of an end-stage lung fibrosis and is the leading cause of death in SSc patients (30,31,37,38).

The heart is involved in 40% of patients, where the damage is characterized by a progressive fibrosis that compromises both the functionality and conduction of the myocardium. This damage may lead to cardiomyopathy, which may progress to heart failure and myocardial electrical instability, at times triggering ventricular arrhythmia (32,33).

#### *The manifestations of the gastro-intestinal tract.*

Scleroderma patients had higher prevalence of upper gastro-intestinal (GI) tract symptoms (heartburn, nausea and vomiting, dysphagia and epigastric pain).

This has been reported in 50-90% of patients and it is typically result of the fibrosis that characterizes this disease. SSc can affect numerous sites within the GI tract, and patients may experience substantial dysfunction in the processes of motility, digestion, absorption and excretion. Oesophageal dysfunction is the most common GI manifestation, even if patients may also have dysfunction of the stomach, small intestine, colon or rectum, each of which may be responsible for severe and distressing symptoms (8,34,35).

The oesophagus is the frequently involved in SSc. The fibrous replacement of the parietal smooth muscle causes a worsening hypotonia, with marked alteration and a reduction in peristalsis. The first symptom is dysphagia for solid foods follow by liquids. Patients also report having epigastric pain, gastroesophageal reflux, frequently secondary to a hiatal hernia (8,34,35).

#### *The renal manifestations*

Kidney disease is also common and the most frequent is a reduction in renal function due to chronic disease. Whilst the most serious clinical manifestation is scleroderma



renal crisis (SRC). This life-threatening complication occurs in up to 15% of dcSSc cases. SRC is characterized by an abrupt rise in blood pressure over days to weeks and a rapidly progressive renal failure if left untreated, usually within the first 5 years after disease onset. Apart from diffuse disease, other risk factors include the use of corticosteroids/cyclosporine A, or the presence of anti-RNA polymerase III antibodies. The spectrum of presentation ranges from normal or mildly elevated blood pressure to malignant hypertension, causing elevated plasma renin levels, elevated serum creatinine (observed in 50% of patients), proteinuria or microangiopathic hemolytic anemia (seen in 50% of patients). Urine sediment may contain mild proteinuria, a few cells or casts. Mortality can be reduced by the administration of angiotensin converting enzyme (ACE) inhibitors (8,36).

### *Evolution and prognosis*

SSc has quite a variable evolution. There are forms where the cutaneous sclerosis remains acro-located for years without visceral involvement, whilst other forms involve lesions that rapidly extend to all skin areas, with frequent organ involvement. The disease still has periods of activity alternating with remission.

The prognosis of SSc depends on the visceral lesions; there is roughly a 90% five-year survival rate, which drops to 70% at 10 years. The most frequent causes of death are heart, kidney or lung failure, as well as right heart dysfunction (2,8,13-18).

## **1.5 Treatment outline**

Treating and managing scleroderma remains challenging due to the heterogeneity of the disease manifestations and variable clinical course. In 2009, the EULAR Scleroderma Trials and Research group (EUSTAR) published recommendations for the treatment of SSc, which was updated in 2017 (39,40) (Table II and Figure 1). However, to date, there is no treatment able to block disease progression, reverse the fibrosis or improve long-term outcome. Several studies have reported the efficacy of immune modulating/suppressant drugs in the treatment of this disease, although these have mostly been in open, uncontrolled trials and recent data support the benefit of immunosuppression for skin and lung fibrosis in SSc, especially when

administered during the early stages of disease (39-41). The most commonly used immunosuppressant drugs for SSc management are: methotrexate, mycophenolate mofetil, azathioprine, cyclosporine and cyclophosphamide (39-41). Interestingly, an observational study on the efficacy of five different treatment protocols in early dcSSc did not observe any significant difference in outcomes (42). Summarizing: cyclophosphamide is recommended for skin disease in dcSSc and lung fibrosis, methotrexate can be used in skin disease or in patients with features of overlap inflammatory arthritis, mycophenolate mofetil is being increasingly used for skin and lung disease and azathioprine may be an alternative option (39,40). As aforementioned, the choice of immunosuppressant is dictated by the organ complications.

It is important to consider not only immunosuppression but also symptomatic treatment of symptoms. One of most important symptoms is Raynaud's phenomenon, where treatment/management includes lifestyle changes, such as stopping smoking, avoiding cold environments, wearing layers of warm clothing and gloves. Moreover, calcium channel blockers, angiotensin II receptor blockers and selective serotonin reuptake inhibitors may be helpful. Iloprost IV, phosphodiesterase 5 (PDE5) inhibitors and endothelin receptor antagonists (ERA) should be taken into consideration for severe Raynaud's phenomenon, especially if associated with digital ulceration and/or critical digital ischemia. Sympathectomy may even be considered in severe cases (39-42).

Gastrointestinal symptoms are extremely common and most patients have some element of gastroesophageal reflux, which can be treated with proton pump inhibitors. Many patients have severe symptoms that require higher doses to control symptoms or the addition of histamine 2 receptor antagonists. Prokinetics may be prescribed for dysphagia and rotating antibiotic courses of for small bowel overgrowth to avoid diarrhea (39,40) (Table I, Figure 1).

## **Chapter II**

### **Evaluation of skin damage by the modified Rodnan skin score and skin high frequency ultrasound**

#### **2.1 Introduction**

Skin involvement is a hallmark of systemic sclerosis (SSc), its classification and activity (43-47). Skin damage is related to an excessive dermal deposition of collagenous and non-collagenous extracellular matrix components due to an altered production and remodeling of tissue fibroblasts and myofibroblasts (48-52).

There are 3 stages of scleroderma skin impairment in SSc, i.e. edematous, fibrotic or atrophic. In the edematous phase, there is painless pitting edema of the hands and fingers, which may also affect the feet, legs and the forearms. This situation usually evolves quickly in fibrosis, with a reduction in skin elasticity. The second phase, which may last for many years, is characterized by hard, shiny and taut skin that is adherent to the sub-cutis. In the atrophic phase the skin becomes thin and is bound to the underlying tissue (43-48).

Quantifying skin impairment is a must not only to assess disease activity, but also the severity and therapy response (53). Indeed, skin damage is a vital element as the severity of skin involvement inversely correlates with both survival and prognosis (44-47).

#### **2.2 The modified Rodnan skin score**

Skin manifestation can be identified and studied by the modified Rodnan skin score (mRss), the validated method to evaluate the severity of skin involvement in SSc and to distinguish, as aforementioned, patients with either limited (lcSSc) from diffuse (dcSSc) cutaneous skin involvement (4,43-47). In fact, the skin damage is confined to the extremities (hands, forearms, feet, legs and face) in lcSSc, whilst it is present on the arms, chest, abdomen and thighs in dcSSc patients (4,43-47).

The original Rodnan skin score (RSS) was developed in 1979 by Rodnan et al (54) and the mRSS is now used to evaluate skin thickness and has been applied as the

primary outcome measure in most clinical trials. It is a feasible, reliable, valid method responsive to change in multicenter clinical trials, especially in dcSSc.

The mRss is a summation of ratings obtained from the clinical palpation of seventeen skin areas, i.e. zygoma, fingers, dorsum of hands, forearms, arms, chest, abdomen, thighs, legs and feet (55-57). The total skin score is the sum of the individual skin assessments in the 17 body areas, that provided a score from 0 (=normal skin thickness in entire body) to 51 (severe skin thickness in all 17 areas); the higher the score, the greater the extent and severity of the skin thickening. The mRss does have some drawbacks, as it is extremely dependent on the examiner's skills, requires specific training and experience, cannot differentiate between skin thickness and tightness and it has high intra- and inter-observer variability (12% and 25 % respectively) and is unable to detect small but clinically relevant changes in skin thickness over time, mRss tends to worsen in early disease and improve in late disease, although the time of peak involvement still remains poorly defined (55-57).

### **2.3 Skin high frequency ultrasound**

Recently, several studies have reported the utility of high frequency skin ultrasound (US) for early identification of skin involvement in SSc patients (58-61). Alexander and Miller were the first to use a 15 MHz US probe to assess skin thickness, publishing their findings in 1979 (62).

Most investigators now use a US equipped with a 18-32 MHz probe, even if it has poor penetration, as a high frequency with a good resolution is necessary to obtain a clear visualization and to distinguish epidermis, dermis and subcutaneous fat. US allows not only for the determination of skin thickness but also provides a qualitative assessment (63-67). Several benefits are to be had by using US in the assessment of skin: it is a reliable and reproducible method to assess dermal thickness (intra- and inter-observer variability, 4% and 8% respectively) (58-61). Moreover, it provides early detection of skin involvement and it has been demonstrated that US values of dermal thickness are correlated to the clinical phase (53). Interestingly, a recent study has also demonstrated a correlation between dermal thickness and peripheral blood perfusion (60).

However, US also has drawbacks, for example the operator requires specific training in the correct use of the machine, i.e. setting, the probe positioning and hand pressure. In fact, it takes longer for the US to evaluate the same 17 points as the mRSS does, (15-20 minutes per pt. vs 5 minutes respectively). However, US images can be saved for future evaluation.

The increased sensitivity of US in the early stages of skin involvement may well represent a valuable potential contribution to clinical assessment, especially when researching the pathogenesis and treatment of this disease. In conclusion, US is a promising technique to detect and manage skin impairment in systemic sclerosis and localized scleroderma, even if further studies are required before it can become a validated measure.

#### **2.4 The modified Rodnan skin score vs skin high frequency ultrasound**

Most authors have made a comparison between the mRSS and US, but only Ihn H. et al have compared US to histopathological findings (67). No correlation between local mRSS and US findings has been found, but some authors have demonstrated a correlation between global mRSS and US (63). The US offers a wider range of values for dermal thickness than the semi/quantitative mRSS, which has only 4 integer values. In fact, the mRSS evaluates skin impairment, texture and fixation, whilst US distinguishes the different skin layers and makes a precise measurement of its thickness (45-47,58,59).

A recent 2017 study also demonstrated that high frequency ultrasound is able to identify subclinical diffuse dermal involvement in lcSSc patients. The results of this study showed a higher DT in lcSSc than in healthy subjects in 4 of the 6 skin areas that had a normal mRSS (mRSS=0) in contrast with the criteria that classified these patients as lcSSc. The results of this research are in line with those of recent microarray gene expression studies, suggesting that clinically unaffected skin shares the peculiar gene signatures and pathology of clinically affected skin in SSc (69,70).

The authors also confirmed that US identifies an earlier skin involvement than does the modified Rodnan skin score. In fact, another article demonstrated that US was able to identify the edematous phase preceding the palpable skin involvement in

early disease, thus helping to diagnose precocious skin involvement (61). Whereas few studies have evaluated the sensitivity that US has to perceive changes in DT (71).

## Chapter III

### The role of circulating fibrocytes in systemic sclerosis

#### 3.1 The role of circulating fibrocytes in systemic sclerosis

Progressive fibrosis is one of the most important hallmarks of systemic sclerosis (SSc) which is also characterized by a disorder of the autoimmune system (72,73). Activation of the immune response through autoantibody production, together with the recruitment and transition of endothelial cells and pericytes into active myofibroblasts, seems to play an important role in the progression of fibrosis in almost all organs. Therefore, although the pathogenesis of SSc remains unclear, myofibroblast activation is believed to be the step which follows microvascular damage (74,75). Myofibroblasts are characterized by a high expression of specific phenotype markers and profibrotic molecules, primarily  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and fibroblast-specific protein-1 (S100A4), as well as by an overproduction of extracellular matrix (ECM) proteins, such as fibronectin (FN) and fibrillar collagens (type I and III) (76-78). Various cell types, including endothelial cells, circulating mesenchymal cells and even fibrocytes, may differentiate into myofibroblasts (79).

Fibrocytes are circulating progenitor cells derived from the bone marrow that express specific markers of both hematopoietic (CD34, CD43, CD45, LSP-1 and MHC class II) and stromal cells (collagen I and III), together with the chemokine receptors CCR2, CCR7, and CXCR4, which regulate their migration into inflammatory lesions (80-84). Circulating fibrocytes are recruited through CXCR4/CXCL12 interaction into injured tissues where they differentiate into fibroblasts/myofibroblasts, thereby regulating the healing process (by producing cytokines, chemokines and growth factors), secreting essential ECM proteins and promoting angiogenesis (85-87). Moreover, although fibrocytes are involved in physiological wound repair to local tissue injury, in chronic fibroproliferative disorders they may be the cause of an excessive deposition of ECM molecules (88). In vitro, fibrocytes appear to differentiate from circulating CD14+ monocytes into spindle-shaped, fibroblast-like cells and seem to have an antigen-presenting capability, expressing class II major histocompatibility complex molecules (HLA-DP, -DQ, and -DR) and the CD11a, CD54 (ICAM-1: intracellular adhesion molecule-1), and CD58 adhesion molecules (80,89-91). When cultured in the

presence of a specific antigen, human fibrocytes induce antigen-presenting cell (APC)-dependent T-cell proliferation, which is significantly higher than what is induced by monocytes and nearly as high as the proliferation of purified dendritic cells (91).

Human fibrocytes seem to have an antigen presenting capability and appear to be an important source of fibroblasts/myofibroblasts in the physiological and pathological tissue remodeling that characterizes SSc. Indeed, several recent studies have demonstrated that circulating fibrocytes are correlated with several aspects of fibrosis at the level of different internal organs in many diseases, including systemic sclerosis (92-97).



## **Chapter IV**

### **Experimental Section**

#### **Correlations between mRSS and US and circulating fibrocytes percentage and gene expression in limited cutaneous scleroderma patients**

##### **4.1 Introduction**

Systemic sclerosis (SSc) is a connective tissue disorder characterized by progressive fibrosis of skin and internal organs and microvascular abnormalities (1-7).

Skin involvement may be classified by the modified Rodnan skin score (mRSS), the validated method to evaluate the severity of skin thickening in SSc. The mRSS is also used to distinguish patients with limited (lcSSc: skin damage is confined to the hands, forearms, feet, legs and face) from diffuse (in dcSSc, skin involvement is also present on the upper-arms, chest, abdomen and thighs) cutaneous involvement (54-57).

The mRSS has some drawbacks, as it is unable to identify slight alterations in skin thickness and has high intra- and inter-observer variability (55-57). Conversely, several authors have reported the utility of high frequency ultrasound (US) in the early identification of skin involvement in SSc patients and have also reported that this technique is very reliable (58-66). However, mRSS and US measure different skin properties. The mRSS measures skin thickness, texture and rigidity, whilst US makes an accurate identification of the different skin layers of skin and also measures the dermal thickness (DT) (55-66).

In SSc, fibrosis is caused by the activation of fibroblasts and their transition into profibrotic myofibroblasts (76-83). After their phenotype transition, myofibroblasts acquire an increased persistent capability to synthesize and accumulate extracellular matrix (ECM) proteins, such as type I collagen (COL-1) and fibronectin (FN), initiating an altered process which leads to systemic fibrosis (76-83). Myofibroblast transition and ECM overproduction are known to be induced by several profibrotic mediators, including the transforming growth factor- $\beta$  (TGF $\beta$ ), endothelin-1 (ET-1), cytokines and chemokines, such as IL-6 and CCL18 (76-83).

On the basis of this knowledge, blocking the fibroblast-to-myofibroblast transition, diminishing the profibrotic myofibroblast activity and related ECM overproduction might be important steps in reducing the fibrotic process, at least in SSc (76-83).

The aims of this study were:

- *firstly*: to compare the performance of 2 high range ultrasound transducers (18 MHz and 22 MHz), in evaluating the subclinical DT changes in lcSSc patients and to confirm any correlation between US and mRSS in the evaluation of skin involvement in lcSSc patients.
- *secondly*: to evaluate any correlations between mRSS, DT, measured by US with the 18 and the 22 probes and the percentage and phenotype of circulating fibrocytes in lcSSc patients.

## 4.2 Methods

### *Study population*

In the **first part of the study** we enrolled a total of 48 lcSSc patients (40 females and 8 males), classified on the basis of a normal mRSS (score = 0) at the level of the upper-arms, thighs, thorax and abdomen. The SSc patients met the 2013 ACR/EULAR criteria for SSc (5). A complete medical history was taken for all patients and a clinical examination performed (Table III). The control group consisted of 48 sex- and age-matched healthy subjects (CNT) (Table III). We obtained written informed consent to participate at this study.

In the **second part of the study** a total of 8/48 of the lcSSc patients were selected (7 females and 1 male) (Table IV).

As no evident clinical SSc complications were present other than skin involvement and Raynaud's phenomenon, the lcSSc patients were on vasodilators (mainly cyclic prostanoids) at enrollment. All instrumental examinations (i.e. pulmonary function testing, chest CT, echocardiography, etc.) were normal (Table IV).

Peripheral venous blood samples were taken at time T0 in the 8 lcSSc patients (7 females and 1 male) that had a capillaroscopic "Active" pattern and in the 5 healthy subjects (CNT) (4 females and 1 male) (Table IV).

The study was approved by the local Ethics Committee (protocol number 273-REG-2015).

### *Inclusion criteria:*

- written, signed and dated informed consent before starting the study;
- no evident clinical SSc complications at the time of skin sampling;

- having ceased taking immunosuppressants and/or endothelin-1 inhibitors for at least three months before the study.

*Exclusion criteria:*

- severe organ involvement (respiratory, cardiac, renal or hepatic insufficiency) or a history of recurrent ulcers resistant to available therapies, diabetes, treatment-resistant hypertension, arrhythmia;
- manifestations of other autoimmune diseases (antibody positivity or clinical symptoms) that may lead to the suspicion that they had an overlap form;
- ongoing immunosuppressive therapy with cyclosporine, cyclophosphamide, mofetil mycophenolate, methotrexate etc;
- corticosteroid therapy;
- ongoing therapy with endothelin-1 receptor antagonists.

The Ethics Committee approved the study (protocol number 273-REG-2015).

*The Modified Rodnan skin score (mRSS)*

The mRSS was used for the SSc patients on the standard 17 skin areas (zygoma, fingers, dorsum of the hands, forearms, upper-arms, chest, abdomen, thighs, legs and feet) (54-57). The skin thickness was assessed by skin palpation and graded on a scale from 0 to 3, where 0 = normal, 1 = weak, 2 = intermediate and 3 = severe skin thickening (54-57).

As aforementioned, only patients classified as having lcSSc were enrolled into the study to investigate any correlation between the mRSS and DT values (measured by both 18 and 22 MHz probe) in lcSSc patients and to compare these results to the CNT. The mRSS was equal to zero in all the healthy subjects in the CNT group. The same operator performed the mRSS in all subjects and was blinded to the US assessment.

*Skin high-frequency ultrasound (US)*

Two different US transducers were used in all the 17 skin areas of both patients and healthy subjects: an 18 MHz (MyLab 25, Esaote, Genoa, Italy) and a 22 MHz (MyLab One, Esaote, Genoa, Italy). The US measurements were based on two bi-dimensional B-mode images. As reported in previous studies, an electronic calibre was used to measure the DT (59,60). The ultrasound values were recorded in

millimetres. The same operator performed the US evaluations for both groups and was blinded to the mRSS assessment. Both mRSS and US were performed on the same day in all patients.

#### *Nailfold videocapillaroscopy (NVC)*

In the **first part of the study** NVC was performed by an optical probe, equipped with a 200x contact lens, connected to image analysis software (Videocap, DS Medica, Milan, Italy) to classify all lcSSc patients on the correct microangiopathy pattern ("Early", "Active", or "Late"), according to the classification by Cutolo et al (9-12).

In the **second part of the study** we decided to select only patients with an "Active" microangiopathy pattern to obtain an intermediate microvascular damage, as those with a "Late" pattern are more likely to have organ damage, to avoid any bias due to the need for immunosuppressive therapy (9-12,17).

The same operator performed the NVC evaluations and was blinded to the skin assessment.

#### *Cell culture*

Circulating fibrocytes were obtained from all lcSSc and CNT enrolled. Fibrocytes were isolated from peripheral blood mononuclear cells (PBMCs) and characterized by fluorescence-activated cell sorter analysis (FACS) at baseline (T0) and after 8 days of culture (T8) in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Milan, Italy) at 20% of Fetal Bovine Serum using anti-CD45, CXCR4, CD14, HLA-DRII (Beckman Culter, CA, USA) and anti-COL-1 (Millipore, MA, USA) conjugated primary antibodies, in accordance with several other studies (18,19). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for COL-1, TGF $\beta$ 1 and the myofibroblast phenotype markers  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and fibroblast specific protein-1 (S100A4), using specific primers supplied by Primer Design, on T8-cultured SSc and CNT fibrocytes (97). Gene expression values were calculated using the comparative  $\Delta\Delta$ Ct method and they corresponded to the expression level (fold increase) of the target gene in SSc fibrocytes compared to CNT fibrocytes (taken as the unit value by definition) (90).

### *Statistical analysis.*

The data were processed by non-parametric tests: a Mann-Whitney U test was performed to compare unpaired groups of variables and a Kruskal-Wallis test compared continuous variables with nominal variables that had more than two levels. The Spearman rank correlation test was used to search for any relationships between variables, along with linear regression tests. The Intraclass Correlation Coefficient (ICC) calculated the intra-operator repeatability. Any p-value <0.05 was considered statistically significant. The results are reported as median and interquartile range (IQR) (98).

### **4.3 Results.**

#### ***In the first part of the study.***

The estimated DT values obtained by the 22 MHz transducer were statistically significantly higher than those of the 18 MHz transducer in all body areas in both lcSSc patients and the CNT group (Table V) (60). The median difference in DT values between the two transducer was  $0.12 \pm 0.06$  SD millimetres in lcSSc patients and  $0.01 \pm 0.01$  millimetres in the CNT group.

The median DT values were significantly higher in lcSSc patients than in CNT, in all the six clinically unaffected skin areas when assessed with the 22 MHz transducer (Table V) (60). These differences were statistically significant in only 4/6 skin areas where the mRSS was in the normal range (=0), in agreement with the classification of lcSSc (the arms, chest and abdomen), when the 18 MHz probe was used.

A positive statistically significant correlation was observed between the two transducers in the DT evaluation ( $p < 0.0001$ ,  $r = 0.92$ ), as well as between both probes and mRSS ( $p < 0.0001$  for both,  $r = 0.50$  for the 18 MHz transducer and  $r = 0.58$  for the 22 MHz transducer) (60). The DT in the lcSSc patients increased progressively along with the microangiographic pattern (Early, Active and Late). There was also a positive correlation between the DT values and the MES scores ( $p < 0.05$ ). No correlation was observed between the DT and duration of either the SSc ( $p = 0.70$ ) or Raynaud's phenomenon ( $p = 0.60$ ). No statistically significant correlation was observed between the DT and organ involvement (gastrointestinal tract, lung, heart, kidney, digital ulcer occurrence) in our lcSSc patients.

The time needed for the assessment of DT for all three methods was  $26\pm 3$ ,  $19\pm 2$  and  $10\pm 2$  minutes, for the 18 and 22 MHz probes and mRSS respectively ( $p < 0.0001$ ).

There was a 96% intra-operator reproducibility for the 18 MHz transducer (95%CI 0.92 to 0.98), 98% for the 22 MHz (95%CI 0.97 to 0.98) and 95% for the mRSS (95%CI 0.94 to 0.98).

***In the second part of the study.***

A positive correlation was observed in lcSSc patients between the percentage of circulating fibrocytes at T0 and both mRSS ( $p=0.04$ ) and DT, evaluated by the two probes (22MHz  $p=0.03$ ; 18MHz probe  $p=0.05$ ). No correlations were observed between the T8-cultured fibrocytes and mRSS or US (the two probes) in lcSSc patients. Moreover, no correlation was observed in CNT at both T0 and T8, between the aforementioned values (mRSS vs fibrocytes; US 18 MHz probe vs. fibrocytes and US 22 MHz probe vs. fibrocytes) (Table VI).

The FACS analysis showed an approximately two-fold higher percentage of fibrocyte at T0, characterized as  $CD45^+COL-1^+CXCR4^+$ , in SSc patients than in CNT group (Table VI) (97). Furthermore, there was a higher percentage of fibrocytes in both the SSc and the CNT group from T0 to T8, confirmed previous results (Table VI) (97). T8-cultured SSc fibrocytes were characterized also by a significant increase of basal expression of  $\alpha$ SMA, COL-1, S100A4 and  $TGF\beta 1$ , compared to T8-cultured CNT fibrocytes ( $p < 0.01$ ) (Figure 2) but there were no correlations between these data and the mRSS or the DT (for both probes) ( $p > 0.05$ ) (Table VII). A statistically significant correlation was observed between the mRSS and the total DT, evaluated by the two probes (22MHz  $p=0.05$ ; 18MHz probe  $p=0.05$ ) and between the two transducers in the measure of DT ( $p=0.04$ ) (Table VII). There was no correlation between the DT-US, mRSS and disease or Raynaud's phenomenon duration ( $p > 0.05$ ). The DT evaluated by both probes was significantly higher in lcSSc patients than in the CNT group (Table VI).

#### **4.4 Discussion**

The data of first part of the study demonstrate that lower frequency probes (18 vs 22 MHz) might well lead to an underestimation of DT. Moreover, it was observed that a higher frequency probe, like the 22 MHz probe, is more sensitive in detecting subclinical involvement of the skin (63,67,99). Furthermore, the 22 MHz probe had a lower intra-operator variability than did the 18 MHz probe. This may be due to the fact that 22 MHz allows for a clearer identification of the different skin layers. The 22 MHz transducer was also able to reduce the time required for DT measurement over the 18 MHz (60,68).

Our observations confirmed, as reported in previous studies (60,68,100), that high frequency US, equipped with a 20-30 MHz probe, allows for a better resolution and visualization of derma, providing a more accurate DT determination and qualitative assessment of the skin (60,63,67,99,100). This is especially relevant in the thigh and upper-arm, where the identification of the different skin layers was more difficult with an 18 MHz transducer (60,100).

Furthermore, this study confirms, as did a previous study of ours, that skin high frequency US is able to evaluate subclinical dermal involvement in patients with lcSSc (60,100). This finding is supported by genetic and pathophysiological studies (69,70).

A statistically significant correlation between mRSS and US-DT values was observed with both transducers (68,100). The modified RSS and US different skin properties: i.e. the mRSS is influenced by skin thickness, texture and fixation, whose high frequency US measures DT more accurately (45,46,71,99).

Noteworthy is the fact that several studies have recently demonstrated that skin high frequency US is a valid and reproducible technique to measure DT in patients with SSc and to identify early skin impairment and that it may also help to monitor therapy response (101).

Skin US may be considered valuable and acceptable technique for use in clinical research into the pathogenesis of the disease and treatment efficacy, in as much as it is a non-invasive and safe approach (101). However, US evaluation necessitates training and is more time consuming than mRSS. Therefore, we are of the opinion that high frequency US is very valuable in the evaluation of small DT variations, such

as during clinical trials or for therapy assessment and in the early phase of disease with the aim of obtaining a very early diagnosis (58,61,102).

Our study does have some limitations, such as the relatively small sample size and the fact that the US evaluation was performed by only one operator. Moreover, DT may vary according to age and pre/postmenopausal status (103), but this potential bias was overcome by enrolling sex and age-matched subjects.

In conclusion, the first part of the study shows that high frequency US is able to detect early skin involvement in subclinical stages in lcSSc patients, thus supporting the hypothesis that US is an important tool for the assessment of skin involvement in this disease, in both clinical and research settings.

The results of the **second part of the study** confirm that the percentage of circulating fibrocytes, characterized as CD45<sup>+</sup>COL-1<sup>+</sup>CXCR4<sup>+</sup> cells, was higher in the SSc patients than in the CNT group (97). The study confirmed that SSc circulating fibrocytes show an increased  $\alpha$ SMA, COL-1 and TGF $\beta$ 1 gene expression compared to CNT fibrocytes, suggesting they have a propensity for transition into profibrotic activated myofibroblasts, which are key cells involved in both tissue repair and fibrosis (97). Interestingly, the two patients who had an highest increase in the percentage of circulating fibrocytes from T0 to T8 also showed elevated levels in the  $\alpha$ SMA and TGF $\beta$ 1 gene expression. Furthermore, these two patients also had high US and mRSS values.

This result confirms recent findings reporting that circulating fibrocytes might represent a further important source of activated fibroblasts/myofibroblasts and they might contribute to the increased presence of these cells in the tissues of SSc patients (89,97,104,105). Furthermore, circulating fibrocytes characterized as CD14<sup>-</sup>CD34<sup>+</sup>CD45<sup>+</sup>CXCR4<sup>+</sup>COL-1<sup>+</sup> cells, have been reported to be involved in the ischemic and fibrotic processes of SSc. Indeed, it has been demonstrated that the percentage of these cells is directly correlated with the worsening of idiopathic pulmonary fibrosis and prognosis (84,106-111).

Circulating fibrocytes derive from bone marrow progenitor cells and in response to different stimuli, i.e. chemokine ligand-receptor pairs, they extravasate into sites of tissue injury, differentiate into fibroblasts/myofibroblasts and contribute to the generation of ECM during fibro-proliferation (108-111). Several animal and human studies have investigated the role of fibrocytes in the pathogenesis of chronic



inflammatory and fibrotic disorders in different organs in autoimmune rheumatic diseases, such as rheumatoid arthritis (RA) and systemic ertematosus lupus (SLE): for example cardiac, pulmonary and renal fibrosis (108-111).

Clearly, fibrocytes can be considered effector cells that exert different effects during the course of various autoimmune conditions (108-111). Fibrocytes are potentially involved in disease initiation and progression (108-111). Persistent activation of autoreactive T-cells in autoimmunity may drive fibrocyte differentiation, potentially amplifying disease severity and progression (108-111). Knowing the various factors that contribute to, or antagonize, fibrocyte production and effector functions will provide potential targets for therapeutic intervention strategies in different autoimmune conditions.

The correlation observed in our study is in agreement with other studies, where fibrocytes are correlated with different tissue impairment in autoimmunity diseases and might well also confirm the pivotal role of these cells in the process of fibrosis (108-111).

#### **4.5 Conclusion**

However, larger multicentre studies should be carried out to confirm these preliminary data, as a relatively small number of subjects were enrolled from a single Centre. Moreover, this is a cross-sectional study, whilst a longitudinal study would be beneficial. Despite these drawbacks, the observation that the percentage of circulating fibrocytes is correlated with DT-US and mRSS does carry some weight as skin involvement in SSc is not only critical for the initial diagnosis but also has a prognostic relevance (112).

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**Table I.** 2013 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for the classification of systemic sclerosis.

Criteria	Sub-criteria	Weight/score
Skin thickening of the fingers of both hands extending proximal to MCP joints		9
Skin thickening of the fingers <i>(count the higher of the two)</i>	Puffy fingers	2
	Whole Finger, distal to MCP	4
Fingertip lesions <i>(count the higher of the two)</i>	Digital Tip Ulcers	2
	Pitting Scars	3
Telangiectasia		2
Abnormal nailfold capillaries		2
Lung Involvement	Pulmonary arterial hypertension (on RHC)  and/or  Interstitial lung disease (on HRCT)	2
Raynaud's phenomenon		3
Scleroderma related antibodies	Any of the anti-centromere, anti-topoisomerase I (anti-Scl 70), anti-RNA polymerase III	3

**Legend:** metacarpo phalangeal joints (MCP) right heart catheterisation (RHC), High-resolution computed tomography (HRCT)

\* A total score of  $\geq 9$  is classified as SSc (Systemic sclerosis).



**Table II.** EULAR Scleroderma Trials and Research group (EUSTAR) published the recommendations for treatment of SSc (2009) and **the update (2017)**.

	2009	2017
Raynaud's Phenomenon	Dihydropyridine Calcium channel blockers i.v. Prostaglandins	Dihydropyridine Calcium channel blockers i.v. Prostaglandins <b>PDE-5 Inhibitors</b> <b>Fluoxetine</b>
Digital Ulcers	i.v. Prostaglandins Bosentan	i.v. Prostaglandins <b>PDE-5 Inhibitors</b> Bosentan
PAH	Bosentan, Sitaxentan, Sildenafil, Epoprostenol	<b>ERA (Ambrisentan, Bosentan, Macitentan)</b> <b>sGC Stimulator Riociguat</b> <b>PDE-5-Inhibitors (Sildenafil, Tadalafil)</b> Epoprostenol <b>i.v. Prostaglandins</b>
Skin- and Lung Fibrosis	Methotrexate for skin (early SSc) Cyclophosphamide for ILD	Methotrexate for skin (early SSc) Cyclophosphamide for ILD <b>Hematopoietic Stem Cell Transplantation</b>
SRC	ACE Inhibitors No Steroids	ACE Inhibitors No Steroids
Gastro-Intestinal Tract	PPI Prokinetics (rotating) Antibiotics	PPI Prokinetics (rotating) Antibiotics

**Legend:** i.v.: intravenous therapy; PDE-5 Inhibitors: phosphodiesterase type 5 inhibitors; ERA: endothelin receptor antagonist; sGC: stimulator of soluble guanylate cyclase; ILD: interstitial lung disease; SRC: scleroderma renal crisis; PPI: proton pump inhibitor.

**Table III.** Clinical findings in systemic sclerosis (SSc) patients and healthy subjects (CNT).

	Age (year)	RP duration (years)	SSc duration (years)	Treatment	DT Total 18 MHz Probe (millimetres)	DT Total 22 MHz Probe (millimetres)	mRSS Total (score)
<b>CNT (#48)</b> median [IQR]	66.0 [16.0]	-	-	PPI 6 Antihypertensive drugs 1	14.2 [1.2]	14.9 [0.8]	-
<b>lcSSc (#48)</b> median [IQR]	67.0 [16.3]	8.0 [9.4]	4.5 [7.5]	Vasodilat 40 ERA 10 Cyclosp 7 Mtx 5 Aspirin 44 PPI 38 Antihypertensive drugs 10	17.0 [2.6]	17.5 [4.8]	4.6 [4.2]
<b>lcSSc vs CNT</b> Stat. signif.	n.s.	-	-	-	p<0.01	p<0.01	-
<b>Early (#16)</b> median [IQR]	64.0 [19.4]	4.0 [6.0]	1.8 [2.1]	Vasodilat 9 ERA 1 Cyclosp 1 Mtx 0 Aspirin 14 PPI 9 Antihypertensive drugs 2	16.5 [1.1]	17.5 [2.2]	2 [2.0]
<b>Active (#16)</b> median [IQR]	63.7 [14.5]	7.0 [7.5]	4.2 [3.5]	Vasodilat 15 ERA 3 Cyclosp 3 Mtx 2 Aspirin 15 PPI 14 Antihypertensive drugs 3	17.9 [2.0]	21.1 [2.8]	4 [2.0]
<b>Late (#16)</b> median [IQR]	65.9 [15.7]	11.6 [10.6]	7.5 [5.2]	Vasodilat 16 ERA 6 Cyclosp 3 Mtx 3 Aspirin 15 PPI 15 Antihypertensive drugs 5	18.4 [1.2]	21.6 [2.9]	6 [3.0]
<b>Early vs Late</b> Stat. signif.	n.s.	<0.001	<0.001	-	<0.02	<0.02	<0.01

**Legend:** # = number; lcSSc = limited cutaneous SSc; RP = Raynaud's phenomenon; DT = dermal thickness (ultrasound evaluation measurement in millimetres); mRSS = modified Rodnan skin score; Early, Active, Late = patterns of microangiopathy at nailfold videocapillaroscopy; ERA: endothelin-1 receptor inhibitors (average dosage 125 mg twice/day; cyclosp: cyclosporine (average dosage 150 mg/day); Mtx: methotrexate (average dosage 7.5 mg/week); aspirin (average dosage 100 mg/day); PPI: proton pump inhibitors.

**Table IV.** Clinical findings in in limited cutaneous systemic sclerosis (lcSSc) and healthy subjects (CNT). RP = Raynaud’s phenomenon; DT = dermal thickness (ultrasound evaluation); mRSS = modified Rodnan skin score; DU = digital ulcer presence at the evaluation; GI = gastrointestinal involvement in our patients was represented by alteration of gastrointestinal motility evaluated by manometry; Lung = lung involvement in our group of SSc was represented by mild / moderate fibrosis at the HRCT evaluation; Heart = cardiac involvement was represented by presence of systolic pulmonary artery pressure >40 mm Hg at echocardiography; limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc), antinuclear antibody (ANA) patterns, centrom: centromeric (ACA+), early, active, late = patterns of microangiopathy at nailfold videocapillaroscopy; vasodilat: vasodilators; PPI: proton pump inhibitor. The results were reported as median and interquartile range [IQR], N.A. = not applicable.

	<b>CNT (#5)</b> median [IQR]	<b>lcSSc (#8)</b> median [IQR]	<b>lcSSc vs CNT</b> Stat. signif.
<b>Age</b> (years)	60.0 [3.2]	60.0 [10.7]	p>0.05
<b>RP duration</b> (years)	-	15.5 [11.7]	N.A.
<b>SSc duration</b> (years)	-	7.8 [8.1]	N.A.
<b>mRSS Total</b> (score)	-	17.5 [4.8]	N.A.
<b>Organ involvement</b>	-	DU1,GI 0, Lung 0, Heart 0	N.A.
<b>Skin</b>	-	lcSSc 8 dcSSc 0	N.A.
<b>ANA</b>	-	Centrom 7 Negative 1	N.A.
<b>ENA</b>	-	Centromer 7 Neg 1	N.A.
<b>Capillaroscopy pattern</b>	-	Early 0, Active 8 Late 0	N:A.
<b>Treatments</b>	PPI 2	Vasodilat 8, Aspirin 7 PPI 7	N.A.

**Table V.** Dermal thickness (DT) in healthy subjects (CNT) and in limited cutaneous systemic sclerosis (lcSSc) patients, evaluated by both 18 and 22 MHz transducers in the seventeen skin areas.

Skin area	lcSSc			CNT			lcSSc vs CNT	
	18 MHz Median DT [IQR]	22 MHz Median DT [IQR]	p value 18 vs 22 MHz	18 MHz Median DT [IQR]	22 MHz Median DT [IQR]	p value 18 vs 22 MHz	p value	
							18 MHz	22 MHz
Chees	0.88 [0.16]	0.90 [0.17]	<0.0001	0.68 [0.05]	0.69 [0.06]	0.001	<0.0001	<0.0001
3th right finger	0.88 [0.27]	1.32 [0.55]	<0.0001	0.69 [0.10]	0.7 [0.10]	<0.0001	<0.0001	<0.0001
3th left finger	0.93 [0.21]	1.32 [0.60]	<0.0001	0.70 [0.10]	0.71 [0.10]	<0.0001	<0.0001	<0.0001
Right hand dorsum	0.85 [0.23]	0.97 [0.33]	<0.0001	0.68 [0.09]	0.70 [0.08]	0.008	<0.0001	<0.0001
Left hand dorsum	0.85 [0.23]	0.97 [0.33]	<0.0001	0.69 [0.08]	0.70 [0.07]	0.003	<0.0001	<0.0001
Right forearm	1.00 [0.29]	1.10 [0.43]	<0.0001	0.76 [0.07]	0.77 [0.08]	0.003	<0.0001	<0.0001
Left forearm	1.00 [0.34]	1.05 [0.44]	<0.0001	0.76 [0.07]	0.77 [0.09]	0.003	<0.0001	<0.0001
Right upper-arm	1.10 [0.22]	1.20 [0.31]	<0.0001	0.85 [0.12]	0.86 [0.12]	0.008	<0.0001	<0.0001
Left upper-arm	1.10 [0.20]	1.15 [0.30]	<0.0001	0.85 [0.10]	0.86 [0.12]	0.005	<0.0001	<0.0001
Chest	1.30 [0.30]	1.31 [0.20]	<0.0001	1.12 [0.02]	1.13 [0.09]	0.001	0.005	<0.0001
Abdomen	1.30 [0.20]	1.40 [0.30]	<0.0001	1.12 [0.02]	1.13 [0.05]	0.009	0.005	<0.0001
Right thigh	1.30 [0.41]	1.40 [0.30]	<0.0001	1.07 [0.34]	1.10 [0.34]	0.003	0.1	0.005
Left thigh	1.20 [0.40]	1.40 [0.35]	<0.0001	1.09 [0.34]	1.10 [0.34]	0.006	0.1	0.005
Right leg	1.00 [0.26]	1.10 [0.29]	<0.0001	0.93 [0.03]	0.94 [0.04]	0.004	0.01	0.005
Left leg	1.09 [0.29]	1.10 [0.36]	<0.0001	0.93 [0.03]	0.89 [0.04]	0.005	0.01	0.004
Right foot	0.98 [0.11]	0.99 [0.80]	<0.0001	0.86 [0.03]	0.89 [0.04]	0.009	<0.0001	<0.0001
Left foot	0.98 [0.20]	0.99 [0.22]	<0.0001	0.89 [0.04]	0.90 [0.04]	0.004	<0.0001	<0.0001

**Table VI.** The percentage of fibrocytes at basal time (T0) and after 8 days of culture (T8), and gene expression values of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), type I collagen (COL 1), transforming growth factor- $\beta$ 1 (TGF $\beta$ 1), and fibroblast specific protein-1 (S100A4) at T8 in limited cutaneous systemic sclerosis (lcSSc) patients and healthy subjects (CNT).

	<b>CNT (#5)</b> median [IQR]	<b>lcSSc (#8)</b> median [IQR]	<b>lcSSc vs CNT</b> Stat. signif.
<b>DT Total 18 MHz Probe</b> (millimetres)	13.5 [1.6]	19.5 [4.1]	p<0.01
<b>DT Total 22 MHz Probe</b> (millimetres)	14.1 [1.3]	18.6 [2.7]	p<0.01
<b>Fibrocytes at T0</b> (%)	0.4 [1.2]	0.8 [2.7]	p>0.05
<b>Fibrocytes at T8</b> (%)	60.2 [44.6]	56.7 [40.7]	p>0.05
<b><math>\alpha</math>SMA expression level (T8)</b>	1	11.1 [6.8]	p<0.01
<b>COL 1 expression level (T8)</b>	1	22.9 [18.1]	p<0.01
<b>TGF<math>\beta</math>1 expression level (T8)</b>	1	3.6 [3.5]	p<0.01
<b>S100A4 expression level (T8)</b>	1	2.5 [1.3]	p<0.01

**Table VII.** Correlation between the different methods (modified Rodnan skin score, high frequency skin ultrasound with the 18 and 22 MHz probes) to evaluate dermal tickness (DT) and the percentage of fibrocytes at basal time (T0) and after 8 days of culture (T8), as well as gene expression values of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), type I collagen (COL 1), transforming growth factor- $\beta$ 1 (TGF $\beta$ 1), and fibroblast specific protein-1 (S100A4) at T8 in limited cutaneous systemic sclerosis (lcSSc) patients.

	<b>mRSS Total</b> (score)	<b>DT Total</b> <b>18 MHz Probe</b> (millimetres)	<b>DT Total</b> <b>22 MHz Probe</b> (millimetres)
<b>mRSS Total</b> (score)	-	<b>p=0.05</b> <b>[r=0.59]</b>	<b>p=0.05</b> <b>[r=0.43]</b>
<b>DT Total 18 MHz Probe</b> (millimetres)	<b>p=0.05</b> <b>[r=0.59]</b>	-	<b>p=0.04</b> <b>[r=0.60]</b>
<b>DT Total 22 MHz Probe</b> (millimetres)	<b>p=0.05</b> <b>[r=0.43]</b>	<b>p=0.04</b> <b>[r=0.60]</b>	-
<b>Fibrocytes at T0</b> (%)	<b>p=0.04</b> <b>[r=0.96]</b>	<b>p=0.05</b> <b>[r=0.52]</b>	<b>p=0.03</b> <b>[r=0.66]</b>
<b>Fibrocytes at T8</b> (%)	p=0.9 [r=0.63]	p=0.19 [r=0.46]	p=0.92 [r=0.20]
<b><math>\alpha</math>SMA expression level (T8)</b>	p=0.22 [r=0.48]	p=0.48 [r=0.30]	p=0.67 [r=0.16]
<b>COL 1 expression level (T8)</b>	p=0.18 [r=0.41]	p=0.84 [r=0.29]	p=0.18 [r=44.6]
<b>TGF<math>\beta</math>1 expression level (T8)</b>	p=0.48 [r=0.49]	p=0.94 [r=0.30]	p=0.92 [r=0.71]
<b>S100A4 expression level (T8)</b>	p=0.49 [r=0.63]	p=0.18 [r=0.45]	p=0.26 [r=0.22]

**Figure 1.** Update of 2017 EULAR recommendations for the treatment of systemic sclerosis.

## Recommendations

**Table 1** The final set of 14 recommendations based on both evidence from the literature and expert opinion

No	Recommendation	Strength of recommendation	References
I	SSc-related digital vasculopathy (RP, digital ulcers)		
1	A meta-analysis on dihydropyridine-type calcium antagonists and one meta-analysis on prostanoids indicate that nifedipine and intravenous iloprost reduce the frequency and severity of SSc-RP attacks Dihydropyridine-type calcium antagonists, usually oral nifedipine, should be considered for first-line therapy for SSc-RP, and intravenous iloprost, or other available intravenous prostanoids for severe SSc-RP	A	14 24
2	Two RCT indicate that intravenous prostanoids (particularly intravenous iloprost) are efficacious in healing digital ulcers in patients with SSc. Intravenous prostanoids (in particular iloprost) should be considered in the treatment of active digital ulcers in patients with SSc	A	21 22
3	Bosentan has no confirmed efficacy in the treatment of active digital ulcers in SSc patients. Bosentan has confirmed efficacy in two high-quality RCT to prevent digital ulcers in diffuse SSc patients, in particular in those with multiple digital ulcers Bosentan should be considered in diffuse SSc with multiple digital ulcers after failure of calcium antagonists and, usually, prostanoid therapy	A	30-32
II	SSc-PAH		
4	Two high-quality RCT indicate that bosentan improves exercise capacity, functional class and some haemodynamic measures in PAH. Bosentan should be strongly considered to treat SSc-PAH	A/B	36 37
5	Two high-quality RCT indicate that sitaxentan improves exercise capacity, functional class and some haemodynamic measures in PAH. At present, sitaxentan may also be considered to treat SSc-PAH	A/B	39 47 48
6	One high-quality RCT indicates that sildenafil improves exercise capacity, functional class and some haemodynamic measures in PAH Sildenafil may be considered to treat SSc-PAH	A/B	52
7	One high-quality RCT indicates that continuous intravenous epoprostenol improves exercise capacity, functional class and haemodynamic measures in SSc-PAH. Sudden drug withdrawal may be life threatening Intravenous epoprostenol should be considered for the treatment of patients with severe SSc-PAH	A	29
III	SSc-related skin involvement		
8	Two RCT have shown that methotrexate improves skin score in early diffuse SSc. Positive effects on other organ manifestations have not been established Methotrexate may be considered for treatment of skin manifestations of early diffuse SSc	A	60 61
IV	SSc-ILD		
9	In view of the results from two high-quality RCT and despite its known toxicity, cyclophosphamide should be considered for treatment of SSc-ILD	A	62 63
V	SRC		
10	Despite the lack of RCT, experts believe that ACE inhibitors should be used in the treatment of SRC	C	64-66
11	Four retrospective studies suggest that steroids are associated with a higher risk of SRC. Patients on steroids should be carefully monitored for blood pressure and renal function	C	67-70
VI	SSc-related gastrointestinal disease		
12	Despite the lack of specific RCT, experts believe that PPI should be used for the prevention of SSc-related gastro-oesophageal reflux, oesophageal ulcers and strictures	B	71 72
13	Despite the lack of specific RCT, experts believe that prokinetic drugs should be used for the management of SSc-related symptomatic motility disturbances (dysphagia, GORD, early satiety, bloating, pseudo-obstruction, etc)	C	73-80
14	Despite the lack of specific RCT, experts believe that, when malabsorption is caused by bacterial overgrowth, rotating antibiotics may be useful in SSc patients	D	

ACE, angiotensin-converting enzyme; GORD, gastro-oesophageal reflux disease; PAH, pulmonary arterial hypertension; PPI, proton pump inhibitor; RCT, randomised controlled trial; RP, Raynaud's phenomenon; SRC, scleroderma renal crisis; SSc, systemic sclerosis; SSc-ILD, SSc-related interstitial lung disease; SSc-PAH, SSc-related pulmonary arterial hypertension; SSc-RP, SSc-related Raynaud's phenomenon.

**Figure 2.** Evaluation by quantitative real-time polymerase chain reaction (qRT-PCR) of the gene expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), fibroblast specific protein-1 (S100A4), type I collagen (COL-1) and transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) in cultured human fibrocytes isolated from healthy subjects (CNT) and systemic sclerosis patients (SSc) maintained in Dulbecco's Modified Eagle's Medium (DMEM) growth medium at 20% of Fetal Bovine Serum (FBS) for 8 days (T8). The gene expression of  $\alpha$ -SMA, S100A4, COL-1 and TGF $\beta$ 1 in cultured human SSc fibrocytes was compared to that detected in cultured CNT fibrocytes, which was taken as unit value by definition.

