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TESI DI LAUREA SPERIMENTALE

Microvascular involvement and clinical features in scleroderma patients

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1. INTRODUCTION

1.1 SYSTEMIC SCLEROSIS

Systemic sclerosis is an immune-mediated rheumatic disease that is characterised by vasculopathy and fibrosis of the skin and internal organs [1].

The typical patient is a young or middle-age woman with a history of Raynaud phenomenon who presents with skin induration and internal organ dysfunction [2].

Although systemic sclerosis is a rare disease with a prevalence lower than 5 out of 10.000 cases, it may have a high morbidity and mortality if untreated. Improved understanding of systemic sclerosis has allowed better management of the disease, including improved classification and more systematic assessment and follow-up. Additionally, treatments for specific complications have emerged and a growing evidence base supports the use of immune suppression for the treatment of skin and lung fibrosis. Some manifestations of the disease, such as scleroderma renal crisis, pulmonary arterial hypertension, digital ulceration, and gastro-oesophageal reflux, are now more treatable [1].

Systemic sclerosis has a high mortality, greater than other rheumatic diseases, despite evidence of improved survival, especially for patients with diffuse cutaneous systemic sclerosis [3].

1.2 PATHOGENESIS AND CLINICAL FEATURES

Pathogenesis of systemic sclerosis involves endogenous and exogenous trigger factors which promote epigenetic mechanisms in genetically primed subjects. Some risk factors are known and include combination of persistent Raynaud's phenomenon, steroid hormone imbalance, selected chemicals and silicon breast implants and frequent thermal or other mechanical injuries, mainly at acral regions of the body [4]. The first damages are early microvascular changes with endothelial cell dysfunction, followed by the activation of mechanisms promoting their transition to myofibroblasts. Microvascular damage is followed by a complex autoimmune response, involving innate and adaptive immunity with autoantibody production. Finally, a progressive fibrosis and ischemia involve skin and visceral organs resulting in their irreversible damage. Monocytes and fibrocytes, together with growth factors and cytokines, participate in disease evolution [4].

1.2.1 Microvascular pathophysiology

As previously stated, Raynaud phenomenon (RP) is highly prevalent in patients with SSc and patients with primary RP progress in almost 15% of cases to secondary RP associated with SSc. The initial but reversible microvascular damage induced by primary RP in presence of other risks factors and enhanced immune response progress to secondary RP (SRP), with irreversible micro vessel deletion, capillary destruction, and subsequent increase in tissue fibrosis. Evidence suggests that SRP in SSc results from a vasculopathy involving all layers of the peripheral blood vessels and in part is caused by the dysfunction of the endothelium. An abnormal function of the endothelium is due to an imbalance of vasoactive factors with overproduction of the vasoconstrictor endothelin-1 (ET-1) in skin, lung tissue and serum and

underproduction of the vasodilator nitric oxide (NO) and prostacyclin. The alteration of the microvascular tone is a noxious trigger to the endothelial barrier leading to opening of the endothelial junctions, further inflammatory cells homing, increased micro vessel permeability and continuous vascular leak. This phenomenon causes micro haemorrhages and local oedema [5]. In addition, severe vasospasm of digital arteries and cutaneous thermoregulatory vessels is observed in SSc with repeated bouts of vasoconstriction that potentially may cause severe obstacle to the microcirculation and ischemia-reperfusion injury of tissues [6]. The microvascular damage is paralleled by an increased production of pro-angiogenic factors (i.e. VEGF-A, ET-1) and, on the other hand, a defective response of the damaged endothelial cells [7]. Despite the increase of VEGF-A in SSc skin and serum, there is a clear evidence of an insufficient angiogenic response [8]. Microvascular endothelial cell injury and apoptosis is a central event in the pathogenesis of SSc vasculopathy that leads to immune system activation [9].

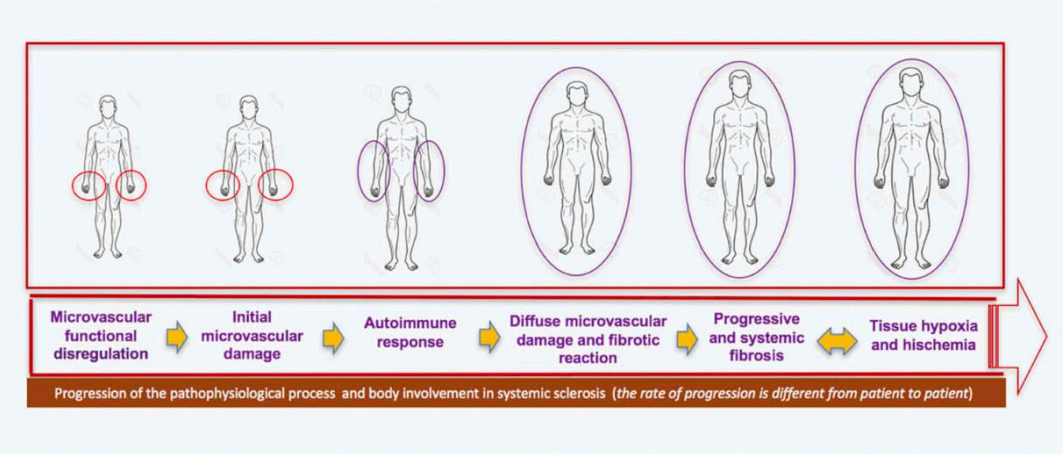

Figure 1: progression of the pathophysiological process and body involvement from: ("Pathophysiology of systemic sclerosis: current understanding and new insights by M. Cutolo, S. Soldano, V. Smith")

1.2.2 The immune system response in SSc

The alteration of both innate and adaptive immune response plays a prominent role in early SSc pathophysiology and includes the altered functions of inflammatory cells and cytokines in target tissues, such as skin and lungs. Evidence demonstrated that polymorphisms in IFN-regulatory factors confer an increased risk of SSc, and IFN excess is evident in the blood and skin of a large percentage of SSc patients [10]. Among T cells ($CD4^+$ T cells), the type 2 T helper (TH2) cells characterised by secretion of IL-4 and IL-13, are more expressed in SSc than TH1 cells, which primarily secrete anti-fibrotic IFNγ [11]. In early SSc, circulating progenitor cells, such as monocytes recruited from the bone marrow, migrate in the tissues together with T cells and macrophage precursors, and create a perivascular infiltrate [12]. Macrophages, after differentiation from monocytes, can generate different phenotypes of cells distinguished by different surface markers as classically activated (M1) or alternatively activated (M2) macrophages [12]. Generally, M1 macrophages are effector phagocytes with an increased microbicidal or tumoricidal capacity and they produce proinflammatory cytokines like TNF-alpha, IL-6, IL. In contrast, M2-polarized macrophages produce anti-inflammatory cytokines, mostly IL-4, IL-13 and IL-10 [13]. During the tissues wound healing or at the peak of the profibrotic late immune response, M2 macrophages are considered as inducers of tissue fibrosis in SSc. Therefore, M2 macrophages partially suppress M1 and promote extracellular matrix (ECM) protein synthesis, including profibrotic cytokine release; they also potentiate the anti-inflammatory response by inducing Th2 effector activities [13].On the other hand, an evident B cell activation produces several autoantibodies (AAb) targeting a variety of nuclear, cytoplasmatic, and extracellular autoantigens which are hallmark of

SSc and are observed at diagnosis in more than 95% of patients [14]. Some of these AAbs are highly specific for SSc [15] and, although they usually do not directly contribute to disease pathogenesis, are used as routine markers for diagnosis and prognosis of organ involvement [16]. In diffuse cutaneous SSc (dcSSc) anti-topoisomerase AAbs (ATA), formerly known as anti-Scl70 AAbs, are more prevalent, whereas anticentromere AAbs (ACA) are more frequent in limited cutaneous SSc (lcSSc). A possible pathogenic role of ATA in SSc has been suggested following its binding to fibroblasts and induced adhesion and activation of cocultured monocytes [17]. This observation might provide an explanation for the amplification of the fibrogenic cascade in ATA-positive SSc patients. In patients with isolated Raynaud syndrome, the presence of ACA has been reported to predict the likelihood that these patients will progress to SSc [18]. In general, ACA positive patients have a better prognosis and show lower mortality than SSc patients with other anti- nuclear AAbs (ANA); however, 50% will eventually die from pulmonary arterial hypertension (PAH) [14]. Similarly to ATA, also anti-RNA polymerase I/III AAbs (anti- RNAP) are typically associated with a more rapidly progressive dcSSc. Interestingly, the presence of anti-RNAP, especially the presence of anti-RNAP III, is strongly associated with renal crisis and malignancy [15,16,19]. Other AAbs that are highly specific for SSc include anti-Th/To ribonucleoprotein (RNP) AAbs; anti-fibrillarin/U3RNP and anti- U11/U12RNP AAbs, and anti- U1RNP AAbs [14].

1.2.3 Fibrotic process in SSc

SSc fibrosis represents a failure to terminate the normal tissue repair to the inflammatory stimulus. Fibrosis with progressive tissue accumulation of ECM proteins like collagens, elastin, glycosaminoglycans, tenascin and fibronectin in skin and

multiple organs, is a prominent pathological finding and distinguishing hallmark of clinically overt SSc (limited cutaneous and diffuse cutaneous SSc). Fibroblasts are differentiated in activated myofibroblasts as principal effector cells during the progression of the disease. Among growth factors and cytokines implicated in SSc, TGF- β, a pleiotropic cytokine, is considered the main modulator of fibrosis and it is produced from M2 macrophages and other cells (i.e. endhotelial cells) as inactive precursor, it is accumulated within the ECM and converted to its biologically active form via integrin-mediated activation [20]. Moreover, it was found that ET-1 produced by activated endothelial cells contributes to myofibroblast activation using TGF-β machinery via an ET-1/TGF-β receptor complex and it is now evident that activated fibroblasts also produce ET-1 [21]. This is an important finding as it explains ET-1 role in fibrosis pathogenesis in SSc. As a matter of fact, inhibitors of ET-1/2 receptors (bosentan, macitentan) have been found to interfere with the profibrotic action of TGF-β, blocking in detail the ET-1 receptor portion of the ET-1/TGF-β receptor complex [22]. The progression of the mechanisms implicated in the complex pathophysiology of SSc are clinically mirrored by the patient complications, and partially morphologically reflected by the observation of the microvascular alterations that are scored by nailfold videocapillaroscopy as progressive ‘early’, ‘active’ and ‘late’ patterns [23-27].

1.3 DISEASE MANIFESTATIONS AND VASCULOPATHY

Vascular dysfunction is a major feature of SSc that is understood to be of particular importance to the development of Raynaud’s phenomenon, digital ulceration, scleroderma renal crisis, and pulmonary arterial hypertension (PAH) [28,29].

Raynaud's phenomenon is often the first symptom of the disease. It is characterized by reversible vasospasm of the arterioles in the distal extremities combined with dilation of the capillaries in response to cold or other stimuli, and can be associated with a sensation of burning or severe pain. Digital tip ulcers are understood to result from ischemia, which arises from vasospasm, intimal fibro-proliferation, and thrombosis of the digital arteries [30].

Renal complications and scleroderma renal crisis affect an estimated 5–10% of SSc patients and were formerly the most lethal manifestations in the past [31]. Scleroderma renal crisis, defined as the new onset of severe hypertension associated with rapid increases in serum creatinine concentration, microangiopathic haemolytic anaemia, or both, constitutes one of medical emergencies in SSc [32]. The pathophysiology underlying scleroderma renal crisis is incompletely characterized and may arise following endothelial cell injury in the renal arteries, epithelial-to-mesenchymal trans differentiation and fibrosis in the glomerular and tubulo-interstitial compartments, and dysregulation of endothelin (ET)-1 receptor expression [33,34]. The progression of renal failure can be exacerbated by reduced renal perfusion and hyper-reninemia [32]. In recent years, PAH associated with SSc has been recognized as one of the leading causes of SSc-related mortality [31].

PAH has ben defined as a mean pulmonary artery pressure ≥ 25 mmHg with a pulmonary capillary wedge pressure ≥15 mmHg measured by cardiac catheterization [35]. Consistent with SSc, the mechanisms underlying the pathophysiology of PAH include vasoconstriction, inflammation and fibrosis. An errant expression of ET-1, thromboxane, prostacyclin and nitric oxide has also been implicated in the development of PAH, and ET-1 pathway is targeted by current therapeutic approaches [36].

1.4 OTHER CLINICAL FEATURES

1.4.1 Skin

Considering skin involvement, scleroderma patients can be classified into three different groups with different clinical and serological features: diffuse systemic sclerosis (dcSSc), limited systemic sclerosis (lcSSc) and systemic sclerosis sine scleroderma. Patients with dcSSc are characterized by excess collagen production and consequent skin thickening over large areas of the body, usually fingers, hands, arms, anterior trunk, legs and face (*figure 2*). They usually have a contemporary or very near (< 1 year) development of Raynaud phenomenon and scleroderma and an early internal organ disease. The more prevalent serologic markers of dcSSc are autoantibodies against topoisomerase I (anti-Scl 70).

Patients with lcSSc, in contrast, usually have a long interval between the development of Raynaud phenomenon and scleroderma, which is generally limited to fingers, hands, forearms, legs, feet and face, but can also be absent (sine-scleroderma).

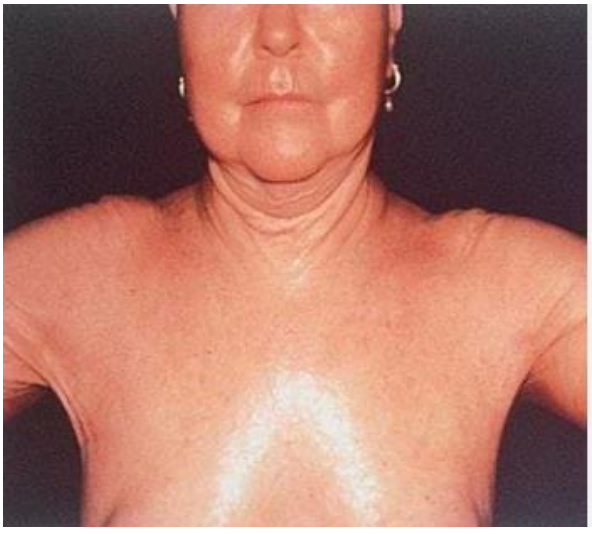

Figure 2: Skin involvement in systemic sclerosis (from MSD Manual)

lcSSc patients can have a late development of PAH and they may present calcinosis and telangiectasias. Autoantibody against centromere (ACA) are the more prevalent serologic marker of this group of patients. There are three evolutionary phases of cutaneous involvement: oedematous, indurative or sclerotic and atrophic. During the advanced sclerotic phase the hardening and tightening of the skin is typical: hairs thin out, lips are thinned and the opening of the oral rhyme is reduced. The tightening of the skin to the underlying plans in the articular regions, together with tendon fibrosis, causes flexion contractures (i.e. claw hands). In these regions, pressure exerted by the underlying bone on the stretched skin can determine the development of ulcers that can be difficult to treat because of superinfection and hypoxia. Other cutaneous manifestations are pigment changes of the skin: "melanoderma" and "pseudo-vitiligo", telangiectasia and calcinosis. The extent of sclerosis varies in the different stages of the disease and can be clinically evaluated by the Rodnan Skin Score, which is based on the evaluation of skin thickening on a scale from 0 to 3 in 17 body areas. The evaluation of this score over time, especially in patients with early stage dcSSc (< 3 years from diagnosis) is useful for assessing the progression of the disease and response to therapies [37].

1.4.2 Gastrointestinal System

Gastrointestinal system, especially oesophagus, is involved in SSc. Oesophageal disease in scleroderma undergoes 3 phases: autonomic neuropathy because of vasculopathy and autoantibody damage (i.e. anti-muscarinic-3 receptor antibodies); replacement fibrosis, particularly of the tunic muscle and finally atrophy of the wall, with progressive hypomobility and organ dysfunction [38]. Initially there is a reduced tone of the lower oesophageal sphincter, which may be asymptomatic, while in some

cases can cause retrosternal burning and acid regurgitation. Therefore hypomotility of the lower 2/3 occurs and causes dilatation and atony with acid gastroesophageal reflux and dysphagia. This can cause peptic esophagitis, associated, in some cases, with Barrett's metaplasia and evolution to cicatricial stenosis. Stomach is less frequently affected, although dilatation and atony may occur and facilitate the development of duodeno-gastric reflux that may cause feeling of early satiety.

The small intestine is affected in 40% of cases, especially in lcSSc and SSc sine scleroderma. Wall fibrosis causes bowel hypomotility which may result in paralytic ileus. Colic wall fibrosis causes hypomotility and is complicated by the development of broad-based diverticula.

Intestinal involvement is manifested by a sense of distension and abdominal cramps. It can give diarrhea in case of superinfection of the hypokinetic small intestine and secondary malabsorption syndrome (SIBO syndrome) [37].

1.4.3 Respiratory system

Pulmonary involvement is manifested by two distinct pulmonary pictures, which can also coexist. In 70% of patients, an inflammatory interstitial disease occurs, which, in most cases, manifests itself with a substrate of NSIP (non-specific interstitial pneumonia) and less frequently UIP (usual interstitial pneumonia) or, even more rarely, other substrates, predominantly in the lower lung fields (*figure 3*). Interstitial disease is expressed with the appearance of dyspnea on exertion, less frequently at rest and with the finding of bibasal crackles on physical examination.

In 10% of patients, 10-20 years after the onset of the disease, pulmonary arterial hypertension occurs: it is caused by vascular disease of the small and medium-sized pulmonary arteries.

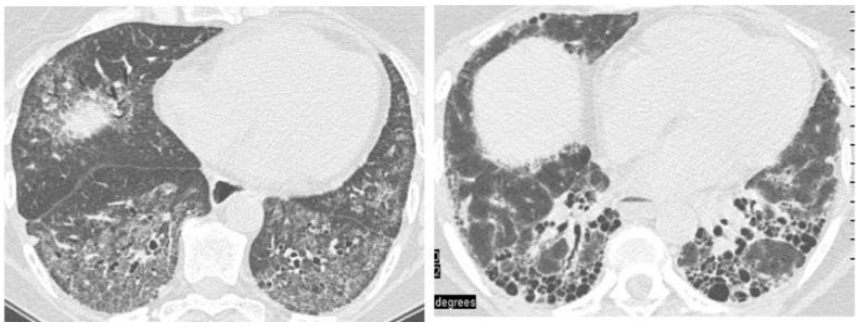

Figure 3: NSIP on the left and UIP on the right.
(from "Interstitial lung disease in systemic sclerosis", Revue des maladies respiratoire 2007)

Pulmonary function tests show a restrictive dysventilatory syndrome with decrease in total lung capacity (TLC), forced vital capacity (FVC), forced expiratory flow in the first second (FEV1), residual volume and alveolar-capillary diffusion of CO (DLCO). The possible development of dry or exudative pleurisy, clinically evident in 15% of patients, should also be considered in patients with SSc [37].

1.4.4 Heart

Scleroderma heart disease is divided into primary or secondary. The manifestations related to direct involvement of the cardiac tissue are primary, while the conditions resulting from a primary extracardiac involvement (pulmonary or renal) are secondary. SSc causes changes in the pericardium, myocardium and, to a lesser degree, valves. Pericarditis can be acute or chronic. Myocardial involvement is characterized by fibrosis, supported both by accumulation of collagen in the interstitium and by vascular disease of the small coronary arteries. Unlike atherosclerotic fibrosis, scleroderma myocardial fibrosis affects both ventricles and is clinically manifested by conduction disturbances such as atrioventricular blocks, bundle branch blocks and supraventricular and ventricular arrhythmias. These can be responsible in fatal cases for sudden death or congestive heart failure, especially diastolic.

1.4.5 Kidney

Kidney disease in systemic sclerosis is expressed essentially with the so-called "scleroderma renal crisis" or scleroderma malignant hypertension, characterized by abruptly worsening renal failure associated with accelerated or malignant hypertension, visual disturbances due to hypertensive retinopathy, headache and possible development of cerebral stroke or pulmonary oedema. In some cases microangiopathic haemolytic anaemia and thrombocytopenia from intravascular coagulation occur.

1.4.6 Joints and Tendons

Arthropathy is a very frequent manifestation in SSc and in some cases can represent the onset of the disease. The clinical presentation, in 10% of cases, is characterized by a symmetrical rheumatoid-like polyarthritis, often associated with carpal tunnel syndrome. More frequently these are polyarthralgias with or without stiffness or joint stiffness only. From a radiological point of view there is reduction of the joint line, juxta-articular osteoporosis, ankylosis, rheumatoid-like erosions.

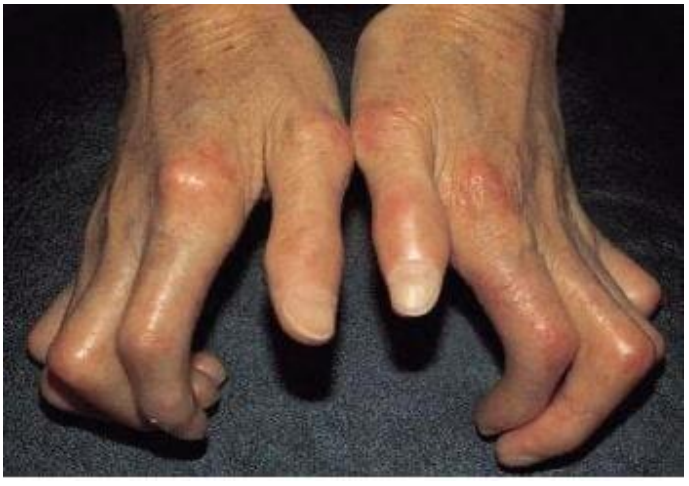

Figure 4: joints contractures in systemic sclerosis (from "MSD manuals")

Three mutually exclusive patterns were identified: an inflammatory rheumatoid-like, an osteoarthritis-like, and a periarticular fibrotic pattern. The latter is characteristic and it may be a consequence of the sclerosis of the skin and surrounding tissues, which constricts joints and determines the development of flexion contractures, configuring a characteristic periarthropathy (*figure 4*) [37].

1.4.7 Bone

The most characteristic finding is constituted by acro-osteolysis of the distal phalanges on an ischemic basis (*figure 5*). An increased incidence of osteoporosis associated with vitamin D deficiency, very frequent in SSc, is described.

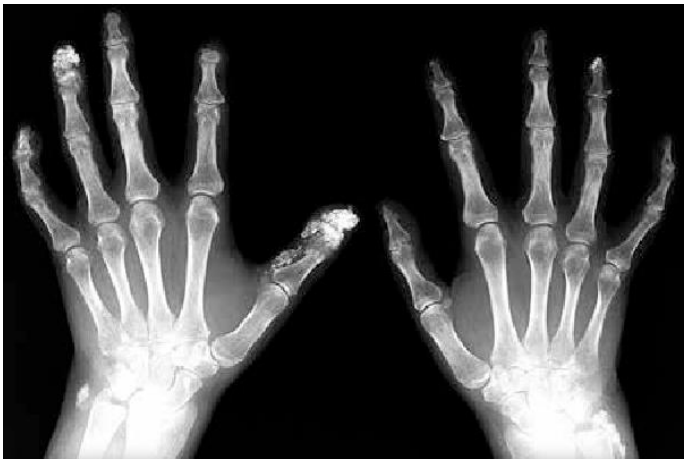

Figure 5: acroosteolysis of distal phalanges in patient with systemic sclerosis (from: Dermatology Review, 2016)

1.4.8 Muscle

Three types of muscle involvement can occur: disuse myopathy, which is expressed only with mild muscle weakness; primary myopathy, characterized by proximal muscle weakness and mild elevation of muscle enzymes (CPK, LDH) and

polymyositis in overlap. The latter is associated with the presence in the serum of specific antibodies, called anti-Pm-Scl [37]

1.5 DIAGNOSIS

Once systemic sclerosis is suspected on the basis of the above reported clinical signs and symptoms, patients should be assessed so that a definite diagnosis can be ascertained. A definitive diagnosis is confirmed with the fulfilment of the 2013 European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) classification criteria (*figure 6*) [39,40].

Items	Sub-items	Weight
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints		9
Skin thickening of the fingers *(only count the highest score)*	Puffy fingers Whole Finger, distal to MCP	2 4
Finger tip lesions *(only count the highest score)*	Digital Tip Ulcers Pitting Scars	2 3
Telangiectasia		2
Abnormal nailfold capillaries		2
Pulmonary arterial hypertension and/or Interstitial lung Disease		2
Raynaud's phenomenon		3
Scleroderma related antibodies (any of anti-centromere, anti-topoisomerasel [anti-ScL 70], anti-RNA polymerase III)		3
	TOTAL SCORE:	
Patients having a total score of 9 or more are being classified as having definite systemic sclerosis.		

Figure 6:ACR 2013 classification criteria for systemic sclerosis
(from "Classification Criteria for Systemic Sclerosis: Preliminary Results", BMJ, 2014)

1.6 NAILFOLD VIDEOCAPILLAROSCOPY IN SYSTEMIC SCLEROSIS

1.6.1 Definition and clinical use

Morphological capillary abnormalities are a peculiar feature of scleroderma microangiopathy and nailfold videocapillaroscopy (NVC) represents the best non invasive method to detect and analyse the microvascular abnormalities.

Nailfold is the skin that overlaps the edge of a fingernail and the dermis at this site is as thin as the capillary can be seen directly. The gold-standard to perform NVC is the optical videocapillaroscope equipped with a 200 magnification probe and connected to image analysis software, after a drop of immersion oil is applied to the nailfold. In order to prevent vasospasm, the patient should be inside a room with a temperature of 20–22 degrees for a minimum of 15 minutes before the nailfold is examined. Nailfolds of the second, third, fourth and fifth fingers are examined. Many capillaroscopic parameters are evaluated: density, dimension, morphology and presence of haemorrhages. A mean number of 7 capillaries per linear mm, measured in the distal row, is accepted as stereotype normal.

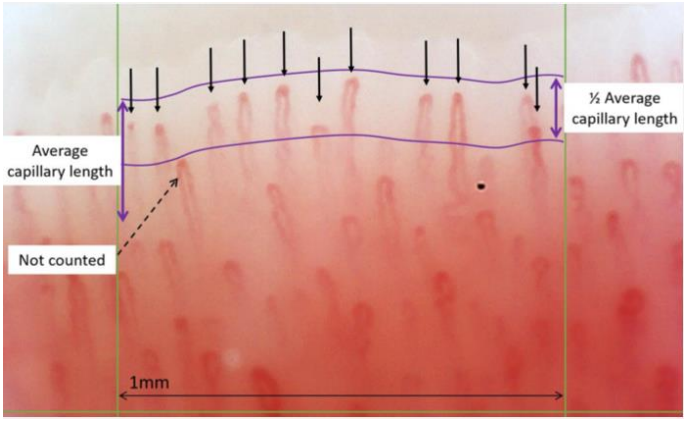

Figure 7: Normal videocapillaroscopic picture. (from "Fast Track alghorithm: How ro differentiate a "scleroderma pattern" from a non scleroderma pattern" Autoimmunity Reviews 2019, , Smith V., Cutolo M. et al)

The normal dimension measured at the transitional limb of a capillary is 20 μm (*figure* 7). Non-specific abnormalities are between 20-50 μm, while a normal shaped capillary with an apical diameter ≥ 50 μm is called a "giant" capillary. The detection of even a single giant capillary should be considered a potential marker of microangiopathy [41]. Capillaries with "hairpin" shape, crossing shape or tortuous shape (the afferent and efferent limb undulate but do not cross) are defined as being "normal" on the condition that the tip of the capillary is convex. All other shapes are defined as being "abnormal" (*figure 8).*Increased capillary diameters, abnormal direction, ramifications and reduced number of capillaries are seen in patients with SSc. These sequential capillaroscopic changes, which are the so-called NVC scleroderma spectrum abnormality, are typical of microvascular involvement in SSc.

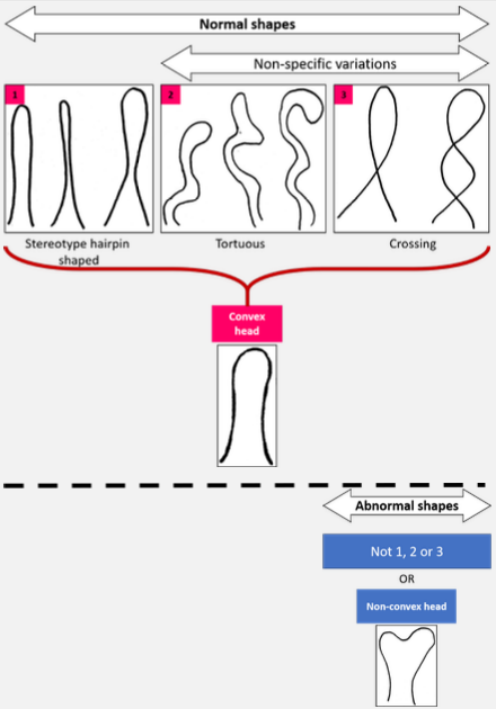

Figure 8: normal and abnormal capillaries shapes (from: "Fast Track alghorithm: How ro differentiate a "scleroderma pattern" from a non scleroderma pattern" Autoimmunity Reviews 2019, , Smith V., Cutolo M. et al)

NVC is established as a validated method to evaluate microvascular damage and is considered to be indispensable in the diagnosis and treatment of SSc [42,43]

One of the most significant changes of ACR/EULAR criteria proposed in 2013 was that nailfold capillary abnormalities were listed as one of the new items [44,45].

1.6.2 The scleroderma patterns

"Non-scleroderma patterns" consist of stereotype normal or combination of non-specific abnormalities, while "scleroderma patterns" are characterised by either the presence of giant capillaries ("early" and "active" scleroderma pattern) or either by a combination of extremely lowered density combined with "abnormal shapes" ("late pattern") [41]. See table I for more details.

The early pattern is characterized by few giant capillaries, few capillary microhaemorrhages and no evident loss of capillaries (*figure 9*). The active pattern comprises frequent giant capillaries, frequent capillary microhaemorrhages and moderate loss of capillaries (*figure 10*). The late pattern is characterized by irregular enlargement of capillaries, almost absent giant capillaries and microhaemorrhages, severe loss of capillaries with extensive avascular areas, ramified capillaries and intense disorganization of the normal capillary array (*figure 11)* [43,46,47].

Capillaroscopic characteristics	**Category 1**					**Category 2**		
	Non-scleroderma pattern					**Scleroderma Pattern**		
	Normal	**Non Specific Abnormalities** If any of the capillaroscopic characteristics is abnormal, alone or in any combination, as highlighted in yellow				**Early**	**Active**	**Late**
Density (/mm)	≥ 7	↓				≥ 7	Lowered density (4-6)	Further lowered density (≤3)
Dimension (µm)	Normal		20-50			> 50 (giant)	> 50 (giant)	-
Abnormal morphology	-			+		-	+	++
Haemorrhages	-				+	+/-	+/-	-

Table I. NVC characteristics of non scleroderma and scleroderma pattern

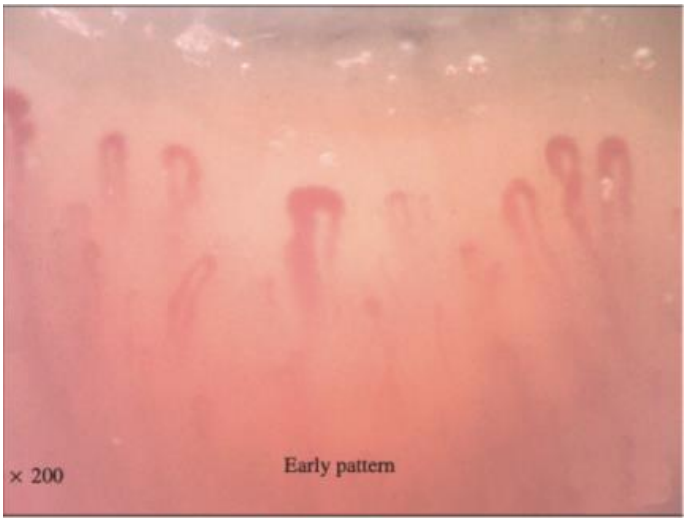

Figure 9: Scleroderma-pattern: Early pattern.

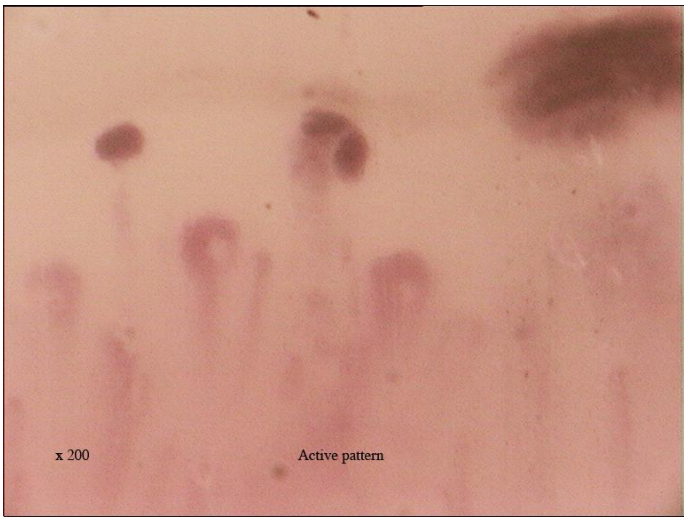

Figure 10: Scleroderma-pattern: Active pattern.

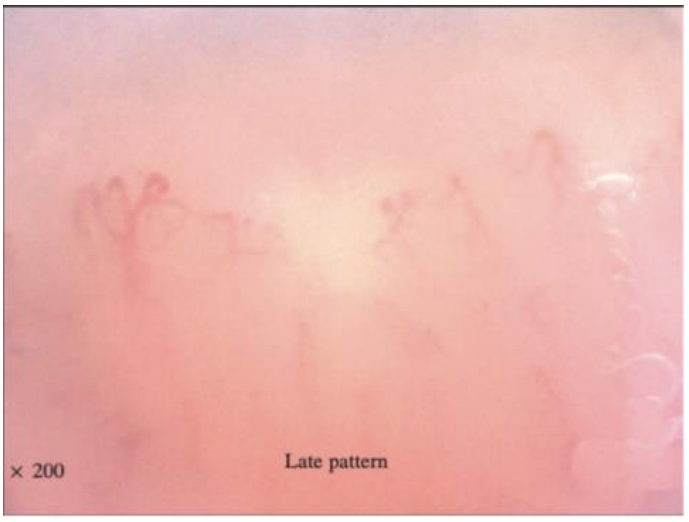

Figure 11: Scleroderma-pattern: Late pattern.

Natural progression of microvascular damage starts from the early pattern and proceeds to the late pattern [48][49]. The presence of anti- Scl70 antibodies seems to be related to the earlier expression of the active and late patterns. On the other hand, the anti-centromere antibody seems to be related to the delayed expression of the late NVC pattern [50].

1.6.3 Correlation between NVC scleroderma patterns and organ involvement

Pattern classification of nailfold capillary changes in SSc has a strong correlation with organ involvement [51]. Interstitial lung disease and pulmonary artery hypertension and skin ulcers are common in cases with advanced NVC abnormalities [52]. In particular, the loss of capillaries with extensive avascular areas is an important finding, and there are many reports that a late NVC pattern is related to the presence of organ involvement. The largest retrospective multicentre collaborative study by EULAR scleroderma trials and research (EUSTAR) database showed a correlation between organ involvement and vasculopathy [53]. In this study, the researchers investigated

the correlation between the pattern of NVC scleroderma spectrum abnormalities and clinical findings and they found out that complications of organ involvement, such as skin ulcer, pulmonary artery hypertension, interstitial lung disease and joint contractures increased with the progression of NVC scleroderma spectrum abnormalities. This can be thought of as indirect evidence that organ involvement of SSc occurs, based on vasculopathy.

The prognostic value of NVC scleroderma spectrum abnormalities in predicting SSc disease progression has been also investigated. For example, it has been reported that NVC abnormalities predict skin ulcers [54].

1.7 SKIN ULTRASOUND IN SYSTEMIC SCLEROSIS

Skin involvement is a cardinal feature of systemic sclerosis (SSc), and its extent and rate of progression are associated with visceral involvement, functional disability, and survival [55]. The modified Rodnan Skin Score (mRSS) is the current gold standard for the assessment of the skin in SSc, frequently elected as the primary or secondary endpoint in clinical trials [56]. However, this score has considerable limitations, with emphasis on the low intra- and inter-rater reproducibility and poor sensitivity to change, especially in the limited cutaneous form of the disease. There is an urgent need for more sensitive and objective measurement tools of skin involvement to support the evaluation and development of new drugs. This will certainly also facilitate an earlier diagnosis of SSc, allowing the initiation of treatment before irreversible damage of the skin and other organs is established [57].

The use of ultrasound in SSc has been extensively investigated over the last 4 decades [58,59]. However, despite these advances, the role of ultrasound in clinical practice and research in SSc is not yet established.

Recently, a systematic literature review, performed accordingly to the OMERACT (Outcome Measures in Rheumatology) filter, summarized all available evidence about the use of ultrasound to assess skin involvement in SSc [60]. This review showed that ultrasound has some advantages over mRSS, including its higher intra and inter- rater reproducibility and sensitivity to change [60].

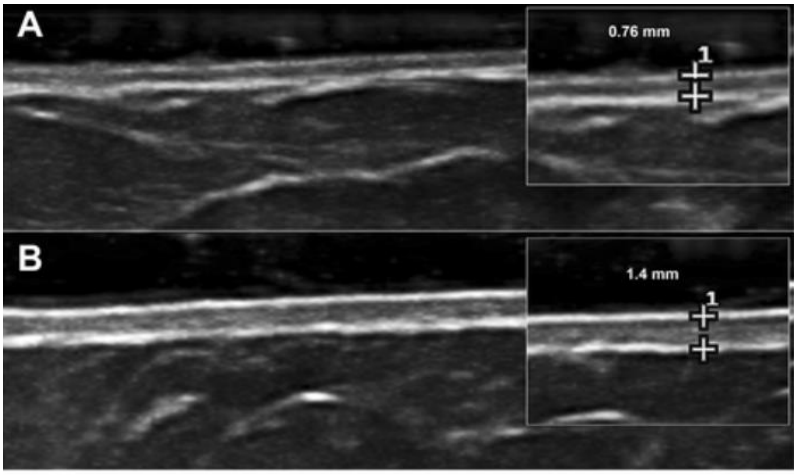

Figure 12: Dermal thickness measured with high-frequency ultrasound (from: Arthritis Research & Therapy, 2017 by Sulli A. et al)

Moreover, high-frequency ultrasound demonstrated validity against mRSS and pathological skin findings and can identify early subclinical skin involvement in areas with a normal mRSS [61].

A crucial step to further consolidate the contribution of ultrasound was the development of the first recommendations for technical execution and reporting of skin ultrasound studies by the World Scleroderma Foundation Working Group [62]. Their implementation will certainly expand standardization and harmonization of the

technical procedures, and, consequently, improve the interpretability, reproducibility, and applicability of the study results. This will represent a major advance in topics such as identification of subclinical or early disease, and assessment of response to immunosuppressive or antifibrotic therapies. Feasibility and the number and location of body sites that should be used for disease assessment and monitoring, responsiveness to change and appropriate threshold of meaning should be addressed in future good-quality and well-designed observational studies and randomized clinical trials. The clarification of the contextual factors that impact on ultrasound measures is also of crucial importance to allow their use in future clinical trials. Gender and age were found to be relevant factors influencing ultrasound dermal thickness and skin stiffness in skin Rodnan sites [57].

2. EXPERIMENTAL SECTION

Microvascular involvement and clinical features in scleroderma patients: an updated investigation

2.1 Introduction

Microvascular involvement is a dynamic event in systemic sclerosis (SSc) and it is one of the best evaluable predictors of the disease development [63]. It may be easily assessed by nailfold videocapillaroscopy (NVC) which allows to classify and score the different patterns of microvascular damage [46-48].

The extent of nailfold microangiopathy, assessed by NVC patterns and microvascular evolution score (MES), was demonstrated to correlate with organ involvement in SSc, and NVC has been proposed as a biomarker of the disease [48,51].

NVC parameters also correlate with the modified Rodnan skin score (mRSS) which is employed to evaluate the extension and severity of skin involvement [65,66]. The mRSS was found progressively greater in patients with higher MES, as well as in 'Early', 'Active' and 'Late' patterns of microangiopathy [67]. Several studies also reported the utility of skin high-frequency ultrasound (HF-US) in the early identification of the oedematous phase of the disease that may precede palpable skin involvement in SSc patients as well as its capability to reflect the overall severity of the skin involvement [68–70].

As a matter of fact, HF-US was employed to assess dermal thickness in SSc patients which was found progressively higher in SSc patients with 'Early', 'Active' and 'Late' NVC patterns of microangiopathy [66]. Moreover, a statistically significant positive

correlation between dermal thickness and MES was demonstrated by HF-US, as well as between dermal thickness and mRSS [67].

SSc patients enrolled in the above-reported studies were mainly taking aspirin, cyclic prostanoids and endothelin-1 receptor antagonists. Nowadays several drugs, such as Mycofenolate mofetile, Sildenafil, Tadalafil, Rituximab, Selexipag, Riociquat have a fundamental role in the treatment of SSc in tertiary centres, allowing a better management of the disease.

2.2 Aim

The aim of this study was to investigate if the previously reported correlations between NVC parameters and organ involvement, including dermal thickness, are still valid in SSc patients treated with a modern wider range of disease-modify drugs.

2.2 Methods

Fifty-two SSc patients were enrolled (7 males and 45 females, mean age 63±13 SD years, mean disease duration: 9,32±6,39), after written informed consent to enter the study. SSc patients met the 2013 ACR criteria for SSc classification [71]. SSc patients were consecutively recruited at the Scleroderma Clinic of the University of Genova. Complete medical history and laboratory and medical assessments were obtained (including presence of active digital ulcers). The duration of both RP and SSc was calculated by clinical interview and from the time of onset of clinical signs or symptoms clearly related to SSc other than RP. Skin involvement was assessed by mRSS and presence of limited (lcSSc) or diffuse (dcSSc) skin involvement was recorded [26]. Pulmonary function was assessed by lung volume testing, measurement of diffusing capacity for carbon monoxide (DLCO) and HRTC; renal function was

assessed by laboratory testing and arterial Doppler echography, while cardiac performance was investigated by Doppler echocardiography. Cardiac catheterization was only performed in patients with Doppler echocardiography with estimated pulmonary arterial pressure > 40 mmHg in order to confirm the diagnosis of pulmonary artery hypertension [72,73]. Finally, oesophageal evaluation was performed by manometry. Abnormal findings included the presence of oesophageal dysmotility, presence of lung alveolitis or fibrosis, forced vital capacity and/or DLCO <75% of predicted, mean pulmonary artery pressure > 25 mmHg at rest (with a pulmonary arterial wedge pressure > 15 mmHg and a pulmonary vascular resistance >3 Wood units), creatinine level >1.3 mg/dl and renal artery resistive indexes (RRI) >0.70 [72, 73]. NVC was performed in each patient to assess morphological microvascular damage using a videocapillaroscope optical probe, equipped with a 200× contact lens, connected to image analysis software (Videocap, DS Medica, Milan, Italy). The same operator performed the NVC examination in all SSc patients, according to previous published methods [46,47,63,74]. Each capillary abnormality was scored by a validated rating scale by considering the average of eight fingers [43,47,75]. The appropriate NVC pattern of microangiopathy was defined in the SSc patients on the basis of their nailfold capillary abnormalities: 'Early', 'Active' and 'Late' NVC patterns [43,46,47,49,74]. In addition, the microangiopathy evolution score (MES) was calculated by the sum of three scores: loss of capillaries, disorganisation of the microvascular array and abnormal capillary shapes [47,74]. Each abnormality was scored as follows: 0 = absent, 1 = present in ≤ 33%, 2 = present in > 33% to ≤ 66%, 3 = present in > 66% of the field [47].

Dermal thickness (DT) was assessed by mRSS in SSc patients at the level of the dorsum of the middle phalanx of the third finger on both hands, hand dorsum, forearms, arms, thighs, legs, feet, forehead, chest and abdomen [65,69,76].

DT was also assessed by high-frequency ultrasound (HF-US) in the same areas in which we calculated mRSS. The mean value for bilateral areas was calculated, as well as the total mean value. A My Lab 25 ultrasound system equipped with an 22 MHz probe was used (Esaote, Genoa, Italy).

The same operator performed the ultrasound in all subjects. Patients were treated with variable drugs, also in combination. Treatments of the enrolled scleroderma patients are reported in Table II.

Drug	# treated patients	Drug	# treated patients
Aminaphtone	36	Nintedanib	2
Mycophenolate mofetil	7	Bosentan	7
Sildenafil	5	Idroxychloroquine	7
Macitentan	3	Rituximab	2
Methotrexate	8	Aspirin	27
Selexipag	1	Ciclic prostanoids	33

Table II. Treatments of the enrolled scleroderma patients.

Statistical analysis was carried out by parametric procedures, and confirmed by non-parametric tests. Student's t test, not assuming equal variances, and Mann–Whitney U test, were performed to compare unpaired groups of variables, and Kruskal– Wallis test was used to compare continuous variables with nominal variables with more than two levels. Multiple and linear regression, along with Spearman's rank correlation tests, were employed to search for possible relationships between variables.

The p values lower than 0.05 were considered statistically significant. Results are reported as mean ±SD and box blots with percentiles.

2.4 Results

A positive correlation was detected between the extent of microvascular damage assessed by NVC and the degree of organ involvement. In particular, organ involvement was progressively more severe in SSc patients with the Early, Active or Late pattern of microangiopathy.

In this study we found out that patients with lung disease, pulmonary arterial hypertension, oesophagus and gastrointestinal involvement have a statistically significant progressively more severe grade of microangiopathy among 'Early', 'Active', and 'Late' patterns. Also, a progressively higher DT assessed by HF-US (statistically significant) was found in 'Early', 'Active' and 'Late' patterns of microangiopathy. Similarly, a progressively higher mRSS was found in 'Early', 'Active' and 'Late' patterns (see table II for statistical details).

SSc patients with skin involvement had a significantly higher MES than those without skin involvement (sine-scleroderma), as well as MES was significantly higher in dcSSc than lcSSc patients (see table III for further details).

MES was found significantly higher in patients with pulmonary arterial hypertension, oesophagus, kidney and gastro-intestinal involvement than in those without. SSc patients with lung and heart involvement had an higher MES, although non-statistically significant (respectively p=0,09 and p=0.75). In addition, a positive and statistically significant correlation was found between MES and PAPs, as well as between MES and RRI, although non statistically significant (table IV).

Furthermore, MES had a positive correlation with dermal thickness in different skin areas as assessed by HF-US. Similarly, a positive correlation between MES and mRSS was detected (table IV for more details).

Finally a positive correlation was found between DT measured by HF-US and mRSS.

	Early	**Active**	**Late**	**p value**
Esophagus (%)	14	27	53	**0.05**
Digital ulcers (%)	47	33	35	0.70
Lung (%)	47	25	82	**0.05**
Heart (%)	17	17	17	0.99
PAH (%)	6	8	23	0.07
Kidney (%)	23	17	41	0.09
Gastro-intestinal (%)	0	8	18	**0.05**
Mean dermal thickness (mm±SD)	0.99±0,1	1.01±0,08	1.11±0,21	**0.05**
Finger dermal thickness (mm±SD)	0.81±0,14	0.85±0,21	1.02±0,27	**0.03**
Hand dermal thickness (mm±SD)	0.84±0,1	0.88±0,14	0.97±0,16	**0.05**
mRSS	3±3,86	4.67±2,64	6.87±5,6	**0.05**

Table III. Clinical involvement in SSc patients with different capillaroscopic pattern of microangiopathy. Dermal thickness was assessed by high-frequency skin ultrasound (PAH = pulmonary arterial hypertension, mRSS = modified Rodnan skin score).

	MES		
	Yes	**No**	**p value**
Skin (mean±SD)	4.5±2.2	2.4±2.6	**0.02**
Esophagus (mean±SD)	5.2±2.5	3.8±2.3	**0.05**
Digital ulcers (mean±SD)	3.7±2.3	4.3±2.5	0,30
Lung (mean±SD)	4.5±2.6	3.5±2	0.09
Heart (mean±SD)	4.22±2.9	4.0±2.3	0.75
PAH (mean±SD)	6±2	3.0±2.2	**0.03**
Kidney (mean±SD)	5±2	3.4±2.3	**0.03**
Gastro-intestinal (mean±SD)	5.5±1	3.5±2.2	**0.05**
Sine scleroderma (mean±SD)	2.4±2.6	-	
lcSSc (mean±SD)	4.0±2.16	-	**0.04**
dcSSc (mean±SD)	5.4±1.7	-	

Table IV. Microangiopathy evolution score (MES) in patients with or without organ involvement, as well as in patients with different skin involvement (PAH = pulmonary arterial hypertension, lcSSc = limited cutaneous SSc dcSSc = diffuse cutaneous)

	MES	
	r	**p**
PAP	+0.37	**0.01**
RRI	+0.08	0.87
Mean dermal thickness	+0.26	**0.05**
Finger dermal thickness	+0.33	**0.03**
Hand dermal thickness	+0.34	**0.03**
mRSS	+0.43	**0.03**
	mRSS	
	r	**p**
Mean dermal thickness	+0.37	**0.008**
Finger dermal thickness	+0.47	**0.0005**
Hand dermal thickness	+0.35	**0.01**

Table V. Correlations between microangiopathy evolution score (MES) and clinical involvement. Correlations between modified Rodnan skin score (mRSS) and dermal thickness assessed by high-frequency skin ultrasound are also reported. (PAP = pulmonary artery pressure, RRI = renal resistive index, r = correlation coefficient, p = statistical significance)

2.4 Discussion

This study confirms the relationships between nailfold microangiopathy extent and both skin and internal organ involvement in SSc patients, in particular with kidney, oesophagus, gastro-intestinal, lung disease and PAH. Moreover, present results show that nailfold microangiopathy severity, evaluated by NVC, is progressively more severe in patients with higher DT, assessed by both high-frequency ultrasound and mRSS. Therefore, these findings confirm previous studies which correlated nailfold microangiopathy with SSc clinical features, thus suggesting the role of NVC as a possible biomarker in SSc [51,67].

This is an important achievement because SSc patients enrolled in our study were treated with a wider range of new drugs, than those used in the past. These drugs (i.e. Mycophenolate mofetil, Rituximab, Bosentan, Sildenafil, Selexipag, Nintedanib) have demonstrated to improve clinical symptoms and quality of life of SSc patients, possibly interfering with disease progression. For this reason the role of NVC as a possible biomarker in SSc had to be reconfirmed.

Since the correlations between NVC parameters and clinical features are still maintained even in patients treated with a wide range of new drugs, we can confirm that nailfold microangiopathy extent is an excellent disease biomarker in SSc.

One limit of this study was that the group of patients enrolled was small and heterogeneous in terms of organ involvement, and few comparisons and correlations demonstrated in the past were found not statistically significant, even if the same trend was detected.

By considering a larger group of patients it would have been of interest to investigate if a specific drug had a statistically significant role in modifying the comparisons/correlations that we observed between microangiopathy and clinical

features. The heterogeneous use of new drugs among our small cohort of patients did not allowed this evaluation.

Moreover it would be worthy to investigate if, after a period of treatment with one of the drug mentioned above, NVC parameters would change, in order to assess possible effectiveness of the drug on microangiopathy. This issue is matter of future ongoing investigation.

In conclusion, in our study nailfold microangiopathy assessment by NVC seems to be confirmed as a reliable biomarker in SSc, worthy to be performed in all patients.

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