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SYSTEMIC SCLEROSIS – EXPLORING NEW PATHOGENIC VIEWS

INÊS JOÃO DA SILVA CHORA

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To my parents, who constantly stood for my education.

Prologue

https://www.wikiart.org/en/paul-klee

Paul Klee. *Dream City* (1921).

My favorite definition of Internal Medicine is “the Medicine of complexity”: complexity in *quantity*, in patients with multiple concomitant diseases, whose management must be plural; and complexity in *quality*, in patients with poorly known clinical conditions, who need both a wise knowledge and a meticulous regard, as well as perseverance on diagnosis pursuance, optimally developed by internist’s endeavor.

Systemic sclerosis is a prototype of the latter and has soon triggered my inquisitive concern. The disease constitutes a fair example of how multiple organ systems link, interact and cause disease, reinforcing the role of a holistic approach.

“With research, possibilities are limitless. Be part of the change.”

Slogan of Rare Disease Day 2017

Researching is intrinsic to the curious internist, who recognizes that the whole one can know is still scarce. I believe that every tiny discover is a further cobblestone on human path to knowledge. And the most valuable learnings reside on the journey, rather than on the end.

Financial Support

The Student was endowed with a Foundation for the Development of Internal Medicine (FDIME) Grant for Research into Rare Diseases in Adults, in 2013 (10 000€).

Young Internists (under 35 years old) from countries affiliated to the European Federation of Internal Medicine, who have done or are currently undertaking a PhD Program, are given the opportunity to work for a period of up to one year on a Rare Disease project outside their home country. The FDIME Grant intends to support the living costs on the host country. Applications are assessed by FDIME Research Grants Selection Board, chaired by Prof. Loïc Guillevin. In 2013, from 12 candidate-projects, three were successful.

The laboratory work developed in Departmento de Biomedicina, Unidade de Bioquímica, Faculty of Medicine, University of Porto, Portugal and I3S, Instituto de Investigação e Inovação em Saúde, University of Porto, Portugal was co-funded by Fundação para a Ciência e a Tecnologia (FCT: UID/BIM/04293/2013).

Thesis Outline

In Chapter **I. Introduction**, a review of the literature will be presented, including an overview of systemic sclerosis and very early diagnosis of systemic sclerosis, focusing on the currently known pathogenesis of systemic sclerosis microvasculopathy. This chapter will also comprise the review paper:

- **First Paper** – Chora I, *et al*. "Vascular biomarkers and correlation with peripheral vasculopathy in systemic sclerosis." Autoimmun Rev. 2015 Apr;14(4):314-22.

Reference will be made to a recently published Commentary, that will be available at the Appendix:

- **Fourth Paper** – Matucci-Cerinic M, Manetti M, Bruni C, Chora I, *et al*. "The 'myth' of loss of angiogenesis in systemic sclerosis: a pivotal early pathogenetic process or just a late unavoidable event?" Arthritis Res Ther. 2017 Jul 6;19(1):162.

In Chapter **II. Aims**, the main objectives of the study will be pointed out.

In Chapter **III. Methods**, the study design and methodology will be succinctly introduced.

Chapter **IV. Results** will present the papers reporting the findings of the study:

- **Second Paper** – Romano E, Chora I, *et al*. "Decreased expression of neuropilin-1 as a novel key factor contributing to peripheral microvasculopathy and defective angiogenesis in systemic sclerosis." Ann Rheum Dis 2016;75:1541-1549.
- **Third Paper** – Chora I, *et al*. "Evidence for a Derangement of the Microvascular System in Patients with a Very Early Diagnosis of Systemic Sclerosis." J Rheumatol. 2017 Aug;44(8):1190-1197.

Chapter **V. General Discussion** will debate and summarize the importance of the results described in this project.

Chapter **VI. Conclusions and Future Perspectives** will highlight the main contribution of these findings to the state of the art in systemic sclerosis pathogenic views and raise further concerns for future research.

The **References** will be enumerated at the end of the document.

An **Appendix** containing the fourth paper and other supplementary files will be provided. These documents will be denoted in the text as [Doc.].

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List of Acronyms

ACA – anticentromere antibodies

ANA – antinuclear antibodies

AOUC – Azienda Ospedaliero-Universitaria Careggi

CHSJ – Centro Hospitalar de São João

CTGF – connective tissue growth factor

dcSSc – diffuse cutaneous systemic sclerosis

ECs – endothelial cells

ECM – extracellular matrix

EPC – endothelial progenitor cell

ET-1 – endothelin 1

EULAR – European League Against Rheumatism

EUSTAR – European League Against Rheumatism Scleroderma Trials and Research

Fli1 – Friend leukaemia integration 1

H-MVECs – healthy microvascular endothelial cells

IL – interleukin

lcSSc – limited cutaneous systemic sclerosis

MVECs – microvascular endothelial cells

NRP1 – neuropilin 1

NRPs – neuropilins

NVC – nailfold videocapillaroscopy

PAH – pulmonary arterial hypertension

PDGF – platelet-derived growth factor

RNA – ribonucleic acid

RP – Raynaud's phenomenon

Sema3 – class 3 semaphorins

Sema3A – semaphorin 3A

SSc – systemic sclerosis

TGF-β – transforming growth factor-beta

uPAR – urokinase-type plasminogen activator receptor

VEDOSS – very early diagnosis of systemic sclerosis

VEGF – vascular endothelial growth factor

VEGFRs – vascular endothelial growth factor receptors

vSMCs – vascular smooth muscle cells

I. ABSTRACT/RESUMO

Paul Klee. *Abstraction with Reference to a Flowering Tree* (1925).

Abstract

Systemic sclerosis (SSc) is a rare and heterogeneous autoimmune disorder of unknown etiology, characterized by widespread vascular injury and dysfunction, impaired angiogenesis, immune deregulation and progressive fibrosis of the skin and internal organs.

SSc is typically diagnosed late. The establishment of preliminary criteria for very early diagnosis of systemic sclerosis (VEDOSS) (Raynaud's phenomenon [RP] and puffy fingers, together with abnormal nailfold videocapillaroscopy and positive SSc-specific antinuclear antibodies) intend to counteract this tendency, as earlier identification and treatment of VEDOSS patients may slow disease progression.

A growing body of evidence supports the concept that SSc is primarily a vascular disease, mediated by autoimmunity and evolving into tissue fibrosis. The deregulation of vascular tone control, clinically evident as RP, and microcirculatory abnormalities are the earliest clinical manifestations of SSc and may precede cutaneous and visceral involvement by months or years.

Despite the hypoxic conditions of microangiopathy, angiogenesis is paradoxically impaired in SSc. It has been shown that vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR) signaling pathway is profoundly disturbed in SSc, namely due to a switch into VEGF-A_{165} anti-angiogenic isoforms. Altered expression of neuropilin 1 (NRP1), a receptor for both class 3 semaphorins and VEGF-A, might play a role on this process and had not been studied in SSc, to the date of this project writing.

The aim of the thesis was to explore the microvasculopathy and deregulated angiogenesis, namely the possible involvement of the axis NRP1/semaphorin 3A (Sema3A), in 55 SSc patients and 25 VEDOSS patients from two hospital centers.

Serum circulating levels of Sema3A and soluble NRP1 (sNRP1) were measured by enzyme-linked immunosorbent assay (ELISA). NRP1 and Sema3A expression in skin biopsies was evaluated by immunofluorescence and Western blot. NRP1 expression was assessed in dermal microvascular endothelial cells (MVECs) of SSc (SSc-MVECs) and healthy controls, in endothelial progenitor cell (EPC)-derived endothelial cells (ECs) of SSc and controls and in healthy MVECs (H-MVECs) stimulated with

patients' sera, by Western blot. The possible impact of transcription factor Friend leukaemia integration 1 (Fli1) deficiency on endothelial NRP1 expression was investigated by gene silencing. The binding of Fli1 to NRP1 gene promoter was evaluated using chromatin immunoprecipitation. Capillary morphogenesis was performed on Matrigel. Cell proliferation was assessed by 5'-bromodeoxyuridine assay and migration capacity by *in vitro* wound-healing assay.

Serum levels of pan-VEGF were increased in SSc and VEDOSS patients *versus* controls, while serum levels of sNRP1 were significantly reduced in those patients, compared to controls. In SSc, decreased sNRP1 levels were associated with "active" and "late" nailfold videocapillaroscopy patterns and digital ulcers.

NRP1 expression was constitutively downregulated in SSc-MVECs, both *ex vivo* and *in vitro*, but not in SSc peripheral blood EPC-derived ECs. The expression of NRP1 was significantly reduced in H-MVECs after treatment with patients' sera, being upregulated after stimulation with recombinant VEGF-A_{165}.

No differences were found for Sema3A serum levels or expression between patients and controls.

Fli1 was significantly decreased in H-MVECs challenged with SSc sera, but not in SSc EPC-derived ECs. Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs. NRP1 gene silencing in H-MVECs resulted in a significantly impaired angiogenic capacity, comparable to that of cells treated with SSc sera.

Capillarogenesis, migration and proliferation were decreased in H-MVECs stimulated with SSc and VEDOSS sera, compared to cells stimulated with healthy sera.

In conclusion, NRP1 deficiency may be an additional factor in the perturbed VEGF-A/VEGFR-2 signaling, contributing to peripheral microvasculopathy and defective angiogenesis in SSc, which are evident from the very early stage of the disease.

Key-words: systemic sclerosis; very early diagnosis of systemic sclerosis; angiogenesis; biological markers.

Resumo

A esclerose sistémica (SSc) é uma doença autoimune rara e heterogénea, de etiologia desconhecida, caracterizada por lesão e disfunção vasculares, desregulação imune e fibrose progressiva cutânea e visceral.

O diagnóstico de SSc é habitualmente tardio. Os critérios preliminares para diagnóstico muito precoce de esclerose sistémica (VEDOSS) (fenómeno de Raynaud [RP] e edema digital, com alterações capilaroscópicas e anticorpos antinucleares específicos de SSc positivos) pretendem contrariar esta tendência, uma vez que a identificação e tratamento precoce dos doentes com VEDOSS poderão retardar a progressão da doença.

Evidência científica crescente sugere que o atingimento vascular é o evento patogénico primário na SSc, mediado por fenómenos de autoimunidade e progredindo posteriormente para fibrose tecidular. A disfunção do tónus vascular, evidente no RP, e as alterações da microcirculação são as manifestações clínicas mais precoces de SSc e podem preceder o envolvimento cutâneo e visceral por meses a anos.

Apesar da hipóxia determinada pela microangiopatia, a angiogénese encontra-se paradoxalmente comprometida na SSc. A desregulação da via de sinalização do fator de crescimento endotelial vascular (VEGF)-A e seu recetor (VEGFR) tem implicações importantes neste paradoxo, nomeadamente pela sobre-expressão da isoforma anti-angiogénica do VEGF-A_{165}. O possível envolvimento da neuropilina 1 (NRP1), recetor quer da classe 3 das semaforinas quer do VEGF-A, constituía uma lacuna de investigação na patogénese da SSc, até à data do presente trabalho.

O objetivo da tese consistiu em explorar a microvasculopatia e a angiogénese desregulada, nomeadamente o possível envolvimento do eixo NRP1/semaforina 3A (Sema3A), em 55 doentes com SSc e 25 doentes com VEDOSS, seguidos em dois centros hospitalares.

Os níveis séricos de Sema3A e NRP1 solúvel (sNRP1) foram medidos por ELISA. A expressão de NRP1 e Sema3A em biópsias cutâneas foi avaliada por imunofluorescência e *Western blot*. A expressão de NRP1 foi avaliada em células endoteliais microvasculares (MVECs) cutâneas de SSc (SSc-MVECs) e de controlos saudáveis, em células endoteliais (ECs) derivadas de células progenitoras endoteliais

(EPC) e em MVECs saudáveis (H-MVECs) estimuladas com soros de doentes, por *Western blot*. O possível impacto do défice do fator de transcrição *Friend leukaemia integration 1* (Fli1) na expressão endotelial de NRP1 foi investigado por silenciamento de genes. A ligação do Fli1 ao promotor do gene da NRP1 foi avaliada utilizando a técnica de imunoprecipitação da cromatina. A capilarogénese foi avaliada através do ensaio de formação de capilares em Matrigel. A proliferação celular foi avaliada pelo ensaio de incorporação de 5'-bromodeoxiuridina e a migração pela capacidade de cicatrização de cultura danificada.

Os níveis séricos de VEGF total estavam aumentados nos doentes com SSc e VEDOSS *versus* controlos saudáveis, enquanto que os níveis séricos de sNRP1 se encontravam significativamente reduzidos nesses doentes, comparados com os controlos. Na SSc, a diminuição de sNRP1 associou-se aos padrões capilaroscópicos "ativo" e "tardio" e à presença de úlceras digitais.

A expressão de NRP1 estava constitutivamente diminuída nas SSc-MVECs, *ex vivo* e *in vitro*, mas não em ECs derivadas de EPC de SSc. A expressão de NRP1 foi significativamente reduzida após estimulação das H-MVECs com soros de doentes, e sobre-regulada após estimulação com VEGF-A_{165} recombinante.

Não se registaram diferenças nos níveis séricos e expressão de Sema3A entre doentes e controlos.

O Fli1 encontrava-se significativamente diminuído nas H-MVECs estimuladas com soro de SSc mas não em ECs derivadas de EPC de SSc. O Fli1 ligou-se ao promotor do gene da NRP1 e o seu silenciamento reduziu a expressão de NRP1 nas H-MVECs. O silenciamento da NRP1 resultou no compromisso da capacidade de angiogénese das H-MVECs, comparável ao das células estimuladas com soro de SSc.

A capilarogénese, migração e proliferação encontravam-se diminuídas nas H-MVECs estimuladas com soros de SSc e VEDOSS, em comparação com células estimuladas com soro saudável.

Em conclusão, o défice de NRP1 parece ser um fator patogénico adicional na via de transdução de sinal do VEGF-A/VEGFR-2, contribuindo para a microvasculopatia e comprometimento da angiogénese na SSc, evidentes desde a fase muito precoce da doença.

Palavras-chave: esclerose sistémica, diagnóstico muito precoce de esclerose sistémica, angiogénese, biomarcadores.

II. INTRODUCTION

Paul Klee. *Rising Sun* (1907).

1. Systemic Sclerosis

1.1 Definition and Epidemiology

Systemic sclerosis (SSc, previously known as scleroderma) (ORPHA90291) is a rare systemic autoimmune connective tissue disorder. This complex chronic illness has substantial interindividual variability in clinical manifestations and outcomes (1). SSc has long represented one of the greatest challenges on the management of autoimmune rheumatic diseases. Due to its unpredictable course and resistance to therapy, SSc has the highest disease-related mortality and morbidity (2, 3).

SSc is characterized by widespread vascular dysfunction and injury, immunological abnormalities and excessive extracellular matrix (ECM) accumulation. The most apparent and almost universal clinical features of SSc are related to the progressive fibrosis of the skin, the microvasculature and of numerous internal organs (4).

SSc has a prevalence of 5-24:100 000 and an incidence of 1-5:100 000, varying significantly in different geographic areas and time points (5, 6). There is a trend towards increasing prevalence and incidence rates in more recent studies (6).

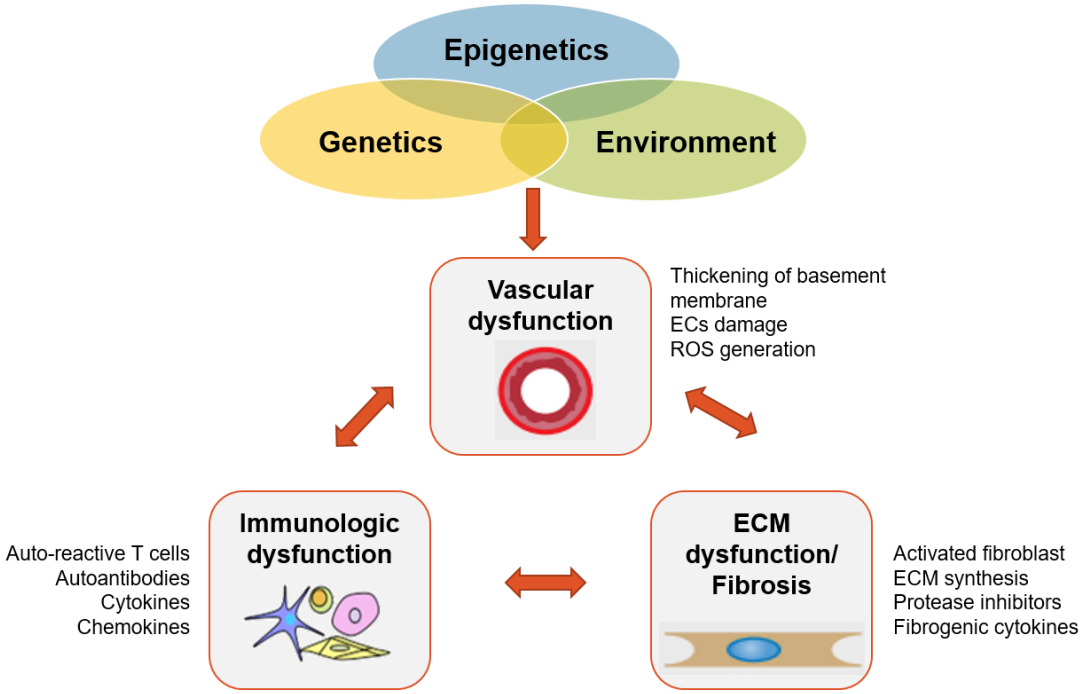
In the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) register on 9149 SSc patients, the female/male sex ratio was 6:1 (7). The peak incidence lies between the third and fifth decades and the age at disease onset differs according to gender and ethnic background (5, 6). Multiple studies have suggested that subjects of African ancestry have a higher susceptibility to SSc and develop more severe disease (6).

1.2 Pathogenesis

The etiology of SSc remains largely unknown but is likely to involve the interaction of environmental factors in a genetically primed individual, under a regulatory epigenetic mechanism (8-12). Generation of mouse models allowed the identification of the main players in different phases of the pathophysiology (13). SSc is characterized by three cardinal pathogenic features: 1) microvascular endothelial cell/small vessel fibroproliferative vasculopathy, 2) immune system abnormalities and

3) connective tissue-producing cells (namely fibroblasts/ myofibroblasts) dysfunction. All of these processes interact and affect each other (***Figure 1***) (10, 13-15).

Figure 1 – Main cornerstones in the pathogenesis of systemic sclerosis. *Adapted from (Eckes B et al. 2014.) and* (*Katsumoto TR et al. 2011*) (13, 15). ECs – endothelial cells; ECM – extracellular matrix; ROS – reactive oxygen species.



Pathogenesis is dominated by early microvascular changes targeting endothelial cells (ECs), with the release of several mediators promoting an inflammatory response and vascular remodeling. As suggested by several lines of evidence, autoimmunity constitutes a key perpetuating event, with activation of both innate and adaptive immunity and with production of distinct autoantibodies. Autoantibodies to ECs, ischemia-reperfusion injury following Raynaud's phenomenon (RP), generation of reactive oxygen species with inflammatory cell infiltration and subsequent cytokine production trigger myofibroblastic transformation of ECs and fibroblasts, inducing excessive production of collagens and other ECMs and ultimately leading to tissue fibrosis, under autocrine and paracrine control (9, 16).

A complex interplay between these major components establishes and maintains the inability of the vasculature to adequately react to the need for dilatation, constriction and growth of new vessels, causes increased deposition of ECM constituents and

facilitates immunological derangement (17). Various cell types involved in different pathophysiological events must be considered as part of an environment in which cell-cell and cell-ECM interaction controls cellular activities (13).

The roles played by other ubiquitous molecular entities (such as lysophospholipids, endocannabinoids and their receptors and vitamin D) are just beginning to be understood and studied and may provide insights into new therapeutic approaches in SSc (14).

1.2.1 Genetics and Epigenetics

There is evidence for genetic factors influencing susceptibility and familial clustering, more recently from genetic linkage and genome-wide association studies; different human leukocyte antigen (HLA) alleles and genetic polymorphisms have been associated with the predisposition for developing SSc and to SSc-specific autoantibodies in different populations (5, 13, 18). The heterogeneous clinical presentation probably reflects the influence of different genetic or triggering factors (14). It may be that the clinical course of the disease is influenced by the expression of micro-ribonucleic acids (miRNAs), which are epigenetically altered by body environmental factors (12, 19).

SSc is not inherited in a Mendelian fashion, with a genetic heritability of only 0.008 (20). However, a positive family history significantly increases the relative risk by 15 to 19-fold in siblings and by 13 to 15-fold in first-degree relatives (12, 21). Some ethnic groups have higher prevalence of SSc compared to the general population. Native American tribe Choctaw display a more homogeneous clinical and immunological phenotype than general population, supporting the role of genetics in SSc (22).

A recent systematic review provides an update on genomic and genetic studies of SSc (18). There is evidence that chromosomal breakage is a main feature in members of SSc families. HLA and non-HLA studies showed that alleles in the HLA-DRB1, HLA-DQB1, HLA-DQA1, HLA-DPB1 genes and variants in signal transducer and activator of transcription 4 (STAT4), interferon-regulatory factor 5 (IRF5) and CD247 are frequently associated with SSc. The known variants are mostly located in non-coding regions of the genes and do not predict regulatory functions. Thus, no causal variant was identified and the biological consequences of the variants remain uncertain.

Moreover, most of the genes shown are also associated with other autoimmune diseases, defining what is called "shared autoimmunity" (9). DRB1*1101 and DPB1*1301 alleles were associated with the presence of anti-topoisomerase I antibodies, while DRB1*0401-22 and DRB1*0801-11 were associated with anticentromere antibodies (23). The combination of STAT4 and IRF5 was associated with an increased risk of pulmonary fibrosis (24).

Genome-wide association studies have identified the single-nucleotide polymorphisms (SNPs) rs35677470 in DNASE1L3, rs12528892 in TAP2, rs9494883, rs5029939 and rs7749323 in TNFAIP3 and rs7574865 in STAT4 as those showing the strongest association with SSc subgroups. Regarding non-HLA gene interaction networks, nuclear factor of kappa light polypeptide gene enhancer in B cells 1, colony stimulating factor 3 receptor, STAT4, interferon-gamma, prolactin and interleukins (eg. IL-10, IL-13, IL-6, IL-2, IL-1B, IL-2RA, IL-12RB2) appear to be the main pivots mediating inflammation (18).

A functional genomic approach has lately performed a comprehensive comparison of available gene expression data from five different therapeutic trials in SSc (25). Molecular phenotyping of SSc patients prior to treatment may increase the likelihood of meaningful clinical response.

Epigenetic mechanisms are thought to represent the crossroad between genetics and environmental factors (12). The nature of the specific stimuli that trigger epigenetic modifications among patients with SSc remains mainly uncharacterized, but may include external factors (e.g. diet, chemicals, exposure to silica, toxins and drugs) and internal factors (e.g. ageing, sex hormones, hypoxia and oxidation injury) (26).

Current evidence demonstrates alterations in DNA methylation, histone code modifications and changes in miRNAs expression levels in SSc cells, particularly in fibroblasts, microvascular endothelial cells (MVECs), B and T cells (12). Epigenetic variation appears to target landmark pathways involved in SSc pathogenesis, such as transforming growth factor-beta (TGF-β) and downstream signaling cascades (27).

Hypermethylation of the promoter region of friend leukaemia virus integration gene (FLI1) leads to repression of friend leukaemia integration 1 (Fli1), a transcription factor which inhibits collagen gene expression. Epigenetic repression of FLI1 may play a key role in collagen deposition and tissue fibrosis. TGF-β signaling pathway is further

activated by miRNAs through upregulation of profibrotic molecules, such as Smad3 and Smad4, or by downregulation of antifibrotic molecules, such as Smad7, that contribute to increased collagen synthesis and ECM expansion. miRNAs also modulate collagen gene expression; for instance, underexpression of miR-196a, Let-7a and miR-29 are examples of post-transcriptional modification of collagen genes (26).

DNA hypermethylation and repression of key genes in SSc MVECs is maintained by upregulation of DNA (cytosine-5-)-methyltransferase 1 expression in MVECs. Hypermethylation of the promoter region of bone morphogenic protein receptor II and nitric oxide synthetase 3, and consequently underexpression of these genes in MVECs, leads to a cascade of events characterized by ECs apoptosis, vasoconstriction, recruitment of inflammatory cells, oxidation injury and ultimately activation of fibroblasts (26).

Epigenetic modifications are potentially reversible. Inherited epigenetic modifications can vanish after a variable number of cell divisions and epigenetic risk factors could be counteracted by treatment with currently available epigenetic modifier molecules, such as histone deacetylase inhibitors, DNA methyltransferase inhibitors and synthetic miRNAs (27).

1.2.2 Environment

Several environmental factors have been related to an increased SSc susceptibility, such as the exposition to chemical compounds (silica, chlorinated solvents, trichloroethylene, welding fumes for men, aromatic solvents and ketones for women and white spirit for both genders), infectious agents or pregnancy, although the involvement of these factors in SSc development needs further exploration (24, 28).

Occupational exposure to silica/solvents is correlated with more severe forms of SSc, characterized by diffuse cutaneous involvement, interstitial lung disease, digital ulcers, myocardial dysfunction and association with cancer (29).

1.2.3 Vascular Dysfunction

Microvascular dysfunction is believed to be the cornerstone of SSc pathogenesis and its mechanisms will be more extensively reviewed elsewhere (**II.3**).

1.2.4 Immunologic Dysfunction

Although the pathogenic relationship between systemic autoimmunity and the clinical manifestations of SSc remains uncertain, SSc patients display abnormal immune activation, in both innate and adaptive systems (30).

Macrophages/monocytes are involved in the pathogenesis of SSc, especially during its early stage, but their precise role in the disease is still unclear. Monocytes are one of the first type of immune cells infiltrating SSc skin lesions (31). The presence of both M1 phenotype (pro-inflammatory) and strong M2 phenotype (classic activation and profibrotic/anti-inflammatory) signatures observed in the skin, blood, and lungs of SSc patients is very clear (32). M2 phenotype is characterized by low secretion of interleukin (IL)-12 and IL-23 and elevated IL-10 and by an increased synthesis of cytokines such as IL-4, IL-10, IL-13 or IL-6, connective tissue growth factor (CTGF) and arginase-1, which can stimulate the synthesis of collagen. Circulating and tissue macrophages/monocytes, plasmacytoid dendritic cells and stromal cells express a type 1 interferon signature that reflects an activation involving toll-like receptors (TLRs). Among TLRs, TLR-4 recognizes constitutive molecules of ECM, such as hyaluronic acid, fibronectin fragments or heparan sulfate. In animal models of TLR4 knock-out mice, exposure to bleomycin induced less dermal and pulmonary fibrosis than in wild mice (9, 14). Proteome-wide analysis showed that CXCL4 is the predominant protein secreted by plasmacytoid dendritic cells in SSc, especially in early disease, both in the circulation and in the skin, predicting the risk of SSc progression (16).

Observations over several decades strongly implicate the adaptive immune system in SSc pathogenesis (14).

B cell homeostasis is disturbed during SSc (33, 34). Quiescent B cells are activated by four main mechanisms: B cell receptor stimulation (by autoantigens such as topoisomerase I) and deregulation (by abnormal expression of regulating co-receptors, autoantibodies such as anti-C22 and genetic polymorphisms), survival signal

stimulation by B cell activating factor (BAFF) and proliferation-inducing ligand (APRIL) (secreted by peripheral blood mononuclear cell and myofibroblasts), TLR stimulation by endogenous ligands (nucleosomes released from apoptotic cells, ECM degradation products) and T helper (Th) 2 cell stimulation by Th2 cytokines and direct cell-to-cell contact (34). An increased CD19/CD22 ratio may facilitate the sustained activation of B cells (16).

Activated B cells are mostly contained in the memory subset and display an increased susceptibility to apoptosis, which is responsible for their decreased number. This chronic loss of B cells enhances bone marrow production of the naïve subset, accounting for their increased number in peripheral blood. Activated B cells participate in the inflammatory and fibrotic events observed during SSc through increased production of pro-inflammatory and profibrotic cytokines (that promote activation of T cells and fibroblasts), decreased production of anti-inflammatory cytokines, secretion of pathogenic autoantibodies (targeting fibroblasts, ECM proteins or ECs), and direct cell-to-cell contact (notably through T cell co-stimulatory molecules) (33, 34). IL-10-producing regulatory B cells, which induce generation of regulatory T cells (Treg) and can ameliorate autoimmune diseases, were found to be reduced in SSc, favoring autoaggression of B cells in this disease (35).

Antinuclear antibodies (ANA) are found in the sera of the vast majority of SSc patients and their antigenic specificity significantly correlates with clinical characteristics (16). Autoantibodies such as anti-topoisomerase I, anti-RNA polymerase III, anti-U3 RNP, anticentromere, anti-Th/To and anti-U1 RNP antibodies are currently the most reliable biomarkers for diagnosis, classification and prediction of specific clinical features of SSc (16, 24). Many other functional autoantibodies targeting ECs, intercellular adhesion molecule 1, endothelin-1 (ET-1) type A receptor, angiotensin II type I receptor, platelet-derived growth factor (PDGF) receptor and fibrillin-1 are known to induce activation or apoptosis of ECs. This facilitates inflammatory cell infiltration, cytokine production and myofibroblastic transformation of fibroblasts and ECs (16, 36).

These functional autoantibodies in SSc, as opposed to the classic antibodies often used as biomarkers, may actually be responsible for many of the key symptoms and manifestations of the disease, highlighting the potential value of treatment with

intravenous immunoglobulin, rituximab and/or newer therapies targeting B-cells and/or plasma cells (30, 36).

CD4+T cell activation is a key factor in the pathogenesis of SSc, with a predominance of Th 2 cells (which are mainly profibrotic) over Th1 cells (largely responsible for the secretion of inflammatory cytokines and growth factors) (9, 37). Activated T cells can release various cytokines (namely IL-4 and IL-13), resulting in inflammation and B cells stimulation, microvascular damage and fibrosis (16, 38). Th17 and Treg activities are a hallmark of SSc, as Th17-type cytokines can induce both inflammation and fibrosis. More recently, several studies have reported new T cell subsets, including Th9 and Th22 cells, along with their respective cytokines, in the peripheral blood, serum and skin lesions of individuals with SSc. The role of T follicular helper cells in SSc is not yet known (38).

In SSc, T cell infiltration is more obvious in the edema stage than in the hardening stage. CD4+ T cell infiltration is significantly increased in skin lesions and peripheral blood of patients with SSc, and most of the T cell clones in skin lesions can express the CD4 co-receptor. T cell proliferation and clonal expansion in response to unknown specific antigens may occur. Activated T cells can activate adjacent fibroblasts via direct cell-cell contact or via paracrine cytokine and chemokine production. Furthermore, autoreactive T cells may interact with B cells to promote the production of characteristic autoantibodies (38).

There is a general agreement regarding the decreased functional capacity of circulating Tregs in SSc. Some patients, particularly those with active disease, may have increased numbers of circulating Tregs, which parallel the expansion of the total pool of activated CD4+ cells, represent the inhibitory response of the immune system to its inappropriate activation, or occur as a compensatory move for their decreased suppressive activity. A decreased pool of circulating Tregs can be seen in other SSc patients, with even lower Treg numbers seen in patients with long-standing disease. Similarly, skin resident Tregs are depleted in advanced SSc but are active and can have a role in earlier disease stages. In addition, conversion of circulatory Tregs to Th17 cells and skin-resident Tregs to Th2 cells, producing corresponding inflammatory and profibrotic cytokines, has been demonstrated in SSc (39).

IL-17-producing Th17 cells are significantly increased in the peripheral blood, skin lesions and lung tissues of patients with SSc. Serum-derived IL-17A from SSc patients can promote proliferation, migration, collagen synthesis and secretion of SSc patient-derived dermal vascular smooth muscle cells (vSMCs), further aggravating vasculopathy in SSc. A positive feedback loop may exist in SSc whereby IL-17 can either directly or indirectly promote the activation of fibroblasts, ECs and vSMCs, and in turn the cytokines secreted by these cells can enhance Th17 cell differentiation (38).

1.2.5 Extracellular Matrix Dysfunction and Fibrosis

The end of the pathogenic sequence of SSc is fibrosis (9). Fibrogenesis is a consequence of a multistage process, initiated by deregulated tissue repair responses, in which aberrantly sustained production of cytokines, growth factors and angiogenic factors tilt tissue homeostasis towards interstitial hyperplasia and excessive accumulation of ECM (37).

Fibrosis in SSc is typically characterized by prolonged and/or exaggerated activation of fibroblasts, a key feature of which is the differentiation of fibroblasts, vSMCs or ECs into myofibroblasts (9, 24, 37). Endothelial-to-mesenchymal transition (EndoMT) consists on a nonmalignant phenomenon of cellular trans-differentiation, by which ECs undergo a phenotypical conversion, loosing vascular EC markers and acquiring myofibroblast-like features (40). EndoMT, which is enhanced by the synergistic action of endohelin-1 and TGF-β, appears to be a critical event linking endothelial dysfunction and the development of dermal fibrosis in SSc (40-42). ET-1 antagonists seem able to antagonize this phenomenon *in vitro* (41).

Activated myofibroblasts, which are resistant to apoptosis, synthesize and deposit ECM components, leading to ECM accumulation, increased collagen crosslinking, contraction and fibrosis. Fibrotic tissues exhibit increased tissue pressure and hypoxia, further activating resident fibroblasts and escalating fibrotic mechanisms (9, 37).

Cells of the innate and adaptive immune system elaborate a variety of cytokines and chemokines in addition to TGF-β and IL-4 (such as IL-6, PDGF, IL-1, IL-13, IL-17, IL-5, monocyte chemoattractant protein-1 and CTGF) that have been found to be increased in serum or in tissues in which excessive ECM is accumulating in SSc.

These cytokines/chemokines are at the interface between the immune system and fibroblasts (14). The close interaction between the immune system and fibrosis gives opportunities for intervention (31).

In SSc, representative profibrotic growth factors and cytokines encompass TGF-β, CTGF, PDGF, IL-6 and IL-4/IL-13 and play a pivotal role in collagen production from myofibroblasts (16).

TGF-β is considered the central regulatory factor of fibrosis in SSc (9). It binds the TGF-β receptors and downstream signaling occurs through the canonical Smad pathway, inducing gene transcription of type I collagen, α smooth muscle actin (αSMA) and CTGF. TGF-β causes increased collagen and ECM in dermal fibroblasts and induces epithelial to mesenchymal transition, being clearly perturbed in SSc (31). The expression of αSMA is a hallmark of myofibroblastic transformation of activated fibroblasts, which is also frequently detected in SSc (16).

The expression of CTGF is induced by TGF-β, ET-1 and hypoxia. The levels of CTGF are markedly elevated in the lesional skin of SSc patients and in SSc mouse models (16).

The expression of PDGF and its receptors is elevated in SSc fibroblasts and in the lesional skin. Agonistic antibodies to the PDGF receptor were found in SSc patients and appear to induce a myofibroblast phenotype in dermal fibroblasts, although further research is required (16, 31).

In SSc, activated B cells secrete IL-6, which directly stimulates fibroblasts and induces type 1 collagen expression via enhancing TGF-β-Smad3 signaling pathway. Both IL-4 and IL-13 likely activate fibroblasts and induce type 1 collagen synthesis via a TGF-β-independent approach, and serum levels of IL-4 and IL-13 were significantly higher in patients with SSc compared to healthy controls (16).

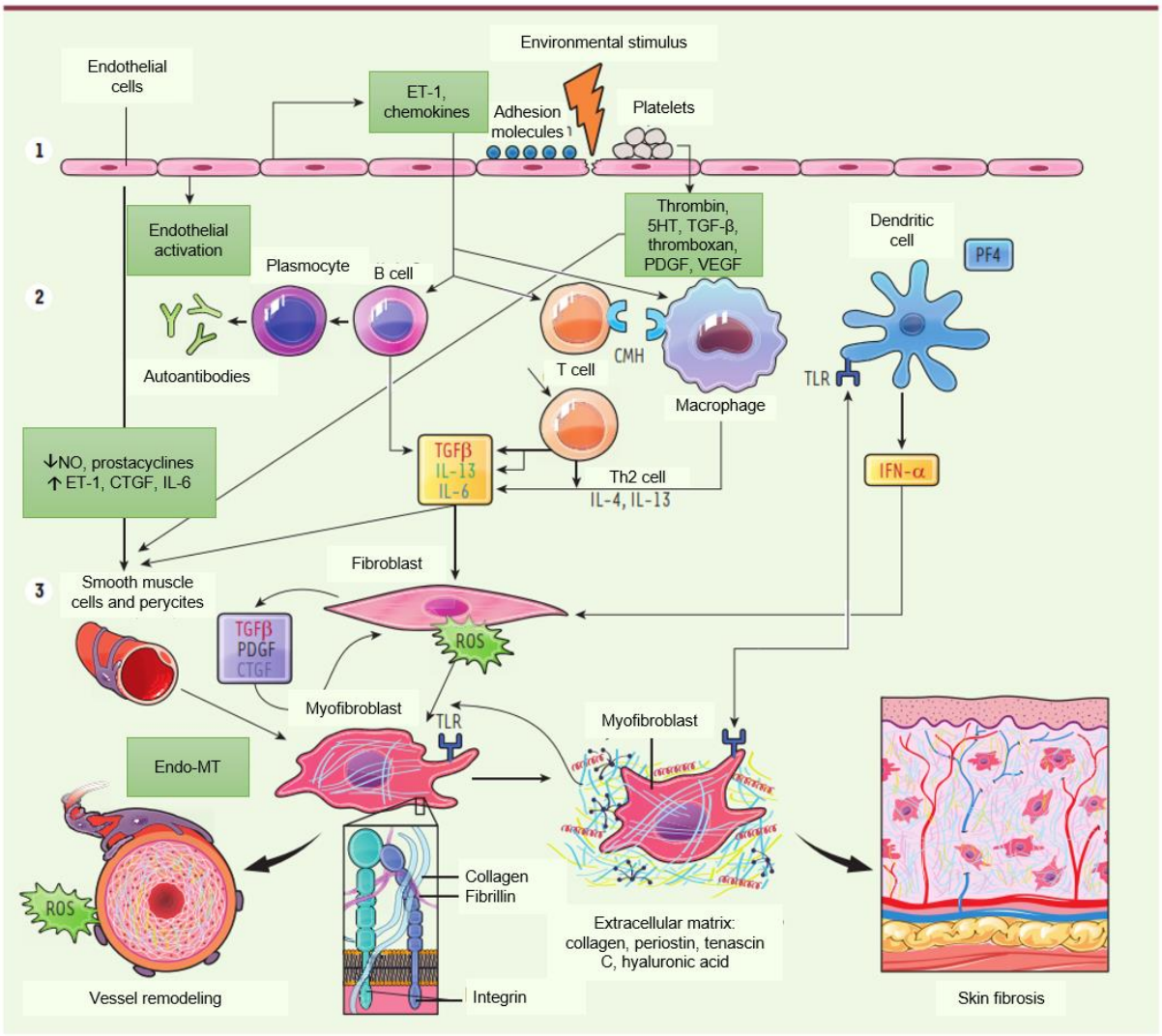
Platelets may be actively involved in the pathogenesis of SSc by activating immune responses and facilitating fibrosis (43). Platelets can be activated by the damaged ECs and subsequently release bioactive molecules stored in their granules, such as TGF-β and serotonin. Serotonin may bind to and activate $5HT_{2B}$ receptors on nearby fibroblasts, leading to enhanced collagen production. TGF-β released from activated platelets at sites of endothelial damage may be involved in EndoMT. Furthermore, activated platelets express CD40L on their surface and may interact with B cells by

providing co-stimulatory signals. Theoretically, this may facilitate a breach in immunologic tolerance and auto-antibody production (43).

The transcription factor Fli1 is a negative regulator of fibrosis, and its expression has been shown to be substantially reduced in SSc fibroblasts and SSc skin, whereas overexpression of Fli1 results in decreased collagen synthesis. Interestingly, the tyrosine kinase inhibitor imatinib has been shown to alter the DNA-binding ability and protein stability of Fli1 thereby reducing collagen levels (31). In a recent study, gene silencing of Fli1 induced a SSc-like molecular phenotype in keratinocytes (44). Keratin 14-expressing epithelial cell-specific Fli1 knockout mice spontaneously developed dermal and esophageal fibrosis with epithelial activation. Furthermore, they developed remarkable autoimmunity with interstitial lung disease derived from thymic defects with downregulation of autoimmune regulator (Aire). Fli1 directly regulated Aire expression in epithelial cells. Epithelial Fli1 deficiency might be involved in systemic autoimmunity and selective organ fibrosis in SSc.

In ***Figure 2***, a schematic representation of the main mechanisms involved in SSc pathogenesis is provided.

Figure 2 – Synthesis of systemic sclerosis pathogenesis. *Adapted from (Allanore Y. 2016.)* (9). 5HT – serotonin; CMH – major histocompatibility complex; CTGF – connective tissue growth factor; ET-1 – endothelin-1; Endo-MT – endothelial-to-mesenchymal transition; IFN-α – alpha interferon; IL – interleukin; NO – nitric monoxide; PDGF – platelet-derived growth factor; PF4 – platelet factor 4; ROS – reactive oxygen species; TGF-β – transforming growth factor-beta; TLR – toll-like receptor; VEGF – vascular endothelial growth factor.



1.3 Classification Criteria

New American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria were published in 2013 (***Table 1***) (45).

Table 1 – The American College of Rheumatology/European League Against Rheumatism criteria for the classification of systemic sclerosis. *Adapted from (van den Hoogen et al. 2013.)* (45).*

Item	Sub-item(s)	Weight/ score†
Skin thickening of the fingers of both hands extending proximal to the MCP joints (*sufficient criterion*)	-	9
Skin thickening of the fingers *(only count the higher score)*	Puffy fingers Sclerodactyly of the fingers (distal to MCP joints but proximal to the PIP joints)	2 4
Fingertip lesions *(only count the higher score)*	Digital tip ulcers Fingertip pitting scars	2 3
Telangiectasia	-	2
Abnormal nailfold capillaries	-	2
Pulmonary arterial hypertension and/or ILD (*maximum score is 2)*	Pulmonary arterial hypertension ILD	2 2
Raynaud's phenomenon	-	3
SSc-related autoantibodies (anticentromere, anti-topoisomerase I [anti-Scl-70], anti-RNA polymerase III) *(maximum score is 3)*	Anticentromere Anti-topoisomerase I Anti-RNA polymerase III	3

*These criteria are applicable to any patient considered for inclusion in a systemic sclerosis study. The criteria are not applicable to patients with skin thickening sparing the fingers or to patients who have a scleroderma-like disorder that better explains their manifestations (eg, nephrogenic sclerosing fibrosis, generalized morphea, eosinophilic fasciitis, scleredema diabeticorum, scleromyxedema, erythromyalgia, porphyria, lichen sclerosis, graft-versus-host disease, diabetic cheiroarthropathy).

†The total score is determined by adding the maximum weight (score) in each category. Patients with a total score of ≥9 are classified as having definite systemic sclerosis.

ACA – anticentromere antibodies; ILD – interstitial lung disease; MCP – metacarpophalangeal; PIP – proximal interphalangeal; SSc – systemic sclerosis.

In a cohort of patients with mild/early SSc, the new ACR/EULAR classification criteria showed higher sensitivity (79.6%) and classified more patients as definite SSc patients than the previous ACR criteria (53.3%) (46). This current classification system is also characterized by a high specificity but still cannot identify patients in the very early phases of the disease process (47).

The two subset criteria first proposed by LeRoy in 1988 and modified in 2001 ***(Table 2)*** have been the most influential for the sub-classification of SSc (48).

Table 2 – Criteria of classification of limited and diffuse cutaneous SSc according to LeRoy *et al*. (49).

Limited cutaneous SSc	Diffuse cutaneous SSc
• RP for years (occasionally decades) • Skin involvement limited to hands, face, feet, and forearms (acral) or absent • Late incidence of pulmonary hypertension, with or without ILD, trigeminal neuralgia, skin calcifications and telangiectasia • ACA (70–80%) • Dilated nailfold capillary loops, usually without capillary dropout	• Onset of RP within 1 year of onset skin changes (puffy or hidebound) • Truncal and acral skin involvement, tendon friction rubs • Early and significant incidence of ILD, oliguric renal failure, diffuse gastrointestinal disease and myocardial involvement • Absence of ACA • Nailfold capillary dilatation and capillary destruction • Anti-topoisomerase I antibodies (30%)

ACA – anticentromere antibodies; ILD – interstitial lung disease; RP – Raynaud's phenomenon; SSc – systemic sclerosis.

Systemic sclerosis *sine scleroderma* is an uncommon form of the disease (2%), without clinically detectable skin involvement, yet with visceral and immunological manifestations that are characteristic of SSc (50, 51).

1.4 Clinical Presentation and Diagnosis

The diagnosis of SSc is made clinically and generally suggested by the presence of typical skin thickening and hardening (sclerosis), which usually begins in the fingers. RP usually precedes skin sclerosis by several weeks to several years. The diagnosis is supported by the presence of additional extra-cutaneous features ***(Table 3)***, nailfold videocapillaroscopy (NVC) SSc patterns and characteristic autoantibodies (49).

Data from the EUSTAR database (7655 patients from 174 centers) showed that the most prominent clinical hallmarks of SSc were RP (96.3%), ANA (93.4%) and typical capillaroscopic patterns (90.9%). Scleroderma was more common on fingers and hands than on any other part of the skin (52).

The presence of characteristic autoantibodies is supportive of the diagnosis of SSc. Specific autoantibodies include anticentromere (ACA) (15-43%), anti-topoisomerase I (anti-Scl-70) (21-34%) and anti-RNA polymerase III (5%). Other autoantibodies are more rarely found in SSc patients, including anti-Th/To, anti-U3 RNP, anti-PM/Scl and anti-U1 RNP (24, 49). Autoantibodies in SSc are highly heterogeneous, reflecting the multiplicity of the disease. An autoantibody profile is a useful aid in refining disease

subsets (10). Moreover, autantibodies are important prognostic indicators and valuable for predicting skin, vascular and internal organ involvement (13, 49, 53). ACA are associated with limited cutaneous systemic sclerosis (lcSSc), pulmonary arterial hypertension (PAH), longer time to onset of visceral complications and lower mortality rates, while anti-topoisomerase I antibodies are associated with diffuse cutaneous systemic sclerosis (dcSSc), increased lung involvement and higher mortality rates (53). The presence of anti-RNA polymerase III is associated with dcSSc, scleroderma renal crisis and tendon contractures (24). Classification of the disease by identifying the dominant SSc specific autoantibody, in addition to skin and organ involvement, has been proposed (54, 55).

Table 3 – Main clinical manifestations of SSc (2, 5, 6, 24, 52, 56-58).

Involvement	**Clinical Presentation**	**Prevalence** (%)		**Main Diagnosis Tests**
		LcSSc	*DcSSc*	
Vascular	Raynaud's phenomenon	97	96	NVC
	Digital ulcers	33	42	
Cutaneous	Sclerosis	98		Modified Rodnan Skin Score
	Telangiectasia	75		
	Calcinosis	25		
Pulmonary	Interstitial lung disease	31	52	Chest X-ray, chest HR-CT, pulmonary function tests with DLCO, 6-minute walking test, TTE, RHC
	Pulmonary arterial hypertension	21	22	
Renal	Scleroderma renal crisis	1	4	Blood pressure, renal ultrasound, GFR, urine protein/creatinine ratio
Cardiac	Pericardial disease	6	12	ECG, TTE with Doppler, Holter-ECG, coronary angiography, cardiac MRI
	Conduction block	10	12	
	Diastolic dysfunction	17	18	
Digestive	Esophageal	66	70	EGD, esophageal manometry, colonoscopy
	Gastric	22	27	
	Intestinal	23	24	
Musculo-skeletal	Myositis	5	13	Joint X-ray/MRI, muscle enzymes, electrodiagnostic testing, muscle biopsy
	Arthritis	13	20	
	Tendon friction rubs	5	18	
	Weakness	19	24	

DcSSc – diffuse cutaneous systemic sclerosis; DLCO – diffusing lung capacity for carbon monoxide; ECG – electrocardiography; EGD – esophagogastroduodenoscopy; GFR – glomerular filtration rate; HR-CT – high resolution computed tomography; LcSSc – limited cutaneous systemic sclerosis; MRI – magnetic resonance imaging; NVC – nailfold videocapillaroscopy; RHC – right heart catheterization; TTE – transthoracic echocardiography.

Some patients with SSc have signs of other defined connective tissue diseases, such as systemic lupus erythematosus, rheumatoid arthritis or polymyositis. This is called overlap syndrome and occurs in roughly 10% of SSc patients (8). SSc has an increased association with certain malignancies and, like other autoimmune diseases, it can present as a paraneoplastic syndrome, the latter being associated with a more severe/refractory disease course (5).

1.5 Management

The multifaceted nature of SSc, with variable extension of skin and internal organ involvement, creates significant challenges for the management of this disorder. Moreover, there is a lack of good quality randomized studies on most of the organ-based manifestations of the disease (10). A comprehensive guideline of the British Society for Rheumatology and British Health Professionals in Rheumatology has been published in 2016 (59).

Because SSc is a heterogeneous disease with an unpredictable course, treatment must be tailored to the single patient. Effective management depends on secure diagnosis and subset classification (based upon the extent of skin thickening), stage of disease, extent of organ-based complications and presence of overlap features. All potential problems should be addressed with early identification and appropriate therapy (6, 59).

Both limited and diffuse cases should be treated for vascular manifestations. Active early diffuse cutaneous SSc requires immunosuppression. In all cases of SSc, vigilant follow-up to determine significant organ-based complications is mandatory (59).

Treatment options for SSc are still limited to specific aspects of organ involvement, especially for scleroderma renal crisis, interstitial lung disease and PAH (6, 48, 59). Therapies targeting the vasculature (namely ET-1 receptor antagonists, phosphodiesterase-5 inhibitors, angiotensin-converting enzyme inhibitors, prostacyclins), the immune system and/or the fibrotic process (methotrexate, cyclosporine, cyclophosphamide, azathioprine, mycophenolate mofetil, rituximab, tocilizumab, abatacept) have been or are being evaluated in SSc. Autologous

haematopoietic stem cell transplantation and intravenous immunoglobulins are reserved to unresponsive SSc patients (19). Current established therapeutic approaches focus mainly on vascular or inflammatory components; however, at least one putatively antifibrotic substance, nintedanib, is being tested for SSc interstitial lung disease (60).

Besides pharmacological treatments, the eviction of aggravating factors (smoking, vasoconstrictive drugs, cold) remains a major issue (24). Patients should be educated to risk factors and prognosis and allowed to actively participate in treatment; this should follow a holistic approach, recognizing the impact of the disease on lifestyle and relationships (3, 6).

Despite many advances and an increasing number of clinical trials in SSc, no treatment has been shown to modify mortality on controlled clinical trials. No drug has been so far labelled to reduce skin fibrosis or organ involvements. The interactions between vasculopathy, immune disturbances and fibroblast activation are complex and may hamper the recognition of a single effective therapeutic agent. A better comprehension of disease pathogenesis may lead to the development of targeted approaches, in order to attenuate key mediators of signaling pathways (6, 61).

Targeted therapies (antifibrotic agents, anti-CD20 antibodies, etc.) are currently being investigated (24). Refocusing immunotherapies already used in other diseases makes sense and is in line with genetic data that demonstrated the shared autoimmunity between autoimmune disorders (61). Two novel approaches – the IL-6 receptor blocker tocilizumab and nintedanib – are being evaluated in phase III clinical trials.

Ideal disease-modifying therapy should address the inflammatory, vascular, and fibrotic aspects of SSc (1). Combining immune and antifibrotic therapies might be a very efficient treatment for the disease (61).

1.6 Prognosis

In a meta-analysis, the pooled standardized mortality ratio for SSc was measured as 3.53 (95% CI 3.03 to 4.11), without significant changes over time (62). Nevertheless,

improved survival rates for SSc have been reported in recent studies (24, 63). In an Italian study, the 10-year survival showed a clear-cut increase from 69% to 81%, in the same center (63). Possible explanations include improved therapeutic management, changes in the natural history of the disease, earlier referral of the patients and, even more likely, a better recognition of patients with milder disease (61).

SSc requires multicenter collaboration to reveal comprehensive details of disease-related causes of morbidity and mortality (52). Among 987 patients with SSc, recruited from the nationwide Spanish Scleroderma Registry (RESCLE) between 1990 and 2009, SSc-related factors were responsible for 72% of all deaths in patients diagnosed within 1990-99, compared to 48% within 2000-09. Pulmonary involvement was the leading cause of death in both decades, while renal causes decreased since 1990 and cardiac causes tripled their ratio (64).

Independent risk factors for mortality have been identified, namely elevated age at RP onset, increased modified Rodnan skin score, positive anti-topoisomerase I antibodies, proteinuria, PAH on echocardiography, pulmonary restriction, dyspnea, decreased diffusing lung capacity for carbon monoxide and extent of lung disease on chest high resolution computed tomography scan (24, 65).

A single clinical manifestation, even the extent of cutaneous sclerosis, is not *per se* a sufficient prognostic indicator in each patient. The thorough clinical assessment and the early detection of organ damage are decisive for a correct prognostic evaluation and effective therapeutic interventions (63).

2. Very Early Diagnosis of Systemic Sclerosis

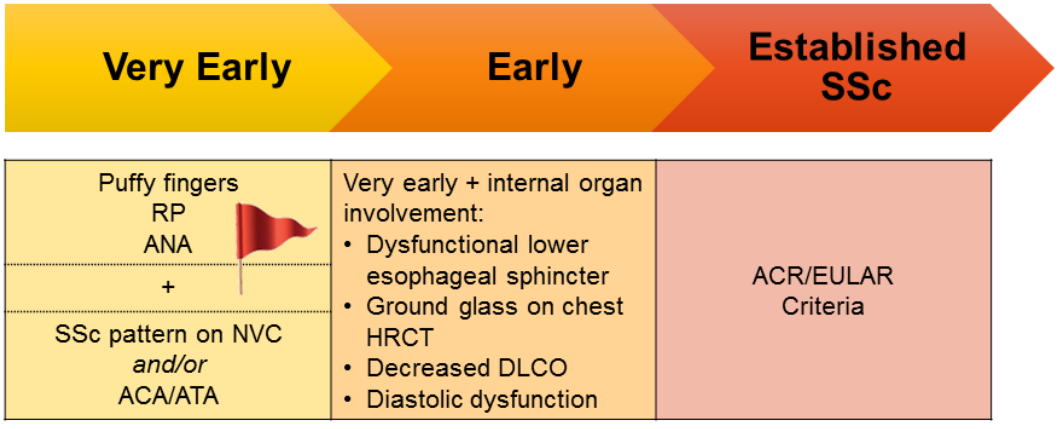
Early diagnosis of SSc may allow an earlier treatment and a slower disease progression, being of pivotal importance (66).

RP is the clinical reflection of diffuse microvascular damage in SSc and often precedes skin and visceral involvement by years or decades (67). In 1996, the term “pre-scleroderma” intended to identify patients with RP plus digital ischemic changes and typical NVC changes or disease-specific circulating ANA (68). In 2001, LeRoy and

Medsger proposed that patients with RP who had *both* a SSc pattern on NVC and SSc-specific autoantibodies be classified as having “early-SSc” (69). Seven-years later, a prospective study by Koenig *et al*. has validated these criteria: patients with RP in whom both predictors were present at baseline were 60 times more likely to develop definite SSc than were patients without these predictors, with a sensitivity of 47%; when the presence of an abnormal NVC *and/or* SSc autoantibodies at baseline was considered, the sensitivity increased to 89% (67). This study has also shown that the incidence of progression from isolated RP to definite SSc was 12.6 % and that patients classified as early-SSc would ultimately develop definite SSc.

EUSTAR proposed a set of preliminary criteria for the very early diagnosis of systemic sclerosis (VEDOSS) that are currently under validation (70). The presence of positive ANA, RP and puffy swollen fingers turning into sclerodactyly constitute a “red flag” to suspect of SSc; if these patients additionally have positive SSc-specific ANA (ACA, anti-topoisomerase I, anti-RNA polymerase III) and/or NVC with SSc pattern, EUSTAR criteria for VEDOSS are fulfilled (***Figure 3***). In a preliminary analysis of VEDOSS EUSTAR multicenter study, almost 90% of patients with “red flags” had ACA or anti-topoisomerase I antibodies and/or a NVC SSc pattern (71).

Figure 3 – Progression of systemic sclerosis. *Adapted from (Matucci-Cerinic M et al. 2012.) (72).*



ACA – anticentromere antibodies; ACR – American College of Rheumatology; ANA – antinuclear antibodies; ATA – anti-topoisomerase I antibodies; DLCO – diffusing lung capacity for carbon monoxide; EULAR – European League Against Rheumatism; HRCT – high resolution computed tomography; NVC – nailfold videocapillaroscopy; SSc – systemic sclerosis.

VEDOSS Programme has been designed to anticipate the diagnosis of SSc and to examine whether this may change the disease prognosis (66, 72, 73). In order to

achieve very early/early diagnosis of SSc, patients with RP who present to a specialized center should be evaluated both for NVC abnormalities and for SSc-specific autoantibodies, including anti-Th/To and anti–RNA polymerase III (67).

In early SSc, marker autoantibodies and NVC patterns may be related to different clinical-preclinical features and circulating activation markers at presentation (74). Indeed, a label of “Undifferentiated Connective Tissue Disease at risk for SSc”, with three different subsets (i.e. RP associated to marker autoantibodies and SSc NVC abnormalities; RP associated to marker autoantibodies in the absence of SSc NVC abnormalities; and RP associated to SSc NVC abnormalities without any detectable marker autoantibody) has been proposed (75).

Early clinical symptoms (i.e. RP) and biomarkers (i.e. serum autoantibodies) appear to represent the best “early signals” of possible SSc to be considered (19). In a retrospective study of 497 patients with primary RP, SSc NVC pattern and anti-topoisomerase I antibodies were independent risk factors for SSc. The presence of both SSc NVC pattern and anti-topoisomerase I or SSc NVC and ACA was also a good predictor for the development of SSc (76). It is crucial to focus on these signs to look for valid predictors of disease evolution and to conduct a close follow-up of the patients, in order to capture the slightest change in clinical condition, as soon as possible (47).

A subclinical scleroderma-related internal organ involvement (heart, lung, esophagus) was detected in patients with early-SSc (without clinical manifestations other than RP but with SSc marker autoantibodies and/or typical NVC abnormalities) (68). There is also evidence for esophageal and anorectal involvement in VEDOSS and a history of digital ulcers significantly correlated with gastrointestinal involvement in these patients (77, 78). This corroborates the impending need to screen for visceral involvement in VEDOSS patients, so it can be early managed, efficiently controlling disease progression (73).

In the EUSTAR cohort, 695 SSc patients with a baseline visit within one year after RP onset were evaluated for incident non-RP manifestations (79). The most frequent were skin sclerosis (75%), gastrointestinal symptoms (71%) and diffusing lung capacity for carbon monoxide <80% of predicted (65%); of note, cardiac involvement was already present in 32%, mainly diastolic dysfunction. Approximately half of all incident

organ manifestations occurred within two years and had a simultaneous rather than a sequential onset.

The time gap between the appearance of early signs and the development of internal organ fibrosis, should be considered as the “window of opportunity” for SSc patients (72, 73). The choice of an aggressive treatment before the patient fulfills SSc validated criteria may expose some patients to overtreatment and risks connected to side effects (72). This might be overtaken when criteria for VEDOSS and predictors of severity in this cohort are validated. One should consider randomized controlled trials to evaluate mild immunosuppression in RP patients with typical SSc NVC changes and anti-topoisomerase I and/or anti-RNA III polymerase autoantibodies, in a well-monitored environment (80).

The identification of genetic, clinical and circulating markers are crucial steps to lift the curtain on the prognosis of a very early-SSc patient without skin sclerosis, burdened by the risk of developing severe organ involvement (73).

The negative predictive value of testing in the very early phase might identify SSc patients who will not progress to major complications, thus sparing them from an aggressive overtreatment (72). On the other hand, positive predictive valued tests would allow high-risk patients to be identified and straightly monitored, to initiate appropriate treatment as soon as SSc manifestations appear. Studies on preventive therapies could also be a reality (73).

The EUSTAR effort to validate the criteria of the VEDOSS project will definitively shed a new light in SSc approach (47). Rather than waiting for the clinical overt disease to set in, eyes are geared to diagnose the disease “early” or “very early”, so that, in the future, selected SSc can be treated before clinical complications occur (19).

3. Microvasculopathy of Systemic Sclerosis

Microvascular lesions are a main feature of SSc and may play a central pathogenic role. Vasculopathy accompanies the pathogenesis of SSc from the early beginning and

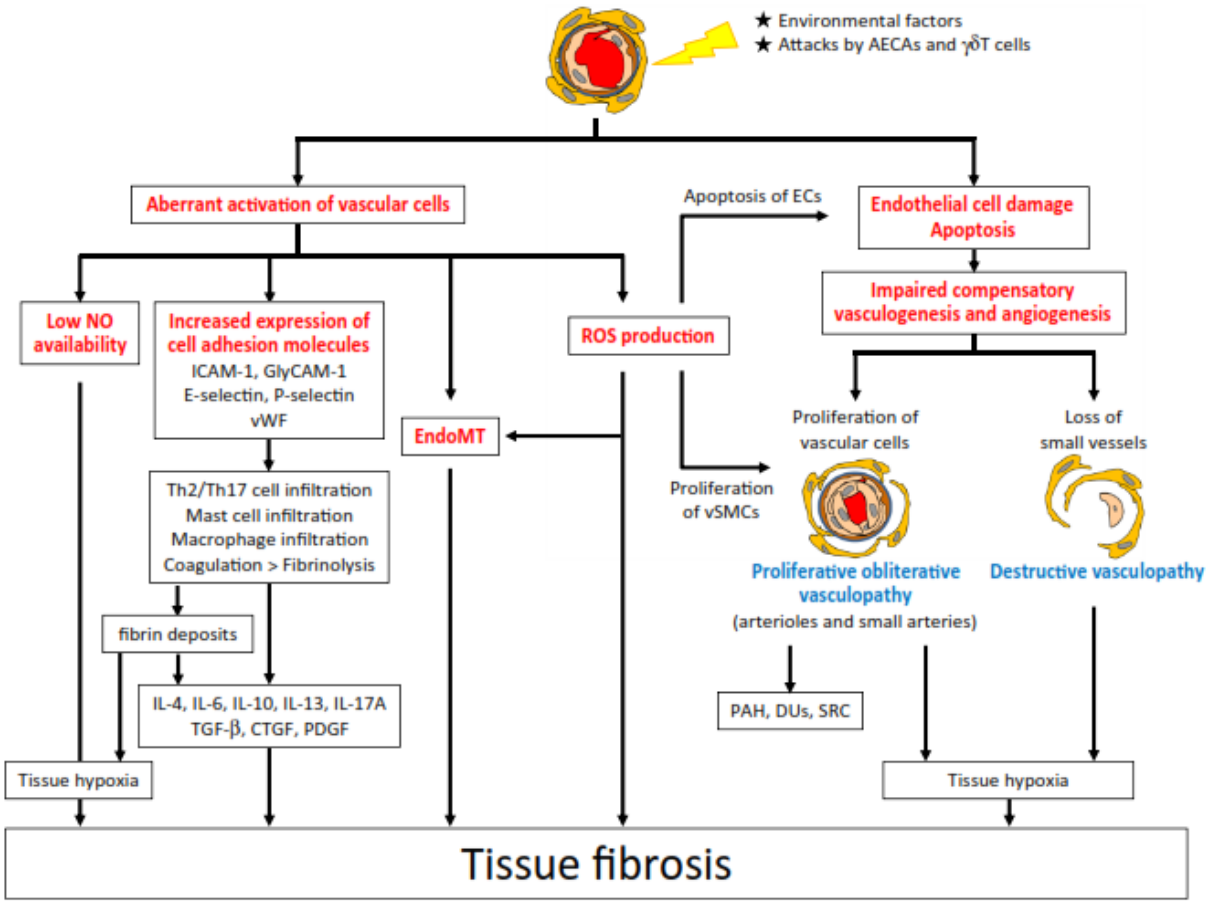
is responsible for the most significant clinical determinants of morbidity and mortality, including PAH, digital ulcers and scleroderma renal crisis.

The etiology of the initial vascular damage in SSc is not known. Viral agents (especially human cytomegalovirus) and other environmental factors, cytotoxic T cells, antibody-dependent cellular cytotoxicity, anti-endothelial cell antibodies and ischemia-reperfusion injury are all suggested mechanisms for ECs damage (14).

Initial vascular injury generates structural changes (destructive and proliferative obliterative vasculopathy caused by impaired compensatory vasculogenesis and angiogenesis) and functional abnormalities of the vasculature (altered expression of cell adhesion molecules, ECs dysfunction, activation of endothelial-to-mesenchymal transition, impaired coagulation/ fibrinolysis system), eventually leading to constitutive activation of fibroblasts in various organs (***Figure 4***) (81). ECs injury is proposed as the crucial initiating event leading to vascular remodeling, with intimal proliferation of arterioles, capillary breakdown and blood vessel occlusion. ECs dysfunction is present and prominent in distinct aspects of cell survival, angiogenesis and vasculogenesis, and disturbed interactions between ECs and various other cells contribute to SSc vasculopathy (42).

The participation of functional autoantibodies in endothelial apoptosis/activation of SSc deepens the understanding about the pathophysiologic mechanisms underlying autoimmune vasculopathies (36, 82). Anti-endothelial cell antibodies cause endothelial cell apoptosis (83). Anti-ICAM1 antibodies induce the production of reactive oxygen species and expression of VCAM-1, which may facilitate the attachment of immune cells (84). Antibodies against angiotensin II type I receptor (anti-AT1R) and ET-1 type A receptor (anti-ETAR) are detected in most SSc patients (16). These agonistic antibodies upregulate ECs expression of TGF-β, IL-8 and VCAM-1 and cause fibrosis, vasoconstriction and recruitment of immune cells. The expressions of anti-ETAR and anti-AT1R are found to be highest in patients with early SSc (85, 86).

Figure 4 – The potential mechanism of SSc vasculopathy leading to tissue fibrosis. *Adapted from* (*Asano Y, Sato S. 2015)* (81). CTGF – connective tissue growth factor; DUs – digital ulcers; ECs – endothelial cells; GlyCAM-1 – glycosylation dependent cell adhesion molecule-1; ICAM-1 – intercellular cell adhesion molecule-1; PAH – pulmonary arterial hypertension; PDGF – platelet-derived growth factor; SRC – scleroderma renal crisis; TGF-β – transforming growth factor-β; vSMCs – vascular smooth muscle cells; vWF – von Willebrand factor.



The activation of ECs leads to the expression of adhesion molecules, intercellular adhesion molecule-1 (ICAM) and VCAM-1, which promote the recruitment of inflammatory cells (87).

Capillary damage of SSc is characterized by endothelial apoptosis, intimal and medial fibrous thickening and adventitial fibrosis, with perivascular infiltration of macrophages, B cells and T cells. Precapillary arterioles then show endothelial proliferation and mononuclear inflammatory infiltrates, followed by intimal proliferation and luminal narrowing (16). The evolution of the lesions leads to capillary rarefaction and to obliteration of the small vessels, which is responsible for hypoxia and oxidative

stress (9). Pericytes also proliferate and contribute to increased vascular wall thickness (14).

Both activated ECs and recruited inflammatory cells constitute an important source of pro-inflammatory cytokines and profibrotic molecules, which stimulate the proliferation of vSMCs and the synthesis of ECM components (9, 81). ECs exhibit substantial plasticity and can undergo endothelial-to-mesenchymal transition, acquiring ECM-producing myofibroblast features (40).

Damaged ECs release several molecular substances into the circulation that interfere with coagulation homeostasis (42). Sub-endothelial tissue forms a nidus for platelets to aggregate and initiates fibrin deposition and intravascular thrombus formation (14).

Impaired vascular permeability and tone are the earliest symptoms of vascular pathology in SSc (88). Increased ET-1 levels and a defective production of prostacyclins and nitric oxide enhance vasoconstriction, inflammation and vascular remodeling and disturb the physiological antithrombotic action of the endothelium (9, 24). The interaction between ECs and platelets also plays an important role in vascular tone regulation in SSc (42).

The absence of compensatory angiogenesis in SSc will be explored in the next sections, as well as the significant role of vascular endothelial growth factor (VEGF), whose synthesis is stimulated by increased endothelial permeability and hypoxia phenomena (**II.3.2** *and* **II.3.3**).

The role of circulating endothelial progenitor cells (EPCs) in SSc vasculopathy is unclear (81). Contradicting studies have reported their decrease or increase in the circulation of SSc patients (88). EPCs may be inadequately recruited and therefore may not contribute to vascular repair or there may be an early apoptosis of these cells (14).

Microangiopathy appears to be the best evaluable predictor of SSc development and may precede symptoms of internal organ involvement by many years. Therefore, the activity and severity of digital vascular disease in patients presenting with RP – often the earliest clinical sign of vascular involvement in SSc – must be measured and monitored (2). A recent study evaluated post-occlusive reactive hyperemia (PORH) by

laser speckle contrast analysis; a statistically significant difference was detected in the PORH peak flow between VEDOSS and established SSc and PORH peak flow decreased according to the capillaroscopic pattern from “early” to “late” (89). POHR might prove a tool to separate pre-clinical from full-blown SSc.

3.1 Biomarkers of microvasculopathy in Systemic Sclerosis

A biological marker (biomarker) was defined as “a characteristic marker that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (90). A biomarker should be easily obtainable, preferably by noninvasive means and eventually be validated in clinical studies (4).

There is an unmet need for validated biomarkers in SSc (4, 91). The discovery and validation of biomarkers can help on identifying disease risk, improving early diagnosis and prognosis, better designing clinical trials and assessing response to treatment, as well as on further elucidating the underlying pathogenic mechanisms of this disease (92).

Biomarkers for SSc can be categorized into activity biomarkers, severity biomarkers, predictive biomarkers and biomarkers for specific clinical features (skin fibrosis, lung fibrosis, PAH, peripheral vasculopathy, gastrointestinal involvement and malignancy) (91).

The first and best developed biomarkers in SSc are assessments of disease status or severity at a point in time (93). At present, SSc-specific autoantibodies are the most useful biomarkers for diagnosis and predicting clinical features (91). Otherwise, biomarkers specific only for SSc have not yet been identified (94). A recent review by Matsushita T. *et al* highlighted the biomarkers most strongly associated to SSc, which include anti-RNA polymerase III as a predictive biomarker of malignancy and gastric antral vascular ectasia, CXCL4 as a predictive biomarker of worsening skin sclerosis and lung fibrosis, the DETECT algorithm as a predictive biomarker for PAH, CCL2 as an activity biomarker of the skin and lungs and interferon-inducible chemokine score as a severity biomarker (91).

Current research is focusing on the discovery of useful mechanistic biomarkers, reflecting ongoing inflammatory or fibrotic activity in the skin and internal organs, as well as being predictive of future disease course (92, 95, 96). Aggregate indices of multiple biomarkers are being applied for improving prognostic discrimination, and similarly genomic expression patterns and disease trajectory analysis have demonstrated early promise in grouping patients with shared clinical characteristics (97). Biomarkers predicting future disease course are particularly important, so that patients with spontaneously regressive disease are not entered into clinical trials and are not treated aggressively with disease-modifying medications (91, 93).

3.1.1 First Paper

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Review

Vascular biomarkers and correlation with peripheral vasculopathy in systemic sclerosis

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ABSTRACT

Vascular disease is a hallmark of systemic sclerosis (SSc). It is present in every patient, being responsible both for the earliest clinical manifestations and the major life-threatening complications of the disease, and thus determining important morbidity and mortality.
In SSc, progressive vascular injury leads to vascular tone dysfunction and reduced capillary blood flow, with consequent tissue ischemia and chronic hypoxia. These phenomena are often accompanied by abnormal levels of vascular factors.
Microangiopathy in SSc may be easily assessed by nailfold videocapillaroscopy. The variety of derangements detected in the nailfold capillaries is accompanied by abnormal levels of different vascular mediators and appears to be the best evaluable predictor of the development of peripheral vascular complications, such as digital ulcers.
The purpose of this review is to summarize in SSc the most relevant vascular biomarkers and the main associations between vascular biomarkers and capillaroscopic parameters and/or the presence of digital ulcers.
Vascular biomarkers could become useful predictive factors of vascular damage in SSc, allowing an earlier management of vascular complications.

Contents

Abbreviations: AAVA, anti-annexin V antibodies; AECA, anti-endothelial cell antibodies; Ang, angiopoietin; ANGPTL, angiopoietin-like protein; AT_1R, antibodies against angiotensin II type 1 receptor; CCR, chemokine receptor; dcSSc, diffuse cutaneous systemic sclerosis; DcR, decoy receptor; DU, digital ulcers; ECs, endothelial cells; ECM, extracellular matrix; ENG, endoglin; ET, endothelin; ET_AR, entdothelin-1 type A receptor; EUSTAR, EULAR Scleroderma Trials And Research; IL, interleukin; JAMs, junctional adhesion molecules; MMPs, matrix metalloproteinases; NVC, nailfold videocapillaroscopy; PAH, pulmonary arterial hypertension; PBMCs, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; PlGF, placental growth factor; RBP, retinol binding protein; RP, Raynaud's phenomenon; sENG, soluble endoglin; sICAM, soluble intercellular adhesion molecule; SSc, systemic sclerosis; sVCAM, soluble vascular cell adhesion molecule; TGF-β, transforming growth factor-β; TWEAK, TNF-like weak inducer of apoptosis; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptors

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1. Introduction—systemic sclerosis is a vascular disease from its early onset

Systemic sclerosis (SSc; scleroderma) is a life-threatening connective tissue disorder of unknown etiology, characterized by widespread vascular injury and dysfunction, impaired angiogenesis, immune dysregulation and progressive fibrosis of the skin and numerous visceral organs [1–4].

A growing body of evidence supports the concept that SSc is primarily a vascular disease mediated by autoimmunity and evolving into tissue fibrosis [5,6]. The dysregulation of vascular tone control, clinically evident as Raynaud's phenomenon (RP), and microcirculatory abnormalities are the earliest clinical manifestations of SSc and may precede skin and visceral involvement by months or years [6–9]; the interval between RP onset and the first non-RP sign in SSc will predict the prognosis, as a short interval (weeks or months) is usually associated to a more aggressive disease course [10]. Telangiectasias, pitting scars, digital ulcers (DUs) and pulmonary arterial hypertension (PAH) may occur later in the disease process, severely affecting the quality of life of SSc patients [11].

In SSc, the progressive vascular injury is characterized by a persistent activation/damage and apoptosis of endothelial cells (ECs), intimal thickening and narrowing of the vessel which may evolve to lumen obliteration. Vascular remodeling leads to vascular tone dysfunction and reduced capillary blood flow, with consequent tissue ischemia and chronic hypoxia—a vicious cycle of ischemia–reperfusion injury, further exacerbated by extracellular matrix (ECM) accumulation due to fibrosis [4,9,10]. The whole process is characterized by an uncontrolled regeneration of the vasculature and subsequent microvascular loss, due to defects in both vascular repair and expected increase in new vessel growth (angiogenesis) [5,11]. The occurrence of overt vascular inflammation is less frequently observed [12].

In SSc, several studies have addressed the utility, in the clinical setting, of vascular biomarkers to evaluate the evolution of the pathological process affecting the vessels as well as to predict the outcome and the treatment response [13]. In this review, we focus the attention on the involvement of the microvasculature and present the main vascular biomarkers and their reported associations with the vascular features of the disease.

2. Peripheral vascular features of systemic sclerosis

2.1. Nailfold videocapillaroscopy: an open-window for detecting microvasculopathy in SSc

The damage of the microvessels evolves progressively from the early to the late stages in SSc, with different morphological abnormalities that are clearly shown by nailfold videocapillaroscopy (NVC) changes during the disease evolution [14–18]. These modifications are often accompanied by abnormal levels of angiogenic/angiostatic factors and markers of EC activation and injury.

The variety of NVC changes parallel the different degree of vascular disturbances in SSc [6,9]. In the early SSc, a pro-inflammatory state (giant capillaries) and an increased production of pro-angiogenic factors may stimulate angiogenesis (new abnormal and tortuous capillaries). This pro-angiogenic response is followed by a significant modification of the angiogenic process, which might in part be explained by the action of several angiostatic factors, ultimately resulting in a loss of angiogenesis characterized by a reduced capillary density and extensive avascular areas [9]. The most frequently observed NVC changes (giant capillaries, hemorrhages, avascular areas, ramified/bushy capillaries) are known as the "scleroderma pattern" [19]. According to their different proportions, they may distinguish an "early", an "active" and a "late" pattern of SSc capillaroscopy (Table 1) [20].

In SSc, NVC is a very useful clinical tool to achieve an early diagnosis, monitoring disease progression and predicting organ involvement [21–23]. The scleroderma pattern of nailfold capillary changes is used as a clinical diagnostic tool that enables physicians to distinguish patients with SSc from patients with uncomplicated primary RP [6]. High avascular scores were recently found to be an independent predictor of death in SSc [24] and patients with "late" NVC pattern had a higher frequency of pulmonary and esophageal involvement, compared to patients with the "early"/"active" patterns [25]. Therefore, the changes of the NVC pattern may represent a morphological reproduction of the evolution of SSc at microvascular level [20]. In RP patients, NVC can be used to monitor the modifications of the microcirculation thus establishing precisely the activity and severity of digital vascular disease [8].

2.2. Digital ulcers: an early consequence of vascular involvement

In SSc, DUs are an early manifestation of vasculopathy (vasomotor dysregulation and vascular histological changes) [10,26,27] and represent a considerable burden [28]. They are often extremely painful and cause significant impairment of hand function and activities of daily living, having a major impact on quality of life [28]. In SSc, DUs are frequent, averaging 30% prevalence according to the EULAR Scleroderma Trials And Research (EUSTAR) registry [29]. They result from ischemia due to vasospasm, intimal fibro-proliferation and thrombosis of the digital arteries; additional co-factors as sclerodactyly, calcinosis and local trauma may further contribute to their genesis [30]. The presence of persistent and severe DUs significantly increases the need for hospitalization of patients and for antibiotic treatment [31]. In addition, DUs are thought to be a clinical parameter of severe vasculopathy that can be associated with or predict other vascular lesions. Treatment of DUs remains challenging and the identification of reliable predictors of this complication is still an unmet clinical need in SSc [32].

3. Vascular biomarkers and correlations with NVC changes and DUs

Several vascular biomarkers have been studied in SSc and correlated with NVC changes and the presence of DUs (Table 2, Fig. 1).

3.1. Endothelial cell adhesion molecules

EC adhesion molecules play a pivotal role in angiogenesis, often acting in concert with angiogenic cytokines [33]. Adhesion molecules involved in cell–cell and cell–ECM interactions are important in the pathogenesis of the earlier stages of vascular alterations in SSc and have been suggested as potential biomarkers for SSc vasculopathy [1].

Table 1
"Early", "active" and "late" patterns of capillary microscopic changes in SSc [89].

	"Early" pattern	"Active" pattern	"Late" pattern
Architecture	Well preserved	Mildly disorganized	Disorganized
Focal missing of capillaries	+	++	+++
Avascular areas	−	−	+++
Hemorrhages	+	+++	(+)
Megacapillaries	+	+++	(+)
Elongated capillaries	+	++	+++
Ramified/bushy capillaries	−	+	+++

Table 2
Vascular biomarkers and correlations with NVC changes and DUs.

Authors (year)	Vascular biomarkers	Correlation with NVC changes				Correlation with the presence of DUs
		"Early"	"Active"	"Late"	Others	
Sfikakis et al. (1993)	sICAM-1	–	–	–	–	↑
Sugiura et al. (1999)	Anti-annexin V antibodies	–	–	–	–	↑
Hebbar et al. (2000)	Endostatin	–	–	–	–	↑
Distler et al. (2002)	VEGF, b-FGF, endostatin	Not found			↓ endostatin if giant capillaries	↓ VEGF
Choi et al. (2003)	VEGF	–	–	–	inverse correlation with capillary density	–
Riccieri et al. (2003)	IL-13	–	↑	–	↑ if hemorrhages and enlarged capillaries	–
Valim et al. (2004)	Soluble E-selectin	–	–	–	↑ if capillary loss	–
Del Rosso et al. (2005)	Tissue kallikrein, kallistatin	↑ kallikrein		–	↑ kallikrein if giant capillaries and microhemorrhages	Not found
Riccieri et al. (2008)	AECA	–	–	↑	–	–
Wipff et al. (2008)	Soluble endoglin	–	–	–	–	↑
Bielecki et al. (2009)	PBMCs production of TWEAK	–	–	–	↓ in more severe patterns	–
El Serougy et al. (2009)	Anti-annexin V antibodies (IgG)	–	–	–	–	↑
Solanilla et al. (2009)	Activated-platelets release of VEGF, PDGF, PDGF-BB, TGF-β1, Ang-1 and Ang-2	–	–	–	↑ VEGF if giant capillaries	–
Sulli et al. (2009)	ET-1	–	–	↑ (vs "early")	↑ if "ramified" and enlarged capillaries; ↑ if capillary loss	↑
Habeeb et al. (2010)	Anti-annexin V antibodies	–	–	–	Absent in "early" pattern	↑
Jinnin et al. (2010)	Soluble VEGF receptor-2	–	–	–	Not found (for microhemorrhages)	Not found
Kim et al. (2010)	ET-1	–	–	–	↑ with increased capillary dimension	↑
Yanaba et al. (2010)	CCL13	–	–	–	–	Not found
Bassyouni et al. (2011)	Soluble CD36	–	–	–	–	↑
Bielecki et al. (2011)	PBMCs production of VEGF	–	–	–	↑ in less severe patterns	↓
Gambichler et al. (2011)	CCL13	–	–	–	–	Not found
Manetti et al. (2011)	IL-33	–	↑ (vs "late")	–	–	Not found
Michalska-Jakubus et al. (2011)	Ang-1, Ang-2	–	–	↑ Ang-2	–	↓ Ang-2
Rabquer et al. (2011)	CXCL9, 10 and 16	–	–	–	–	Not found
Riccieri et al. (2011)	VEGF, Ang-2, PDGF-BB, G-CSF, sPECAM-1, leptin, HGF, folistatin, IL-8	–	–	↑ Ang-2	–	↑ PDGF-BB and sPECAM-1
Riemekasten et al. (2011)	Anti-AT_1R and anti-ET_AR	–	–	–	–	↑
Yanaba et al. (2011)	CCL23	–	–	–	–	Not found
Avouac et al. (2012)	VEGF, sVCAM, PlGF	–	–	–	–	↑ PlGF (new DUs)
Bandinelli et al. (2012)	CCL 2, 5 and 3	Not found			–	Not found
Manetti et al. (2012)	MMP-12	–	↑ (vs "early")	↑ (vs "active")	–	↑
Taniguchi et al. (2012)	Galectin-3	–	–	–	–	↑
Yamada et al. (2012)	DcR-3	Not found				Not found
Aozasa et al. (2013)	Apelin	–	–	–	–	↑
Avouac et al. (2013)	ET-1, VEGF, PlGF, sVCAM, Tie-2, Ang-2, endostatin, endoglin	–	↑ ET-1	↑ VEGF	–	–
Bielecka et al. (2013)	Ratio soluble CD163/soluble TWEAK	–	–	–	–	↓
Cozzani et al. (2013)	ET-1	–	–	–	–	↓
Fakour et al. (2013)	VEGF, endostatin	–	–	–	–	↓ VEGF; ↑ endostatin
Ichimura et al. (2013)	CXCL5	–	–	–	–	↓
Manetti et al. (2013)	Soluble JAM-A, soluble JAM-C	↑	↑	–	–	↑
Manetti et al. (2013)	$VEGF_{165b}$	–	↑(vs controls)	↑	↓ if microhemorrhages; ↑ if bushy capillaries and avascular areas	–
Noda et al. (2013)	Soluble Tie-1	Not found				Not found
Terras et al. (2013)	IL-33	–	–	–	–	↑
Toyama et al. (2013)	RBP-4	–	–	–	Not found (for microhemorrhages)	Not found
Valentini et al. (2013)	Soluble E-selectin	–	–	–	↑ if NVC SSc pattern present (in early-SSc)	–
Ichimura et al. (2014)	ANGPTL-3	–	–	–	Not found (for microhemorrhages)	↑
Vettori et al. (2014)	CCL2,CXCL8	Not found			–	–
Yanaba et al. (2014)	Galectin-1	–	–	–	–	↓

Abbreviations
↑: higher levels or increased; ↓: lower levels or decreased; –: not evaluated; AECA: anti-endothelial cell antibodies; Ang: angiopoietin; ANGPTL: angiopoietin-like protein; AT_1R: angiotensin II type 1 receptor; b-FGF: basic fibroblast growth factor; CD: cluster of differentiation; DcR: decoy receptor; DUs: digital ulcers; ET-1: endothelin-1; ET_AR: ET-1 type A receptor; G-CSF: granulocyte colony stimulating factor; HGF: hepatocyte growth factor; IL: interleukin; JAM: junctional adhesion molecule; MMP: matrix metalloproteinase; NVC: nailfold videocapillaroscopy; PBMCs: peripheral blood mononuclear cells; PDGF: platelet derived growth factor; PDGF-BB: PDGF, homodimeric form BB; sPECAM: soluble platelet endothelial cell adhesion molecule; PlGF: placental growth factor; RBP-4: retinol binding protein-4; sICAM: soluble intercellular adhesion molecule; SSc: systemic sclerosis; sVCAM: soluble vascular cell-adhesion molecule; TGF: transforming growth factor; TWEAK:TNF-like weak inducer of apoptosis; VEGF: vascular endothelial growth factor; vs: versus

There is evidence that increased levels of soluble adhesion molecules released by ECs, such as soluble E-selectin, soluble vascular cell adhesion molecule (sVCAM)-1 and soluble intercellular adhesion molecule (sICAM)-1 may reflect the ongoing EC activation state in early stages of SSc and correlate to the presence and severity of specific organ complications [2,34]. Circulating levels of ***sICAM-1*** [35] and plasma

levels of ***soluble platelet endothelial cell adhesion molecule-1*** [16] were significantly higher in SSc patients with DUs, compared to those without. On the other hand, high serum levels of sVCAM-1 were not independent predictors for the occurrence of new DUs in SSc [32] and no association was observed between serum levels of ***sVCAM-1*** and NVC patterns [36].

Serum levels of soluble ***E-selectin*** were found to correlate with the presence of NVC avascular areas, within the first 48 months from the diagnosis of SSc, suggesting that it might be a useful biochemical marker of disease activity. [37]. No association was observed between serum levels of soluble E-selectin and giant capillaries [37]. In patients with early-SSc (RP with SSc marker autoantibodies and/or a NVC scleroderma pattern without any clinical manifestation of definite SSc), serum levels of soluble E-selectin were increased in those who presented a NVC scleroderma pattern [38].

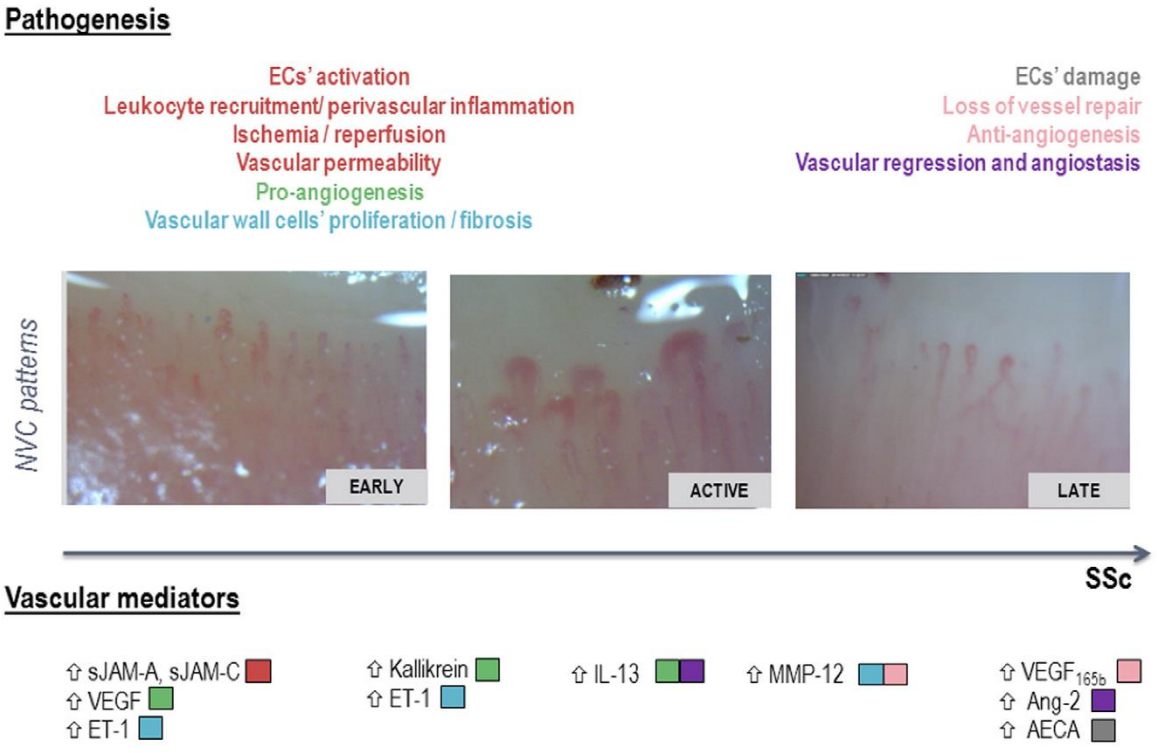
The possible role of junctional adhesion molecules (JAMs) in SSc pathogenesis was also recently investigated [2]. JAMs regulate leukocyte recruitment to sites of inflammation, ischemia–reperfusion injury, vascular permeability and angiogenesis. JAM-A is crucial for a correct EC motility, directional movement and focal contact formation during angiogenesis, and JAM-C is also a pro-angiogenic molecule [2]. Serum levels of both ***soluble JAM-A*** and ***soluble JAM-C*** were significantly higher in SSc patients with "early" or "active" NVC patterns (versus "late" pattern) and in those with active DUs (compared with patients without DUs). These results suggest the participation of JAMs in early EC activation and perivascular inflammatory processes, in defective angiogenesis and in the loss of microvessels at a later stage.

3.2. Chemokines

Cytokines inducing chemotaxis or chemokines are a family of small molecules that are classified according to the position of the NH(2)-terminal cysteine motif. Chemokines mediate leukocyte chemotaxis and migration through endothelia into the organ tissues, leading to the interaction between leukocytes and fibroblasts [39]. They participate actively in inflammation and accumulation of ECM. In SSc, chemokines may be critical in initiating and developing fibrosis, by attracting into the tissues leukocytes and mononuclear cells that, in turn, might release pro-fibrotic growth factors [40].

In SSc, chemokines may also modulate angiogenesis. CXC chemokines containing the ELR motif (Glu-Leu-Arg), such as IL-8 (CXCL8) and growth-related oncogenes α, β and γ (CXCL1-3), have strong chemotactic effects on ECs and can induce neovascularization in vivo, even in the absence of leukocytes. By contrast, CXC chemokines lacking the ELR motif, such as platelet factor 4 (CXCL4) and monokine induced by IFN-γ (MIG, CXCL9), are potent inhibitors of angiogenesis by binding CXCR3. In addition, CXCL16 promotes angiogenesis by binding its unique receptor CXCR6 [41,42].

Several chemokines have been studied in SSc, and some works have addressed their possible correlation with peripheral vasculopathy. Serum levels of ***CXCL10*** and ***CCL2*** were evaluated in patients with SSc but no correlation was found with the peripheral vascular involvement [43]. Moreover, anti-angiogenic ***CXCL10*** and ***CXCL9*** were elevated in SSc serum and highly expressed in SSc skin, but their receptor CXCR3 was decreased on ECs in SSc skin. Conversely, pro-angiogenic ***CXCL16*** was elevated in SSc serum and increased in SSc patients and its receptor



Abbreviations: ⇧: higher levels or increased; ⇩: lower levels or decreased; AECA: anti-endothelial cell antibodies; Ang: angiopoietin; ECs: endothelial cells; ET-1: endothelin-1; IL: interleukin; MMP: matrix metalloproteinase; NVC: nailfold videocapillaroscopy; sJAM: soluble junctional adhesion molecules; SSc: systemic sclerosis; VEGF: vascular endothelial growth factor.
(NVC images from Autoimmunity Outpatient Clinic of Internal Medicine Department – Centro Hospitalar São João, Porto, Portugal.)

Fig. 1. Nailfold videocapillaroscopy (NVC) changes parallel the different degree of vascular disturbances in SSc: an initial state characterized by endothelial cell (EC) activation, inflammation and an increased production of pro-angiogenic factors, is progressively replaced by a state of vascular regression and angiostasis, ultimately resulting in reduced capillary density and extensive avascular areas.

CXCR6 was overexpressed on ECs in SSc skin. None of these chemokines correlated with the presence of DUs [42].

Serum levels of the pro-angiogenic ***CXCL5*** were significantly lower in SSc patients versus healthy subjects [44]. This may be explained by its role as a neutrophil chemo-attractant, not induced during the fibrotic process of SSc (perivascular infiltration of mononuclear cells). In non-early stage diffuse cutaneous SSc (dcSSc) (>1 year), decreased serum CXCL5 levels were linked to the development of DUs and suggested as a marker of DU development in mid and late stage of dcSSc. CXCL5 is suppressed at least partially due to the deficiency of Fli1 (a member of Ets transcription factor family) in SSc ECs. Since Fli1 deficiency is deeply related to aberrant angiogenesis in SSc, it is plausible that serum CXCL5 levels inversely reflect the severity of SSc vasculopathy [44].

In a recent study, ***CCL2*** and ***CXCL8*** were found to be increased in all SSc patients as compared to controls and paralleled the severity of the disease subset (early SSc < limited cutaneous SSc < dcSSc). NVC findings did not affect the serum profile of the investigated soluble factors in early SSc patients [45].

No significant correlation of ***CCL2***, ***CCL5*** and ***CCL3*** levels was found in patients with/without DUs, telangiectasias or NVC patterns, either before or after therapy with prostaglandin E1 [40].

CCL13 directs the migration of monocytes / macrophages, T lymphocytes and eosinophils through its functional ligands CC chemokine receptor (CCR)2 and CCR3 [46]. Serum CCL13 levels did not correlate with clinical features as DUs, pitting scars or telangiectasias in SSc [46,47].

CCL23 interacts with its functional ligand CCR1 and has chemotactic activity for monocytes/macrophages, dendritic cells, lymphocytes and ECs. It is thought to play an important role in angiogenesis, as it enhances the up-regulation of matrix metalloproteinase-2, inducing EC migration and proliferation [48]. Serum CCL23 levels were elevated in SSc patients compared with healthy individuals [48] and were associated with disease activity and shorter disease duration in SSc patients. CCL23 levels did not correlate with the presence of DUs, pitting scars or telangiectasias. Raised CCL23 levels were associated with a higher frequency of PAH.

3.3. Pro-angiogenic factors

Vascular endothelial growth factor (**VEGF**) is strongly overexpressed in the skin and serum of SSc patients, together with the VEGF receptors (VEGFR-1 and -2), although no effective angiogenesis is observed. VEGF levels are mainly increased in the earliest stages of the disease, which may be related to compensatory mechanisms and may have deleterious effects on the vascular network [14–16,18]. Although elevated levels of VEGF are consistent with active angiogenesis, an uncontrolled chronic overexpression throughout various disease stages, as seen in SSc patients, might contribute to disturbed vessel morphology rather than to promote new vessel formation [14].

Serum levels of VEGF were inversely correlated with the capillary density of nailfold [49]. Recently, higher levels of VEGF were reported to be independently associated with "late" NVC pattern and history of DUs, suggesting a role for VEGF in endothelial injury and repair in SSc [36]. It was hypothesized that VEGF up-regulation might be an insufficient compensatory mechanism to stimulate angiogenesis and a prolonged overexpression of VEGF might have deleterious effects on the vascular network. High VEGF levels might serve as a surrogate marker of capillary damage in SSc [49].

Patients without DUs were found to have higher serum levels of VEGF than patients with DUs [18,50]. Thus, VEGF appeared to be protective against ischemic manifestations, when its concentrations exceeded a certain threshold level [18]. Accordingly, high serum levels of VEGF did not predict new DUs [32].

Peripheral blood mononuclear cells (PBMCs) from patients with SSc were shown to produce significantly greater amounts of VEGF as compared to controls [51]. The same result was found in the group of patients without active DUs and in those with a less severe capillaroscopic pattern, when compared to controls, suggesting that SSc patients with less vascular damage produce more VEGF than controls. There was no significant difference in the production of VEGF between SSc patients with greater or lesser NVC damage. Another group investigated the role of platelets as a source of VEGF and other angiogenic mediators in SSc [52] and showed that they secrete large amounts of VEGF when activated, independently from disease duration. Patients with giant capillaries had higher levels of VEGF in platelet releases and plasma, compared to patients without.

Serum levels of soluble VEGFR-2 were also studied in patients with SSc. Only female patients had significantly higher VEGFR-2 levels in comparison to healthy controls [11]. No correlations were found between levels of soluble VEGFR-2 and DUs or nailfold bleeding.

Endoglin (ENG) is a co-receptor for transforming growth factor-β (TGF-β) family members that is highly expressed in ECs and has a critical function in the development of the vascular system, being required for efficient VEGF-induced angiogenesis [53]. On the other hand, soluble ENG (sENG) acts as an anti-angiogenic protein that interferes with the binding of TGF-β to its receptor. Serum levels of sENG were shown to be increased in a large cohort of SSc patients compared to healthy controls [54]. In multivariate analysis, serum levels of sENG were significantly increased in SSc patients with DUs, in comparison to those without. In a recent study, no association was observed between serum levels of ENG and NVC patterns [36].

An important role in the pathogenesis of SSc is played by the endothelins and in particular by ***endothelin-1*** (ET-1), which is a potent endogenous vasoconstrictor and also mediates vascular wall cell proliferation, fibrosis, and inflammation [55]. Significant correlations were observed between plasma levels of ET-1 and DUs in SSc, as well as for single NVC measures (capillary number, "ramified" capillaries and enlarged capillaries) [56]. Plasma levels of ET-1 were significantly lower in patients with "early" pattern compared to patients with "late" NVC pattern [56]. These results support the involvement of ET-1 in the progression of microvascular/fibrotic damage in SSc. In another study [36], serum levels of ET-1 were significantly higher in patients with "active" NVC pattern compared to those with "early" and "late" patterns (in univariate analysis but not in multivariate one). Plasma levels of ET-1 were notably higher in patients with DUs and PAH, compared to those without, suggesting that it can be closely related to SSc progression and severity [57]. Differently, a recent pilot-study [55] has reported higher serum levels of ET-1 in patients without DUs and ET-1 levels did not correlate with the development of new DUs.

No correlation was found between serum levels of ***placental growth factor*** (PlGF) and the different NVC patterns [36]. Conversely, high serum levels of PlGF were reported to be predictors of new DUs in SSc, highlighting the critical role of angiogenesis in this vascular outcome [32]. These markers may improve DU risk stratification and possibly allow earlier therapeutic intervention. Plasma levels of platelet derived growth factor-bb were higher in SSc patients with DUs compared to those without [16].

The endothelial cell-specific receptor tyrosine kinases ***Tie-1*** *and* ***Tie-2*** and their ligands ***angiopoietin-1*** (Ang-1) and ***Ang-2*** critically regulate both vasculogenesis and angiogenesis. Ang-1-mediated Tie-2 signaling controls vascular quiescence and is essential for maturation of vessels. Ang-2 antagonizes Ang-1 and may be the principal switch controlling the transition from resting to activate endothelium, and so facilitates the inflammatory response [58]. Tie-1 receptor is believed to limit the ability of Ang-1/-2 to bind and activate Tie-2 [59]. Lower serum levels of Ang-1 and higher serum levels of Ang-2 were found in SSc, compared to healthy controls [60]. Levels of Ang-2 were significantly increased in SSc patients with a "late" NVC pattern with respect to those with "early"/"active" ones [16,60]. The independent negative correlation between DUs and Ang-2 levels in SSc suggested that it is more likely

that increased Ang-2 levels reflect ongoing processes of angiogenesis rather than have a deleterious effect on vasculature [60]. Dunne et al. [58] showed higher ratios of soluble Ang-2/Ang-1 and soluble Ang-2/Tie-2 in SSc when compared to healthy controls, suggesting a pro-inflammatory state in an active endothelium and a shift toward vascular regression and angiostasis. Serum levels of soluble Tie-1 were comparable among SSc patients and healthy controls [59]. However, decreased serum levels of soluble Tie-1 in patients with disease longer than six years were associated with higher prevalence of organ involvements due to proliferative vasculopathy. Recently, another study did not find any differential concentration of serum levels of Ang-2 and Tie-2 between the different NVC patterns [36].

The clinical significance of the pro-angiogenic ***angiopoietin-like protein-3*** (ANGPTL-3), a secreted glycoprotein structurally similar to angiopoietins but not binding Tie-1 and -2, was evaluated in SSc [61]. While no differences were found between SSc patients and controls, the prevalence of DUs was significantly higher in patients with elevated serum levels of ANGPTL-3 (versus patients with normal levels) and might contribute to proliferative vasculopathy in SSc. ***Retinol binding protein-4***(RBP-4), an adipocytokine that is likely to be associated with fibrosis, vasodilation and angiogenesis, was also investigated in SSc [62]. No associations were found with nailfold bleeding or DUs but decreased serum levels of RBP-4 were associated with the prevalence of RP and the degree of pulmonary vascular involvement in limited cutaneous SSc, suggesting a possible contribution to SSc pathogenesis.

Serum levels of ***tissue kallikrein*** (a potent angiogenic agent) were increased in SSc patients with respect to controls [63]. Levels of tissue kallikrein were higher in SSc with "early" and "active" NVC patterns, compared to "late" pattern, and were associated with the presence of giant capillaries and microhemorrhages. These data suggest that increased tissue kallikrein levels correlate with an early derangement of microcirculation and might be a reaction to chronic ischemia and/or a frustrated attempt to provide effective angiogenesis in SSc. Conversely, no differences in ***kallistatin*** levels were detected between SSc patients and controls.

It has been investigated whether serum levels of ***interleukin (IL)-13*** correlated with different NVC findings in patients with SSc [64]. Elevated levels of IL-13 were associated with a more frequent "active" NVC pattern, the presence of hemorrhages and sludging of blood, as well as with larger total loop and arterial capillary diameters, suggesting a microvascular role for this cytokine, besides the immunological and fibrotic ones.

Interleukin-33 belongs to the IL-1 family and induces gene expression of Th2-associated cytokines [65]. IL-33 induces IL-13-dependent cutaneous fibrosis and stimulates angiogenesis and vascular permeability [66]. Manetti et al. [66] evaluated serum levels of IL-33 and their possible correlation with clinical features and microvascular involvement in patients with SSc. Serum levels of IL-33 were significantly higher in SSc than in controls. An "active" pattern in NVC was associated with higher levels of IL-33 (versus "late" pattern), suggesting that it might participate in the active derangement of the microcirculation in SSc, probably in response to pro-inflammatory triggers and following EC activation/damage. In a large cohort of SSc patients, there was a significant correlation between detectable IL-33 serum levels and the occurrence of DUs [65].

Galectin-3 is a multifunctional protein implicated in fibrosis, pro-angiogenesis and immune activation and has been evaluated in SSc by Taniguchi et al. [67]. In SSc, serum levels of galectin-3 were significantly lower with respect to controls but were significantly higher in patients with DUs versus those without, suggesting a role in the development of this complication. Galectin-1, another member of β-galactoside-binding lectins, was also recently studied in SSc [68]. Although no significant differences on serum levels of galectin-1 were found between SSc patients and controls, lower levels were associated with the presence of DUs, suggesting a protective role, possibly by preventing ECs from apoptosis.

Apelin is a bioactive peptide with pro-angiogenic and pro-fibrotic effects, and its role in SSc was recently studied [69]. Although no differences were found in SSc patients versus controls, in patients with disease longer than 10 years the prevalence of severe vascular involvements (intractable DUs, scleroderma renal crisis and PAH) was significantly higher in patients with elevated serum apelin levels versus those without. Apelin may be associated with altered and activated angiogenesis prior to fibrotic responses in early SSc and with the development of proliferative vasculopathy in later stages.

The ***TNF-like weak inducer of apoptosis*** (TWEAK) is a multifunctional cytokine, which regulates inflammation, angiogenesis and tissue remodeling [70]. The production of TWEAK by PBMCs was significantly diminished in SSc patients with more severe microvascular damage, as indicated by the presence of an "active" NVC pattern, compared to patients with "early" pattern and to controls, and correlated inversely with the duration of RP [71]. Cluster of differentiation (CD) 163 is a member of the scavenger receptor cysteine rich family that binds TWEAK and inhibits its actions. In a recent study, high serum levels of soluble CD163 and a high ratio of soluble CD163/soluble TWEAK were associated with a lower risk of DUs in SSc [70], suggesting that elevated expression of CD163 plays a crucial role in this phenomenon and the effects are not associated with its ability to neutralize TWEAK.

Decoy receptor (DcR)-3, a member of the tumor necrosis factor receptor family, has been associated with aberrant activation of autoimmunity and angiogenesis under pathological conditions and was also assessed in SSc [72]. Significantly higher serum levels of DcR-3 were found in SSc compared to controls but it was not associated with NVC changes or DUs.

3.4. Anti-angiogenic factors

Endostatin is a main inhibitor of angiogenesis and significantly increased serum concentrations of circulating endostatin were found in SSc with respect to healthy controls [73]. Higher endostatin levels were reported in patients with DUs compared to those without and, furthermore, patients who had the highest endostatin concentrations frequently had larger ulcers. Accordingly, a role for endostatin in the occurrence of ischemic manifestations in SSc was hypothesized. However, in another study [18], serum levels of endostatin were not increased in SSc compared to controls, nor showed any association with DUs; instead, lower levels of endostatin were associated with the presence of giant capillaries in NVC. More recently [50], significantly higher levels of serum endostatin in SSc patients with DUs were again reported. No correlations were found between serum levels of endostatin and the different NVC patterns [36].

VEGF pro-angiogenic ($VEGF_{165}$) and ***anti-angiogenic ($VEGF_{165b}$)*** isoforms have been uncovered and appear to be generated by alternative splicing mechanisms in the terminal exon of VEGF pre-mRNA. A switch from pro-angiogenic to anti-angiogenic VEGF isoforms may play a crucial role in the defective angiogenic and vascular repair processes that characterize SSc [74,75]. Plasma circulating levels of $VEGF_{165b}$ were shown to be increased in SSc [74]. Almost all VEGF detected in SSc skin and circulation is the anti-angiogenic $VEGF_{165b}$ and increased levels of $VEGF_{165b}$ are both early and persistent features of the disease. A subsequent study [76] showed significantly higher plasma levels of $VEGF_{165b}$ in SSc patients with "late" NVC pattern (versus "early" and "active" patterns); $VEGF_{165b}$ levels were significantly raised in SSc patients either with "active" or "late" NVC patterns with respect to controls, suggesting that the $VEGF_{165b}$ splice variant might actively participate in the loss of microvessels. Increased plasma levels of $VEGF_{165b}$ correlated significantly with the absence of microhemorrhages and the presence of ramified/bushy capillaries and avascular areas.

Matrix metalloproteinases (MMPs) by degrading collagen and other extracellular macromolecules, cytokines, growth factors and their receptors exert various biological effects on cell migration, on ECM and vascular remodeling, as well as on inflammatory and immune

processes. They are also known to play an important role in aberrant fibrotic tissue remodeling [3]. MMP-12 has been correlated to impaired EC proliferation, migration and angiogenesis [77]. Its anti-angiogenic role depends on the ability to convert plasminogen into angiostatin, a potent inhibitor of angiogenesis, as well as on the cleavage of endothelial urokinase-type plasminogen activator receptor [3]. Manetti et al. investigated whether MMP-12 could serve as a biomarker of vascular disease in SSc [3]. Circulating levels of MMP-12 were significantly increased in patients with SSc compared to controls and increased serum levels of MMP-12 were associated with the presence of DUs and severity of NVC abnormalities, suggesting that it may actively participate in the derangement of peripheral microcirculation in patients with SSc.

The ***anti-angiogenic receptor CD36*** has been shown to correlate with the severity of vascular disease in SSc [5]. The highest levels of serum soluble CD36 were detected in patients with DUs and digital gangrene.

3.5. Autoantibodies

Autoantibodies directed against a variety of nuclear, cytoplasmic and extracellular autoantigens are a serological hallmark of SSc [78]. Unfortunately, the available methods for identification of autoantibodies other than anti-centromere and anti-topoisomerase I are cumbersome and not suitable for routine use. Practical and rapid assays to detect some secondary antibodies in SSc are being tested [79]. Autoantibodies have been associated with distinct disease subtypes and with differences in disease severity, clinical manifestations and prognosis [78].

Anti-endothelial cell antibodies (AECA) have been identified in SSc and their participation in vascular injury is postulated [80,81]. Serum levels of AECA were significantly higher in the "late" NVC pattern, with respect to the "early" and "active" ones [82]. More severe NVC findings were more frequently found in AECA positive cases with higher levels of these antibodies, regardless of the disease duration. Although not directly linked with the progression of the disease, AECA may have a role in the endothelial damage of SSc and their presence and titer shall be considered as an adjunctive risk factor for a more severe disease [82].

Agonistic ***antibodies against angiotensin II type 1 receptor*** (AT_1R) and ***ET-1 type A receptor*** (ET_AR) have been identified in a large proportion of patients with SSc [83]. In addition to ECs, AT_1R and ET_AR are expressed on fibroblasts, epithelial and immune cells and could represent a link between autoimmunity, endothelial injury and fibrosis. Patients with elevated serum levels of anti-AT_1R and anti-ET_AR antibodies had a higher risk for the development of DUs, as well as for more severe disease manifestations, and elevated AT_1R and anti-ET_AR levels predicted SSc-related mortality.

Annexins are a group of twelve highly conserved proteins which exert several regulatory functions on cell biology [84]. Annexin V is highly expressed in vascular ECs, where it is thought to play an antithrombotic role [84]. Anti-annexin V antibodies (AAVA) were detected in patients with SSc [78,84,85]. In one study, 75% of SSc patients with AAVA had digital ischemia (versus 24% of those without this antibody) [86]. AAVA of IgG type were positively correlated with DUs in an Egyptian cohort [87]. The association between AAVA and the presence of severe ischemic manifestations in SSc, including DUs, has also been reported in another study [88]. AAVA were negative in the patients with "early" NVC pattern and positive in all patients with "active" and "late" NVC patterns, without significant difference between these two groups but somewhat higher in patients with "late" pattern [88].

4. Conclusion

Microvascular abnormalities are the earliest events and key features of SSc responsible for the clinical manifestations of peripheral vasculopathy that are among the major life-threatening complications of the disease.

SSc microangiopathy seems to be strictly related to an impairment of selective factors reflecting disturbances of angiogenesis and endothelium damage. Many of these markers have been shown to clinically correlate with disturbed NVC capillary architecture and/or the occurrence of ischemic DUs. This suggests an active role in SSc pathogenesis, namely at its onset. Identifying these vascular mediators and their pathways may be clinically extremely relevant as they could become targets for novel specific therapies, aiming to prevent further vascular injury and to stimulate vascular repair.

Since these correlation studies between vascular biomarkers and clinical features reflect a single moment of the disease and as the NVC phenotype can change during SSc evolution, a prospective follow-up of these biomarkers' levels is warranted to confirm a possible predictive value in the development of microvascular complications. Further studies in SSc are necessary to assess if vascular biomarkers could become useful for risk stratification and as predictive factors of peripheral vasculopathy in the next future, thus allowing an earlier therapeutic intervention.

Take-home messages

- In SSc, microvascular abnormalities are responsible both for the earliest clinical manifestations of peripheral vasculopathy and the major life-threatening complications of the disease.
- Several vascular mediators are dysregulated in SSc, reflecting endothelial damage and disturbances of angiogenesis.
- Some of these biomarkers correlate with NVC changes and/or the occurrence of ischemic DUs.
- Vascular mediators and their pathways could become targets for novel specific therapies, aiming to prevent further vascular injury and to stimulate vascular repair.

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Serial ANA testing is not useful in predicting biologics-induced autoimmune diseases in patients with rheumatoid arthritis

It is known that biological disease-modifying agents, i.e. TNF inhibitors (TNFi), can induce autoimmune diseases, with consequent pathological autoantibody production. However, at present, no consensus has been achieved on the utility of ANA monitoring in order to predict the development of autoimmunity following TNFi therapy. To gain insight into such controversial debate, Takase *et al.* (**Ann Rheum Dis 2014;73:1695-9**) performed a prospective study on the incidence of ANA/dsDNA seroconversion, clinical data and EULAR response in a single-centre cohort of 454 patients with rheumatoid arthritis (RA), that, since 2005 received: i) a first TNFi (etanercept, infliximab, or adalimumab) and ii) tocilizumab and/or abatacept. During a median 5-year observation period (range 0.6-8.0 years), ANA seroconversion occurred more frequently in infliximab-treated patients (31.2%) than in etanercept- (11.8%) or adalimumab-treated patients (16.1%). New onset anti-dsDNA antibodies were observed in six out of 83 ANA seroconverters, 3 of them developing drug-induced systemic lupus erythematosus following the treatment with first-choice or second-choice TNFi. More than 60% of the patients discontinued the first TNFi. Of note was the significantly higher ANA seroconversion rate in secondary non-responders (21.6%) compared with that observed in primary non-responders (5.4%). The study demonstrated that routine ANA/dsDNA testing is not useful in predicting biologics-induced autoimmune diseases; otherwise, ANA seroconversion seems to predict secondary non-response to TNFi.

Anna Ghirardello

Several other promising biomarkers related to the evaluation and management of SSc patients have more recently been presented (91, 92).

A systematic review from Mostmans Y. *et al* identified reliable biomarkers of ECs dysfunction in SSc vasculopathy (42). The most representing biomarkers described were adhesion molecules for ECs activation; anti-endothelial cell antibodies for ECs apoptosis; VEGF, its receptor VEGFR-2 and endostatin for disturbed angiogenesis; endothelial progenitor cells for defective vasculogenesis; ET-1 for disturbed vascular tone control; von Willebrand Factor (vWF) for coagulopathy; and IL-33 for disturbed communication between ECs and the immune system (occurring early in SSc). Emerging biomarkers included $VEGF_{165}b$, IL-17A and the adipocytokines (namely resistin and chemerin). The role of endothelial-to-mesenchymal transition in the pathogenesis of SSc vasculopathy still needs clarification.

Other studies have corroborated that endoglin and VEGF serum levels are potential risk factors for the occurrence of new digital ulcers (DUs), with VEGF showing a predictive value (98), and that ET-1 is a strong predictor of new DUs in SSc patients (99, 100). Asymmetric dimethylarginine has also been implicated as an independent predictor of new DUs (100).

Endostatin was significantly increased in all NVC stages, while angiostatin was only elevated in “active” and “late” phases (101). Besides peripheral vasculopathy, endostatin was also found to represent a marker of renal scleroderma-associated vasculopathy (102). A negative correlation was observed between serum levels of endostatin and estimated glomerular filtration rate. Moreover, in SSc patients with high resistive index in renal Doppler ultrasound, serum levels of endostatin were significantly higher than in patients with normal resistive index.

The role of angiopoietin(Ang)/Tie2 system in SSc was lately readdressed (103). SSc dermal microvessels abundantly expressed Ang-2, but not Ang-1, compared to healthy controls. Membrane bound Tie2, which ensures vessel stability, was profoundly decreased in SSc microvessels, while the levels of soluble Tie 2, which attenuates Tie2 signaling, were increased already in early disease. Both in skin and sera of SSc patients, the Ang1/2 ratio was reduced, being lowest in patients with DUs.

Decreased serum levels of the pro-angiogenic epidermal growth factor-like domain 7 (EGFL7) were significantly correlated with the severity of NVC abnormalities in SSc

(104). Patients with the most severe capillary changes and DUs had serum EGFL7 levels significantly lower than healthy controls, while the EGFL7 levels did not differ significantly between controls and SSc patients with less capillary damage and lack of DUs.

Serum vaspin levels were significantly decreased in SSc patients with DUs compared to those without, suggesting the potential contribution of this adipokine implicated in vascular inflammation and remodeling (105).

In a study analyzing the correlation between serum levels of uric acid and some clinical variables of SSc, the mean value of uric acid increased with the severity of NVC damage (106).

Identification of biomarkers of future disease remains the next great frontier in SSc, as they are keys for proper clinical management and for selecting patients for drug trials (94). Patients with RP without an established connective tissue disease were studied at their first evaluation, to correlate the levels of endothelial markers with the subsequent development of an overt disease (107). Plasma levels of tissue-type plasminogen activator (t-PA), vWF and IL-6 were higher in patients with normal NVC than in healthy controls and even much higher in patients with NVC scleroderma pattern. After 36 months, among 48 RP patients with normal basal NVC, 24 were diagnosed as primary and 24 as secondary RP. In secondary RP, basal levels of t-PA, IL-6 and particularly vWF were higher than in primary RP and healthy controls. Further larger, multicenter, prospective, longitudinal studies will be needed to identify and validate critical biomarkers of SSc (94).

3.2 Loss of angiogenesis in Systemic Sclerosis: paradox or myth? [Doc. 1]

Angiogenesis is a complex and finely balanced process of formation of new vessels from the pre-existing ones, mainly triggered by tissue hypoxia. Sprouting angiogenesis encompasses an increase in vasopermeability, leading to the extravasation of plasma proteins that function as a temporary scaffold for migrating ECs. Matrix metalloproteinases, secreted by the endothelium, break down the vascular basement membrane and allow the invasion of the surrounding stroma by ECs, in the direction

of the pro-angiogenic stimulus. ECs migration and invasion are accompanied by proliferation and the organization of newly formed ECs into three-dimensional tubular structures. Lumen formation and vessel wall stabilization by pericytes are the final processes of sprouting angiogenesis and lead to the formation of a functional network of new capillary vessels (108).

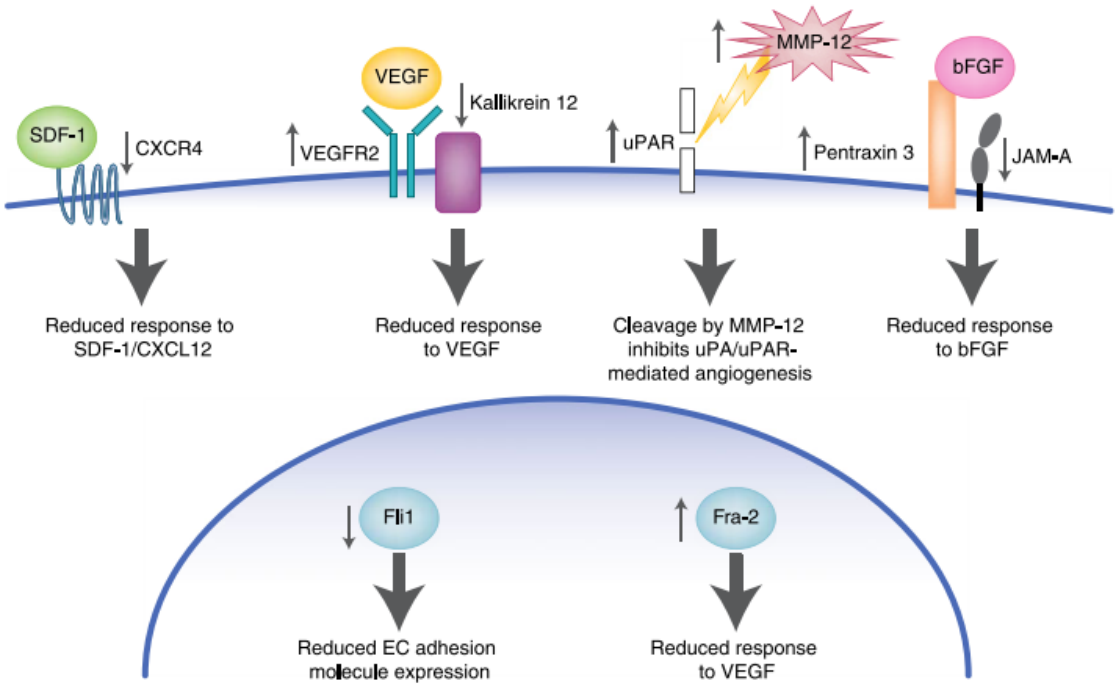
Physiological angiogenesis is tightly regulated by the opposing activities of stimulating (pro-angiogenic) and inhibiting (anti-angiogenic) factors (109). VEGF is a central regulatory factor, which controls several cellular and molecular steps in the angiogenic cascade. Indeed, under VEGF priming, ECs increase their migration and initiate the proliferative process which will stop only when a complete tube structure will be formed (110). Under normal conditions, the levels of angiogenesis inducers and inhibitors are balanced and angiogenesis does not occur in healthy tissues (111). If their activity becomes altered under pathological conditions, abnormal growth of the vasculature and defective repair processes may occur (109).

Defective angiogenic pathways have been identified in SSc patients and several mechanisms have been implicated (***Figure 5***) (108):

- a defective contribution of SSc immune cells to angiogenesis;
- a severe imbalance between pro-angiogenic and anti-angiogenic factors in SSc;
- platelet activation, aggregation and release of bioactive molecules into the circulation and injured endothelium;
- overexpression of pro-angiogenic transcripts and a variety of genes with negative effect on angiogenesis by SSc MVECs;
- a change in the endothelial phenotype of residual microvessels, favoring anti-angiogenic mechanisms.

Existing studies point to the presence of intrinsic pro-angiogenic factors residing in SSc vasculature and adjacent tissues. The inability to regenerate injured vessels might result from the failure of subsequent stages of angiogenesis, such as lumen formation or vessel maturation or stabilization. Alternatively, persistent injury may interfere with this process (112).

Figure 5 – Potential mechanisms of deregulated angiogenesis in SSc. *Adapted from (Rabquer BJ, Koch AE. 2012.) (113).* bFGF – basic fibroblast growth factor; Fli1 – Friend leukemia integration1; JAM-A – junctional adhesion molecule-A; MMP-12 – matrix metalloproteinase-12; SDF-1 – stromal cell-derived factor 1; uPAR – urokinase plasminogen activator receptor; VEGF – vascular endothelial growth; VEGFR-2 – VEGF receptor 2.



Whether deregulated levels of circulating angiogenic factors or angiostatic factors (or both) are a cause or a consequence of an ongoing vascular disease is presently unknown (112).

In a recent study, dermal MVECs isolated from SSc patients were unable to respond to pro-angiogenic chemokines, despite their increased expression in serum and ECs (114). The signaling pathways and transcription factor machinery activated by chemokines are impaired in SSc ECs.

The significance of "time"

Although most studies point to a *lack of angiogenesis* in SSc, it does not correspond to the whole truth during SSc evolution.

In the early stage of the disease, a pro-inflammatory state and an increased production of pro-angiogenic factors may stimulate angiogenesis. Capillaroscopic

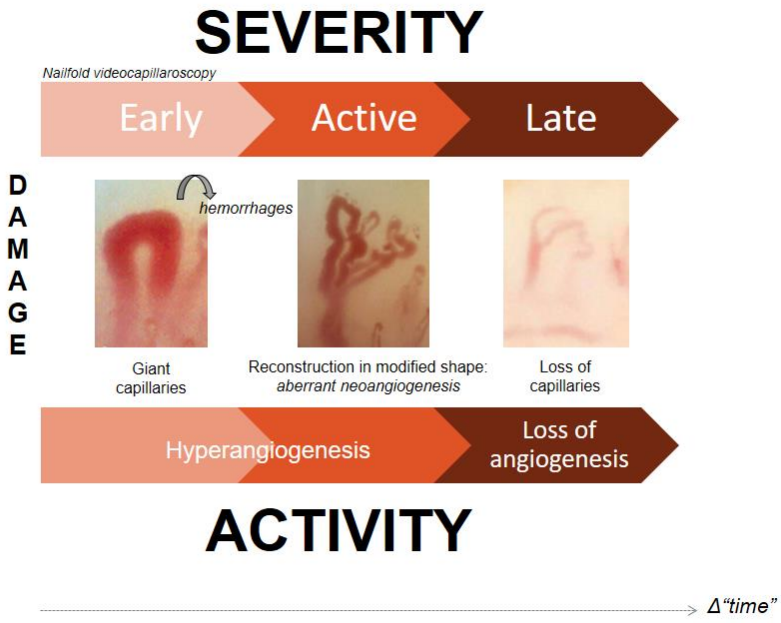
analysis of the nailfold beds demonstrates the presence of tortuous, giant capillary loop clusters that are surrounded by normal capillary loops of varied shapes, with some detectable micro-hemorrhages, reminiscent of immature and unstable newly formed microvessels, during an uncontrolled angiogenic response (108, 115).

This short pro-angiogenic response is followed by an extensive reduction in capillary density, leaving large avascular areas. The dramatic switch from pro- to anti-angiogenic characteristics suggests an impairment of the angiogenic process which might in part be explained by the action of several angiostatic factors ($VEGF_{165b}$, angiopoietin-2), ultimately resulting in profound loss of capillaries along with the absence of visible new normal vessel formation (108, 115, 116). An alternative explanation for the loss of angiogenesis in the course of SSc is that the upregulated pro-angiogenic factors could be exceeded by an even greater upregulation of angiostatic factors (108).

Cumulative data support an initial VEGF-related pro-angiogenic event in SSc patients but suggest that the angiogenic process is then aborted (115). As disease progresses, a marked loss of the microvasculature occurs in several organs. The reduction in capillaries leads to a decrease in the supply of oxygen and nutrients and thus to a hypoxic state. Tissue hypoxia is normally a trigger for angiogenesis; however, vascular recovery is impaired in SSc, and avascular areas are prominent (113).

The severity of microvascular damage is sequential (112), as testified by morphological changes in NVC (capillary enlargement followed by aberrant capillary shapes and then by capillary loss) and is paralleled by a different activity of vascular mediators through disease progression over time. Indeed, in SSc, the meaning of “time” in disease evolution is determined by its severity and activity, instead of measured by the years from diagnosis (***Figure 6***). The associations between specific NVC patterns and severe internal organ involvement represent a shift in interest from the diagnostic to the prognostic utility of NVC in SSc (117).

Figure 6 – The significance of “time” in systemic sclerosis.



Defective angiogenesis, due to a combination of several pathological mechanisms, accompanies SSc evolution and ranges from an early pro-angiogenic state to a late impairment of the angiogenic process, ultimately resulting in reduced capillary density and extensive avascular areas.

In vitro effects on dermal MVECs from sera of patients treated with intravenous cyclophosphamide (CYC) or sera of treatment-naïve patients have been studied (118). When dermal MVECs were challenged with sera from CYC-treated SSc patients, their angiogenic capacity was comparable to that of cells treated with healthy sera, while wound healing capacity and chemotaxis did not show significant differences between CYC-treated and treatment-naïve sera. Contrarily to treatment-naïve sera, MVECs proliferation was not impaired in the presence of sera from CYC-treated patients and CYC-treated SSc sera did not induce MVECs apoptosis. The authors concluded that CYC treatment might boost angiogenesis and consequently improve peripheral microangiopathy.

Recognizing the different phases of angiogenic response in SSc is essential, as the complex imbalance between pro-angiogenic and angiostatic factors might be a therapeutic target. In this regard, strategies for dosing and timing of angiogenic factors might be of major relevance.

3.3 The VEGF Family and its co-receptors Neuropilins

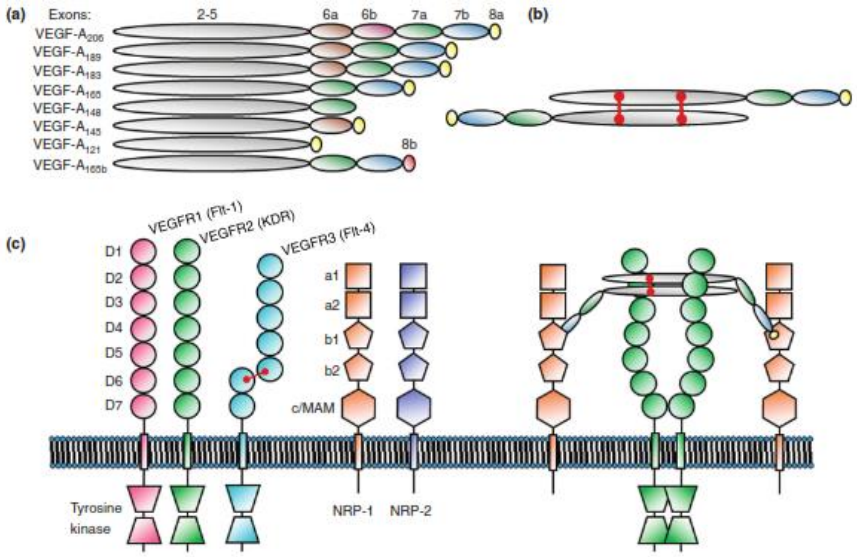
Among the most potent pro-angiogenic cytokine families is the **VEGF family**, which includes VEGF-A to D and placental growth factor (PlGF) (119). VEGF-A is alternatively spliced to generate VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165} and VEGF-A_{189} (its main isoforms), which are endowed with different biological properties (120).

VEGFs are master regulators of vascular development and of blood and lymphatic vessel function, during health and disease, in adults. These ligands act through three tyrosine kinases receptors (VEGFR-1 to -3), that dimerize and become activated upon ligand-binding (121, 122). VEGFR-1 is critical in the regulation of migration of endothelial precursors as well as mature monocyte/macrophages; VEGFR-2 is the key transducer of VEGF function in vascular ECs; and VEGFR-3 is required for lymphatic endothelial function. The active VEGF receptors (VEGFRs) initiate signal transduction pathways. Signaling is modulated through co-receptors such as heparan sulfate, neuropilins (NRPs) and integrins. Importantly, angiogenic signaling is accomplished only through the coordinated activity of VEGF, VEGFR and NRP. While binding of VEGF to VEGFR weakly activates its intracellular kinase activity, NRP is required for strong and sustained kinase activation, leading to the initiation of the pro-angiogenic cascade (119, 120, 122) (***Figure 7***).

VEGF-A-induced VEGFR-2 homodimerization and consequent kinase activation is assumed to underlie most of the known VEGF biology in vascular ECs. VEGFR-2 is critical for vascular development, as gene inactivation of Vegfr2 results in early embryonic lethality (122). VEGFR-2 plays essential roles not only in endothelial differentiation/proliferation and vascular permeability but also in a wide range of other ECs activities, such as survival and motility.

VEGF-A is secreted by a variety of cells and acts in a paracrine manner on ECs to stimulate signal transduction and regulate cellular function. Additionally, endothelial secretion of VEGF-A is essential for promoting cell survival in an autocrine manner (123). VEGF is produced as a consequence of relative hypoxia in a growing tissue and mediates angiogenesis mainly through VEGFR-2 and NRP1 (120).

Figure 7 – VEGF family. (a) VEGF-A splice isoforms indicating the exon-based origin of the domain organization. (b) VEGF proteins are disulphide-crosslinked (red) antiparallel homodimers, indicated here for VEGF-A. (c) Outline domain structure of VEGFR and NRP isoforms, drawn as transmembrane monomers (left). Representation (right) illustrating how VEGF-A_{165} might crosslink VEGFR-2 and NRP1 to effect signaling. *Adapted from (Djordjevic S, Driscoll PC. 2013.) (124).*



VEGF-A binding to VEGFR-2 triggers dimerization and trans-autophosphorylation of several cytoplasmic tyrosine residues. VEGFR-2 activation initiates downstream intracellular signaling events, such as p38 mitogen-activated protein kinase (involved in controlling cytoskeletal dynamics, actin remodeling and ECs migration), extracellular signal-regulated kinases 1/2 with subsequent activating of transcription factor 2 (determining regulation of cell proliferation, migration, tubulogenesis and ECs-leukocyte adhesion) and phosphoinositide-3-kinase with sequential activation of Akt and endothelial nitric oxide synthase (causing increased cell survival and nitric oxide-induced vascular permeability). Increased VEGFR-1 expression and calcium-regulated plasma membrane translocation upon VEGF-A mediated VEGFR-2 activation constitutes a negative feedback loop (123).

VEGF dysfunction is implicated in pathological angiogenesis, leading to major chronic disease states including atherosclerosis, diabetes and cancer. In addition to directly regulating pro-angiogenic signal transduction pathways and subsequent gene expression, VEGFR activation influences metabolic homeostasis and plays essential roles in non-vascular systems, such as the immune system, epithelium and brain (123). In rheumatoid arthritis (RA), VEGF and angiopoietins are the most potent pro-

angiogenic molecules promoting synovial angiogenesis, an early and critical event in disease pathogenesis (125). Moreover, VEGF genetic polymorphisms as well as VEGF levels may be associated with the susceptibility to RA (126). In systemic lupus erythematosus, serum levels of VEGF were higher than in controls, significantly different according to disease activity degree and directly inter-related to abnormal NVC patterns and a more active disease (127).

The spectrum of cell types and tissues susceptible to regulation by VEGFs and VEGFRs highlights their therapeutic potential (123). Increased insights into VEGF signaling may allow the development of drugs that specifically inhibit certain signal transduction pathways, responsible for malfunctioning blood and lymphatic vessels in disease, while preserving vessel survival (122).

The **NRP family** consists of essential multifunctional vertebrate cell surface receptors, functioning in many key biological processes including in the cardiovascular, neuronal and immune systems (128).

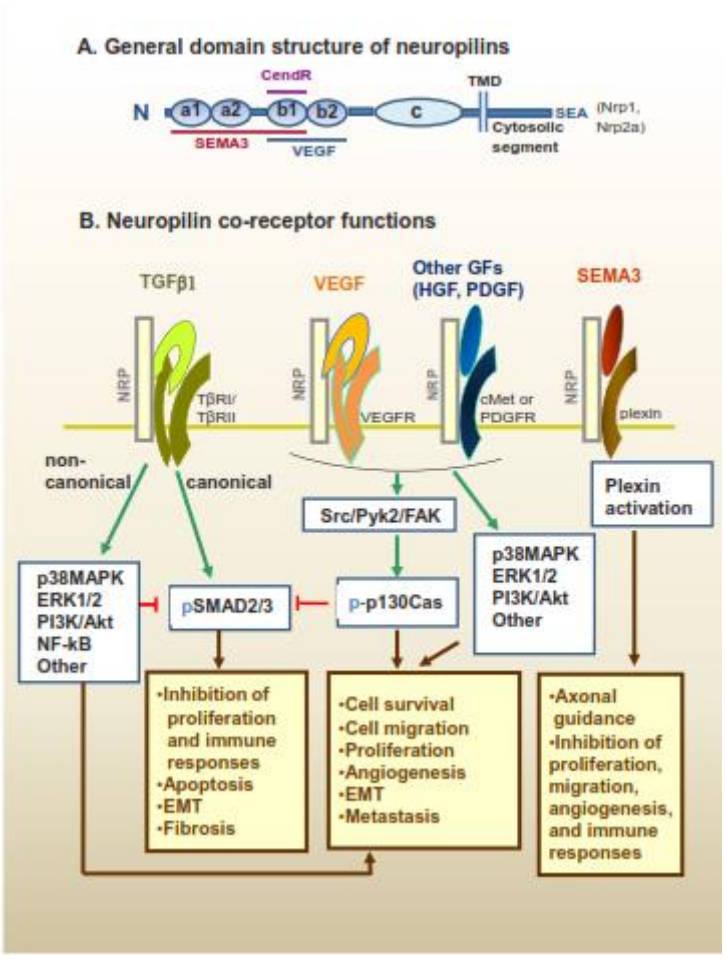
NRPs were initially characterized as receptors for class 3 semaphorins (Sema3) family, functioning in axon guidance. NRPs have also been shown to be critical for VEGF-dependent angiogenesis. Intriguingly, NRP function in these seemingly divergent pathways is critically determined by ligand-mediated cross-talk, which underlies NRP function in both physiological and pathological processes. Multiple general mechanisms have been found to directly contribute to the pleiotropic function of NRP (119) (***Figure 8***). Global NRP1 deletion results in embryonic lethality and severe neuronal and cardiovascular defects (129).

There are two conserved NRP members in vertebrates: NRP1 and NRP2. The NRPs similarly consist of a large extracellular domain, one transmembrane domain and a relatively short cytoplasmic tail. The extracellular domains of both NRPs are divided further into the a1–a2 subdomain, which binds semaphorins; the b1–b2 subdomain, which binds VEGF and supports semaphorin binding to the a1–a2 subdomain; the c subdomain is dispensable for ligand binding but essential for ligand-dependent signaling and mediates NRP dimerization, together with the transmembrane domain (119, 120).

NRP1 was initially identified as a VEGF-A_{165} splice form specific receptor but it has recently been demonstrated that NRP1 can also bind other VEGF-A isoforms, yet uniquely and specifically physically engages VEGF-A_{165} (119). In particular, NRP1 has been shown to be essential in VEGF-A-induced vessel sprouting and branching (120).

NRP1 binds VEGF with an approximately three-fold higher affinity than VEGFR-2. Furthermore, NPR1 additionally binds PlGF and VEGF-B, whereas NRP2 binds PlGF, VEGF-A and VEGF-C. NRPs interact with different receptors: NRP1 is a co-receptor for VEGFR-1 and 2, whereas NRP2 is a co-receptor for VEGFR-3. Soluble NRPs function as natural inhibitors, with sNRP1 acting as a competitive antagonist of $VEGF_{165}$ (130). It remains to be shown whether NRP1 transduces VEGF signals independently of VEGFR-2 or whether it modulates VEGFR-2 signaling (120).

Figure 8 – Neuropilin (Nrp) structure and hypothetical model of interaction with multiple growth factors. *Adapted from (Prud'homme GJ, Glinka Y. 2012.)* (131).



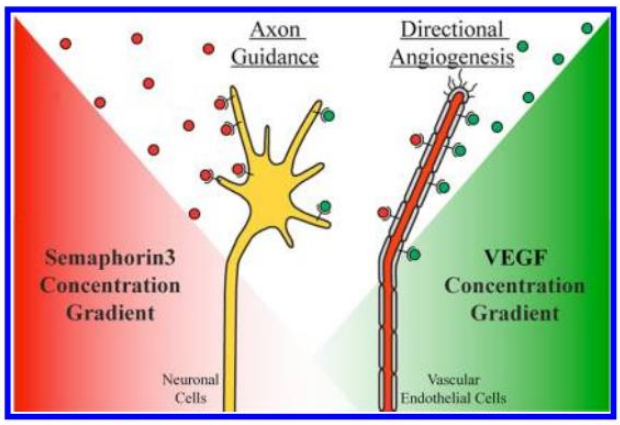
NRP is emerging as a multifaceted vascular regulator. In the vasculature, NRP1 is expressed by ECs, vSMCs, pro-angiogenic tissue macrophages and vascular precursor mesenchymal stem cells, controlling several signaling pathways critical for blood vessel development and function (129).

Besides its role as VEGF co-receptor in ECs, NRP1 has been linked to multiple VEGF-independent vascular signaling pathways, including those driven by fibroblast growth factor, TGF-β and PDGF (132). NRP1 promotes angiogenic sprouting either by amplifying VEGFR-2 signaling in tip cells and by inhibiting TGF-β signaling in stalk cells. On the other hand, NRP1 can promote PDGF signaling and PDGF-dependent migration and proliferation of vSMCs (129).

Semaphorins are grouped into eight classes based on their structural domains; they are characterized by an amino-terminal Sema domain, essential for signaling, and can play a repulsive or attractive role depending on the cell types and biological context. Sema3 family exerts chemorepulsive and anti-angiogenic activity in ECs. All Sema3 proteins (except Sema3E) signal through two major receptor families, Plexins and NRPs, by forming a holoreceptor complex consisting of NRPs as ligand binding, and Plexins as signal transducing subunit (133).

Sema3 can directly compete with VEGF-A for their shared C-terminal arginine binding pocket in the NRP b1 subdomain, inhibiting VEGF-induced ECs proliferation and migration (***Figure 9***).

Figure 9 – Crosstalk between NRP ligands. *Adapted from (Parker MW et al. 2012.)* (119).



Sema3A and F also influence vascular development and angiogenesis by inhibiting integrin-mediated adhesion of ECs to the ECM and enabling the de-adhesion required for vascular remodeling and also by inducing ECs apoptosis (130). The interplay of VEGF and Sema3 is important for diseases associated with angiogenesis (119). Furin processing of Sema3 family members has been demonstrated to be critical for potent and selective engagement of the NRP1 b1 domain, which is critical for competitive binding with VEGF (128).

Sema4A (which also binds to NRP1) and Sema4D were the first semaphorin family members found to be expressed on immune cells. These semaphorins appear to play critical roles in diverse physiological and pathological processes, namely in the pathogenesis of autoimmune diseases (rheumatoid arthritis and systemic lupus erythematosus), which makes them molecules of interest for a potential immunotherapy (134).

The VEGF Family in Systemic Sclerosis

VEGF is strongly overexpressed in the skin and sera of SSc patients, by unclear mechanisms, and its receptors (VEGFR-1 and -2) are upregulated, although non-compensative new vessel formation is observed. Moreover, serum levels of VEGF significantly correlate with the development of SSc digital ulcers (DUs) and higher levels appear to be protective. VEGF shows higher levels mainly at the earliest stages of the disease, which may be related to compensatory mechanisms (135-137).

Prolonged overexpression of VEGF might have deleterious effects on the vascular network, while very high concentrations of pro-angiogenic VEGF isoforms are needed to overcome the inhibitory effects of anti-angiogenic factors (24). Serum levels of VEGF were inversely correlated with NVC capillary density (138) and higher levels of VEGF were reported to be independently associated with “late” NVC pattern and history of DUs (139). High VEGF levels might serve as a surrogate marker of capillary damage in SSc (138).

Patients without DUs were found to have higher serum levels of VEGF than patients with DUs (140, 141) and VEGF appeared to be protective against ischemic

manifestations, when its concentrations exceeded a certain threshold level (140). High serum levels of VEGF did not predict new DUs (142).

The paradox of VEGF overexpression in SSc despite clear evidence of an insufficient angiogenesis has lately gained new insights. VEGF-A pro-angiogenic (VEGF-A_{165}) and anti-angiogenic (VEGF-A_{165}b) isoforms have been uncovered and appear to be generated by alternative splicing mechanisms in the terminal exon of VEGF pre-messenger RNA. A switch from pro-angiogenic to anti-angiogenic VEGF-A isoforms appears to play a crucial role in the defective angiogenic and vascular repair processes that characterize SSc (143-145).

$VEGF_{165}$b appears selectively overexpressed in different cell types of SSc dermis and SSc dermal MVECs express and release elevated levels of $VEGF_{165}$b (143). Additionally, increased plasma levels of the $VEGF_{165}$b are associated with the severity of nailfold capillary loss in SSc (146). A recent study assessed the contribution of SSc platelet-derived factors to the angiogenesis of human dermal MVECs (147). In SSc platelet releasates, $VEGF_{165}$b and $VEGF_{165}$b/VEGF ratio were significantly higher *versus* control subjects. These findings suggest that platelets may be a major source of circulating $VEGF_{165}$b in SSc, following their activation on contact with the injured endothelium (145).

Urokinase-type plasminogen activator receptor (uPAR) has been assumed as another player involved in SSc pathogenesis. Its interaction with VEGFR-2 is determinant for VEGFR-2 pro-angiogenic signaling in ECs and VEGF-A-induced angiogenesis is prevented in uPAR-deficient mice (148, 149). uPAR inactivation/deficiency may significantly contribute to VEGF-A/VEGFR-2 system abnormalities in SSc (145).

In an avian-model of SSc, ischemic skin lesions treated locally with $VEGF_{121}$-fibrin showed clinical improvement *versus* fibrin treated controls, suggesting that cell-demanded release of $VEGF_{121}$ from fibrin matrix induces controlled angiogenesis by differential regulation of VEGFR-1 and VEGFR-2 expression, shifting the balance towards the pro-angiogenic VEGFR-2 (150).

VEGF may also represent a molecular link between vascular involvement and fibrosis (9). VEGF+/+, but not VEGF+/- transgenic mice, spontaneously developed significant skin fibrosis, indicating the profibrotic effect of VEGF in a gene-dosing

manner, highlighting the links between TGFβ and VEGF pathways (151). Moreover, a higher number of microvessels was observed in VEGF+/- transgenic mice than in VEGF+/+ transgenic mice, reinforcing the possible involvement of VEGF in the perturbation of angiogenesis secondary to fibrosis. Indeed, inhibition of VEGF with bevacizumab prevented the bleomycin-induced dermal fibrosis in mice (152), reinforcing a causative role of VEGF in SSc pathogenesis.

The Neuropilin Family and the Semaphorin Family in Systemic Sclerosis

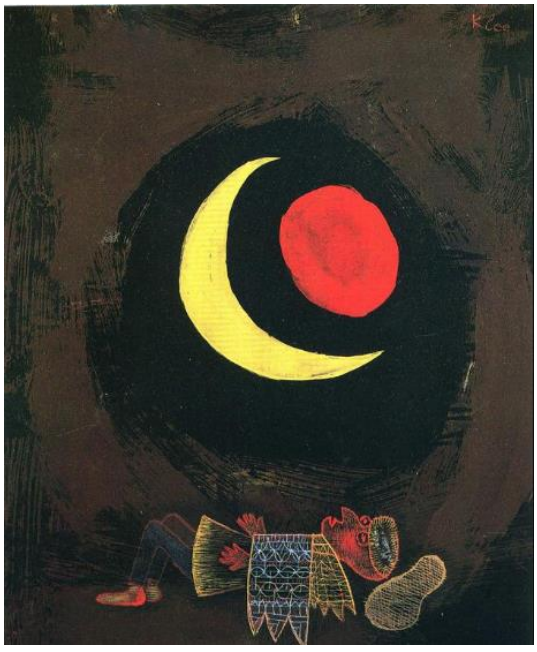
Besides its role in VEGF-mediated angiogenesis, NRP is now known to bind to members of the fibroblast growth factor family, galectin-1, hepatocyte growth factor/scatter factor, anti-thrombin III, prion protein, TGF-β1 and PDGF. It has been found that VEGF and NRP1 directly promote epithelial-mesenchymal transition. A role for NRP in fibrosis has also been proposed, with NRP1 found to regulate TGF-β1 and PDGF signaling (129, 130).

Accumulating evidence indicates that semaphorins and NRPs have distinct biological activities in various phases of immune responses, from immune initiation to terminal inflammatory immune responses (153). They have been studied in systemic lupus erythematosus (154, 155), rheumatoid arthritis (156) and Sjögren syndrome (157) but, to date of project writing, there was no published data about NRP expression in SSc. Considering its link to both VEGF and TGF-β cascades, which are key players in SSc pathogenesis, respectively in vasculopathy and fibrosis, a causative role of NRP1 in SSc was meant to be explored.

More recently, the unique member of Sema3 family that binds and signals directly through Plexin-D1, independently of NRPs (Sema3E) has been investigated in SSc (158). Serum Sema3E levels were found to be higher both in primary RP subjects and SSc patients than in controls, and higher levels correlated with “early” NVC pattern and the absence of digital ulcers. Sema3E expression was strongly increased in SSc dermal microvascular endothelium and cultured SSc-MVECs showed higher levels of phosphorylated Plexin-D1 and Sema3E expression than healthy MVECs. Stimulation with SSc sera increased phosphorylated Plexin-D1 and Sema3E in healthy microvascular endothelial cells (H-MVECs) and the addition of Sema3E-binding

Plexin-D1 soluble peptide significantly attenuated the antiangiogenic effect of SSc sera on H-MVECs.

III. AIMS



Paul Klee. *Strong Dream* (1929).

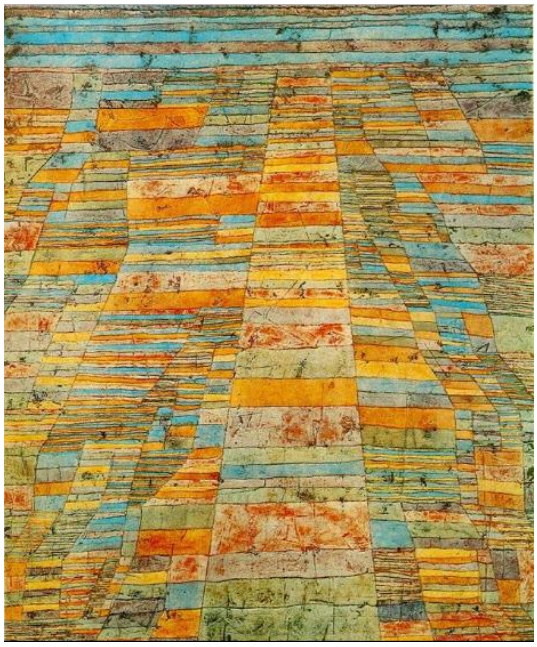
According to the research gap identified in the Introduction, the **meaning** of this project has been to:

- Explore further explanations for deregulated angiogenesis in SSc;
- Lift the curtain of the neurovascular interactions in SSc;
- Discover the “vascular environment” in patients with VEDOSS;
- Improve knowledge on the molecular and cellular pathways that contribute to SSc vasculopathy, so that, in the future, specific therapies for reversing vascular injury or direct normal vascular repair can be developed.

The **specific objectives** of this thesis were to:

- Study the possible involvement of the axis NRP1/Sema3A in the pathogenesis of SSc, by investigating whether the levels of NRP1 and Sema3A could be altered in the circulation, skin and ECs of patients with VEDOSS and SSc, as well as the mechanism explaining the deregulated expression of these molecules and their possible contribution to the disturbed angiogenesis of SSc;
- Investigate whether patients with VEDOSS may already present circulating markers and *in vitro* signs of microvascular dysfunction.

IV. METHODS



Paul Klee. *Highway and byways* (1929).

1. Chronogram of the Activities

The chronogram of the activities developed during the PhD Program is presented in ***Table 4***.

Table 4 – Chronogram of PhD Activities.

<table>
<tr><th></th><th>Jan</th><th>Feb</th><th>Mar</th><th>Apr</th><th>May</th><th>Jun</th><th>Jul</th><th>Aug</th><th>Sep</th><th>Oct</th><th>Nov</th><th>Dec</th></tr>
<tr><td>2011</td><td colspan="9"></td><td colspan="3">PhD Lectures</td></tr>
<tr><td>2012</td><td colspan="8">PhD Lectures</td><td colspan="4">Project writing/ submission...</td></tr>
<tr><td>2013</td><td colspan="3">…Ethical approval</td><td colspan="6">Applying to Grants
Requiring Residency interruption</td><td colspan="3">Working in…
First Article Writing</td></tr>
<tr><td>2014</td><td colspan="3">…Florence</td><td>Interview
Meeting</td><td colspan="8">Working in Porto
Second Article writing</td></tr>
<tr><td>2015</td><td colspan="12">Working in Porto
Fourth Article writing</td></tr>
<tr><td>2016</td><td colspan="5">Third Article writing</td><td colspan="5"></td><td>Thesis…</td></tr>
<tr><td>2017</td><td colspan="2">…writing</td><td colspan="10"></td></tr>
</table>

2. Study Design and Participants

This was a cross-sectional study, recruiting patients followed regularly at two centers:

I. Department of Experimental and Clinical Medicine, Division of Rheumatology, Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy;
II. Autoimmunity Outpatient Clinic of the Department of Internal Medicine, Centro Hospitalar de São João (CHSJ), Porto, Portugal.

Table 5 summarizes the inclusion and exclusion criteria of the subjects, according to the three groups of participants: 1) SSc patients, 2) VEDOSS patients and 3) Healthy controls.

Table 5 – Inclusion and exclusion criteria for study participants.

<table>
<tr><th></th><th>SSc patients</th><th>VEDOSS patients</th><th>Healthy controls</th></tr>
<tr><td rowspan="4">Inclusion criteria</td><td>Presence of both:
Score ≥9 in 2013 ACR/EULAR classification criteria for SSc
Well-defined clinical subset (limited cutaneous/diffuse cutaneous SSc)</td><td>Presence of all three:
RP
Puffy fingers/ sclerodactyly
Positive ANA
And ≥1 of:
NVC with SSc pattern
Positive SSc-specific antibodies (anticentromere, anti-topoisomerase I, anti-RNA polymerase III)
Plus
Score <9 in 2013 ACR/EULAR classification criteria for SSc</td><td>Age, race and gender-matched subjects</td></tr>
<tr><td colspan="3">Written informed consent for chart review and for performing blood tests (and skin biopsy when appropriate)</td></tr>
<tr><td colspan="2">Clinical information available for chart review</td><td></td></tr>
<tr><td colspan="3"></td></tr>
<tr><td rowspan="3">Exclusion criteria</td><td colspan="2">Presence of another concomitant autoimmune disease</td><td>Presence of any autoimmune disease
Primary RP</td></tr>
<tr><td></td><td>Any internal organ involvement due to SSc</td><td></td></tr>
<tr><td colspan="3">Inability to give written informed consent</td></tr>
</table>

ACR – American College of Rheumatology; ANA – antinuclear antibodies; EULAR – European League Against Rheumatism; NVC – nailfold videocapillaroscopy; RP – Raynaud's phenomenon; SSc – systemic sclerosis; VEDOSS – very early diagnosis of systemic sclerosis.

The variables of clinical information obtained by chart review are presented in the ***Appendix*** section of this document [Doc. 2].

The global characterization of patients included is presented in ***Table 6***.

In SSc group, 49 patients were from Florence and six from Porto. In VEDOSS group, all the patients included were from Florence. We included 55 age-matched and sex-matched healthy individuals (51 women; median age 52 years, range 29 to 70 years).

For late-outgrowth endothelial progenitor cell-derived ECs, peripheral blood was also collected from 15 SSc patients (13 women; n=9 with lcSSc and n=6 with dcSSc; median age 60 years, range 42 to 78 years) and eight healthy individuals (all women; median age 55 years, range 30 to 65 years), at Cochin Hospital, Paris, France.

Table 6 – Demographic and clinical characteristics of the patients with SSc and VEDOSS included for collection of serum samples.

	***SSc patients* (n=55)**	***VEDOSS patients* (n=25)**
Demographic		
Age, years, median (range)	64 (37-81)	50 (19-77)
Gender		
Male	6 (11)	4 (16)
Female	49 (89)	21 (84)
Race		
Caucasian	54 (98)	25
Asian	1 (2)	-
Disease **duration**, years, median (range)[a]	10 (1 to 31)	1 (0 to 8)
Clinical manifestations *(ever before)*		
Disease subset		
lcSSc	38 (69)	-
dcSSc	17 (31)	-
Puffy fingers	47 (85)	11 (44)
Sclerodactyly	50 (91)	-
Digital ulcers	32 (58)	1 (4)
Digital tip pitting scars	16 (29)	1 (4)
Telangiectasia	18 (32)	-
Arthritis	10 (18)	1 (4)
Pulmonary arterial hypertension[b]	5 (9)	-
Interstitial lung disease[c]	33 (60)	-
Gastrointestinal involvement[d]	36 (65)	6 (24)
Cardiac involvement[e]	13 (24)	-
Renal involvement[f]	19 (35)	-
Clinical manifestations *(at the time of blood withdrawal)*		
Digital ulcers	6 (11)	-
Modified Rodnan Skin Score, median (range)	6 (0-35)	0 (0-4)
Autoantibodies		
Antinuclear	55 (100)	25
Anticentromere	32 (58)	11 (44)
Anti-topoisomerase I	17 (31)	6 (56)
Anti-RNA polymerase III	-	-
Anti-PM/Scl	2 (36)	1 (4)
Other laboratory exams		
Erythrocyte sedimentation rate, average±SD	22±17	12±10
Hypocomplementemia (C3 or C4)	1 (2)	1 (4)
Elevated creatin kinase	3 (5)	1 (4)
Nailfold videocapillaroscopy pattern		
Normal	4 (8)	7 (28)
“Early”	11 (18)	13 (52)
“Active”	23 (47)	5 (20)
“Late”	17 (27)	0
2013 ACR/EULAR score, median (range)	14 (10-28)	7 (7-8)

Table 6 – (*cont.*).

	***SSc patients* (n=55)**	***VEDOSS patients* (n=25)**
Therapy		
Corticosteroids, n *(average current daily dose of prednisolone-equivalent, mg)*	6 (4)	-
Angiotensin-converting enzyme inhibitors/ ARB	15 (27)	-
Calcium channel blockers	13 (24)	5 (20)
Prostacyclin analogs *(ever before)*	42 (76)	1 (4)
Endothelin antagonists *(ever before)*	8 (15)	-
Phosphodiesterase inhibitors *(ever before)*	9 (16)	2 (8)
Immunosuppressants *(ever before)*	39 (71)	-

Except where indicated otherwise, values are the number (%) of subjects.
[a]Disease duration was calculated since the first non-Raynaud's symptom of SSc.
[b]Diagnosed by right-sided heart catheterization according to standard definitions.
[c]Determined by thoracic high-resolution computer tomography.
[d]Evidence of esophageal hypomotility, incompetence of the lower esophageal sphincter, gastric antral vascular ectasia, pseudo-obstruction, bacterial small bowel overgrowth with malabsorption, fecal incontinence.
[e]Pericardial involvement, myocardial fibrosis, diastolic dysfunction, conduction disturbances, arrhythmias non-attributable to other causes.
[f]History of SSc renal crisis and/or evidence of microalbuminuria and/or elevation in plasma creatinine non-attributable to other causes.
ACR – American College of Rheumatology; ARB – angiotensin II receptor blockers; dcSSc – diffuse cutaneous SSc; EULAR – European League Against Rheumatism; lcSSc – limited cutaneous SSc.

3. Data and Samples Collecting

Patients were provided written information on study design and goals and, when intended, signed informed consent. Interview, physical exam with NVC and chart review were performed at the evaluation center. Blood samples (and skin biopsies) were collected and stored, as specified in ***Results*** section. Before blood sampling, patients were washed out for ten days from oral vasodilating drugs and for two months from intravenous prostanoids.

4. Laboratory Assays

The following assays were performed:

- Assessment of **serum circulating levels** of pan-VEGF, soluble NRP1 (sNRP1) and Sema3A in patients with VEDOSS, patients with SSc and healthy controls, by sandwich enzyme-linked immunosorbent assay (ELISA).

- Assessment of **protein expression** of NRP1 and Sema3A in skin biopsies from SSc patients and healthy donors, by immunofluorescence and Western blot.

- Measurement of the **expression** of NRP1 in H-MVECs, at basal condition and after stimulation with recombinant human VEGF-A_{165}, VEDOSS sera, SSc sera and healthy controls sera, by Western blot. NRP1 protein expression was also evaluated at basal level in dermal MVECs from SSc patients and in late-outgrowth endothelial progenitor cell(EPC)-derived ECs from SSc patients and healthy controls, by Western blot.

- Evaluation of Fli1 **protein expression** in H-MVECs and late-outgrowth EPC-derived ECs from patients with SSc and healthy controls, by Western blot. Assessment of the **effect of** Fli1 **gene silencing** on mRNA levels of the NRP1 gene in H-MVECs transfected with Fli1 siRNA or non-silencing scrambled RNA, by quantitative real-time protein chain reaction (PCR). Evaluation of the binding of Fli1 to NRP1 gene promoter by chromatin immunoprecipitation.

- Assessment of *in vitro* **capillary morphogenesis** in dermal MVECs from SSc patients and in H-MVECS incubated with sera from VEDOSS patients, SSc patients and healthy controls, by Matrigel assay.

- Assessment of the ability of **cell proliferation** in H-MVECs stimulated with VEDOSS sera, SSc sera and healthy controls sera, by 5'-bromodeoxyuridine (BrdU) assay.

- Evaluation of the capacity of **cell migration** to an injured spot in H-MVECs stimulated with VEDOSS sera, SSc sera and healthy controls sera, by wound-healing assay.

A comprehensive description of these assays is included in the Methods section of Second Paper and Third Paper.

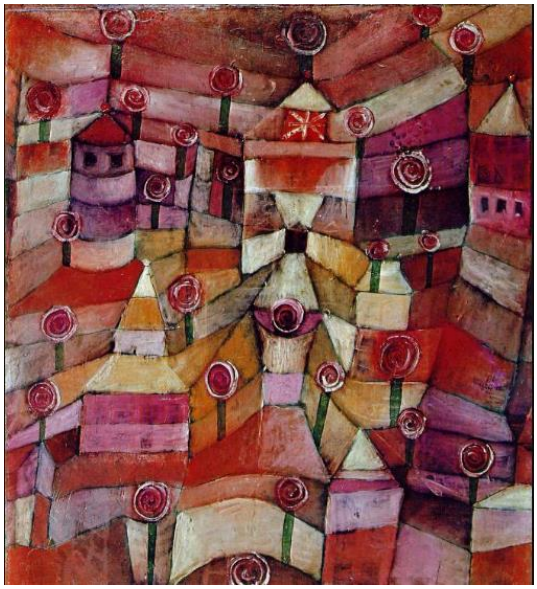
5. Statistical Analysis

Clinical correlations and statistical analysis were performed with Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, IL, USA).

6. Ethical Approval

The study was approved by the local institutional review board of AOUC, Florence, Italy (AOUC 69/13), as well as by the Health Ethical Committee of CHSJ, Porto, Portugal (CHSJ 84/13) – the approval letters are accessible in the ***Appendix*** section of this document [Doc. 3 and 4]. The study was also approved by the local institutional review board at the Cochin Hospital, Paris, France. All subjects provided written informed consent.

V. RESULTS



Paul Klee. *Rose garden* (1920).

1. Second Paper

EXTENDED REPORT

Decreased expression of neuropilin-1 as a novel key factor contributing to peripheral microvasculopathy and defective angiogenesis in systemic sclerosis

Eloisa Romano,[1] Inês Chora,[1,2] Mirko Manetti,[3] Celestina Mazzotta,[1] Irene Rosa,[1,3] Silvia Bellando-Randone,[1] Jelena Blagojevic,[1] Raquel Soares,[4] Jerôme Avouac,[5] Yannick Allanore,[5] Lidia Ibba-Manneschi,[3] Marco Matucci-Cerinic,[1] Serena Guiducci[1]

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ABSTRACT

Objectives In systemic sclerosis (SSc), vascular involvement is characterised by vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR) system disturbances. Neuropilin-1 (NRP1), a receptor for both class-3 semaphorins (Sema3s) and VEGF-A, is required for optimal VEGF-A/VEGFR-2 signalling. Here, we investigated the possible involvement of Sema3A/NRP1 axis in SSc.

Methods Circulating Sema3A and soluble NRP1 (sNRP1) were measured in patients with SSc and controls. NRP1 and Sema3A expression in skin biopsies was evaluated by immunofluorescence and western blotting. NRP1 expression was assessed in SSc and healthy dermal microvascular endothelial cells (SSc-MVECs and H-MVECs), and in SSc and control endothelial progenitor cell (EPC)-derived endothelial cells (ECs). The possible impact of transcription factor Friend leukaemia integration 1 (Fli1) deficiency on endothelial NRP1 expression was investigated by gene silencing. The binding of Fli1 to NRP1 gene promoter was evaluated using chromatin immunoprecipitation. Capillary morphogenesis was performed on Matrigel.

Results Decreased sNRP1 levels in SSc were associated with active and late nailfold videocapillaroscopy patterns and digital ulcers. No difference in Sema3A was found between patients and controls. NRP1 was significantly decreased in SSc-MVECs both ex vivo and in vitro. NRP1 and Fli1 significantly decreased in H-MVECs challenged with SSc sera, while they were not different in SSc and control EPC-derived ECs. Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs. NRP1 gene silencing in H-MVECs resulted in a significantly impaired angiogenic capacity comparable to that of cells treated with SSc sera.

Conclusion In SSc, NRP1 deficiency may be an additional factor in the perturbed VEGF-A/VEGFR-2 system contributing to peripheral microvasculopathy and defective angiogenesis.

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is a life-threatening connective tissue disorder of unknown aetiology, characterised by widespread vascular injury and dysfunction, impaired angiogenesis, immune dysregulation and progressive fibrosis of the skin and internal organs.[1 2] The dysregulation of vascular tone control, clinically evident as Raynaud's phenomenon, and microcirculatory abnormalities paralleled by nailfold capillaroscopic changes are the earliest clinical manifestations of SSc and may precede skin and visceral involvement by months or years.[1–3] The whole process is characterised by an uncontrolled regeneration of the microvasculature and subsequent loss of microvessels, due to defects in both vascular repair and expected increase in new vessel growth (angiogenesis), leading to severe peripheral ischaemic manifestations, such as digital ulcers and gangrene.[2 3]

Recent studies have highlighted the anatomical and structural similarities between blood vessels and nerves.[4] The two networks are often aligned, with nerve fibres and blood vessels following parallel routes. Furthermore, both systems require precise control over their guidance and growth. Several molecules with attractive and repulsive properties have been found to modulate the guidance both of nerves and blood vessels.[4] These include the neuropilin (NRP) receptors and their semaphorin (Sema) ligands, as well as netrins, slits and their receptors.[4] Among these, NRP1 was initially described as an axonally expressed receptor for secreted class-3 Semas (Sema3s), a family of soluble molecules which modulate the development of the nervous and vascular systems.[5 6] NRP1 also serves as specific vascular endothelial growth factor-A (VEGF-A) co-receptor on endothelial cells (ECs) and regulates VEGF receptor (VEGFR) signalling, leading to enhanced migration[7] and survival of ECs in vitro.[8 9] Furthermore, NRP1 has been implicated in VEGFR-2-mediated endothelial permeability[10] and in VEGF-A-induced three-dimensional EC biology, such as vessel sprouting and branching.[11] The absence of functional NRP1 in mice results in embryonic death due to impaired heart and blood vessel development, thus suggesting that this receptor plays a central regulatory role during developmental angiogenesis.[4] The exact molecular mechanisms by which NRP1 modulates VEGF-A biology remain to be elucidated. It has, however, been shown that NRP1 potentiates the VEGF-A/VEGFR-2 signalling pathways implicated in the migratory response of ECs.[12]

The evidence that NRP1 functions as a receptor for both VEGF-A and Sema3s suggests that the latter may also play a role in the modulation of angiogenesis. In particular, it has been reported that Sema3A acts as an antiangiogenic molecule impairing EC adhesion,

migration and survival in vitro[13–15] and regulates tumour-induced angiogenesis in vivo.[16] Moreover, Sema3A null mice exhibit defects in blood vessel reshaping.[15 17 18] The molecular mechanisms underlying the antiangiogenic effects of Sema3A are complex. Hence, it was initially suggested that Sema3A competes with VEGF-A for NRP1 binding, thus inhibiting VEGF-A-induced angiogenesis. However, recent reports have also shown that Sema3A increases vascular permeability, inhibits EC proliferation and induces apoptosis even in the absence of VEGF-A, suggesting that Sema3A may activate its own signalling pathways.[19 20]

On these bases, we hypothesised that the Sema3A/NRP1 axis might play a role in the pathogenesis of SSc-related microvascular abnormalities. Therefore, the aim of the present study was to investigate whether the levels of Sema3A and NRP1 could be altered in the circulation, skin and ECs of patients with SSc, as well as the mechanism explaining the dysregulated expression of these molecules and their possible contribution to the disturbed angiogenesis of SSc.

METHODS

An extended methods section is provided in the online supplementary material.

Patients, controls, serum samples and skin biopsies

Serum samples were obtained from 49 patients with SSc[1] (45 women and 4 men; median age 64 years, range 37–80 years, and median disease duration 10 years, range 2–31 years) classified as limited cutaneous SSc (lcSSc; n=32) or diffuse cutaneous SSc (dcSSc; n=17),[21] and from 39 age-matched and sex-matched healthy individuals. All patients were clinically assessed as described elsewhere.[3 22 23] Clinicodemographical characteristics of patients with SSc used for collection of serum samples are shown in online supplementary table S1. Full-thickness skin biopsies were obtained from the clinically involved skin of one-third of the distal forearm of 18 patients with SSc (15 women, 3 men; median age 48.5 years, range 29–73 years, and median disease duration 7.2 years, range 1–18 years). Skin samples from the same forearm region of 11 age-matched and sex-matched healthy donors were used as controls. Each skin biopsy was divided into two specimens and processed for immunohistochemistry and biomolecular analysis as described elsewhere.[23] The study was approved by the local institutional review board at the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written informed consent.

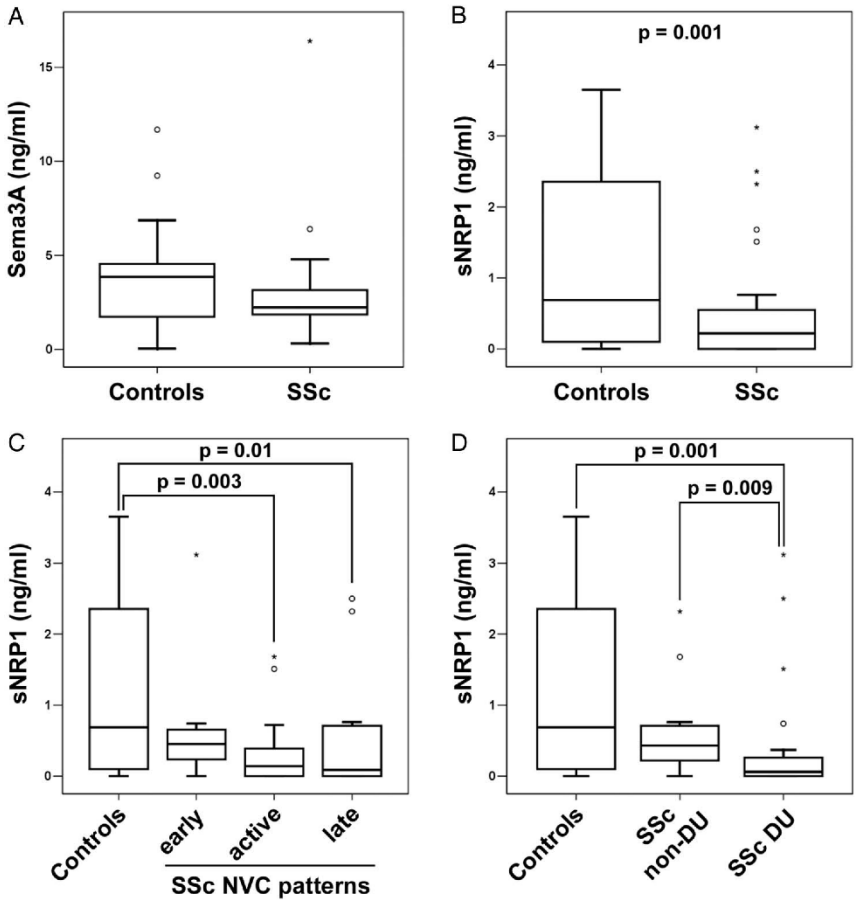

Figure 1 Serum levels of semaphorin3A (Sema3A) and soluble neuropilin-1 (sNRP1) determined by colorimetric sandwich ELISA. (A) Serum Sema3A levels in healthy controls and patients with systemic sclerosis (SSc). (B) Serum sNRP1 levels in healthy controls and patients with SSc. (C) Serum sNRP1 levels in healthy controls and patients with SSc according to nailfold videocapillaroscopy (NVC) pattern (early, active and late). (D) Serum sNRP1 levels in healthy controls and patients with SSc according to the presence/absence of digital ulcers (DU). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers, and asterisks indicate the extreme values. Mann–Whitney U test was used for statistical analysis.

Isolation, culture and stimulation of dermal microvascular ECs

Dermal microvascular ECs (MVECs) were isolated from biopsies of the involved forearm skin from five patients with dcSSc and from five healthy subjects, as described elsewhere.[3 23] MVECs from healthy subjects (H-MVECs) and patients with SSc (SSc-MVECs) were used between the third and seventh passages in culture. For stimulation experiments, H-MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB 131 medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 2% fetal bovine serum (FBS). Medium was removed and cells were incubated with 2% FBS-MCDB medium containing recombinant human VEGF-A165 (10 ng/mL; R&D Systems, Minneapolis, Minnesota, USA), or 10% serum from patients with SSc (n=5) and healthy subjects (n=5) for 24 h.

Late-outgrowth peripheral blood endothelial progenitor cell-derived ECs

Late-outgrowth endothelial progenitor cell (EPC)-derived ECs were obtained from the peripheral blood of 15 patients with SSc (13 women and 2 men; n=9 with lcSSc and n=6 with dcSSc; median age 60 years, range 42–78 years) and eight healthy individuals (all women; median age 55 years, range 30–65 years), as described elsewhere.[3 24 25]

ELISA for serum Sema3A and soluble NRP1

The levels of Sema3A and soluble NRP1 (sNRP1) in serum samples were measured by commercial quantitative colorimetric sandwich ELISA (catalogue number ABIN481720 and ABIN415191, respectively; Antibodies-Online, Atlanta, Georgia, USA), according to the manufacturer's protocol. Each sample was measured in duplicate.

Immunofluorescence

Immunofluorescence on paraffin-embedded skin sections was performed as previously described.[3] For primary and secondary antibodies, refer to the online supplementary material.

Western blotting

Proteins were extracted from skin biopsies, dermal MVECs and late-outgrowth peripheral blood EPC-derived ECs as described elsewhere.[3 25 26] Western blotting was carried out according to previously published protocols.[3] For primary antibodies, refer to the online supplementary material.

Gene silencing of Friend leukaemia integration 1 and NRP1

MVECs were seeded shortly before transfection. The cells were transfected with 10 nM of Friend leukaemia integration 1 (Fli1) small interfering RNA (siRNA), 10 nM of NRP1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology, Dallas, Texas, USA) using HiPerfect transfection reagent (Qiagen, Milan, Italy) for 72 h.

RNA purification, cDNA synthesis and quantitative real-time PCR

Total RNA isolation from MVECs, first strand cDNA synthesis and mRNA quantification by SYBR Green real-time PCR were performed as reported elsewhere.[27] For predesigned oligonucleotide primer pairs obtained from Qiagen, refer to the online supplementary material.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out as previously described[28] using a rabbit polyclonal anti-Fli1 antibody (catalogue number ab15289, Abcam, Cambridge, UK). Putative Fli1 transcription factor binding site was predicted by Tfsitescan. The primers were as follows: NRP1 Forward, 5′-CTAGGGGTGCAGAGCGAG-3′; NRP1 Reverse, 5′-GAAGG AAGGCGCTGGGAG-3′.

In vitro capillary morphogenesis assay

In vitro capillary morphogenesis assay on Matrigel was performed according to previously published protocols,[27] as detailed in the online supplementary material.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, V.20.0 (SPSS, Chicago, Illinois, USA). Data are expressed as mean±SD or

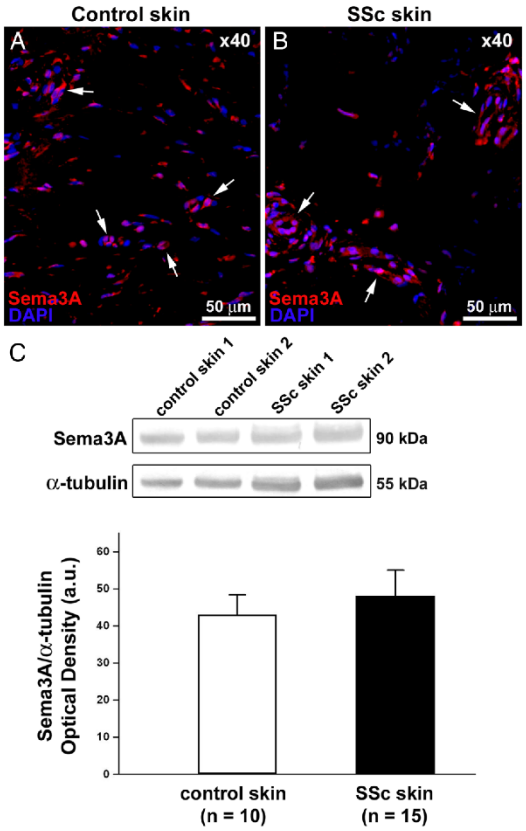

Figure 2 Expression of semaphorin3A (Sema3A) in skin biopsies. (A and B) Representative microphotographs of skin sections from (A) healthy controls (n=11) and (B) patients with systemic sclerosis (SSc) (n=18) immunostained for Sema3A (red) and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. Original magnification: ×40. Scale bar=50 μm. (C) Western blotting of total protein extracts from the skin of healthy subjects (n=10) and patients with SSc (n=15). A protein band with the expected molecular weight of 90 kDa was detected with the anti-Sema3A antibody. Representative immunoblots are shown. The densitometric analysis of the bands normalised to α-tubulin is reported in the histograms. Data are mean±SD of optical density in arbitrary units (a.u.).

median and IQR. The Student's t test and non-parametric Mann–Whitney U test were used where appropriate for statistical evaluation of the differences between two independent groups. A p value of <0.05 was considered statistically significant.

RESULTS

Serum Sema3A and sNRP1 levels in SSc

No significant differences in serum levels of Sema3A were detected between patients with SSc (median 2.22 ng/mL, IQR 1.84–3.17 ng/mL) and healthy controls (median 3.86 ng/mL, IQR 1.64–4.73 ng/mL) (figure 1A). Circulating sNRP1 levels were significantly reduced in patients with SSc (median 0.22 ng/mL, IQR 0.0–0.6 ng/mL) compared with healthy individuals (median 0.69 ng/mL, IQR 0.0–2.5 ng/mL; p=0.001) (figure 1B). Next, we evaluated the possible correlation of serum sNRP1 levels with the nailfold videocapillaroscopy (NVC) pattern as a measure of peripheral microvascular involvement. sNRP1 levels were significantly decreased in patients with SSc having active (median 0.14 ng/mL, IQR 0.0–0.4 ng/mL) or late (median 0.09 ng/mL, IQR 0.0–0.72 ng/mL) NVC patterns than in controls (p=0.003 and p=0.01, respectively) (figure 1C). Conversely, no difference in serum sNRP1 was found between patients with SSc having early NVC pattern (median 0.45 ng/mL, IQR 0.22–0.66 ng/mL) and healthy controls (figure 1C). Moreover, sNRP1 levels were significantly decreased in patients with SSc having digital ulcers (median 0.06 ng/mL, IQR 0.0–0.27 ng/mL) compared both with patients without digital ulcers (median 0.43 ng/mL, IQR 0.17–0.71 ng/mL; p=0.009) and controls (p=0.001) (figure 1D). No significant association was found with other clinicodemographical and laboratory parameters or with clinical SSc subset.

Decreased expression of NRP1 in SSc dermal ECs ex vivo and in vitro

The expression of Sema3A and NRP1 protein in forearm skin biopsies from patients with SSc and controls was investigated by immunofluorescence and western blot. No significant differences in Sema3A expression were detected between SSc and control skin (figure 2A–C). On the contrary, NRP1 expression was decreased in clinically affected skin biopsies from patients with SSc compared with healthy skin, in particular in dermal ECs and perivascular stromal cells (figure 3A–F). The localisation of NRP1 staining in vascular ECs was confirmed by NRP1/CD31 double immunofluorescence staining (figure 3C, F). Moreover, western blot analysis confirmed that NRP1 protein expression levels were significantly reduced in SSc skin with respect to control skin (p<0.001) (figure 3G, H).

Western blot analysis on cultured dermal MVECs revealed that NRP1 protein expression levels were significantly reduced in SSc-MVECs compared with H-MVECs (p<0.005) (figure 4A). Moreover, NRP1 expression in H-MVECs significantly increased after treatment with healthy sera compared with basal condition, while it decreased after challenging with SSc sera (both p<0.005 vs basal H-MVECs). As expected, stimulation with recombinant human VEGF-A165 strongly upregulated NRP1 expression in H-MVECs (p<0.005 vs basal H-MVECs) (figure 4A). On the contrary, no obvious differences in NRP1 protein levels could be found between late-outgrowth EPC-derived ECs from patients with SSc and healthy controls (figure 4B).

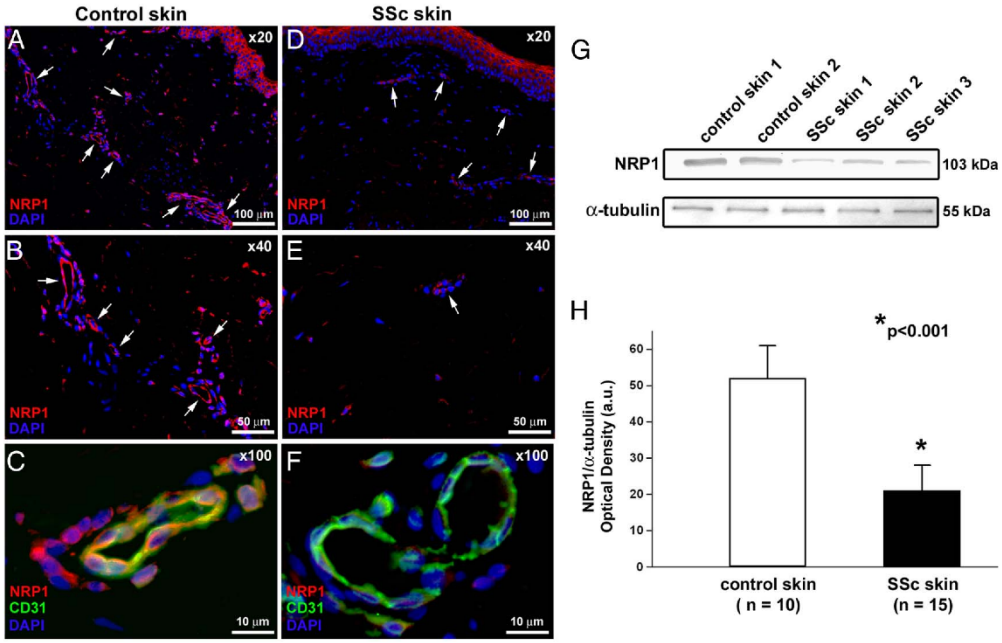

Figure 3 Expression of neuropilin-1 (NRP1) in skin biopsies. (A, B, D and E) Representative microphotographs of skin sections from (A and B) healthy controls (n=11) and (D and E) patients with systemic sclerosis (SSc) (n=18) immunostained for NRP1 (red) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. (C and F) Representative microphotographs of skin sections from healthy controls (C) and patients with SSc (F) double immunostained for NRP1 (red) and the pan-endothelial cell marker CD31 (green) and counterstained with DAPI (blue). Original magnification: ×20 (A and D), ×40 (B and E), ×100 (C and F). Scale bar=100 µm (A and D), 50 µm (B and E), 10 µm (C and F). (G and H) Western blotting of total protein extracts from the skin of healthy subjects (n=10) and patients with SSc (n=15). A protein band with the expected molecular weight of 103 kDa was detected with the anti-NRP1 antibody. Representative immunoblots are shown. The densitometric analysis of the bands normalised to α-tubulin is reported in the histograms. Data are mean±SD of optical density in arbitrary units (a.u.).

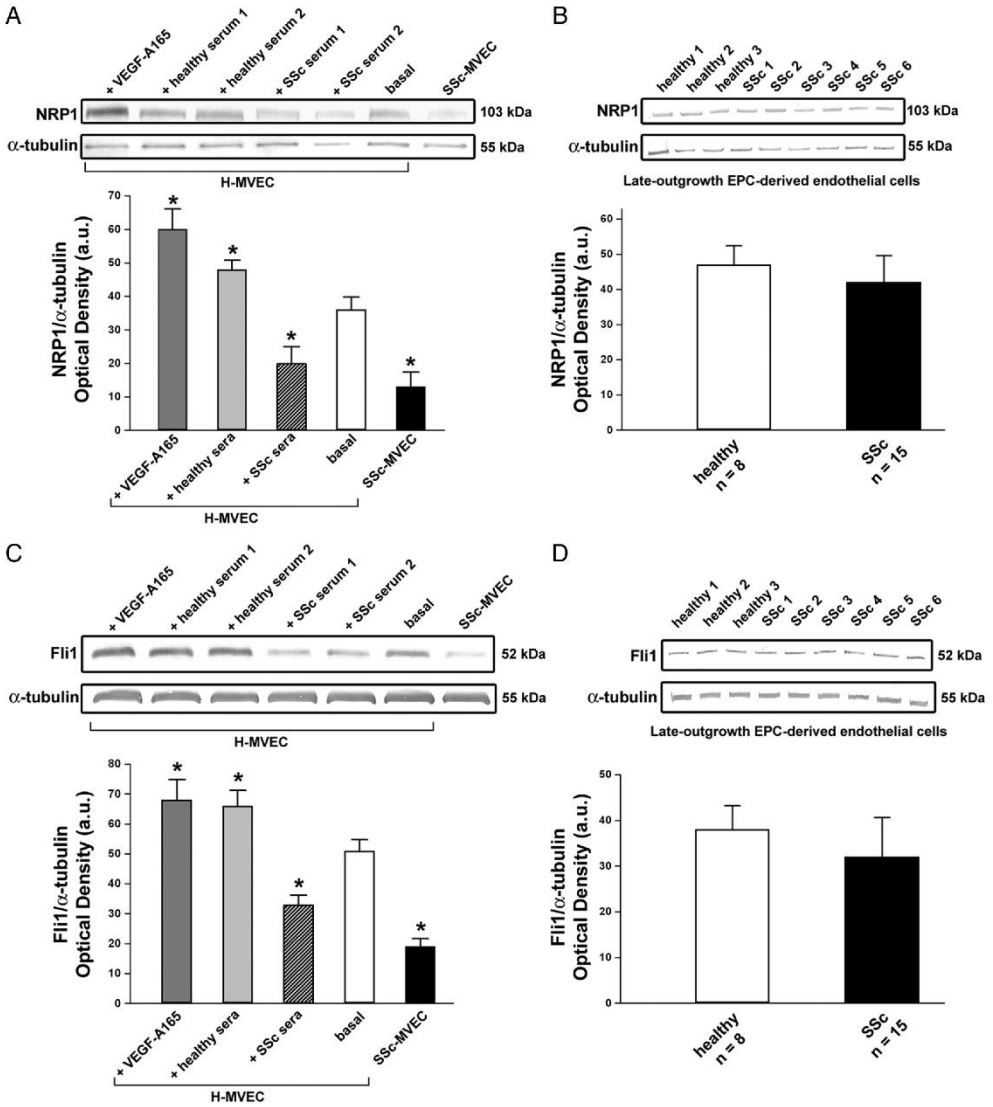

Figure 4 (A–D) Expression of neuropilin-1 (NRP1) and Friend leukaemia integration 1 (Fli1) in dermal microvascular endothelial cells (MVECs) and late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived endothelial cells. (A and C) Western blotting of total protein extracts from healthy MVECs (H-MVECs) at basal condition and treated with recombinant human vascular endothelial growth factor-A165 (VEGF-A165) or 10% serum from patients with systemic sclerosis (SSc) (n=5) and healthy subjects (n=5) for 24 h, and from basal SSc-MVECs assayed with anti-NRP1 (A) and anti-Fli1 (C) antibodies. Representative immunoblots are shown. The densitometric analysis of the bands normalised to α-tubulin is reported in the histograms. Data are mean±SD of optical density in arbitrary units (a.u.). Student's t test was used for statistical analysis; *p<0.005 versus basal H-MVECs. Results are representative of three independent experiments performed with each one of the five H-MVEC and five SSc-MVEC lines. (B and D) Western blotting of protein lysates from controls (n=8) and patients with SSc (n=15) late-outgrowth EPC-derived endothelial cells assayed with anti-NRP1 (B) and anti-Fli1 (D) antibodies. Representative immunoblots are shown. The densitometric analysis of the bands normalised to α-tubulin is reported in the histograms. Data are mean±SD of optical density in arbitrary units (a.u.). (E) mRNA levels of the Fli1 and NRP1 genes in H-MVECs transfected with Fli1 siRNA or non-silencing scrambled RNA (SCR) were measured by quantitative real-time PCR and normalised to expression levels of the 18S ribosomal RNA gene. The relative values compared with SCR are expressed as mean±SD of three independent experiments. Statistical analysis was carried out with Student's t test; *p<0.01 versus SCR. (F) Chromatin was isolated from H-MVECs and immunoprecipitation was conducted with rabbit anti-Fli1 antibody or rabbit IgG. To exclude exogenous DNA contamination, negative controls were obtained by omitting the cell preparation in some specimens. PCR amplification was carried out using NRP1 promoter-specific primers. One representative of three independent experiments is shown.

Fli1 deficiency contributes to the downregulation of NRP1 gene in SSc-MVECs

We next examined the potential mechanism by which NRP1 expression is downregulated in dermal SSc microvessels. As the expression of the transcription factor Fli1 is markedly downregulated at least partially via an epigenetic mechanism in SSc dermal ECs, and experimental endothelial Fli1 deficiency reproduces the histopathological and functional abnormalities characteristic of SSc vasculopathy,[28–30] we hypothesised that endothelial Fli1 deficiency could inhibit the expression of NRP1 in SSc-MVECs.

First, we analysed Fli1 protein expression in cultured dermal MVECs and late-outgrowth EPC-derived ECs from patients

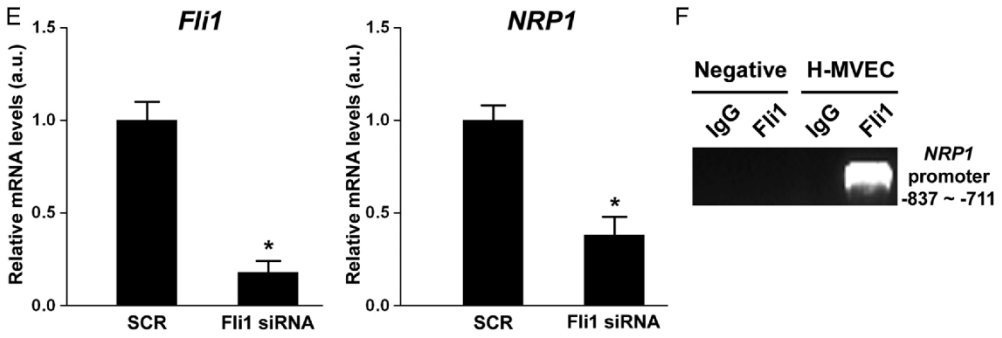

Figure 4 Continued.

with SSc and controls (figure 4C, D). As shown in figure 4C, Fli1 protein expression in H-MVECs closely paralleled that of NRP1 in the different experimental conditions assayed and was strongly downregulated in SSc-MVECs. Similarly to what was observed for NRP1 expression, Fli1 protein levels did not differ between SSc and control late-outgrowth EPC-derived ECs (figure 4D).

Moreover, we examined the effect of Fli1 gene silencing on mRNA levels of the NRP1 gene in H-MVECs. As displayed in figure 4E, gene silencing of Fli1 significantly suppressed the mRNA expression levels of the NRP1 gene in H-MVECs ($p<0.01$). In addition, ChIP analysis revealed that Fli1 occupied the promoter region of the NRP1 gene in H-MVECs (figure 4F). These results indicate that Fli1 directly targets the NRP1 gene promoter and is required for homeostatic NRP1 expression in ECs.

NRP1 deficiency contributes to the impaired angiogenesis of SSc-MVECs

To verify whether endothelial NRP1 deficiency has a role in the modulation of angiogenesis, we carried out in vitro capillary morphogenesis on Matrigel matrix. Consistent with previous findings,[26] capillary morphogenesis was significantly impaired in SSc-MVECs compared with H-MVECs ($p<0.01$) (figure 5). H-MVECs stimulated with healthy sera produced an abundant network of branching cords (figure 5). On the contrary, as previously reported,[31 32] angiogenesis was significantly reduced on challenge with SSc sera ($p<0.01$ vs basal H-MVECs) (figure 5). The addition of recombinant human VEGF-A165 or anti-VEGF-A165b blocking antibodies to SSc sera significantly increased H-MVEC angiogenesis compared with cells treated with SSc sera alone (both $p<0.05$) (figure 5). NRP1 gene silencing in H-MVECs resulted in a significant impairment of angiogenic capacity comparable with that of cells treated with SSc sera ($p<0.01$ vs basal H-MVECs) (figure 5). Stimulation of NRP1-silenced H-MVECs with recombinant human proangiogenic VEGF-A165 or antiangiogenic VEGF-A165b could only slightly increase or decrease angiogenesis, respectively (figure 5).

DISCUSSION

Here, we investigated for the first time the possible involvement of the Sema3A/NRP1 axis in the pathogenesis of SSc. Our present findings clearly demonstrate that serum levels and dermal expression of NRP1 are significantly decreased in patients with SSc and that lower circulating sNRP1 levels correlate with the severity of NVC abnormalities and the presence of digital ulcers. In contrast to constitutive endothelial expression of NRP1 in healthy skin, NRP1 was found to be strongly reduced ex vivo in SSc dermal microvessels, and NRP1 downregulation was maintained in vitro in MVECs obtained from SSc dermis. On the contrary, we could not find any difference in NRP1 protein levels between peripheral blood EPC-derived ECs from patients with SSc and healthy controls. This evidence suggests that the dysregulated expression of this receptor is restricted to locally injured microvasculature in an overt disease without affecting bone marrow-derived circulating endothelial progenitors. As far as Sema3A is concerned, no difference in its expression was observed between SSc and controls either in the circulation or in the cutaneous tissue.

The importance of NRP1 for vascular development is well established and shown by the generation of knockout mice, which display an embryonic lethal phenotype characterised by severe vascular defects due to impaired angiogenic sprouting and branching very much resembling the disturbed vessel morphology seen in patients with SSc.[4 33 34] Moreover, conditional NRP1 knockout in ECs is associated with important cardiac and vascular defects, thus suggesting a crucial role of NRP1 in EC functions.[35] Even though NRP1 was originally identified as an adhesion molecule in the nervous system, it is more commonly studied as receptor for the neuronal guidance molecule Sema3A and as co-receptor for the VEGF-A165/VEGFR-2 complex with key roles in neuronal and vascular development.[12 36] Of note, several studies have implicated a dysfunctional VEGF-A/VEGFR-2 system in the impaired angiogenic process characteristic of SSc.[2 26 37–39] Moreover, besides VEGF-A, it is well known that a dysregulated expression of a large array of proangiogenic and antiangiogenic (angiostatic) factors present in the circulation of patients with SSc may be mostly responsible for such angiogenic deficit.[2 37–40]

In our study, we observed that the proangiogenic NRP1 receptor was constitutively downregulated in dermal SSc-MVECs and that treatment with SSc sera could significantly reduce NRP1 expression in H-MVECs, which is in line with the reported antiangiogenic properties of SSc sera.[2 31 32 37 41] Strikingly, we also found that NRP1 gene silencing in H-MVECs resulted in a significantly impaired angiogenic process comparable to that of cells treated with SSc sera, further supporting the implication of NRP1 deficiency in the disturbed angiogenesis of SSc.

Consistent with previous studies, stimulation with recombinant proangiogenic VEGF-A165 strongly upregulated NRP1 expression in H-MVECs, suggesting that this growth factor not only can activate ECs directly, but can also contribute to angiogenesis by a mechanism that involves upregulation of its homologous receptor NRP1.[36] The findings of NRP1 downregulation in H-MVECs on challenge with SSc sera are in agreement with the evidence that the majority of VEGF-A detected in SSc circulation is not the

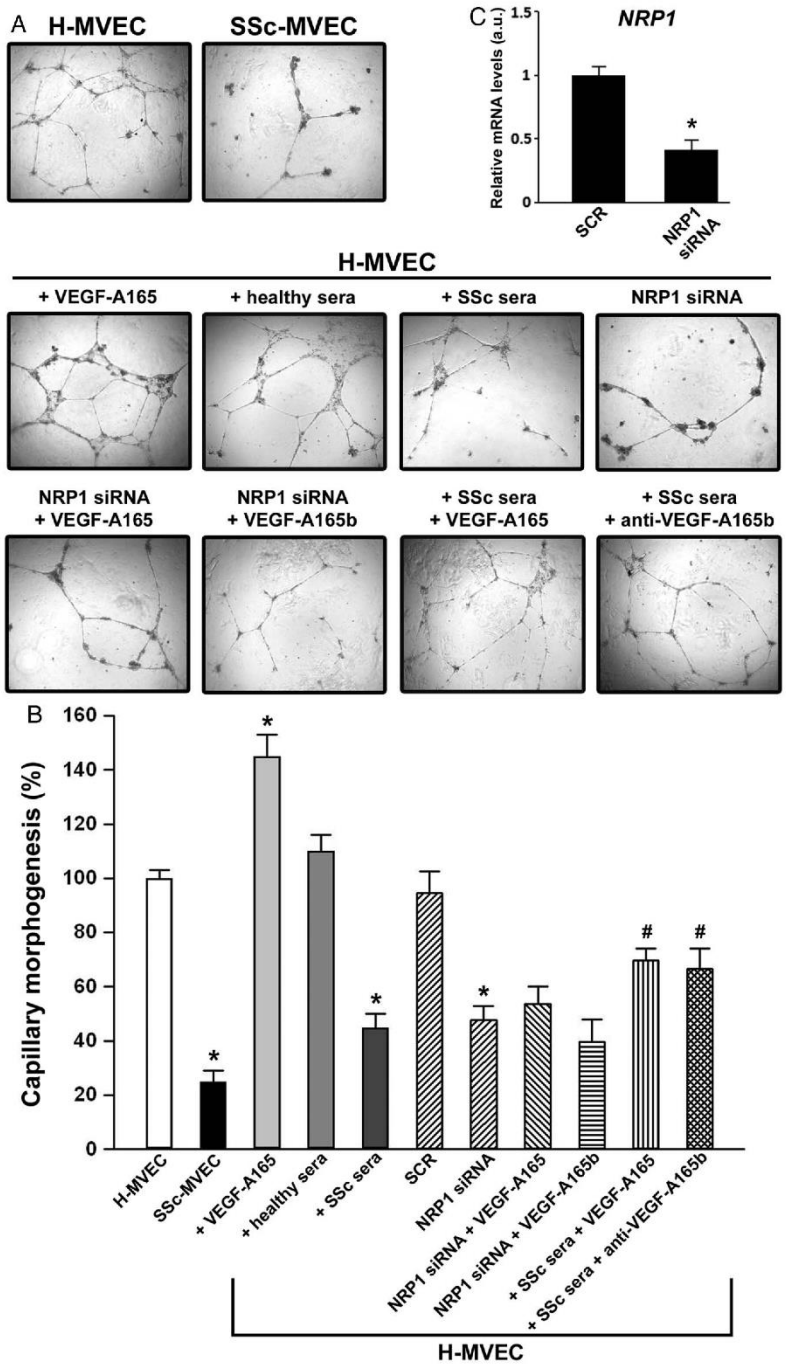

Figure 5 In vitro angiogenesis. (A) Representative images of capillary morphogenesis on Matrigel after 24 h. (B) Capillary morphogenesis of healthy and systemic sclerosis (SSc) dermal microvascular endothelial cells (H-MVECs and SSc-MVECs, respectively) quantified as percentage field occupancy of capillary projections. Capillary morphogenesis of H-MVECs was evaluated at basal condition and after stimulation with sera from healthy subjects (n=5) and patients with SSc (n=5), these latter alone or in combination with recombinant human vascular endothelial growth factor-A165 (VEGF-A165) or anti-VEGF-A165b blocking antibodies. Stimulation with VEGF-A165 added to basal medium was used as positive control of angiogenesis. H-MVECs transfected with neuropilin-1 (NRP1) siRNA, at basal condition or challenged with recombinant human VEGF-A165 or VEGF-A165b, as well as cells transfected with non-silencing scrambled RNA (SCR) were also assayed. Capillary morphogenesis of H-MVECs at basal condition was set to 100%; the other results are normalised to this value. Data are the mean±SD of three independent experiments performed in triplicate with each one of the five H-MVEC and five SSc-MVEC lines. Six to nine photographic fields from three plates were scanned for each experimental point. Student's t test was used for statistical analysis; *p<0.01 versus basal H-MVECs; #p<0.05 versus H-MVECs challenged with SSc sera. (C) mRNA levels of the NRP1 gene in H-MVECs transfected with NRP1 siRNA or non-silencing SCR were measured by quantitative real-time PCR and normalised to expression levels of the 18S ribosomal RNA gene. The relative values compared with SCR are expressed as mean±SD of three independent experiments. Statistical analysis was carried out with Student's t test; *p<0.01 versus SCR.

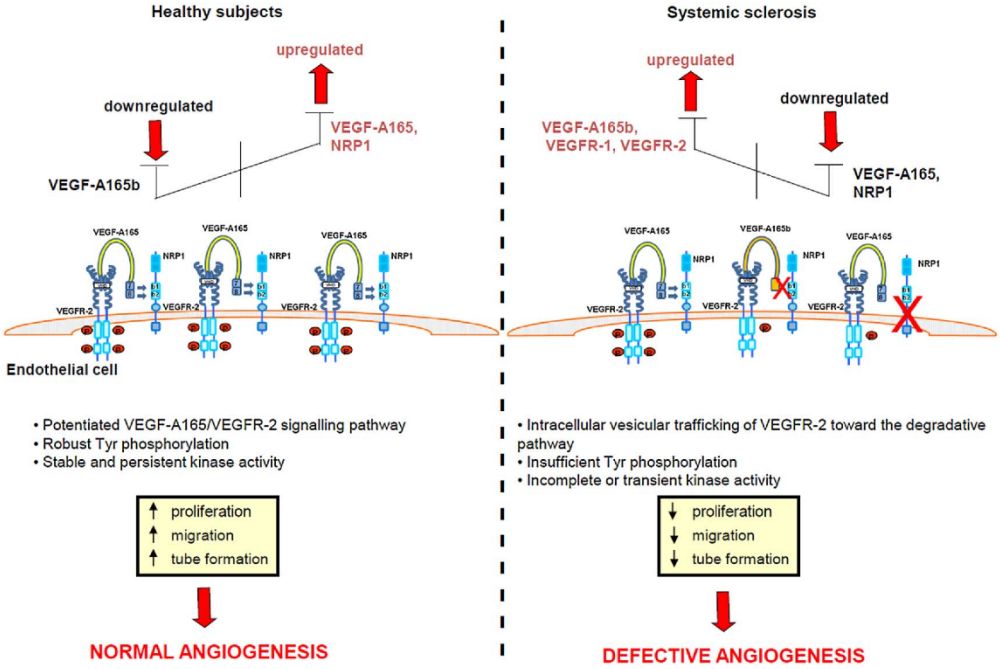

Figure 6 Schematic illustration of the potential mechanisms steering dysregulated angiogenesis in systemic sclerosis (SSc). SSc is characterised by progressive loss of peripheral microvasculature and lack of compensatory angiogenesis. In most angiogenic states, vascular endothelial growth factor-A165 (VEGF-A165) interacts with VEGF receptor-2 (VEGFR-2) via the VEGF homology domain (VHD) and with the b1 and b2 domains of neuropilin-1 (NRP1) via exons 7 and 8. Co-expression of NRP1 and VEGFR-2 enhances VEGF-A165 binding to VEGFR-2, VEGFR-2 phosphorylation and downstream signalling cascades. In non-angiogenic conditions, such as SSc, both a switch from proangiogenic VEGF-A165 to antiangiogenic VEGF-A165b isoform, which is unable to bind the co-receptor NRP1, and concomitant NRP1 downregulation may result in an insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient downstream signalling along with a differential intracellular vesicular trafficking of VEGFR-2 towards the degradative pathway, ultimately leading to an impaired angiogenic response.

proangiogenic VEGF-A165, but rather the antiangiogenic VEGF-A165b isoform.[26 42] Further, it has been reported that VEGF-A165b is unable to bind the co-receptor NRP1 because the basic C-terminal amino acids essential for NRP1 binding are absent in this splice variant.[43 44] Interestingly, we observed that stimulation of NRP1-silenced H-MVECs with proangiogenic VEGF-A165 slightly increased angiogenesis, while stimulation with VEGF-A165b isoform resulted only in a slight decrease in their angiogenic capacity. These data are consistent with the anti-angiogenic action of VEGF-A165b being mainly dependent on its inability to recruit VEGFR-2/NRP1 co-receptor complex and activate downstream signalling. In a recent study, it was demonstrated that VEGF-A165 and VEGF-A165b may control the balance between VEGFR-2 recycling, degradation and signalling. In particular, due to the lack of NRP1 co-receptor binding, VEGF-A165b may induce differential intracellular vesicular trafficking of VEGFR-2 towards the degradative pathway.[45] Thus, both a switch from the proangiogenic to the antiangiogenic VEGF-A isoform and the concomitant NRP1 co-receptor downregulation may have a crucial role in the insufficient angiogenic response found in SSc (figure 6). Indeed, here we also demonstrated that the addition of recombinant human VEGF-A165 or anti-VEGF-A165b blocking antibodies could significantly dampen the antiangiogenic effects of SSc sera on H-MVECs.

The clinical correlation of serum sNRP1 levels with the severity of SSc-related peripheral microvasculopathy also deserves discussion. Indeed, circulating levels of sNRP1 progressively decreased reaching the lowest values in patients with SSc having the active and late NVC patterns, which are characterised by severe architectural changes of microvessels and progressive capillary loss with formation of avascular areas.[46] In addition, patients with active/late NVC patterns and digital ulcers showed serum sNRP1 levels significantly lower than healthy controls, whereas sNRP1 levels did not differ significantly between controls and patients with SSc with early NVC pattern and lack of digital ulcers. However, since sNRP1 may be largely released by ECs, we should also consider that the reduction in circulating levels of sNRP1 might be either a cause or a consequence of the disease, which is characterised by progressive loss of the peripheral microcirculation.[2 3 46] Circulating levels of sNRP1 could even serve as a biomarker reflecting the severity and progression of SSc microvasculopathy. Accordingly, further prospective studies on larger cohorts of patients with SSc are warranted.

Finally, our mechanistic findings indicate that in SSc, endothelial NRP1 expression is suppressed at least partially due to Fli1 transcription factor deficiency. In fact, here we provide the first evidence that NRP1 is a member of the angiogenesis-related gene programme regulated by Fli1 in dermal MVECs. In this context, the impact of Fli1 deficiency in the loss of EC integrity and the development of peripheral microvasculopathy during SSc has been well established.[28–30] Of note, it has been demonstrated that in SSc Fli1 expression is markedly suppressed at least partially through an epigenetic mechanism.[29 30] Thus, such an epigenetic modification might partly explain the persistence of the multiple downstream effects of Fli1 deficiency in an in vitro culture system, as supported by the downregulation of endothelial NRP1 observed ex vivo in SSc dermal microvessels and maintained in cultured dermal SSc-MVECs. Interestingly, NRP1 was also found to be a target of the antiangiogenic microRNA miR-320, and a dysregulated microRNA profile is being increasingly reported in SSc.[47 48]

In conclusion, we shed light on NRP1 deficiency as a novel key factor contributing to peripheral microvasculopathy and defective angiogenesis in SSc. Further studies are warranted to decipher whether therapeutic modulation of VEGF-A/VEGFR-2/NRP1 co-receptor signalling might pave the way for boosting angiogenesis and blocking the progression of peripheral microvasculopathy in SSc.

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Contributors Study conception and design: ER, MM, LI-M, MM-C and SG. Acquisition of data: ER, IC, MM, CM, IR, SB-R, JB, JA and YA. Interpretation of data: ER, IC, MM, RS, LI-M, MM-C and SG. Manuscript preparation: ER, IC, MM, IR, LI-M, MM-C and SG.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The study was approved by the local institutional review board at the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written informed consent.

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Online supplementary material

METHODS

Patients, controls, serum samples and skin biopsies

Serum samples were obtained from 49 systemic sclerosis (SSc) patients [1] (45 women and 4 men; median age 64 years, range 37 to 80 years, and median disease duration 10 years, range 2 to 31 years) classified as limited cutaneous SSc (lcSSc; n = 32) or diffuse cutaneous SSc (dcSSc; n = 17) [2], and from 39 age-matched and sex-matched healthy individuals. All patients reported the occurrence of Raynaud's phenomenon. At the time blood was drawn, the presence of digital ulcers was recorded. Nailfold videocapillaroscopy (NVC) was performed on eight fingers by a single rheumatologist and images were scored blindly to divide patients into three capillaroscopic patterns (i.e., early, active and late) [3,4]. Clinicodemographic characteristics of SSc patients used for collection of serum samples are shown in online supplementary table S1. Patients were not on immunosuppressive medications, corticosteroids or other disease-modifying drugs. Before blood sampling, they were washed out for 10 days from oral vasodilating drugs and for 2 months from intravenous prostanoids. Fresh venous blood samples were drawn, left to clot for 30 minutes before centrifugation at 1,500 *g* for 15 minutes, and serum was collected and stored in aliquots at −80°C until used. Full-thickness skin biopsies were obtained from the clinically involved skin of one-third of the distal forearm of 18 patients with SSc (15 women, 3 men; median age 48.5 years, range 29 to 73 years, and median disease duration 7.2 years, range 1 to 18 years). Skin samples from the same forearm region of 11 age-matched and sex-matched healthy donors were used as controls. Each skin biopsy was divided into two specimens and processed for immunohistochemistry and biomolecular analysis, respectively. For immunohistochemistry, the specimens were fixed in 10% buffered formalin, dehydrated in graded alcohol series and embedded in paraffin. For protein extraction, skin specimens were immediately immersed in liquid nitrogen and stored at −80°C until use. The study was approved by the local institutional review board at the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written informed consent.

Isolation, culture and stimulation of dermal microvascular endothelial cells (MVECs)

Dermal MVECs were isolated from biopsies of the involved forearm skin from 5 patients with dcSSc and from 5 healthy subjects, as described elsewhere [4,5]. Patients were not taking immunosuppressive or disease-modifying drugs at the time of biopsy. Briefly, skin biopsies were

mechanically cleaned of epidermis and adipose tissue in order to obtain a pure specimen of vascularised dermis, and were treated as previously described [4]. Colonies of polygonal elements were detached and CD31-positive cells were subjected to immunomagnetic isolation. Isolated cells were further identified as MVECs by labelling with anti-factor VIII-related antigen and anti-CD105, followed by reprobing with anti-CD31 antibodies. MVECs were maintained in MCDB medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 30% fetal bovine serum (FBS), 20 µg/ml endothelial cell growth supplement (Calbiochem, Nottingham, UK), 10 µg/ml hydrocortisone, 15 IU/ml heparin, and antibiotics. MVECs from healthy subjects (H-MVECs) and SSc patients (SSc-MVECs) were used between the third and seventh passages in culture. For stimulation experiments, H-MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB medium supplemented with 2% FBS. Medium was removed and cells were incubated with 2% FBS-MCDB medium containing recombinant human vascular endothelial growth factor (VEGF)-A165 (10 ng/ml; R&D Systems, Minneapolis, Minnesota, USA), or 10% serum from SSc patients (n = 5) and healthy subjects (n = 5) for 24 hours.

Late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived endothelial cells (ECs)

Late-outgrowth EPC-derived ECs were obtained from the peripheral blood of 15 SSc patients (13 women and 2 men; n = 9 with lcSSc and n = 6 with dcSSc; median age 60 years, range 42 to 78 years) and 8 healthy individuals (all women; median age 55 years, range 30 to 65 years), as described elsewhere [5-7]. The study was approved by the local institutional review board at the Cochin Hospital, Paris, France, and all patients and control subjects provided written informed consent. Briefly, EPC isolation was performed on a 50-ml heparinised venous blood sample obtained from the forearm. Samples from hospitalised patients were obtained in the morning, at rest, during routine analysis. Patient and control samples were immediately transported to the laboratory for testing. The blood mononuclear cell fraction was collected by Ficoll density-gradient centrifugation and was resuspended in complete endothelial cell growth medium 2 (EGM-2; Lonza, Basel, Switzerland). Cells were then seeded onto separate wells of a 12-well tissue culture plate ($2x10^7$ cells/well) precoated with type I collagen (rat tail; BD Biosciences, Le Pont de Claix, France) and stored in an atmosphere of 5% CO2 at 37°C. After 24 hours of culture, non-adherent cells and debris were aspirated, adherent cells were washed once with phosphate-buffered saline (PBS), and complete EGM-2 was added to each well. The medium was changed daily for 7 days

and then every other day until the first passage. Colonies of ECs appeared between 8 and 26 days of culture and were identified as well-circumscribed monolayers of cells with a cobblestone appearance. After the third passage, phenotyping of EPC-derived cells was performed by flow cytometry, as previously described [6]. After confirmation of the endothelial phenotype, cells were suspended in FBS supplemented with 20% dimethyl sulfoxide, frozen in liquid nitrogen, and stored until used.

Enzyme-linked immunosorbent assay (ELISA) for serum semaphorin3A (Sema3A) and soluble neuropilin-1 (sNRP1)

The levels of Sema3A and sNRP1 in serum samples were measured by commercial quantitative colorimetric sandwich ELISA (catalogue number ABIN481720 and ABIN415191, respectively; Antibodies-on line, Atlanta, Georgia, USA) according to the manufacturer's protocol. Each sample was measured in duplicate.

Immunofluorescence

For antigen retrieval, paraffin-embedded skin sections (5 μm thick) were deparaffinised and boiled for 10 minutes in sodium citrate buffer (10 mM, pH 6.0). The sections were washed three times in PBS, incubated in 2 mg/ml glycine for 10 minutes to quench autofluorescence caused by free aldehydes, and then blocked for 1 hour at room temperature with 1% bovine serum albumin in PBS. The slides were incubated overnight at 4°C with rabbit monoclonal antihuman NRP1 antibody (1:50 dilution; catalogue number ab81321, Abcam, Cambridge, UK) or rabbit polyclonal antihuman Sema3A antibody (1:50 dilution; catalogue number ab23393, Abcam). After extensive washing in PBS, the sections were incubated with Rhodamine Red-X-conjugated goat anti-rabbit IgG (1:200 dilution; Molecular Probes, Eugene, Oregon, USA) for 45 minutes at room temperature in the dark. Irrelevant isotype-matched and concentration-matched rabbit IgG (Sigma-Aldrich) were used as negative controls. For double immunofluorescence staining, we used a mouse monoclonal antibody against CD31/pan-endothelial cell marker (1:25 dilution; catalogue number ab9498, Abcam) followed by Alexa Fluor-488-conjugated goat anti-mouse IgG (1:200 dilution; Molecular Probes). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The immunolabelled sections were then observed under a Leica DM4000 B microscope equipped with fully automated fluorescence axes (Leica Microsystems, Mannheim, Germany). Fluorescence images were captured using a Leica DFC310 FX 1.4-megapixel digital colour camera equipped with the Leica software application suite LAS V3.8 (Leica Microsystems).

Western blotting

Proteins were extracted from skin biopsies, dermal MVECs and late-outgrowth peripheral blood EPC-derived ECs as described elsewhere [7,8]. Twenty micrograms of total proteins were electrophoresed on NuPAGE 4 to 12% Bis-Tris Gel (Invitrogen, Carlsbad, California, USA) and blotted onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked with blocking solution included in the Western Breeze Chromogenic Western Blot Immunodetection Kit (Invitrogen) for 30 minutes at room temperature on a rotary shaker and incubated for 1 hour at room temperature with rabbit monoclonal antihuman NRP1 (1:1,000 dilution; catalogue number ab81321, Abcam), rabbit polyclonal antihuman Sema3A (1:1,000 dilution; catalogue number ab23393, Abcam), rabbit polyclonal antihuman Friend leukaemia integration 1 (Fli1) (1:1,000 dilution; catalogue number ab180902, Abcam) and rabbit polyclonal anti-α-tubulin (1:1,000 dilution; catalogue number #2144, Cell Signaling Technology, Danvers, Massachusetts, USA) antibodies, assuming α-tubulin as control invariant protein. Immunodetection was performed as described in the Western Breeze Chromogenic Immunodetection protocol (Invitrogen). Densitometric analysis of the bands was performed using the free-share ImageJ software (NIH, Bethesda, Maryland, USA; online at http://rsbweb.nih.gov/ij) and the values were normalised to α-tubulin.

Gene silencing of Fli1 and NRP1

MVECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 small interfering RNA (siRNA), 10 nM of NRP1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology, Dallas, Texas, USA) using HiPerfect transfection reagent (Qiagen, Milan, Italy) for 72 hours.

RNA purification, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from MVECs using the RNeasy Micro Kit (Qiagen). First strand cDNA was synthesised using the QuantiTect Reverse Transcription kit (Qiagen). For mRNA quantification, SYBR Green real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Milan, Italy) with melting curve analysis. Predesigned oligonucleotide primer pairs were obtained from Qiagen (QuantiTect Primer Assay). The assay IDs were QT00023009 Hs_NRP1, QT00078372 Hs_FLI, and QT00199367 Hs_RRN18S. The PCR mixture contained 1 µl cDNA, 0.5 µM sense and antisense primers, 10 µl 2× QuantiTect SYBR Green PCR Master Mix containing SYBR Green I dye, ROX passive reference dye, HotStarTaq DNA Polymerase, dNTP mix and $MgCl_2$ (Qiagen). Amplification was performed according to a

standard protocol recommended by the manufacturer. Non-specific signals caused by primer dimers or genomic DNA were excluded by dissociation curve analysis, non-template controls and samples without enzyme in the reverse transcription step. 18S ribosomal RNA was measured as an endogenous control to normalise for the amounts of loaded cDNA. Differences were calculated with the threshold cycle (Ct) and comparative Ct method for relative quantification. All measurements were performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, New York, USA). Briefly, cells were treated with 1% formaldehyde for 10 minutes. The cross-linked chromatin was then prepared and sonicated to an average size of 300-500 bp. To exclude exogenous DNA contamination, negative controls were obtained by omitting the cell preparation in some specimens. The DNA fragments were immunoprecipitated with rabbit polyclonal anti-Fli1 antibody (catalogue number ab15289, Abcam) or normal rabbit IgG (catalogue number ab37415, Abcam) at 4°C. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific region of the NRP1 gene promoter. Putative Fli1 transcription factor binding site was predicted by Tfsitescan. The primers were as follows: NRP1 Forward, 5'-CTAGGGGTGCAGAGCGAG-3'; NRP1 Reverse, 5'-GAAGGAAGGCGCTGGGAG-3'. The amplified DNA products were resolved by agarose gel electrophoresis.

***In vitro* capillary morphogenesis assay**

In vitro capillary morphogenesis assay was performed in 96-well plates covered with Matrigel (BD Biosciences). Matrigel (50 µl; 10-12 mg/ml) was pipetted into culture wells and polymerised for 30 minutes to 1 hour at 37°C. H-MVECs ($30x10^3$ cells/well) were incubated in basal MCDB medium containing 10% healthy serum (n = 5) or 10% SSc serum (n = 5), this latter used alone or in combination with recombinant human VEGF-A165 (10 ng/ml; R&D Systems), mouse monoclonal anti-human VEGF-A165b antibody (10 µg/ml; catalogue number ab14994, Abcam) or irrelevant mouse IgG. SSc-MVECs and H-MVECs transfected with NRP1 siRNA, at basal condition or challenged with recombinant human VEGF-A165 or VEGF-A165b (both 10 ng/ml; R&D Systems), or non-silencing SCR, were also assayed. Stimulation with recombinant human VEGF-A165 added to basal MCDB medium was used as positive control of angiogenesis. Plates were photographed at 6 and 24 hours. Results were quantified at 24 hours by measuring the percent field occupancy of

capillary projections, as determined by image analysis. Six to nine photographic fields from 3 plates were scanned for each experimental point.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, Illinois, USA). Data are expressed as mean ± SD or median and interquartile range (IQR). The Student's *t*-test and nonparametric Mann-Whitney *U*-test were used where appropriate for statistical evaluation of the differences between two independent groups. A p-value less than 0.05 was considered statistically significant.

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Supplementary Table S1. Demographic and clinical characteristics of the 49 patients with systemic sclerosis (SSc) included for collection of serum samples.

Characteristics	SSc patients
Age, years, median (range)	64 (37 to 80)
Sex	
Male	4 (8)
Female	45 (92)
Disease subset	
lcSSc	32 (65)
dcSSc	17 (35)
Disease duration, years, median (range)[a]	10 (2 to 31)
Autoantibody positivity	
ANA	49 (100)
ACA	32 (65)
Anti-topo I	11 (22)
Digital ulcers	27 (55)
Digital pitting scars	15 (31)
Nailfold videocapillaroscopy pattern	
Early	11 (22)
Active	21 (43)
Late	17 (35)
Skin score, median (range)[b]	6 (0 to 35)
Interstitial lung disease[c]	27 (55)
2013 ACR/EULAR score, median (range)	14 (10 to 28)

Except where indicated otherwise, values are the number (%) of subjects.
[a]Disease duration was calculated since the first non-Raynaud's symptom of SSc; [b]modified Rodnan skin thickness score; [c]Determined by thoracic high-resolution computer tomography. ACA, anticentromere antibodies; ACR, American College of Rheumatology; ANA, antinuclear antibodies; Anti-topo I, anti-topoisomerase I antibodies; dcSSc, diffuse cutaneous SSc; EULAR, European League Against Rheumatism; lcSSc, limited cutaneous SSc.

2. Third Paper

Evidence for a Derangement of the Microvascular System in Patients with a Very Early Diagnosis of Systemic Sclerosis

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ABSTRACT. ***Objective.*** To investigate whether patients with a very early diagnosis of systemic sclerosis (VEDOSS) may already present circulating markers and *in vitro* signs of microvascular dysfunction.

Methods. Serum samples were obtained from 55 patients with systemic sclerosis (SSc), 25 patients with VEDOSS, and 55 matched healthy controls (HC). Serum levels of pan-vascular endothelial growth factor (VEGF) and soluble neuropilin-1 (sNRP-1) were measured by ELISA. Human dermal microvascular endothelial cells (H-MVEC) were cultured and stimulated with SSc, VEDOSS, and HC sera. Protein expression of NRP-1 was analyzed by Western blotting, cell proliferation by 5′-bromodeoxyuridine assay, migration capacity by wound-healing assay, and capillary-like tube formation by Matrigel assay.

Results. Serum levels of pan-VEGF were increased in patients with VEDOSS and SSc versus HC ($p = 0.05$ and $p = 0.003$, respectively). Serum levels of sNRP-1 were significantly reduced in patients with VEDOSS and SSc compared with controls ($p = 0.012$ and $p = 0.027$, respectively). NRP-1 expression was decreased in H-MVEC stimulated with VEDOSS sera ($p < 0.001$ vs HC). Proliferation was reduced in H-MVEC stimulated either with VEDOSS or SSc sera in comparison with HC sera ($p = 0.015$ and $p = 0.043$, respectively). Wound healing was compromised in H-MVEC stimulated with VEDOSS and SSc sera versus HC sera ($p < 0.01$ for both). Capillarogenesis was decreased in H-MVEC stimulated with VEDOSS sera ($p < 0.01$) and SSc sera ($p < 0.001$) compared with cells stimulated with HC sera.

Conclusion. Similar to patients with SSc, patients with VEDOSS already present biological signs of endothelial dysfunction. Our data demonstrate that VEDOSS sera significantly modify endothelial cell behavior and impair the angiogenic potential of the microvascular system. (J Rheumatol First Release May 15 2017; doi:10.3899/jrheum.160791)

Key Indexing Terms:
SYSTEMIC SCLEROSIS ANGIOGENESIS
VERY EARLY DIAGNOSIS OF SYSTEMIC SCLEROSIS

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Systemic sclerosis (SSc) is characterized by widespread vascular injury and dysfunction, impaired angiogenesis, immunological abnormalities, and progressive fibrosis of the skin and internal organs[1,2,3,4]. In the pathogenetic cascade of SSc, the vascular system seems primarily affected, together with the derangement of the immune system. In this scenario, endothelial cell activation, damage, and apoptosis are the main features that favor vascular tone dysfunction and ischemia-reperfusion injury, vessel wall remodeling, and reduced capillary blood flow. These events progressively lead to extracellular matrix accumulation and fibrosis[4,5,6,7,8]. The involvement of the microvascular system is also characterized by capillary loss, mainly from an aberrant regeneration of capillaries and a defective angiogenesis[5,9]. In SSc, it has been shown that vascular endothelial growth factor (VEGF)-A/VEGF receptor (VEGFR) system is profoundly disturbed. Moreover, a deficiency in the coreceptor neuropilin (NRP)-1 (initially described as an axonally expressed receptor for secreted class-3 semaphorins) may be an additional factor contributing, with the perturbed VEGF-A/VEGFR-2 system, to peripheral microvasculopathy and defective angiogenesis in SSc[10].

The deregulation of vascular tone control, clinically evident as Raynaud phenomenon (RP), and microcirculatory abnormalities are the earliest clinical manifestations of SSc and may precede skin and visceral involvement by months or years[6,7,11,12]. As evident in nailfold videocapillaroscopy (NVC), vascular damage and angiogenic disturbances are present since the "early" NVC pattern of SSc, and further aggravate in the "active" and "late" patterns, culminating in the loss of capillaries with formation of avascular areas[6,7]. Many vascular biomarkers, including proangiogenic and angiostatic factors, have been linked to peripheral vasculopathy in SSc[13].

VEGF is strongly overexpressed in the skin and serum of patients with SSc, together with the VEGF receptors (VEGFR-1 and -2), although no effective angiogenesis is observed. VEGF levels are mainly increased in the earliest stages of the disease, which may be related to compensatory mechanisms and may have deleterious effects on the vascular network. Although elevated levels of VEGF are consistent with active angiogenesis, an uncontrolled chronic overexpression throughout various disease stages, as seen in patients with SSc, might contribute to disturbed vessel morphology rather than promote new vessel formation[13].

It has been shown that RP and puffy fingers, together with abnormal capillaroscopy and positive SSc-specific antinuclear antibodies, may allow identification of patients with the preliminary criteria for very early diagnosis of SSc (VEDOSS)[14,15,16,17]. These patients already present modifications of the microvasculature and complications such as digital ulcers (DU)[18]. For this reason, the aim of our work was to investigate whether the sera of patients with VEDOSS present modifications of factors involved in angiogenesis and may elicit a reduction of the angiogenic potential of microvascular endothelial cells *in vitro*.

MATERIALS AND METHODS

Patients, controls, and serum samples. Patients were included who were followed regularly at the Department of Experimental and Clinical Medicine, Division of Rheumatology, Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, or at the Autoimmunity Outpatient Clinic of the Department of Internal Medicine, Centro Hospitalar de São João (CHSJ), Porto, Portugal. Inclusion criteria consisted of being classified as SSc[19] or VEDOSS[15], having clinical information available for chart review (demographic, clinical manifestations, imaging, and immunology), and being able to give written informed consent for chart review and for performing blood tests. Patients with a concomitant autoimmune disease were excluded. The presence of primary RP was an exclusion criterion for healthy controls (HC).

Serum samples were obtained from 55 patients with SSc (49 women; median age 64 yrs, range 37–81 yrs; mean disease duration 10 years, range 1–31 yrs) classified as limited cutaneous SSc (n = 40) or diffuse cutaneous SSc (n = 15)[20], from 25 patients with VEDOSS (21 women; median age 50 yrs, range 19–77 yrs; median disease duration 1 yr, range 0–8 yrs), and from 55 age-matched and sex-matched healthy individuals (51 women; median age 52 yrs, range 29–70 yrs).

All patients reported the occurrence of RP. At the time blood was withdrawn, the presence of DU was recorded. NVC was performed on 8 fingers by 2 operators (IC and SG). Recorded images were then saved and scored blindly afterward by both doctors to divide patients into 3 capillaroscopic patterns (i.e., "early," "active," and "late")[21].

The clinical and demographic characteristics of the patients with SSc and VEDOSS are shown in Table 1. Patients were not receiving immunosuppressive medications, corticosteroids, or other disease-modifying drugs. Before blood sampling, patients were washed out for 10 days from oral vasodilating drugs and for 2 months from intravenous prostanoids. Fresh venous blood samples were drawn, left to clot for 30 min before centrifugation at 1500 g for 10 min, and serum was collected and stored in aliquots at –80°C until used.

The study was approved by the local institutional review board of AOUC (AOUC 69/13), as well as by the Health Ethical Committee of CHSJ (CHSJ 84/13). All subjects provided written informed consent.

ELISA for serum pan-VEGF, soluble NRP-1 (sNRP-1), and Semaphorin 3A (Sema3A). Serum levels of pan-VEGF (catalogue number DVE00; R&D Systems), sNRP-1, and Sema3A (catalogue number ABIN415191 and ABIN481720, respectively; Antibodies-on line) were measured by commercial quantitative colorimetric sandwich ELISA, according to the manufacturer's protocol. Each sample was measured in duplicate. For all ELISA assays, the interassay and intraassay variances were < 10%.

Culture and stimulation of human dermal microvascular endothelial cells (H-MVEC). H-MVEC purchased from ATCC were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies), 1.176 g/l of sodium bicarbonate, 4.76 g/l of HEPES, 1 ml/l of EGF, and 1 mg/l of hydrocortisone > 98% (Sigma-Aldrich), and maintained at 37°C in a humidified 5% CO_2 atmosphere. H-MVEC were used between the third and seventh passages in culture. In stimulation experiments, H-MVEC were serum-starved overnight before treatment with sera from patients with VEDOSS, patients with SSc, or HC.

Western blotting analysis. Proteins were isolated from H-MVEC lysates using RIPA buffer (Chemicon International) and 20 μg of protein were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond nitrocellulose membrane (Amersham Life Science). Membranes were then incubated with primary antibodies against NRP-1 (1:1000; Abcam) and α-tubulin (1:1000; Sigma-Aldrich). After overnight incubation at 4°C, membranes were washed with Tris-buffered

Table 1. Demographic and clinical characteristics of the patients with SSc and VEDOSS included for collection of serum samples. Values are n (%) unless otherwise specified.

Characteristics	SSc, n = 55	VEDOSS, n = 25
Age, yrs, median (range)	64 (37–81)	50 (19–77)
Sex		
Male	6 (11)	4 (16)
Female	49 (89)	21 (84)
Race		
White	54 (98)	25
Asian	1 (2)	0
Disease subset		
LcSSc	40 (73)	—
DcSSc	15 (27)	—
Disease duration, yrs, median (range)*	10 (1–31)	1 (0–8)
Autoantibody positivity		
ANA	55	25
ACA	32 (58)	11 (44)
Anti–Scl-70	17 (31)	6 (56)
Digital ulcers	32 (58)	1 (4)
Digital pitting scars	16 (29)	1 (4)
Nailfold videocapillaroscopy pattern		
Normal	4 (8)	7 (28)
Early	10 (18)	13 (52)
Active	26 (47)	5 (20)
Late	15 (27)	0
Skin score, median (range)**	6 (0–35)	0 (0–4)
Interstitial lung disease†	36 (65)	0
2013 ACR/EULAR score, median (range)	14 (10–28)	7 (7–8)

* Disease duration was calculated since the first non-Raynaud symptom of SSc. ** Modified Rodnan skin thickness score. † Determined by thoracic high-resolution computer tomography. SSc: systemic sclerosis; VEDOSS: very early diagnosis of SSc; lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous SSc; ANA: antinuclear antibodies; ACA: anticentromere antibodies; ACR: American College of Rheumatology; EULAR: European League Against Rheumatism.

saline containing 0.1% Tween 20 and incubated with secondary antibodies at room temperature for 1 h. Immunoreactive bands were then visualized by the enhanced chemiluminescence detection system (ECL kit, Amersham Life Science) as previously described[22]. The expression of NRP-1 in H-MVEC was measured at basal condition and after stimulation with 10% VEDOSS sera (n = 8) and HC sera (n = 8) for 24 h.

H-MVEC proliferation assay. H-MVEC (6×10^4 cells/ml) were grown for 24 h and then incubated with 10% sera from patients with SSc (n = 10), patients with VEDOSS (n = 10), and HC (n = 10) for 24 h. Basal or stimulated H-MVEC were then incubated with 5′-bromodeoxyuridine (BrdU) solution at a final concentration of 0.01 mM during the treatment period. Optical density of proliferating cells (positive for BrdU) after ELISA assay using anti-BrdU–specific antibodies (Roche Diagnostics) was evaluated at the microplate reader according to the manufacturer's instructions and as previously reported[22].

H-MVEC migration assay: wound healing. H-MVEC were seeded in 24-well plates precoated with 0.1% gelatin and allowed to grow to 100% confluence. Cell monolayer was injured by a 10-μl tip and cells were washed twice with phosphate buffered saline and then incubated in basal medium or medium containing 10% sera of patients with SSc (n = 10), patients with VEDOSS (n = 10), and HC (n = 10). Cell migration into the wounded area was then visualized and photographed on a phase contrast microscope (Nikon) at a magnification of 40×, after 24, 29, 42, and 46 h of incubation. Wound healing capacity was assessed by comparing the images of the wounded area at the beginning and at 46 h to quantify the migration rate of the cells after wounding.

H-MVEC capillary-like tube formation assay. In vitro capillary morphogenesis assay was performed in 96-well plates covered with Matrigel (BD Biosciences). Matrigel (50 μl; 10–12 mg/ml) was pipetted into culture wells and polymerized for 30 min to 1 h at 37°C, as described elsewhere[23]. H-MVEC (30×10^3 cells/well) were incubated in basal RPMI 1640 medium with 2% FBS or 10% sera from patients with SSc (n = 10), patients with VEDOSS (n = 10), and HC (n = 10). Plates were photographed at 6 h and 24 h. Results were quantified at 24 h by measuring the percent field occupancy of capillary projections, as determined by image analysis.

Statistical analysis. Statistical analyses were performed using the Statistical Package for Social Sciences software for Windows, version 20.0 (SPSS). Categorical variables are presented as frequencies and percentages and continuous variables as mean and SD or median and interquartile range (IQR) for variables with skewed distribution. Normal distribution was checked using Shapiro–Wilk test or skewness and kurtosis. The Student t test and nonparametric Mann–Whitney U test were used where appropriate for statistical evaluation of the differences between 2 independent groups, while ANOVA or nonparametric Kruskal-Wallis tests were used for statistical evaluation of the differences between 3 independent groups. Posthoc analyses were performed with Mann–Whitney U test, considering Bonferroni correction (α/number of comparisons). All reported p values are 2-tailed, with a p value of < 0.05 indicating statistical significance.

RESULTS

Serum levels of pan-VEGF, sNRP-1, and Sema3A in patients with VEDOSS. Serum levels of pan-VEGF were increased either in patients with VEDOSS (median 283.96, IQR 191.01–360.29 pg/ml) or in patients with SSc (median 323.13, IQR 151.28–507.51 pg/ml) compared with HC (median 227.81, IQR 114.94–300.64 pg/ml; p = 0.05 and p = 0.003, respectively; Figure 1A). There were no significant differences in levels of pan-VEGF between the VEDOSS and SSc groups (Figure 1A). Higher levels of pan-VEGF were found in patients with VEDOSS with both "early" and "active" NVC patterns (median 313.47 pg/ml and 296.62 pg/ml, respectively) versus HC, although these differences did not reach statistical significance (Figure 1B).

We next addressed whether circulating levels of sNRP-1 were affected in patients with VEDOSS. Serum levels of sNRP-1 were significantly reduced in patients with VEDOSS (median 0.12, IQR 0.03–0.24 ng/ml) and SSc (median 0.23, IQR 0.0–0.45 ng/ml) compared with HC (median 0.38, IQR 0.05–1.56 ng/ml; p = 0.012 and p = 0.027, respectively; Figure 2A). There were no significant differences in levels of sNRP-1 between the VEDOSS and SSc groups (Figure 2A). Regarding NVC changes in VEDOSS, as an additional measure of peripheral microvascular involvement, we also found no differences in sNRP-1 between the patients with "early" and "active" NVC patterns and those with normal NVC (Figure 2B).

Further, in accordance with data from SSc[10], no significant differences in serum levels of Sema3A were detected between patients with VEDOSS (median 2.44, IQR 0.99–4.31 ng/ml) and HC (median 4.04, IQR 1.94–4.80 ng/ml; p = 0.29; data not shown).

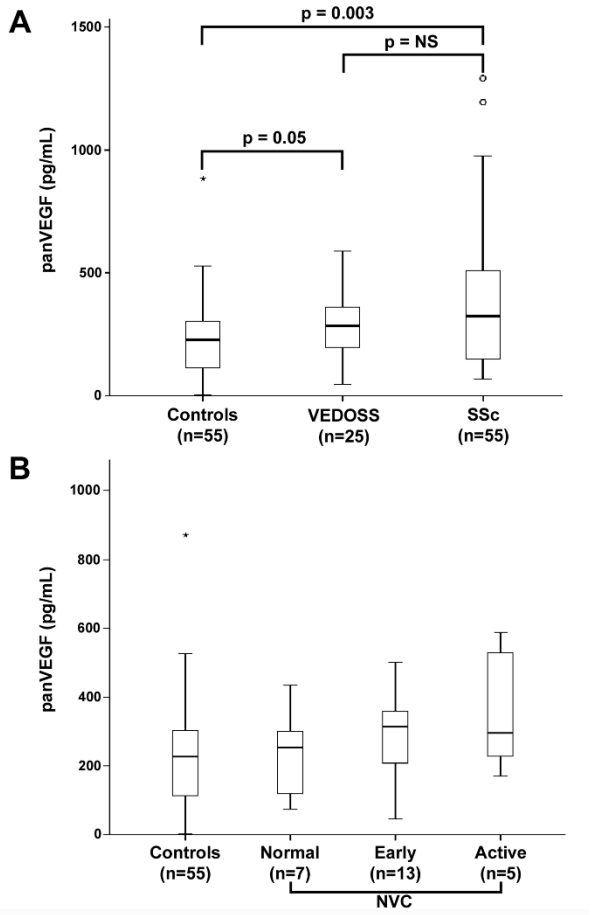

Figure 1. (A) Serum levels of pan-VEGF determined by colorimetric sandwich ELISA in healthy controls and patients with VEDOSS and SSc. (B) Serum levels of pan-VEGF in patients with VEDOSS according to NVC pattern and in healthy controls. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers, and asterisks indicate the extreme values. Kruskal-Wallis test was used for statistical analysis. Pan-VEGF: pan-vascular endothelial growth factor; SSc: systemic sclerosis; VEDOSS: very early diagnosis of SSc; NVC: nailfold videocapillaroscopy; NS: not significant.

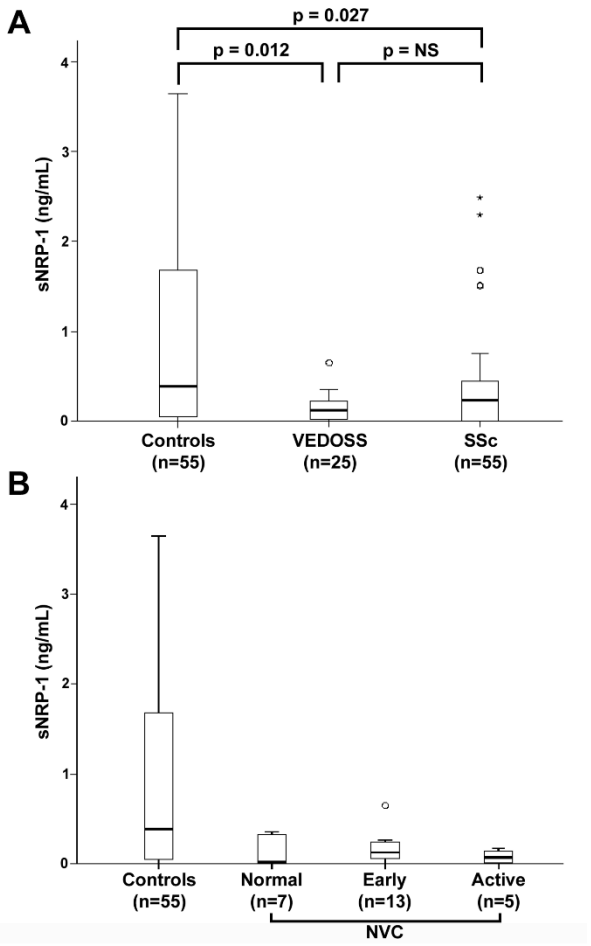

Figure 2. (A) Serum levels of sNRP-1 determined by colorimetric sandwich ELISA in healthy controls and patients with VEDOSS and SSc. (B) Serum levels of sNRP-1 in patients with VEDOSS according to NVC pattern and in healthy controls. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers, and asterisks indicate the extreme values. Kruskal-Wallis test was used for statistical analysis. sNRP-1: soluble neuropilin-1; SSc: systemic sclerosis; VEDOSS: very early diagnosis of SSc; NVC: nailfold videocapillaroscopy; NS: not significant.

Expression of NRP-1 in H-MVEC stimulated with VEDOSS sera. The effect of patients' sera on endothelial cell NRP-1 expression was investigated. In agreement with what was previously reported for SSc[10], the expression of NRP-1 was decreased in H-MVEC after stimulation with VEDOSS sera compared with HC sera ($p < 0.001$; Figure 3).

Proliferation, migration, and capillary-like tube formation of H-MVEC stimulated with VEDOSS sera. Proliferation of H-MVEC was decreased after stimulation either with VEDOSS sera or SSc sera in comparison with HC sera ($p = 0.015$ and $p = 0.043$, respectively; Figure 4A). Further, cell migration was compromised in H-MVEC stimulated with VEDOSS and SSc sera versus HC ($p < 0.01$ for both; Figure 4B and Figure 4C) as determined by wound-healing assay after 46 h of stimulation (the time by which almost complete healing was observed in cultures treated with HC sera).

The assembly of H-MVEC into capillary-like structures was also addressed. Capillarogenesis was decreased in H-MVEC stimulated for 24 h with VEDOSS ($p < 0.01$) and SSc sera ($p < 0.001$) versus HC sera (Figure 5A and Figure 5B). Interestingly, exposure of H-MVEC to VEDOSS sera resulted in a significantly higher angiogenic capacity as compared with cells treated with SSc sera ($p < 0.001$; Figure 5A and Figure 5B).

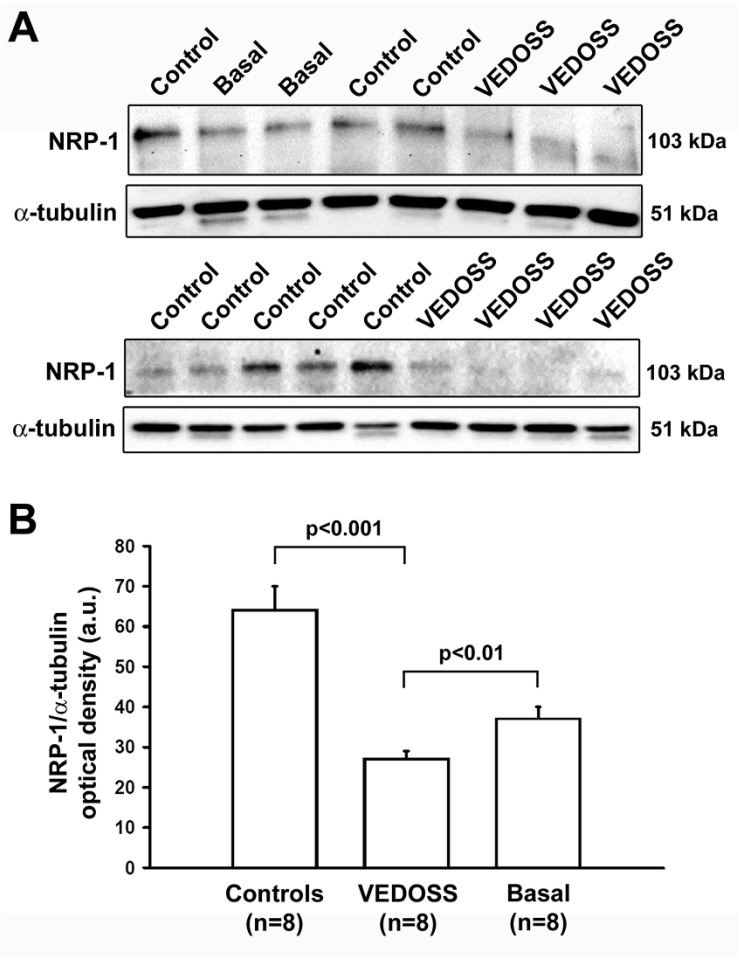

Figure 3. Expression of NRP-1 in H-MVEC. (A) Representative immunoblots. Western blotting was carried out on total protein extracts from H-MVEC at basal condition (n = 8) or treated with 10% sera from healthy control subjects (n = 8) and patients with VEDOSS (n = 8) for 24 h. (B) The densitometric analysis of the bands normalized to α-tubulin is reported in the histograms. Data are means ± SD of optical density in arbitrary units (a.u.). Mann–Whitney U test was used for statistical analysis. NRP-1: neuropilin-1; H-MVEC: human dermal microvascular endothelial cells; VEDOSS: very early diagnosis of systemic sclerosis.

DISCUSSION

SSc is primarily a vascular disease[5,6]. It is generally accepted that initial vascular injury because of autoimmunity and/or environmental factors causes structural and functional abnormalities of microvasculature, resulting in the constitutive activation of fibroblasts in various organs[24]. Because peripheral vasculopathy appears to be present since the very early onset of SSc pathogenesis, our study analyzed the microvascular derangement in patients clinically classified as VEDOSS.

VEGF-A/VEGF receptor signaling pathway, including its coreceptor NRP-1, is involved in the disturbance of angiogenesis in SSc[10]. VEGF has been shown to be strongly overexpressed in the skin and serum of patients with SSc, although without effective angiogenesis, and VEGF levels are mainly increased in the earliest stages of the disease[13]. In our study, both patients with VEDOSS and SSc presented higher serum levels of VEGF when compared with HC, with a tendency to higher levels in SSc versus VEDOSS. In a recent study comparing HC and different stages of SSc[25], VEGF serum levels did not show a highly significant linear trend across the different study groups, while other vascular biomarkers did.

Interestingly, this increase in serum VEGF levels was accompanied by lower circulating levels of sNRP-1 both in patients with VEDOSS and SSc. Moreover, levels of sNRP-1

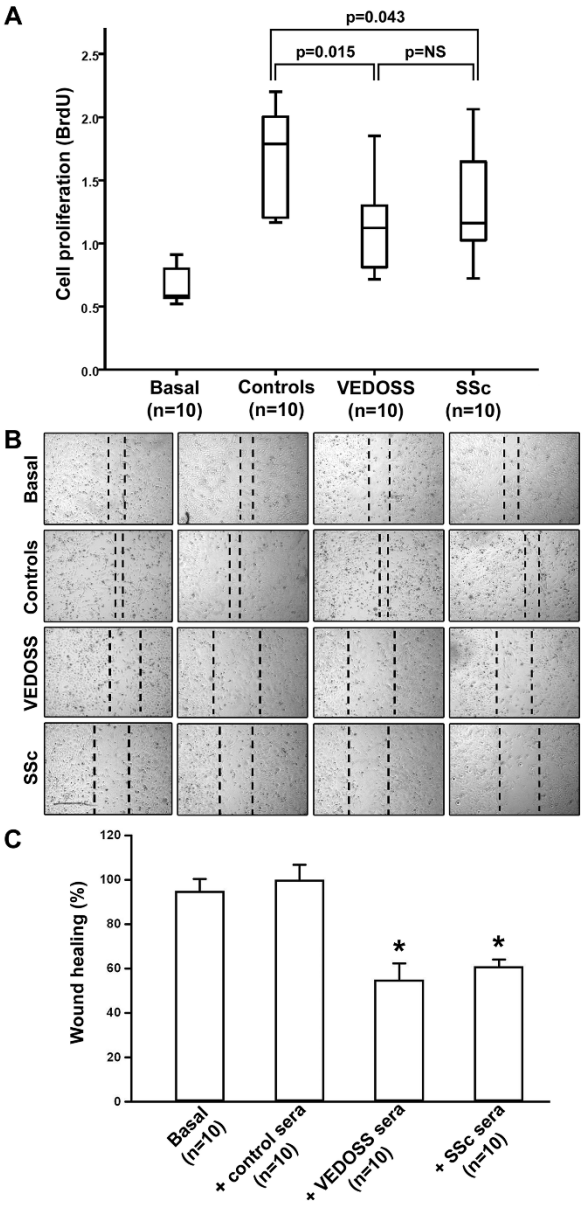

Figure 4. (A) Effect of sera from patients with VEDOSS and SSc on H-MVEC proliferation. Cell proliferation was measured by BrdU assay in H-MVEC at basal condition (n = 10) or treated with 10% sera from healthy controls (n = 10), patients with VEDOSS (n = 10), and patients with SSc (n = 10) for 24 h. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. (B and C) Effect of sera from patients with VEDOSS and patients with SSc on H-MVEC migration. Wound-healing capacity was assessed after 46 h in H-MVEC at basal condition (n = 10) or treated with 10% sera from healthy controls (n = 10), patients with VEDOSS (n = 10), and patients with SSc (n = 10). (B) Four representative images of the wounded area at 46 h are shown for each experimental group; dotted lines represent wound margins. (C) Quantitative analysis of the percentage of wound repair. Data are means ± SD. * $p < 0.01$ vs basal H-MVEC and H-MVEC treated with healthy control sera. SSc: systemic sclerosis; VEDOSS: very early diagnosis of SSc; H-MVEC: human dermal microvascular endothelial cells; BrdU: 5′-bromodeoxyuridine; NS: not significant.

tended to be lower in VEDOSS than in SSc, though not statistically significant. Corroborating ELISA findings, NRP-1 expression was significantly decreased in H-MVEC treated with VEDOSS sera when compared with HC sera. Thus, the higher VEGF serum levels observed in VEDOSS might be a way of compensating for the lack of NRP-1 response in endothelial cells. In contrast to our findings in SSc[10], we found no correlations between serum levels of sNRP-1 and NVC patterns in VEDOSS, perhaps due to either the smaller number of patients in the VEDOSS group or to a less severe peripheral vasculopathy in those patients. Further studies are needed to elucidate this topic. However, regarding the comparison with HC, the findings of circulating sNRP-1 previously reported for SSc were similar to those found in the VEDOSS group of patients in our present study. Of note, there were no significant differences between VEDOSS and SSc results, suggesting that the VEDOSS "environment" already presents characteristics of the established disease, rather than being a "pre-disease". This hypothesis seems further corroborated by the data obtained *in vitro*. In fact, to further analyze vascular derangement in VEDOSS, we performed assays of cell proliferation, migration, and capillarogenesis *in vitro*. Interestingly, stimulation with VEDOSS sera compromised the ability of H-MVEC to proliferate and to perform wound healing. Moreover, Matrigel assay clearly showed a gradual decrease in capillary-like tube formation from VEDOSS to SSc, supporting the progressive antiangiogenic features of the disease.

Of note, our present observations are also in agreement with a recent study showing that distinct SSc subsets have different degrees of vasculopathy and that markers of abnormal endothelial function are increased in the earliest stages of the disease, in which clinical and laboratory findings of advanced disease cannot yet be observed[25].

Patients with VEDOSS already present circulating biomarkers of defective angiogenesis and their sera significantly alter the normal behavior of endothelial cells. This evidence suggests that the involvement of the microvascular system and endothelial cell injury do in fact occur in very early SSc, even when only a few clinical signs and symptoms are present. Further studies, with larger samples of patients with VEDOSS, will be required to identify other potential circulating biomarkers of vascular dysfunction in VEDOSS.

Research into the cellular and molecular basis of SSc has provided new insights into its pathophysiology and potential targets for intervention. Emerging therapies based on immunomodulation, antifibrotic agents, and vasoprotection hold the possibility of preventing end-organ damage and

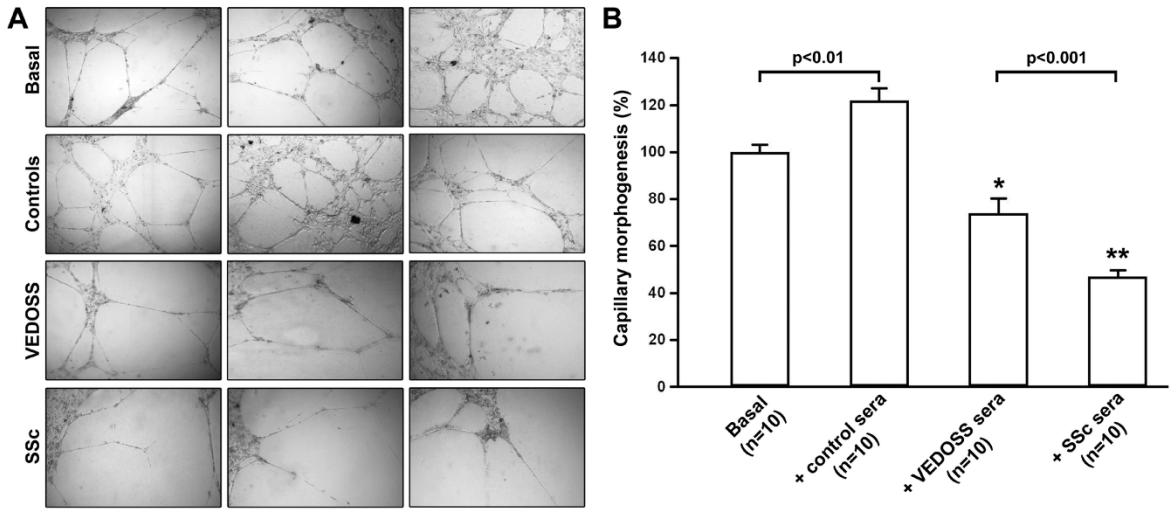

Figure 5. Effect of sera from patients with VEDOSS and SSc on H-MVEC capillary-like tube formation. (A) Representative images of capillary-like tube formation assay on Matrigel after 24 h in H-MVEC at basal condition (n = 10) and after stimulation with 10% sera from healthy controls (n = 10), patients with VEDOSS (n = 10), and patients with SSc (n = 10). Three representative images are shown for each experimental group. (B) Capillary-like tube formation quantified as percentage field occupancy of capillary projections. Statistical analysis was carried out with Mann–Whitney U test. * $p < 0.01$ vs basal H-MVEC and H-MVEC treated with healthy sera. ** $p < 0.001$ vs basal H-MVEC and H-MVEC treated with healthy sera. SSc: systemic sclerosis; VEDOSS: very early diagnosis of SSc; H-MVEC: human dermal microvascular endothelial cells.

improving longterm outcomes in patients with SSc, who can benefit from an early and accurate diagnosis[18]. There must be a window of opportunity for effective therapy for SSc, and this appears to be confined to the very early phase of the disease[26]. In the near future, widening our knowledge about VEDOSS pathophysiology and its pathogenic mechanisms may help to identify new candidate therapeutic agents for this very early phase of SSc.

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VI. GENERAL DISCUSSION

Paul Klee. *The Light and So Much Else* (1931).

Microvasculopathy of Systemic Sclerosis is present from the (Very Early) onset

The vascular hypothesis for SSc was introduced by Campbell and LeRoy, in 1975 (159). The authors proposed that SSc manifestations were due to vascular lesions developed at varying rates and degrees across multiple organs. A cascade of events, including deregulated inflammatory responses and persistent activation of fibroblasts, would lead to decreased blood flow and eventual fibrosis of involved organs.

Several decades after, compelling clinical and biologic evidence still suggests that blood vessels constitute the primary target for both initiating and propagating SSc (116). Some environmental factors may injure ECs, triggering aberrant inflammation and vascular remodeling, leading to the development of SSc in individuals highly predisposed by genetic and epigenetic factors (81). Moreover, despite all the treatments currently available, SSc vasculopathy is responsible for the most significant disease burdens (digital ulcers, renal failure, PAH), leading to increased morbidity and mortality rates in patients with SSc (82).

Multiple studies have focused on the possible players involved in SSc vascular pathogenesis and numerous cellular and molecular entities have been shown to correlate with clinical manifestations of vasculopathy, as revised in the First Paper.

Currently, there are no validated biological measurements to assess the subclinical vascular activity in patients with SSc and the clinician often does not detect the disease until it has already evolved to irreversible fibrotic changes. Searching for new biomarkers of very early disease may help screening patients with early manifestations, allowing an earlier diagnosis of SSc, and may identify patients at risk for major complications, sparing those who are not, from aggressive therapy.

The presence of “red flags”, namely RP and puffy fingers, together with abnormal NVC and positive SSc-specific autoantibodies, may allow to identify patients with the preliminary criteria for VEDOSS (66, 70). These patients already present modifications of the microvasculature and complications like digital ulcers (18).

To our knowledge and to the date of this project writing, there had been no studies exploring VEDOSS vascular “environment”. It would be expectable that recognizing which mechanisms are already deregulated in these patients, and comparing with

findings at established stage, could assist on further comprehension of SSc from the early top of its pathogenic cascade.

More recently, patients classified as having undifferentiated connective tissue disease at risk for SSc (UCTD/SSc) (75) were shown to have increased levels of adhesion molecules, with a gradual increase from UCTD/SSc to fibrotic SSc (160). In another study, markers characterizing endothelial dysfunction were found to be increased in SSc patients from the earliest stages of disease, when clinical and laboratory findings of advanced disease were not yet detected, and linearly increased from early to fibrotic SSc (161).

In our study, patients classified as VEDOSS already presented circulating biomarkers of defective angiogenesis and their sera significantly altered the normal behavior of ECs *in vitro*. After stimulation with VEDOSS sera, the ability of MVECs to proliferate and to perform wound-healing was compromised, although in a lesser extent than after stimulation with SSc sera. We have also shown a gradual decrease in capillarogenesis from VEDOSS to SSc, supporting the progressive antiangiogenic features of the disease.

According to this evidence, the involvement of the microvascular system and ECs injury are in act already in very early SSc, even when only few clinical signs and symptoms are present. Rather than having a “pre-disease”, VEDOSS patients display characteristics of the established microvasculopathy of SSc, which should immediately be targeted.

Translating our findings into clinical practice, the relevance of implementing VEDOSS criteria is superbly evident, to identify and treat these patients without waiting for SSc classification criteria to be met.

If clinicians optimize their awareness to VEDOSS “red flags” and send patients to reference centers for confirmation, therapeutic measures focusing in controlling microvasculopathy can be started much earlier, hopefully postponing the most severe complications of SSc.

Angiogenesis Paradox Unveiled

Microangiopathy in SSc is characterized by a reduced capillary density and an irregular chaotic architecture, leading to chronic tissue hypoxia (which is exacerbated by ECM accumulation due to fibrosis). Despite the hypoxic conditions (which are normally an angiogenic trigger) and the loss of vasculature, there is, paradoxically, no evidence for a sufficient compensative angiogenesis: vascular recovery is impaired and avascular areas are prominent. This is clearly demonstrated by specific NVC morphological changes during disease evolution (113, 135-137).

Among the most potent pro-angiogenic cytokine families is the VEGF family, which includes master regulators of vascular development and of blood and lymphatic vessel function, during health and disease in adults (119). The overexpression of VEGF in SSc, while initially assumed as a compensatory mechanism with deleterious effects on the vascular network, is currently regarded as consequent of switching from pro-angiogenic to anti-angiogenic VEGF-A isoforms (143-145).

In our study, similarly to SSc patients, VEDOSS patients presented higher serum levels of VEGF when compared to healthy controls, with a tendency to higher levels in SSc *versus* VEDOSS. These findings suggest that the normal function of VEGF system in angiogenesis is already disturbed in VEDOSS.

Neuropilin 1: widening pathogenic views

The neuropilin family of multifunctional cell surface receptors was initially described as receptors for Sema3, acting in axon guidance. NRPs are also critical for VEGF-dependent angiogenesis, in addition to VEGFRs, associating with the ligand–receptor signaling complex and modulating the output. Notably, angiogenic signaling is accomplished only through the coordinated activity of VEGF, VEGFRs and NRPs, and NRP is required for strong and sustained kinase activation, allowing the initiation of the pro-angiogenic cascade (119, 120).

NRP1 serves as specific VEGF-A co-receptor on ECs, leading to enhanced migration and survival of ECs *in vitro* (162, 163). NRP1 has been implicated in VEGFR-2-mediated endothelial permeability and in VEGF-A-induced three dimensional ECs biology, such as vessel sprouting and branching (164, 165).

The central role of NRP1 during developmental angiogenesis is exposed by knockout mice, which display an embryonic lethal phenotype with severe vascular defects, very much resembling the disturbed vessel morphology seen in SSc (166).

Despite its coregulation of VEGF and the recognized implication of VEGF system in SSc, as well as the more recent link to TGF-β pathway, NRP role had not yet been explored in this disease setting.

The pathogenesis of RP, a cardinal and very early manifestation of SSc, lays on a disturbance of vascular tone, with an abnormal, long-lasting vasoconstriction, in response to some stimuli. Any cause inducing functional or structural alterations in peripheral nerves, small vessel wall cells and cellular microcirculation may predispose to an abnormal vasoconstriction (167). This close interaction between nerves and vessels networks, from the very beginning of SSc, made even more appealing to study the role of molecules modulating the guidance of both nerves and blood vessels, as NRPs.

Sema3 family exerts chemorepulsive and anti-angiogenic activity in ECs. Sema3A directly competes with VEGF-A for binding to NRP1, inhibiting VEGF-induced ECs proliferation and migration (119). Sema3A can also inhibit integrin-mediated adhesion of ECs to the ECM, enabling the de-adhesion required for vascular remodeling and also by inducing EC apoptosis (130).

On these bases, we hypothesized that the axis NRP1/Sema3A could play a role in the pathogenesis of SSc-related microvascular abnormalities.

In fact, serum levels of NRP1 were significantly decreased in patients with SSc *versus* healthy controls and correlated with the severity of SSc-related peripheral microvasculopathy. Circulating levels of sNRP1 progressively decreased, reaching the lowest values in patients with “active” and “late” NVC patterns. Lower circulating sNRP1 levels correlated with the severity of NVC abnormalities and the presence of digital ulcers. Of note, the reduction in circulating levels of sNRP1 might be either a cause or a consequence of the disease, which is characterized by progressive loss of the peripheral microcirculation.

Serum levels of sNRP1 were also decreased in VEDOSS patients, when compared to healthy controls; however, in contrast to SSc, we found no correlations between

serum levels of sNRP1 and NVC patterns, which could be attributable either to the smaller number of patients in the VEDOSS group or to a less severe peripheral vasculopathy in these patients. Further prospective studies on larger cohorts of patients with SSc may elucidate whether circulating levels of sNRP1 could even serve as a biomarker reflecting the severity and progression of SSc microvasculopathy.

While NRP1 expression was constitutively downregulated in SSc dermal MVECs, both *ex vivo* and *in vitro*, we found no difference in NRP1 protein levels between peripheral blood EPC-derived ECs from patients with SSc and healthy controls. Accordingly, deregulated expression of this pro-angiogenic receptor appears to be confined to the injured microcirculation and not affect bone marrow-derived circulating endothelial progenitors.

No difference in Sema3A serum levels or expression was found between patients and controls, either in SSc and VEDOSS sera and in SSc cutaneous tissue.

The expression of NRP1 was significantly reduced in H-MVECs after treatment with SSc and VEDOSS sera, confirming their anti-angiogenic properties. NRP1 expression in H-MVECs was upregulated after stimulation with recombinant VEGF-A_{165}, suggesting that its pro-angiogenic effects also lay on upregulating NRP co-receptor. The downregulation of NRP1 expression in H-MVECs challenged with SSc and VEDOSS patients' sera supports the evidence that the increased levels of VEGF detected in SSc circulation consist mainly on the anti-angiogenic VEGF-A_{165}b isoform.

Stimulation of NRP1-silenced H-MVECs with VEGF-A_{165} and anti-VEGF-A_{165}b slightly increased angiogenesis. On the other hand, stimulation of NRP1-silenced H-MVECs with VEGF-A_{165}b isoform resulted only in a slight decrease in their angiogenic capacity. VEGF-A_{165}b isoform is unable to bind NRP-1 and its anti-angiogenic properties mostly depend on the inability to recruit VEGFR-2/NRP1 co-receptor complex.

Numerous studies have previously implicated Fli1 in SSc pathogenesis. Fli1 expression is markedly suppressed, at least partially, through an epigenetic mechanism (168, 169). Recently, a new mouse model, with conditional deletion of the transcription factor Fli1 in epithelial cells, recapitulated the disease phenotype observed in patients with SSc (44). Fli1 expression was lower in keratinocytes from SSc patients compared to healthy individuals and gene silencing of Fli1 in

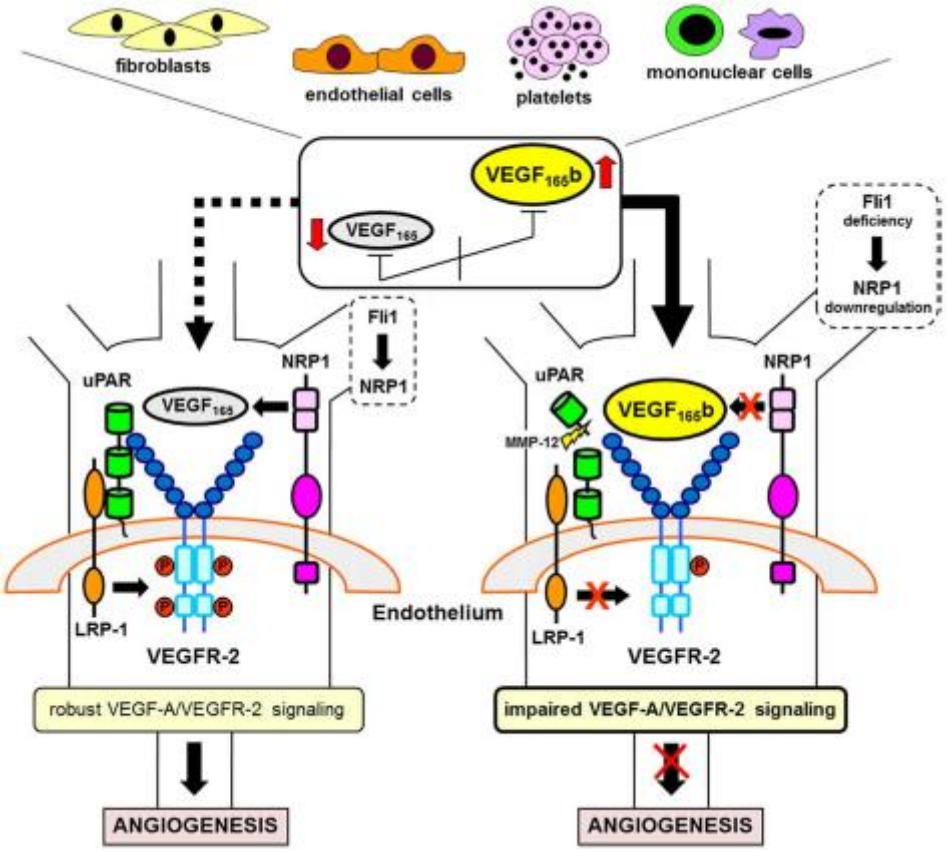
keratinocytes from healthy individuals resulted in a SSc-like gene expression profile. Medullary thymic epithelial cells lacking Fli1 showed a reduced expression of autoimmune regulator (AIRE) and the authors verified that Fli1 directly regulates AIRE transcription. This novel discovery highlights the significant role of Fli1 in autoimmune regulation and marks it out as a potentially therapeutic candidate.

In our study, Fli1 expression was significantly decreased in H-MVECs challenged with SSc sera. NRP1 and Fli1 expression was not different between SSc and control EPC-derived ECs. Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs. The significant impair in angiogenesis after NRP1 gene silencing in H-MVECs was comparable to that of cells treated with SSc sera, further supporting the implication of NRP1 deficiency in the disturbed angiogenesis of SSc. These mechanistic findings allow the assumption of NRP1 as a member of the angiogenesis-related gene program regulated by Fli1 in dermal MVECs.

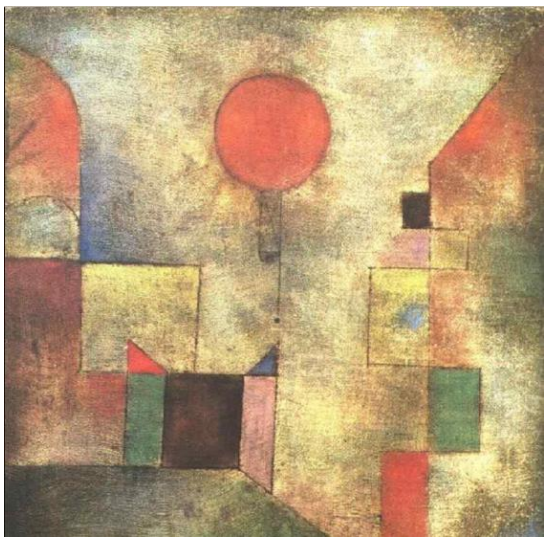
Figure 10 summarizes the current molecular evidence of the pathogenesis of disturbed angiogenesis in SSc.

A switch from pro-angiogenic VEGF-A_{165} to anti-angiogenic VEGF-A_{165}b isoform, and concomitant NRP1 co-receptor downregulation due to Fli1 transcription factor deficiency, result in an insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient downstream signaling along with a differential intracellular vesicular trafficking of VEGFR-2 towards the degradative pathway, ultimately leading to an impaired angiogenic response. Moreover, in SSc ECs, matrix metalloproteinase-12-mediated cleavage/inactivation of uPAR may further impair the VEGF-A/VEGFR-2 system.

Figure 10 – Current pathogenic views of disturbed angiogenesis in systemic sclerosis. *Adapted from (Manetti M et al. 2016.) (145).* Fli1 – Friend leukemia integration 1; LRP-1 – low-density lipoprotein receptor-related protein-1; MMP-12 – matrix metalloproteinase-12; NRP1 – neuropilin 1; uPAR – urokinase-type plasminogen activator receptor; VEGF – vascular endothelial growth factor; VEGFR – vascular endothelial growth factor receptor.



VII. CONCLUSIONS AND FUTURE PERSPECTIVES



Paul Klee. *Red Balloon* (1922).

Considerable progresses have been achieved during the last decade for a better understanding of the cellular and molecular basis of SSc. Significant abnormalities in angiogenesis regulators have been described and may help to explain disrupted mechanisms of vascular repair in SSc, with potential implications for improving clinical practice.

The main findings of this study were:

- Soluble NRP1 levels were decreased in SSc and VEDOSS;
- Decreased NRP1 levels were associated with “active” and “late” NVC patterns and digital ulcers;
- NRP1 was significantly decreased in SSc-MVECs;
- Sema3A levels and expression did not differ in SSc and healthy controls;
- NRP1 expression was significantly decreased in H-MVECs challenged with SSc and VEDOSS sera, while it was not different in SSc and healthy EPC-derived ECs;
- Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs;
- NRP1 gene silencing in H-MVECs resulted in significantly impaired angiogenic capacity;
- VEDOSS patients already present circulating biomarkers of defective angiogenesis;
- VEDOSS sera significantly modify the normal behavior of ECs *in vitro*, in a minor extent than SSc sera.

In conclusion, NRP1 deficiency may be an additional factor in the perturbed VEGF-A/VEGFR-2 signaling, contributing to peripheral microvasculopathy and defective angiogenesis in SSc, evident from the very early stage of the disease.

The study was limited by the number of subjects. These results need to be replicated in other samples and populations, with larger size. Further studies are required to identify additional circulating biomarkers of vascular dysfunction in VEDOSS patients. There is a need to study these potential SSc biomarkers on a prospective cohort of patients, followed longitudinally.

A detailed understanding of SSc pathogenesis from its very early beginning is crucial for the development of novel therapeutic tools. The discovery of key molecular targets may help to develop new and more effective drugs. Normalization of the angiogenic cascade could provide a future therapeutic approach for SSc-related vasculopathy.

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APPENDIX

Document 1 – Fourth paper.

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COMMENTARY Open Access

The "myth" of loss of angiogenesis in systemic sclerosis: a pivotal early pathogenetic process or just a late unavoidable event?

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Abstract

Systemic sclerosis is considered a disease dominated by a "loss of angiogenesis", although in its early phases evidence indicates a disturbed angiogenic response only. In fact, microvascular changes are primarily due to endothelial cell injury, triggering downstream significant *enlargement of the capillary* in an inflammatory environment, followed by capillary rupture (microhemorrhages). Subsequent pro-angiogenic efforts lead to an *aberrant angiogenesis* and, eventually, to a total loss of vessel repair and regeneration (*loss of angiogenesis*). This clearly suggests that the pathogenetic process has a steady progression: from an early excessive pro-angiogenesis, to an aberrant microvascular regeneration, then ending with a late loss of angiogenesis. Herein, we suggest the loss of angiogenesis should not be considered as an overall "myth" characterizing systemic sclerosis but as a very late event of the vascular pathogenesis. Future research should be oriented essentially on the earlier phases dominated by excessive pro-angiogenesis and microvascular aberration.

Keywords: Scleroderma, Systemic sclerosis, Angiogenesis

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Background

In systemic sclerosis (SSc), the loss of angiogenesis has been considered a pivotal event characterizing the disease from its onset. In reality, several pathways of endothelial cell (EC) dysfunction and defective angiogenesis have been identified (Table 1) [1–10]. Paradoxically, in SSc significant concentrations of intrinsic pro-angiogenic factors have been found in the vasculature or adjacent tissues [1, 2, 11]. This evidence is apparently in contrast with the main hypothesis that SSc is fundamentally and originally characterized by a lack or *loss of angiogenesis*. In fact, the eventual inability to regenerate injured vessels may be due to the failure of some angiogenic steps, such as lumen formation or vessel maturation or stabilization. Clearly, the persistent endothelial injury may not only switch on but also significantly perpetuate this process [2]. Whether dysregulated levels of circulating angiogenic or angiostatic factors (or both) are a cause or a consequence of an ongoing vascular disease is still unknown [2].

In early SSc pathogenesis, inflammatory cells have a significant role. In fact, in the edematous tissues, where fibrosis is still absent but inflammation dominates, a significant number of immune cells surround the microvessels. The role of these cells is pivotal in triggering the activation of cells present in the tissues, in particular myofibroblasts, through the release of TGFβ and other cytokines and growth factors [12]. However, other questions remain unanswered. The first is if autoantibodies are pathogenic, contributing to endothelial damage and thus an expression of disease activity, or just innocent bystanders. The second is if endothelial circulating progenitors as well as the resident mesenchymal cells and the cells surrounding the vessel (pericytes, telocytes) may participate in the endothelial dysfunction and the loss of angiogenesis and foster the endothelial to mesenchymal transition (endoMT) process [4, 10, 13].

Table 1 Evidence for endothelial cell dysfunction and defective angiogenic pathways in systemic sclerosis

In vitro studies on peripheral blood mononuclear cells suggest a defective contribution of immune cells to angiogenesis [1]. Greater up-regulation of angiostatic than pro-angiogenic mediators [1, 2]. Microarray studies of microvascular EC gene expression have shown an overexpression of either several pro-angiogenic transcripts or many genes that have a negative effect on angiogenesis [1]
Circulating endothelial progenitor cells, involved in postnatal vasculogenesis, are decreased and functionally impaired [1, 5]. Moreover, these cells show mesenchymal properties that may indicate that they potentially contribute to the accumulation of connective tissue and to vascular malfunction [6]
Bone marrow-derived CD14+ monocytic pro-angiogenic hematopoietic cells (promoting vascular formation and repair and differentiation into mural cells) are significantly increased. They can differentiate into fibroblast-like cells producing extracellular matrix proteins contributing to the fibrotic process [7]
Platelet activation contributes to the pro-angiogenic/angiostatic imbalance by release of bioactive factors and aggregation [8]
A change in the endothelial phenotype of residual microvessels is also present in the skin, favoring anti-angiogenic mechanisms [9]
The endothelial-to-mesenchymal transition process is now clarified and is a novel concept in understanding the significant contribution that ECs may play also in the pathogensis of fibrosis [10]

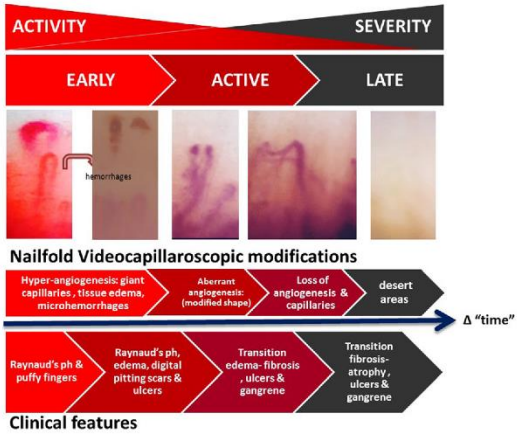

Fig. 1 Clinical and microvascular evolution of systemic sclerosis. *Raynaud's ph* Raynaud's phenomenon

Angiogenesis is a complex and finely balanced physiological process, consisting of the formation of new vessels from pre-existing ones, mainly triggered by damage or tissue hypoxia [1]. Sprouting angiogenesis encompasses an increase in vasopermeability, leading to plasma and protein extravasation, which works as a temporary scaffold for migrating ECs. Matrix metalloproteinases, secreted by the endothelium, break down the vascular basement membrane and allow the invasion of the surrounding stroma by ECs, directed towards the pro-angiogenic stimulus [1]. This process is paralleled by proliferation and organization of newly formed ECs into three-dimensional tubular structures. Lumen formation and vessel wall stabilization by pericytes are the final phases of sprouting angiogenesis and lead to the creation of a functional network of new capillary vessels [1]. Physiological angiogenesis is finely balanced and regulated by stimulating (pro-angiogenic) and inhibiting (anti-angiogenic) factors [1, 2]. Vascular endothelial growth factor (VEGF) has a key role in controlling several cellular and molecular steps in the angiogenic cascade [1]. Indeed, it stimulates ECs to increase their migration and to initiate the proliferative process until a complete tubular structure is formed [1]. Although the majority of studies in the literature highlight the *loss of angiogenesis* in SSc, in practice this condition is present only in the late phase of SSc evolution (Fig. 1). In fact, in the early stage of the disease a vasculopathy with a pro-inflammatory state is evident and an increased production of pro-angiogenic factors (e.g., VEGF, endothelin-1) has been shown [14, 15], despite the defective response of ECs to these stimuli [16]. Moreover, intrinsic abnormal properties of the cellular components of the blood vessels, the presence of fibroblast-derived anti-angiogenic factors, dysfunctional circulating endothelial progenitor cells, and an abnormal expression of transcription factors, including Fra2 and Fli1, may further contribute to SSc vasculopathy [17, 18].

Vascular abnormalities are indeed manifest very early in SSc [3, 19] and are characterized by a progressive involvement of the vessel wall. The main vascular modifications observed with electron and optical microscopy are EC activation/injury and apoptosis, opening of the EC tight junctions allowing inflammatory cell migration, basal membrane duplication, and intimal thickening with vessel narrowing and obliteration [3].

In the early phase, nailfold videocapillaroscopy shows clusters of giant capillaries and tissue edema surrounded by normal capillaries of different shapes. Micro-hemorrhages, derived from the break of mega-capillaries pushed to their upper limits by an *excessive and uncontrolled angiogenesis*, embedded in an inflammatory environment, are clearly detectable [3]. This *"push" of angiogenesis* and the modification of the capillary shape may depend upon the persistent high circulating levels and tissue over-expression of VEGF [11, 14]. These early vascular changes subsequently lead to vascular tone dysfunction, followed by reduced capillary blood flow, with consequent chronic tissue hypoxia, further exacerbated by extracellular matrix accumulation and fibrosis [3]. The process is then characterized by a profoundly *disturbed and aberrant angiogenesis* (tortuous, ramified, and tree-like capillaries; Fig. 1). This phase is followed by a subsequent microvascular loss—known as *loss of angiogenesis*—which is due to defects in both vascular repair and growth of new vessels through vasculogenesis and angiogenesis [1], characterized by a capillaroscopic progressive reduction in capillary density, with large avascular areas (desertification) (Fig. 1).

This evidence demonstrates the switch from an initial pro- to a final anti-angiogenic environment linked to the preponderant action of angiostatic factors (e.g., VEGF165b, angiopoietin-2), resulting in the loss of new normal vessel formation and capillaries. In the early phase, the hypothesis that increased plasma levels of antiangiogenic VEGF165b isoforms profoundly disturb the pro-angiogenic effect of VEGF, being associated with the severity of capillary architectural derangement and loss, has found significant support [14]. The hypothesis that the initial excessive up-regulation of pro-angiogenic factors might lead to an even greater up-regulation of angiostatic factors is also interesting [2]. The reduction of capillary density consequently leads to an impairment in the supply of oxygen and nutrients and thus to a hypoxic state. In this situation, angiogenesis is usually triggered but in SSc the vascular recovery is profoundly disturbed and then impaired and the *loss of angiogenesis* with avascular areas becomes eventually a prominent event [20–22].

In clinics, SSc is characterized by an evolution which is frequently unpredictable, with abrupt acceleration and periods of quiescence. Therefore, awareness of the condition of the microvasculature in the frame of the disease evolution is crucial and may influence the clinical strategy according to a correct evaluation of the disease phase. In practice, it becomes of paramount importance to establish the real disease phase, which should not be centered on the mere measurement of the years from diagnosis but clearly aimed at understanding the "*real time*" of advancement of the microvascular disease (Fig. 1). In SSc, the evolution of microcirculatory modifications and the time to *loss of angiogenesis* may be very fast in diffuse SSc, while it is significantly slower in limited SSc. A switch of research interest to on the early phase of the disease might change the approach to the clinical setting in SSc. In this perspective, the choice of a vasoactive therapeutic strategy aiming at the modulation, in the "time" frame of each phase of microvascular involvement, of the angiogenic process might be a pivotal event changing the approach to SSc therapy in diffuse or limited SSc. In the future, targeting the early inflammatory pro-angiogenic process [22] leading to capillary aberration might be a relevant step to block the disease evolution to prevent the loss of angiogenesis.

Conclusions

The fate of SSc is dictated by the phase of evolution of the microvascular modifications observed in the patient [3]. It is clear that the capacity to define the real advancement of the microvascular involvement during SSc evolution, either in the diffuse or in the limited subset, will be significant for the choice of treatment (immunosuppressive, vasodilatory, vasoactive and its combination, future targeted therapies) to eventually achieve disease remission.

Abbreviations

EC: Endothelial cell; SSc: Systemic sclerosis; VEGF: Vascular endothelial growth factor

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MMC, MM, CB, IC, SBR, GL, ADP, and SG conceived the paper and participated in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Document 2 – Clinical information obtained by chart review.

Demographic	. Age . Gender . Race
Presence of comorbidities	
Disease onset	. Date of diagnosis / date of first visit . Onset of first non-RP manifestation of the disease
Clinical manifestations *(ever before)*	. Skin thickening / clinical subset . Puffy fingers . Sclerodactyly . Digital ulcers/ digital tip pitting scars . Telangiectasia . Arthritis . Pulmonary arterial hypertension . Interstitial lung disease . Gastrointestinal involvement . Cardiac involvement . Renal involvement
Clinical manifestations *(at the time of blood withdrawal)*	. Digital tip ulcers . Modified Rodnan Skin Score
Autoantibodies	. Antinuclear . Anticentromere . Anti-topoisomerase I . Anti-RNA polymerase III . Other
Other laboratory exams *(the closest to the date of blood withdrawal)*	. Erythrocyte sedimentation rate . Hypocomplementemia (C3 or C4) . Creatin kinase . Proteinuria
NVC *(the closest to the date of blood withdrawal)*	Normal SSc pattern: “Early” / “Active” / “Late”
Tests / Functions *(the closest to the date of blood withdrawal)*	. Echocardiography . Electrocardiogram . Chest radiography . Thorax HRCT scan . Pulmonary function tests
Therapy	. Corticosteroids (daily dose) . ACE inhibitors/ ARB . Calcium channel blockers . Prostacyclin analogs . Endothelin antagonists . Phosphodiesterase inhibitors . Immunosuppressive therapy . Others

ACE – angiotensin-converting enzyme; ARB – angiotensin-receptor blockers; NVC – nailfold videocapillaroscopy; RP – Raynaud’s phenomenon.

Document 3 – Ethical Approval (AOUC).

Azienda Ospedaliero Universitaria Careggi

COMITATO ETICO PER LA SPERIMENTAZIONE CLINICA DEI MEDICINALI

Prot. n. 2013/00 24162 **del** 8.7.13

Rif. n. 69/13 (nota da citare sempre in qualsiasi corrispondenza)

Il Comitato Etico dell'AOUCareggi

Riunito in data 17 giugno 2013

Per esprimere il proprio parere sul protocollo **"Valore prognostico della neuropilina 1 (NRP1) e delle semaforine di classe 3 (SEMA3s) come marcatori di danno endoteliale nella Sclerosi sistemica (SSc)".**

Promotore e Responsabile dello studio: Prof. Marco Matucci Cerinic

Accertata

La sussistenza del numero legale

Sentita

La dichiarazione dei componenti del Comitato che dichiarano di astenersi dal pronunciarsi su quegli studi per i quali possa sussistere un conflitto di interesse di tipo diretto o indiretto

Esaminata

La documentazione presentata:

- Richiesta di autorizzazione e di parere al CE;
- Dichiarazione sulla natura osservazionale dello studio;
- Protocollo di studio (Versione nr. 110835008 Febbraio 2013);
- Dichiarazione sulla natura osservazionale dello studio;
- Dichiarazione sul conflitto di interessi dello sperimentatore responsabile dello studio;
- Dichiarazione del Promotore per studi non-profit;
- Foglio Informativo per il paziente;
- Modulo di consenso informato;
- Informativa e manifestazione del consenso al trattamento dei dati personali;
- Lettera al Medico Curante (Versione 1.0 del 27/03/2013);
- Lista Centri partecipanti;
- CV del Medico responsabile dello studio;

Verificati i seguenti aspetti

- conformità ai principi di Buona Pratica Clinica (GCP);
- conformità ai requisiti indicati nella Determinazione AIFA del 20 marzo 2003;
- rispetto delle regole di riservatezza e confidenzialità previste dalle disposizioni normative applicabili;
- salvaguardia dei diritti di sicurezza e benessere dei soggetti partecipanti allo studio;
- validità scientifica e giustificazione etica dello studio;
- correttezza del disegno dello studio e delle procedure di reclutamento dei soggetti;
- completezza e chiarezza del modulo di consenso informato;

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Cod. fisc./Partita iva 04612750481

- appropriatezza delle indagini e/o terapie proposte;
- adeguatezza del rapporto rischi prevedibili/benefici attesi;
- adeguatezza degli spazi e delle strutture e tecnologie disponibili;
- idonea qualificazione del Responsabile dello studio;

Ascoltata

La valutazione dei relatori designati per l'approfondimento dello studio

- HA ESPRESSO **PARERE FAVOREVOLE** ALL'ESECUZIONE DELLO STUDIO presso la SOD Reumatologia sotto la responsabilità del Prof. Marco Matucci Cerinic.

Il parere è stato espresso all'unanimità dei componenti presenti alla seduta.

Il Comitato dovrà essere costantemente aggiornato sull'andamento dello studio, in particolare dovrà essere tempestivamente informato di eventuali modifiche che si intendesse apportare al protocollo.

Inoltre, dovrà essere notificata alla Segreteria del Comitato Etico la conclusione dello studio, accompagnata da una relazione con i risultati ottenuti.

Il Comitato Etico è organizzato ed opera in osservanza a quanto previsto dal D.M.15/7/97, successivo D.M. 18.03.1998 "Linee guida di riferimento per l'istituzione ed il funzionamento dei comitati etici" e dal D.Lgs. 24.06.2003, n. 211 "Attuazione della direttiva 2001/20/CE relativa all'applicazione della buona pratica clinica nell'esecuzione delle sperimentazioni cliniche dei medicinali per uso clinico" e successivo D.M. 12/05/2006 e dalle Linee guida per la classificazione e la conduzione degli Studi osservazionali sui farmaci (G.U. n. 76 del 31.03.08).

Si allega l' elenco dei componenti del Comitato presenti alla seduta.

Il Presidente del Comitato Etico
(Dr. Claudio Galanti)

Document 4 – Ethical Approval (CHSJ).

CES 84-13

Ao CA c/
Inês Chora DC
16.5.2013

Exmo. Senhor
Presidente do Conselho de Administração do
Centro Hospitalar de S. João – EPE

AUTORIZADO
CONSELHO DE ADMINISTRAÇÃO – REUNIÃO DE 25 JUL 2013

Assunto: Pedido de apreciação e parecer para projeto de investigação

Nome do Investigador Principal: Inês João da Silva Chora

Título do projecto de investigação: SYSTEMIC SCLEROSIS – EXPLORING NEW ANGIOGENIC BIOMARKERS

Pretendendo realizar no Serviço de Medicina Interna do Centro Hospitalar de S. João – EPE o projeto de investigação em epígrafe, pelo que solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer.
Para o efeito, anexo toda a documentação referida no dossier dessa Comissão respeitante a estudos/projectos de investigação.

Com os melhores cumprimentos.

Porto, 21 / fevereiro / 2013

O INVESTIGADOR

Inês Chora

Comissão de Ética para a Saúde do Centro Hospitalar de S. João – EPE
Modelo CES 01

SÃO JOÃO

Comissão de Ética para a Saúde do HSJ

Parecer

Projecto de investigação intitulado "Systemic Sclerosis -- exploring new angiogenic biomarkers"

Estudo que se propõe vir a ser desenvolvido no Serviço de Medicina Interna do Centro Hospitalar São João EPE (CHSJ) pela Drª Inês João da Silva Chora, no âmbito da realização de uma dissertação de doutoramento em Medicina Molecular e Oncologia pela FMUP, sob orientação da profª Raquel Soares.

Do ponto de vista científico, o estudo visa avaliar a possibilidade de diversos biomarcadores angiogénicos, designadamente neuropilins, semaphorins e $VEGF_{165b}$ poderem contribuir para identificar um maior risco de progressão da esclerose sistémica.

Para o efeito, a investigadora propõe-se avaliar uma série de marcadores da angiogénese quer em amostras de sangue quer em biopsias cutâneas. *Não é facultada informação sobre a forma como será recrutado o grupo controlo, nem sobre os procedimentos a que este grupo será submetido.*

Não estão previstos benefícios ou incómodos para participantes, com exceção de uma colheita de sangue e de uma biopsia cutânea destinada ao doseamento dos marcadores da angiogénese que se prevê avaliar.

Está prevista a realização de questionários, dos quais se anexam as respetivas cópias e que nos quais é salvaguardado anonimato.

Está previsto o acesso ao processo clínico dos doentes pela investigadora.

Está prevista a obtenção de consentimento informado que é acompanhado de uma informação para o doente que é esclarecedora sobre a natureza do estudo e que salvaguarda as questões éticas pertinentes. *Deverá ser previsto consentimento informado para os controlos.*

A investigadora dispõe da competência científica para a realização do estudo, que está autorizado pelo diretor do serviço de Medicina Interna.

O estudo está basicamente centrado na avaliação da presença/expressão de marcadores da angiogénese em doentes com esclerose sistémica. No entanto, a informação no formulário da CES é a de que o projeto não envolve exames complementares e que não é financiado. Na medida em que os parâmetros avaliados na investigação não fazem parte da avaliação de rotina dos doentes, a *CES questiona a forma como será garantido o financiamento relativo ao doseamento dos marcadores angiogénicos cuja avaliação está prevista no projeto.*

Em face da análise do protocolo proponho que a aprovação do protocolo pela CES do CHSJ fique a aguardar pela resposta da investigadora às questões em itálico.

Porto, 21 de Março de 2013

O relator

Prof. Manuel Pestana

Em face à resposta da proponente a aprovação do protocolo pela CES, é emitido parecer favorável.
20.04.2013

7. SEGURO

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)
NÃO ☒
NÃO APLICÁVEL ☐

8. TERMO DE RESPONSABILIDADE

Eu, Inês João da Silva Chora, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsinquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 21 / fevereiro / 2013

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

Inês Chora

O Investigador Principal

2013.03.22

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO

emitido na reunião plenária da CES de ___/___/___

Aguarda que forma seja satisfeita o esclarecimento prestado pelo investigador

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

2013.04.22