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Identification of predictive risk factors for peripheral microvascular complications in patients with Raynaud's Phenomenon

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IDENTIFICATION OF PREDICTIVE RISK FACTORS FOR PERIPHERAL MICROVASCULAR COMPLICATIONS IN PATIENTS WITH RAYNAUD'S PHENOMENON

PhD thesis, Instituto de Ciências Biomédicas Abel Salazar – University of Oporto

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IDENTIFICATION OF PREDICTIVE RISK FACTORS FOR PERIPHERAL MICROVASCULAR COMPLICATIONS IN PATIENTS WITH RAYNAUD'S PHENOMENON

Tese de Candidatura ao grau de Doutor em Ciências Médicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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"…we are all subjects of Raynaud Phenomenon to a greater or lesser degree"

J. Hutchinson 1901

To my family

Filipe, my greatest love and encourager
Heitor, for his unfailing support, patience and love
Matilde e Manuel, to whom I owe almost all

together we made it possible

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PREFACE

In 2002, Prof. Doutor Mário Caetano Pereira and Prof. Doutor Carlos Vasconcelos created a multidisciplinary Clinics in Clinical Immunology Unit to follow patients with autoimmune diseases at high risk of developing peripheral vascular complications – Consulta Multidisciplinar de Raynaud. I was invited to participate and as so, visited Royal Free Hospital and Prof. Carol Black Department. A similar multidisciplinary model was started in our Hospital.

As a Raynaud subject, I was always sensible to the suffering of my patients, in particular secondary RP patients with systemic sclerosis. These patients, mostly women in childbearing age, dealt with great difficulties in managing their daily activities and in many cases could not even take care of their children due to the severe digital ulcers. Additionally they had great absenteeism from work, a high impact in quality of life with all the huge negative economic and social consequences.

Over the years, my greatest concern was how could I identify the patients that underlie microangiopathy that inevitably would progress to severe digital ulcers. The greatest challenge was that digital ulcers are not associated neither to severity or progression of underlying disease.

With a strong institutional and patients commitment I began this 3-year long journey. This thesis is the report of this extensive process. It cannot express the long days spent in the clinics, battling to conciliate clinical practice with research, fighting the financial constraints, the hope for good results and the sadness and tiredness with each failed attempt.

I wrote this thesis with the aim to help to clarify what I have perceived as a critical gap in understanding the vascular disease underlying severe ischemic peripheral disease and to use these data in the whole process of prevention and care, as the basis for delivering quality of life to all my patients.

It has been an outstanding and extremely rewarding journey.

ACKNOWLEDGEMENTS

This was the longer, loneliest and most challenging journey of my quarter of century professional life. Throughout the time the work presented here was held, and even before it begun, many contributed to help me achieve it. There are so many people to whom I should thank, but that, I'll have to do summarily.

First and foremost I thank my supervisor, Professor Carlos Vasconcelos for the continuous unconditional support throughout my thesis. I also thank for your confidence and fruitful discussions, important in helping me to accomplish this task. You have shared my thoughts, doubts, disappointments, discouragements, risks and enthusiasms. You have shown your commitment. My gratitude can hardly be expressed in words. It will be between us.

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To Dr. José Carlos Oliveira. I will be forever grateful for your huge collaboration in the evaluation of the biomarkers, your patience, rigor, perseverance, affection, support and endless hours spend with this research. I am also grateful to Dra. Fernanda Bravo and to all the staff of Clinical Chemistry in Clinical Pathology Department, who always attended me with a smile and an encouragement word.

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To Pedro, my colleague and friend, my deepest gratitude for your friendship and for your supportive words. When we feel insecure, those words are precious. Thanks for your partnership and making it possible. Together we will win more challenges.

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To Dr. Carlos Salgueiro and to Actelion for all the bibliographic support. It was precious for the outcome of this work.

To all colleagues in my department and in special to all residents. Without you, it would not have been possible and I will be here to support you if you decide to follow a course like this. Thanks to all who shared with me the effort and dreams. My journey is also a construction made of friendships, challenges, shares, and you all are part of it.

To colleagues in Clinical immunology Unit. I have learned a lot with you. Thanks for transmitting happiness, joy, supporting and encouraging my work and catching my interest in this challenging interest. I must point out the special involvement of some colleagues who gave me important steps, namely: Isabel Almeida with whom I began this Raynaud journey 13 years ago, partner in a parallel investigational route and to António Marinho as a good friend, confident, partner and supporter.

To all nurses in my department, in particular Enf. Salomé and Chief Nurse Enf. Paulo. I could always count with your big effort and friendship, sharing with you my enthusiasm, my distress, and my doubts. To each of you, my dearest warm acknowledgements.

My greatest thanks to all my Raynaud patients that I have seen at the Multidisciplinary Consulta de Raynaud – Clinical Immunology Unit – Centro Hospitalar do Porto. Your contribution to this work was determinant. I am grateful for your trust, support and willingness to help research.

To Mónica Duque, my deepest thanks for your friendship, commitment and support. We were always together in this road of challenge. We both know it.

To all my friends, a special word of thanks for their patience, friendship and encouragement.

To my family, the expression of my love and deepest proud for your trust.

In the end, I believe I did a good job.

ABSTRACT

Background

Raynaud's phenomenon (RP) is a common, well-characterized clinical disorder characterized by recurrent episodes of digital artery vasospasm triggered by exposure to cold or emotional stress. RP can be classified as primary (idiopathic; PRP) or secondary (SRP) when associated with several diseases or conditions. The pathogenesis of RP is still not entirely understood; recent insights into the pathogenic mechanisms underlying RP, particularly vascular, neuronal, and intravascular abnormalities, might help identify crucial key points and potential targets for early therapeutic interventions.

Ninety-five percent of patients with systemic sclerosis (SSc), 20-30% of those with systemic lupus erythematous (SLE), 20-30% of those with Sjögren's syndrome, and 25% of those with myositis will have RP as the first clinical manifestation of the disease. The co-existence of RP, positive antinuclear antibody (ANA), and microvascular abnormalities diagnosed by nailfold videocapillaroscopy (NVC) is a core diagnostic triad of associated autoimmune connective tissue disease (CTD). Koenig et al. demonstrated in a prospective study of 586 patients, followed up for 3,197 person-years, that the incidence of progression of isolated RP to definite SSc was 12.6% and 13.6% to other CTDs.

Ischemic digital ulcers (DUs) are a true burden for all patients with SRP and, in particular, SSc patients. DUs are very painful, with prolonged and slow healing, have a high risk of infection, and are extremely disabling, with severe impairment of simple daily activities. In SSc patients, ~30% of the patients per year develop ischemic digital tip ulcerations, 35-60% of SSc patients will experience at least one ulcer in the course of the disease, and about 75% of the affected patients have their first DU episode within 5 years of their first non-Raynaud symptom. Data from the French registry identified 44% of SSc patients as having one or more ischemic DUs, resulting in hospitalization for 33% of cases, with 46% requiring systemic antibiotics; 31–71% of patients have recurrent DUs.

There is an unmet need to properly measure vascular disease activity in SRP patients. Currently available instruments still have many limitations. It could be challenging to identify simple, applicable clinical diagnostic tools to allow the early identification of RP patients at high risk for peripheral ischemic vascular complications and thus enable early preventative target therapy.

Objectives

Measures of disease activity can be used to describe and compare study populations and identify potential risk factors for microangiopathy. Given that research methods in RP and DU are rapidly advancing, they could be used to

determine eligibility for diagnosis and management and as a tool to evaluate outcomes.

The main objectives of this thesis were: i) identification of risk factors for DU in patients with RP, and ii) to evaluate SSc patients' risk of developing a first episode of DU or to have a recurrence of DU during a 3-year-long follow-up period.

Methods

This research study was designed to provide an analysis of the clinical characteristics, endothelial dysfunction parameters, and vascular biomarkers in RP patients. An effort was made to consolidate knowledge and contribute to the discussion of the contradictory findings described in the literature.

A prospective observational cohort study with a 3-year clinical follow-up was conducted to evaluate 109 selected patients that attended the Multidisciplinary Raynaud Outpatient Clinic of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal (77 SSc patients and 32 with primary RP). Thirty-four control patients (healthy, sex/age matched, non-obese, and without self-reported cardiovascular risk factors) participated in the study.

SSc patients were divided into two groups: the DU group, which included 38 patients with an active ulcer at the beginning of our follow-up study, with or without a past history of DU; and a DU-naïve group that included 39 patients with no history of DU until enrolment.

The following were assessed in all patients and controls: clinical and demographic parameters, Allen test, flow-mediated dilatation (FMD), NVC, autoantibody screening and endothelial dysfunction, and angiogenesis vascular biomarkers. When included in the study cohort, patients were instructed to come to the hospital clinics whenever a new digital tropic lesion occurred. If no DU developed, patients were seen on a regular basis at 3- to 6-month intervals, as indicated by disease severity. Final observations were in the fourth trimester of 2014. We lost no patients during the entire course of our follow-up period.

The primary outcome was the occurrence of at least one or more new ischemic fingertip DUs in the 3-year follow-up period. In addition, we applied survival analysis to new occurrences of DU during the study period, regarding endothelium dysfunction (FMD, endothelin-1 (ET-1), and asymmetric dimethylarginine (ADMA)), angiogenic biomarkers (vascular endothelial growth factor (VEGF), endostatin, and endoglin), and microvascular damage (NVC and microangiopathy evolution score (MES)).

The institutional ethical review board of Centro Hospitalar do Porto approved this study. All subjects signed informed consent forms before inclusion in the study. Data were collected by analysis of clinical file data and by clinical interview.

Results

This is the first study that demonstrates that both endothelial dysfunction and an angiogenic stimulus are present in PRP. Clearly, our findings suggest that endothelial dysfunction suggested by increased serum levels of ET-1 and a pro-angiogenic state due to increased serum levels of VEGF might be triggered by the repeated vasospasms; however, when comparing PRP and SRP associated with SSc but without DU, no major differences were found regarding the vascular biomarkers investigated. We show that severe obliterative peripheral vasculopathy is present only in SRP patients with DU as expressed by increased peripheral resistance, low FMD response to shear stress, decreased peak systolic velocity (PSV) and end diastolic velocity (EDV), and high resistive índex (RI), mostly due to endothelial cell injury with endothelial dysfunction associated with impaired angiogenesis.

Regarding risk factors for DU in RP patients, we identified that FMD, PSV, and EDV were significantly lower in patients with DU than in SSc non-DU, PRP, and control patients. Regarding qualitative NVC patterns, FMD levels were statistically significantly different between groups. FMD, PSV, and EDV were significantly lower in the late pattern than in active and early patterns; no differences were found between active and early patterns. We observed successive decreases of PSV and EDV and increased vascular resistance, measured by resistive index (RI), with progression of the microvascular damage diagnosed by NVC. Regarding vascular biomarkers, we report high serum levels of ET-1 and ADMA, low serum levels of VEGF, and increased endoglin serum as independent risk factors of DU in patients.

We identified the following as potential predictive factors of new ischemic DU episodes in SRP: history of at least one DU before enrolment in the study, anti-scleroderma-70 autoantibodies, telangiectasia, NVC late pattern, high MES score, low FMD%, a positive Allen test, and increased ET-1 serum levels. Multivariate Cox analysis confirmed increased MES score, low FMD%, increased ET-1, and ADMA serum levels as independent predictors of the development of a new DU. With respect to angiogenic biomarkers, only VEGF was identified as a predictive risk factor for the occurrence of at least one new DU during the three-year follow-up.

Patients with FMD levels ≤ 9.5% (*p=0.001*), ET-1 serum levels > than 11.9 pmol/ml (*p<0.001*), ADMA serum levels > than 0.49 µmol/l (*p=0.075*), and MES scores greater than 2 (*p<0.001*) had significantly more new DUs in our 3-year survival analysis. Low VEGF serum levels < 422.47 pg/ml had significantly more DUs (*p=0.028*).

Our study identified FMD > 9.41% (HR: 0.37 95%CI: 0.14-0.99), ET-1 >11.85 pmol*/L* (HR: 3.81 95%CI: 1.41-10.26), and late NVC pattern (HR: 2.29 95%CI 0.97-5.38) as predictors of DU recurrence in the 3-year follow-up and when estimating the probability of occurrence of the first DU in DU-naïve patients; only late NVC pattern (HR: 12.66 95%CI: 2.06-77.89) was an independent predictor.

Conclusions

In our investigation, we confirmed that macrovascular disease, endothelium-dependent flow-mediated dilation, ET-1, microvascular damage detected in capillaroscopy, and the angiogenic biomarker VEGF are risk factors for DU events.

Late NVC pattern was the most robust predictor of new DU episodes, recurrent or the first event. Additionally, endothelial dysfunction parameters were strong predictors of DU recurrence.

RESUMO

Introdução

O fenômeno de Raynaud (FR) é uma patologia clínica comum, caracterizada por episódios recorrentes de vasospasmo das artérias digitais desencadeados pela exposição ao frio ou pelo stress emocional. O FR pode ser classificado como sendo primário (FRP) idiopático ou secundário (FRS) se associado a outras patologias ou condições. A patogénese do FR não é ainda totalmente conhecida. Os recentes avanços sobre os mecanismos patogénicos subjacentes ao FR, em particular os vasculares, neuronais e intravasculares, permitem ajudar a conhecer melhor a etiopatogenia, permitindo uma melhor identificação dos potenciais alvos para uma intervenção terapêutica precoce.

Tem sido descrito na literatura que 95% dos doentes com esclerose sistémica (ES), 20-30% com lúpus eritematoso sistémico (LES), 20-30% com S. Sjogren e 25% com miosite terão como primeira manifestação clínica da doença o FR. A tríade FR, anticorpo antinuclear positivo (ANA) e alterações microvasculares diagnosticados por videocapilaroscopia periungueal (NVC) são fundamentais para o diagnóstico de doença autoimune associada. Koenig et al demonstraram num estudo prospectivo que nos 586 pacientes com FR seguidos durante 3.197 pessoas-ano, a incidência da progressão do FR isolado para ES foi de 12,6%, e 13,6% evoluíram para outras doenças do tecido conjuntivo.

As úlceras digitais isquémicas (UD) são extremamente incapacitantes estando sobretudo presentes nos doentes com ES. São muito dolorosas, com tempo de cicatrização prolongado e lento, têm um alto risco de infecção e são comprometedoras de uma forma grave da qualidade de vida dos doentes. Aproximadamente ~30% / ano dos doentes com ES desenvolvem UD. Durante a evolução da sua doença 35-60% dos doentes vão ter pelo menos um episódio de UD sendo que em 75% o seu primeiro episódio UD ocorre nos primeiros 5 anos após a primeira manifestação de FR.

Os dados do Registro francês demostraram que 44% dos doentes com ES tiveram um ou mais episódios de UD, de que resultou hospitalização em 33% dos casos e 46% exigiu antibióticos sistêmicos. A recorrência das UD é elevada na ES variando entre os 31 aos 71%.

Há pouca informação sobre a avaliação e medição da doença vascular nos doentes com FRS. Os instrumentos atualmente disponíveis tem ainda muitas limitações. Como tal constitui um desafio a identificação de ferramentas de diagnóstico clínicos simples, capazes de serem aplicada no dia-a-dia de forma a permitir a identificação precoce de doentes com FR em risco de desenvolver

complicações vasculares isquémicas periféricas permitindo a instituição de uma terapêutica- alvo preventiva precoce.

Objetivos

A quantificação da doença vascular pode ser usada para descrever e comparar populações de estudos, como método de diagnóstico, permitir identificar os factores de risco potenciais para as complicações microangiopáticas e servir como ferramenta de medida na avaliação do resultado de novos tratamentos.

Os principais objectivos desta tese foram: i) identificação de fatores de risco para UD em doentes com FR; ii) avaliar o valor preditivo de marcadores vasculares nos doentes com FRS com ES de desenvolver um primeiro episódio de UD ou ter uma recorrência de UD ao longo de um período de seguimento de 3 anos.

Métodos

Este estudo foi desenhado para avaliar as características clínicas, os parâmetros de disfunção endotelial e os biomarcadores vasculares em doentes com FR. Foi nosso objectivo consolidar o conhecimento atual e contribuir para a discussão de resultados contraditórios descritos na literatura.

Realizamos um estudo de coorte observacional, prospectivo, com um seguimento clínico de 3 anos de 109 doentes (77 doentes com FRS e ES e 32 com FRP) da Consulta Multidisciplinar Raynaud da Unidade de Imunologia Clínica do Centro Hospitalar do Porto, em Portugal. Trinta e quatro indivíduos saudáveis, pareados por sexo e idade, não-obesos, sem factores de risco cardiovascular foram convidados a participar como grupo controle.

Os doentes com FRS e ES foram divididos em dois grupos: grupo UD (38 doentes com uma úlcera ativa no início do nosso estudo) e grupo não-UD (39 doentes sem história de UD durante toda a evolução da sua doença até á inclusão no estudo).

Foram analisados parâmetros clínicos e demográficos, avalaida a doença macrovascular com o teste de Allen e a fluxo mediada pelo dilatação (FMD), doença microvascular através da videocapilaroscopia periungueal (NVC), foi feita a pesquisa de autoanticorpos e medidos os biomarcadores de doença vascular de disfunção endotelial (endotelina-1-[ET-1] e dimetilarginina assimétrica- [ADMA]) e de angiogênese (fator de crescimento endotelial vascular- [VEGF], endostatina e endoglina) em todos os doentes e no grupo controle. Os doente foram orientados no sentido de recorrem ao hospital sempre que uma nova lesão vascular

periférica se desenvolvesse e no caso de não ocorrerem episódios de UD os doentes eram avaliados numa base regular com intervalos de 3-6 meses, conforme indicado pela gravidade da doença. A última observação foi no último trimestre de 2014. Não se perderam doentes no seguimento.

O "*endpoint*" primário foi a ocorrência de pelo menos uma ou mais UD isquêmicas no período de acompanhamento de 3 anos. Além disso, aplicamos os testes de análise de sobrevivência, para avaliar a ocorrência de novas UD durante o período do estudo, aos biomarcadores vasculares: i) disfunção endotelial (FMD, ET-1 e ADMA, ii) biomarcadores angiogênicos (VEGF, endostatina e endoglina) e iii) lesão microvascular pela analise qualitativa e semi-quantitativa na capilaroscopia.

A Comissão de Ética institucional do Centro Hospitalar do Porto aprovou este estudo. Todos os sujeitos assinaram o consentimento informado antes da inclusão no estudo. Os dados foram colhidos por consulta de processo clínico e por entrevista clínica.

Resultados

Do que nos é permitido saber, este é o primeiro estudo a demonstrar que a disfunção endotelial e o estímulo angiogénico estão simultaneamente presentes nos doentes com FRP. Estes dados foram demonstradas pelo aumento dos níveis séricos de ET-1 e de VEGF. No entanto, quando comparamos os doentes com FRP com os FRS sem UD não encontramos nenhuma diferença significativa no que respeita aos biomarcadores vasculares investigados. Assim, um dado novo e útil desta investigação é que vasculopatia periférica obliterante grave só está presente nos doentes com FRS e ES com UD. Estes dados são confirmados pelo aumento da resistência periférica, baixa resposta ao "*shear stress*" com índices de resistência elevados, tradutores da lesão da célula endotelial e uma angiogenese ineficaz.

Como fatores de risco para UD identificamos o FMD e a velocidade diastólica final (EDV), dado estarem significativamente dimuidos nos doentes com UD comparados com restantes grupos. Avaliando o FMD nos diferentes padrões qualitativos da capilaroscopia foram encontradas diferenças estatísticas entre os grupos. O FMD e EDV eram significativamente menores no padrão tardio em relação ao padrão ativo e precoce. Não há diferenças significativas entre os padrões ativos e precoce. Com a progressão do dano microvascular observou-se um aumento da resistência vascular periférica. No que respeita aos biomarcadores vasculares, verificamos que níveis aumentados de ET-1, ADMA e

de endoglina e a diminuição do estimulo angiogénico motivada por baixos níveis séricos de VEGF eram como fatores de risco independentes de UD.

Foi nosso objetivo determinar os fatores preditivos de aparecimento de novos episódios de UD nos doentes com FRS. A história de pelo menos uma UD prévia á inclusão no estudo, a presença de auto-anticorpos anti-esclerodermia-70 e de telangiectasias, o padrão tardio na capilaroscopia, uma resposta diminuída ao "*shear stress*", um teste de Allen positivo e os níveis séricos de ET-1 aumentada foram identificados com factores preditivos de novos episódios de UD. A análise multivariada Cox confirmou que a lesão microvascular avançada, a resposte reduzida de FMD%, o aumento dos níveis séricos de ET-1 e de ADMA como fatores preditivos independentes do desenvolvimento de uma nova UD. Relativamente aos biomarcadores angiogénicos, apenas o VEGF foi identificado como tendo valor preditivo para a ocorrência de pelo menos uma nova UD nos 3 anos de seguimento clinico.

Os doentes com FMD% ≤ 9,5% (*p = 0,001*), nível sérico de ET-1 >11,9pmol / ml (*p <0,001*), de ADMA> de 0,49 pmol / l (*p = 0,075*) de VEGF <422,47 pg / ml (*p = 0,028*).e a lesão microvascular com pontuação superior a 2 (*p <0,001*) apresentaram significativamente mais novas UD na análise de sobrevivência aos 3 anos.

O nosso estudo identificou o FMD% > 9,41% (HR: 0,37 IC 95%: 0,14-0,99); ET-1> 11,85 pmol / L (HR: 3,81 IC 95%: 1,41-10,26) e tardia padrão NVC (HR: 2,29 IC 95% 0,97-5,38) como preditivos da recorrência de UD. O padrão tardio na capilaroscopia (HR: 12.66 CI 95%: 2,06-77,89) foi o único factor preditivo de aparecimento da primeira UD nos doentes incluídos sem histórico de complicações vasculares periféricas.

Conclusões

A nossa investigação confirmou que a doença macrovascular, o FMD endotélio-dependente, os níveis séricos de ET-1 e de VEGF e a lesão microvascular são factores de risco para o desenvolvimento de UD:

O padrão tardio na capilaroscopia é o factor preditivo com maior peso quer na recorrência quer no aparecimento de novos episódios de UD. Na recorrência de UD a disfunção endotelial desempenha um papel fulcral.

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ABBREVIATIONS

ACA	Anti centromere autoantibody
ACE	Angiotensin-converting enzymes
ACR	American College of Rheumathology;
ADMA	Asymmetric dimethyarginine
ANA	Antinuclear autoantibodies
Ang-1	Angiopoietin 1
Ang-2	Angiopoietin 2
ANGPTL3	Angiopoetin-like Protein 3
AUC	Area under the curve
AVAs	Arteriovenous anastomoses
CI	Confidence interval
CRP	C reactive protein
CSURI	Capillaroscopic Skin Ulcer Risk Index
dcSSc	Diffuse systemic sclerosis
DDAH	dimethylarginine dimethylamino- hydrolase
DU	Digital ulcers
EC	Endothelial cell
ECM	extracellular matrix
EDHF	endothelium derived hyperpolarising factor
EDV	End diastolic velocity
ENG	Endoglin
EPC	Endothelial progenitor cells
ESR	Erythrosedimentation rate
ET	Endothelin
EULAR	European League against Rheumatism
EUSTAR	Eular scleroderma trials and research
FMD	Flow mediated dilation
HR	Hazard ratio
lcSSc	Limited systemic sclerosis
Medgers DSS	Medgers disease severity score
MES	Microangiopathy evolution score
NA	Non applicable
NO	Nitric oxide
NOS	Nitric oxide synthase
NVC	Nailfold videocapillaroscopy
OR	Odds ratio
PRP	Primary Raynaud phenomenon
PSV	Peak systolic velocity
Q	Quartile
RI	Resistive Index
ROC	Receiver operating characteristic
RP	Raynaud phenomenon
SD	Standard deviation
SRP	Secondary Raynaud phenomenon
SSc	Systemic sclerosis
VEGF	Vascular endothelial growth factor
vSMC	Vascular smooth muscle cells

OUTLINE AND OBJECTIVES

In Portugal, Centro Hospitalar do Porto was a pioneer hospital by creating the Multidisciplinary Raynaud Clinic following Royal´s Free Hospital model. We have had an active role in this clinic as responsible for the evaluation of Raynaud patients with severe peripheral vasculopathy. We focused in the evaluation of SRP with vascular complications, in particular patients with systemic sclerosis (SSc), and this work was the structural basis of the main objectives of this thesis. In order to compare patients with similar disease, we have excluded from this study patients with SRP and other autoimmune disease beyond SSc.

The main objectives of this thesis are:

1. to study endothelial dysfunction, microvascular damage, angiogenesis and vascular biomarkers in patients with RP.
2. to identify risk factors for digital ulcers in patients with RP.
3. to determine potential predictive factors of appearance of new episodes of ischemic digital ulcers in secondary Raynaud patients.

The first chapter focuses on the thesis rationale and research questions.

The second chapter provides two Prisma-driven systemic reviews on main topics of the thesis: RP and predictive risk factors of DU in SRP – SSc associated patients. We also review the vascular pathology highlighting the role of endothelial dysfunction, microvascular damage, angiogenesis and vascular biomarkers in patients with RP.

The third chapter describes patients and methodology.

The fourth chapter displays the research results:

1. Clinical assessment and characterization of our cohort in the beginning of the study.
2. Microvascular damage, endothelial dysfunction and ischemic peripheral vasculopathy in secondary RP.
3. Description of the clinical correlation of endothelial dependent flow-mediated dilatation and capillaroscopic findings, using two different classifications qualitative “scleroderma pattern” and semi-quantitative

"microangiopathy evolution score", with digital ulcers in patients with Secondary RP; comparison of these parameters between primary and secondary Raynaud patients and comparison with control group.

4. Description of impaired angiogenesis as a feature of digital ulcers in systemic sclerosis.
5. Identification of the predictive value of vascular disease biomarkers for digital ulcers in systemic sclerosis patients.
6. Risk factors for peripheral vasculopathy in systemic sclerosis patients.

The fifth chapter summarizes and discusses the results, limitations of the study, conclusions and suggestions for further research.

SCIENTIFIC AND CLINICAL OUTPUTS

PUBLICATIONS

1. FULL TEXT MANUSCRIPTS

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Published in Angiologia e Cirurgia Vascular 03/2011; 7(1):13-20. Indexed to SciELO Citation Index da Web of Knowledge ISSN: 1646-706X and Latindex.

2014 **"A PRISMA-Driven Systematic Review For Predictive Risk Factors Of Digital Ulcers In Systemic Sclerosis Patients"**
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Published in Autoimmun Rev. 2015 Feb;14(2):140-52. doi: 10.1016/j.autrev.2014.10.009.

2015 **"Endothelial dysfunction and nailfold videocapillaroscopy pattern as predictors of digital ulcers in systemic sclerosis: a cohort study and review of the literature"**
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2015 **"Digital ulcers in Systemic Sclerosis: role of flow-mediated dilatation and capillaroscopy as risk assessment tools"**
Silva I, Loureiro T, Teixeira A, Almeida I, Mansilha A, Vasconcelos C:
Accepted in European Journal of Dermatology

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2015 **"Impaired angiogenesis as a feature of digital ulcers in systemic sclerosis"**
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Silva I, Teixeira A, Oliveira J, Almeida I, Almeida R, Vasconcelos C
Submitted

2015 **"Raynaud Phenomenon"**
I Silva, G Teixeira, M Bertão, R Almeida, C Vasconcelos
Submitted

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Silva I, Teixeira A, Oliveira J, Almeida I, Almeida R, Vasconcelos C
Submitted

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Ivone Silva
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4. PRIZES

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CHAPTER 1
INTRODUCTION

1.1 RATIONALE

Raynaud's phenomenon (RP) generally manifests as an exaggerated vasoconstriction of the cutaneous vascular system in response to cold exposure (1). Primary RP (PRP) is an uncomfortable but ultimately benign condition that is mainly caused by an altered functional response of the cutaneous circulation (2, 3). There is a progressive pathological remodeling within the microvasculature, macrovasculature, and extravascular compartments, resulting in substantial tissue pathology and injury (2). A disorder of the vascular thermoregulatory control mechanisms is believed to be the main cause of the vasospastic attacks (1). Although these can be initiated by reflex activation of the sympathetic adrenergic vasoconstrictor system, e.g., in response to stress, they are mainly due to local cold exposure (1).

RP is a common clinical manifestation with a prevalence of 3-5% in the general population (4). The mean age of affected patients ranges from 47.2 years in Europe (5) to 53.5 years in the US (6). An annual incidence of 0.25% in the general population was reported in France, whereas in the US (Framingham offspring cohort study), RP occurred in 2.2% of women and 1.5% of men over 7 years (2). RP has a large geographic variation due to climatic variations, being more frequent in cold and wet climates. It's generally agreed that hormonal factors can play a role in the genesis of the phenomenon, as indicated by the increased incidence of RP-like vasospastic reactions during the pre-ovulatory period and estrogen induction of endothelial dysfunction in secondary RP (SRP); these hormonal factors may thus account for the higher prevalence of RP in women (7). The role of genetic factors is still unclear, although a polygenic predisposition is suggested by studies of familial groups and by the different prevalence of RP among various ethnic groups (8).

Secondary Raynaud phenomenon (SRP) is commonly associated with autoimmune conditions, such as systemic sclerosis (SSc). Cutolo et al. reported that 14.6% of 129 patients with PRP developed abnormal capillary patterns over a mean period of 29.4 months, allowing early identification of the patients most likely to develop a SSc-spectrum disorder (9).

RP, in the context of SSc, frequently progresses to digital ulceration and/or critical digital ischemia in areas of skin with microangiopathy. Digital ulcers (DUs) are frequent among patients with SSc, with 15%–25% having active DU (10) and at least 40–50 % of all patients with SSc experiencing one or more DU at some point in their disease course (11).

DUs are painful, slow to heal, frequently complicated by secondary infections, and have disabling effects on patients, particularly regarding grip, feeding, dressing, and hand hygiene (12). Severe DU episodes frequently need hospitalization and require time away from the workplace (13). Data from the French registry identified 44% of patients as having one or more ischemic DUs, resulting in hospitalization for 33% of cases; 46% required systemic antibiotics (13).

The etiology of DU is multifactorial and may differ depending on DU localization. Fingertip DUs are attributable not only to the underlying vasculopathy but also the persistent vasospasm of RP (14). Instead, DUs on the dorsal aspect of the fingers are, in the majority of cases, due to epidermal thinning and cutaneous retraction, leading to cracks on the skin overlying the joints (15). DUs may develop on the fingers or toes and can occur over the extensor surface of the joint, on the finger creases, under the nails, and, in the majority of cases, on the fingertips. DUs may also develop from a pre-existing calcinosis and sometimes from digital pitting scars (15).

Although new insights into the management of DU have emerged, it still remains challenging to identify patients at risk of developing DUs. Several reports identified vascular biomarkers and capillaroscopic indexes as predictive of new DUs; however, these tools are very expensive and not applicable for routine use. Still, these research studies are fundamental, as the molecular analysis of the vascular mediators associated with SSc and particularly with peripheral microangiopathy may identify new therapeutic targets that lead to the prevention of further vascular injury or the improvement of SSc disease.

1.2 OBJECTIVES

1. To explore endothelial dysfunction in the pathogenesis of peripheral vasculopathy in RP patients
    a. Research question:
        i. Is endothelium-dependent flow-mediated dilatation an early diagnostic tool for severe peripheral vascular complications in patients with RP and what is its predictive value?
2. To analyse the role of capillaroscopy abnormalities in peripheral vasculopathy
    a. Research question:
        i. Can a qualitative “scleroderma pattern” or semi-quantitative “microangiopathy evolution score” identify patients at risk for DU and predict new episodes?
3. To evaluate the role of vascular biomarkers in predicting DU in SSc patients
    a. Research question:
        i. Can serum levels of endothelial dysfunction (*ET-1 and ADMA*) and angiogenic (*VEGF, endostatin, and endoglin*) biomarkers identify patients at risk and/or predict the occurrence of new DUs?

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CHAPTER 2
BACKGROUND

Manuscript submitted

Raynaud Phenomenon

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2.1 RAYNAUD PHENOMENON

ABSTRACT

Raynaud's phenomenon is a well common clinical disorder characterized by recurrent vasospasm episodes of digital arteries, arterioles, pre-capillary and post-capillary venules triggered by exposure to cold or emotional stress. RP can be classified as primary (idiopathic), or secondary to several diseases or conditions. The pathogenesis of RP is still not entirely clear or understood, but recent insights into the pathogenic mechanisms underlying Raynaud's phenomenon include vascular, neuronal and intravascular abnormalities, which may identify crucial key points and potential targets for therapeutic intervention.

In this review we summarize the epidemiology, pathogenesis, clinical manifestations and assessments as well as the recent advances in therapeutic approaches.

An extensive review of recent literature was conducted, according to the guidelines proposed at the PRISMA statement. MEDLINE database (PubMed) and Thomson Reuters Web of Knowledge platform were searched for articles published in peer-reviewed journals with the last search run on February 2015 and published in English language. The keyword search terms included: Raynaud phenomenon, epidemiology, pathogenesis, thermoregulation, endothelium dysfunction, risk factors, clinical features, nailfold videocapillaroscopy treatment.

Inclusion criteria were: (1) Randomized controlled trials; (2) Reviews, systematic reviews and meta-analysis of randomized controlled trials (3) English language. Studies lacking clinical end points of relief, case reports and open label trials were excluded.

Keywords

Raynaud phenomenon / etiology / physiopathology / complications / diagnosis / drug therapy

INTRODUCTION

Maurice Raynaud first described Raynaud's phenomenon (RP) in 1862. By definition RP are bouts of reversible vasospastic ischemia of the extremities which most frequently involves hands and toes, less frequently nose and earlobes, and very rarely tongue (1, 2). Episodic color changes of the fingers classically turn into white (ischemia), blue (cyanosis) and red (reperfusion), but the three color stages are not needed to be present RP diagnosis (3, 4). When a patient presents with RP, the clinician must diagnose whether RP is primary or secondary, due to different clinical significance, treatment and prognosis.

Primary RP (PRP), also known as Raynaud's disease, is a functional vascular disorder that occurs as a sporadic exaggerated response to cold or emotional stress. PRP does not progress to irreversible tissue injury (5, 6) and has usually a benign clinical course (3). Secondary RP (SRP), also known as Raynaud's Syndrome, appears in response to the same triggers, however it occurs in the setting of an underlying structural vascular disease and is often associated with digital ulcers (DU), scarring or gangrene (3).

EPIDEMIOLOGY

The real prevalence of RP varies between studies mostly due to different geographic conditions such as climate. PRP is a common condition, with a prevalence of 3-5% in the general population (2). In a systematic review and meta-analysis based on six studies assessing the general population, the lowest prevalence of PRP was found in Japan, with an overall prevalence of 1,6 (2,1% in women *vs.* 1,1% in men) and the highest overall prevalence was in the USA with a median prevalence of 7,5% (7,8% in women *vs.* 5,8% in men) (7). Onset age is usually below 40 years and a family history of RP may be present (3). By contrast, SRP is much rarer in the general population, but has a high prevalence among patients with connective tissue diseases such as Systemic Sclerosis (SSc) (90%), systemic lupus erythematous (30%), rheumatoid arthritis (20%), Sjögren's syndrome and polymiositis (8, 9). SRP is an important red flag that raises suspicion for underlying very early SSc (10, 11).

Recent reports alert that RP can be considered a sign or precursor of undiagnosed vascular disease. The presence of RP among whites was associated with 1,6 fold increase in the hazard cardiovascular disease (CDV)-related death (12). Risk factors of RP are complex and not consensual. Some studies suggested that smoking increased risk of RP in male, but heavy alcohol consumption and hormonal factors increased risk only in women (2, 13). The contribution of genetic factors is not clear, however familiar aggregation and some rare

syndromes such as hereditary angiopathy with nephropathy, aneurysms, and muscle cramps HANAC syndrome suggest a polygenic etiology (2).

RAYNAUD PATHOGENESIS

Pathogenesis of RP is undoubtedly complex, once both central and 'local' mechanisms are involved. Vascular, neural and intravascular abnormalities can impair blood flow and/or cause endothelial injury. The blood vessel wall (endothelium), the neural control of the vascular tone (deficiency of the vasodilator calcitonin gene-related peptide, α_{2}-adrenoreceptors activation) and many circulating factors (activated platelets, impaired fibrinolysis, increased viscosity, oxidant stress) all together play a role in the pathogenesis of RP (14).

During body cooling or ambient temperature reduction, blood flow falls to markedly low levels in patients with RP and in SRP individuals it can fall to absolute zero (15). In response to local finger cooling patients with RP demonstrate increased sensitivity to cold with more severe and prolonged blood flow reduction. Additionally with local warming blood flow recovery was higher in PRP (15) probably due to structural alterations in SRP.

Vascular mechanisms

Finger blood flow compromises both nutritional blood flow and through arteriovenous anastomoses (AVA). In control subjects total and AVA blood flow are reduced in colder environments but no changes in nutritional blood flow are observed. In contrast, nutritional and AVA blood flow is reduced in RP subjects, with a more marked reduction in nutritional blood flow in SRP SSc-associated patients (15).

The pallor phase of RP is due to cold induced vasoconstriction (arterial and venous vasospasm). Sympathetic neurotransmission can actually be more effective in cutaneous veins than in arteries, due to better penetration of nerve fibers in the low-pressure cutaneous veins. This leads to a direct release of norepinephrine smooth muscle cells in veins wall that have an extremely high activation of α_{2}-adrenoreceptors (α_{2} AR), making them highly responsible for to the local cold-induce amplification of sympathetic constriction (15).

The cyanotic phase is often attributed to local hypoxia. However, it is likely mediated by early vasodilation of venous cutaneous system with deoxygenated blood flow, while AVA remain constricted (15).

Hyperemic phase might be a consequence of delayed dilation of AVA allowing a large influx of fully oxygenated flow into the dilated venous system.

Functional abnormalities

RP occurs when the balance of vascular tone is disturbed, favoring vasoconstriction. Endothelial activation and/or damage lead to underproduction and efficacy of vasodilators and/or overproduction of vasoconstrictors. A complex and still not fully understood response to whole body and local cooling add a challenge to the unraveling pathogenesis of RP (5).

i) Vasodilatation

Doubts persist whether there is underproduction of endothelium vasodilators such as nitric oxide (NO) and prostacyclin, or whether they are impaired in RP (5). Further complicating the role of NO, patients with SSc, have increased plasma levels of an endogenous inhibitor of endothelial NOS—asymmetric dimethyl arginine— (ADMA) leading to reduced NO production (16). Endothelium-dependent vasodilation is preserved in PRP, while in SRP an impaired vasodilation occurs and worsens through disease progression. This might be due to endothelium dysfunction and to structural changes in blood vessels wall.

ii) Vasoconstriction

Injured endothelial cells (ECs) overproduce vasoconstrictors such as endothelin-1 (ET-1) and angiotensin II. Although some studies suggest a role of ET-1 in PRP, this evidence is much weaker than that for SRP (5). Also an imbalance in the renin–angiotensin system, favoring angiotensin II is thought to occur in SRP (5). New insights in the increased understanding of signal transduction pathways in vascular smooth muscle highlights the fact that 'vascular' and 'neural' mechanisms cannot be considered isolated (5, 17).

Structural abnormalities

Microvasculopathy with alterations in morphology and function can easily be detected at the nailfold bed with Nailfold Videocapillaroscopy (NVC). Structural morphological and functional changes of capillaries allow differentiation between PRP and SRP, as major structural abnormalities does not exist in PRP.

Architectural disorganization, as a result of microvascular injury, includes giant capillaries, microhemorrhages, capillary loss, avascular areas and morphological changes with branched/ramified/bushy capillaries suggesting angiogenesis. Homogenous enlarged loops (diameter >50 mm) are probably the first sign of microvascular disease (18) and should be valorised even if only a single giant capillary is present. When the capillary wall ruptures, leakage of red blood cells is observed as microhemorrhages. With disease progression,

increases capillary loss avascular areas reflect areas of hypoxia, and consequently greater risk of microvascular complications, namely digital ulcers. The response to hypoxia activates angiogenesis, resulting in branched/ramified/bushy capillaries, mostly ineffective in tissue perfusion.

Intravascular factors

Among intravascular factors, platelet activation has received the biggest attention, but defective fibrinolysis, increased thrombin generation, reduced red blood cell deformability, white blood cell activation and increased viscosity are all likely to have a role (5). Some mechanisms involved in oxidative stress could be considered 'intravascular', such as hypoxic–reperfusion injury and white blood cell activation. Oxidative stress can also provide a link between vascular and intravascular abnormalities and fibrosis (5).

Polycythemia and macrocryoglobulinemia are paradigmatic examples of how intravascular alteration may predispose to RP. Further factors have been studied: platelet activation released thromboxane A2, thromboglobulin, serotonin, platelet-derived microparticles, and platelet-derived growth factor; impaired fibrinolysis has been documented by the increased serum levels of tissue plasminogen activator and von Willebrand factor; oxidative stress results from repeated episodes of vasospasm followed by reperfusion which may contribute to endothelium damage through lipid peroxidation of endothelial cells membranes; red blood cell lipid membranes are another substrate of intravascular reactive oxygen species and associated to an increased peroxidation of membrane lipids; and leukocyte hyper-activation released substances which affect the microcirculation. The evidence for oxidative stress in PRP is much weaker than that in SRP (2, 5).

Neuronal

The autonomic nervous system regulates the vascular tone through a number of chemical substances (mediators), which trigger either **vasodilation** (calcitonin gene-related peptide, neurokinin A, substance P and vasoactive intestinal peptide) or **vasoconstriction** (adrenergic agonists and nerve growth factor) (19).

i) Local mechanisms

a) Impaired vasodilatation

Calcitonin gene-related peptide (CGRP) is a potent vasodilator produced by central and peripheral neurons, which binds a heterodimer receptor composed of

the G protein-coupled receptor (GPCR) referred as calcitonin receptor-like receptor and to the receptor activity modifying protein (RAMP-1). CGRP triggers the intracellular synthesis of adenylate cyclase in smooth muscle cells (SMC) that increases cyclic adenosine monophosphate (cAMP), leading to SMC relaxation (19). Immunoreactivity of calcitonin gene-related peptide is reduced in the skin of the patient's fingers with SSc and PRP, but this effect is more pronounced in SSc. Other vasodilators including substance P, neurokinin A and vasoactive intestinal peptide have been less well studied (5).

b) Vasoconstriction

Ensuring that blood flow to individual organs matches their individual needs is not achieved by central command (engaged in maintaining blood pressure and restricting blood flow– except brain and heart) but at the local level (17).

Skin circulation has two distinct types of sympathetic nerve fibers: (i) a traditional sympathetic adrenergic innervation, norepinephrine, that initiates cutaneous vasoconstriction by activating two distinct families of receptors on vascular smooth muscle cells (VSM), α_1 and α_2-adrenoceptors (AR) and (ii) a sympathetic cholinergic innervation, acetylcholine, that indices vasodilatation (17).

Cold causes a remarkable functional and spatial rescue of α_{2C}-AR from the *trans*Golgi to the cell surface, where they can respond to stimulation (20). Although α_{2C}-ARs are important effectors in the cutaneous vascular response to cooling, they do not appear to respond directly to cold and therefore should be considered 'thermoeffectors' rather than 'thermosensors'.

Immediately upon cooling, there is increased generation of reactive oxygen species (ROS) from VSM mitochondria in cutaneous arteries (20). This is the earliest detectable cold-induced response in cutaneous arteries, suggesting that the mitochondria may be the 'thermosensors' responsible for initiating cold-induced vasoconstriction in cutaneous blood vessels. These mitochondrial ROS do not appear to be pathogenic, but, as an alternative, activating novel REDOX signaling through the RhoA/Rho kinase (ROCK) pathway (21) that promotes cold-induced translocation of α_{2C}-AR to the cell surface. The most important byproducts of local cellular metabolism such as decreased O_2, increased CO_2 and metabolites such as adenosine can also suppress the activity of sympathetic nerves by inhibiting the release of norepinephrine and counter central command to decrease blood flow – functional sympatholysis (17). Under normal physiological conditions, this ongoing conflict between central command and

local organ requirements creates a highly efficient and effective control system (17).

ii) Central mechanisms

Despite the fact that many patients describe having RP in response to stress, the possible role of central nervous system is not well studied (5).

CLINICAL INVESTIGATION

Clinical assessment

Diagnosis of RP is based on the history of episodic bi- or triphasic color change of fingers or toes - pallor (ischemia), cyanosis (anoxia) and rubor (reactive hyperemia). Accompanying symptoms may be numbness and pain.

In a Delphi exercise round, 12 invited experts agreed recently in three-step outline for a newly proposed diagnostic method. Consensus achieved that at least biphasic color changes are required to make the diagnosis of RP. They also agreed that white/pallor and blue/cyanosis were the two most important colors to make a diagnosis and patients must report cold temperatures as one of the triggers for their RP attacks. Besides cold, other triggers (e.g. emotional stress) were deemed helpful but not required to make a diagnosis of RP. In addition, standardized questionnaires, photographs of episodes provided by patients, history of attacks at other sites other than the hands, numbness and paresthesia were thought to be helpful but not required to make a diagnosis of RP. Color charts, family history and a history of drug-induced or smoking induced attacks were not useful. The minimal number of attacks, duration time, a well demarcated color change and the requirement for a physician assessment did not meet agreement standards (6).

Clinical history and physical examination are very important in RP patient's evaluation. Investigation must include the RP onset, frequency, severity and duration of attacks.

Physical examination should evaluate peripheral pulses (exclude peripheral vascular disease), blood pressure in both arms (unequal blood pressure suggests proximal vascular stenosis/occlusion), seek for palpable cervical rib, associated signs of alopecia, altered skin texture, calcinosis or telangiectasia and trophic skin changes on tips of fingers and toes.

Recent advances in the diagnosis of RP have recognized that abnormalities in nailfold capillary pattern and specific autoantibodies are independent risk factors for CTD (5). One study reported that in 586 RP patients followed for 3,197

patients years, 1.8% with a normal capillary pattern and negative ANA, 25.8% with an abnormal capillary pattern, 5.4% with a specific autoantibody and in 79.5% of those with both an abnormal capillary pattern and an SSc-specific autoantibody developed SSc (5).

PRP onset is more common in the second or third decade, but it can present at any age. The classical criteria proposed by LeRoy and Medsger (22) were: (i) episodic attacks of acral pallor or cyanosis, usually symmetrical (usually affects both hands); (ii) strong symmetric peripheral pulses are present; (iii) no digital pitting scars, tissue necrosis, ulceration or gangrene is found; (iv) normal nailfold, erythrocyte sedimentation rate (ESR) and negative antinuclear antibody tests. Mean age of onset is 14 years and up to 27% have RP onset after the forties (23).

Regarding-PRP, round 2 of the Delphi exercise with expert´s panel, concluded that an age of onset less than or equal to 25 was helpful but not required to make a diagnosis. There was also strong agreement that normal capillaroscopy findings should be incorporated into the diagnostic requirements. This introduces some challenges, as there is a wide range of nailfold patterns seen in healthy individuals (31). Thus, for the purposes of being precise, “normal” is tentatively defined as patterns that fit within clusters 1 and 2, which were described as “normal” and “perfect normal” by Ingegnoli *et al* (31). These two clusters account for 93% of healthy individuals.

Unlike previous RP criteria, the panel agreed that a normal ESR and a normal ANA were not required for a diagnosis of primary RP. The panel felt that these tests have too low specificities to be reliable as specific criteria and those patients with low but nevertheless abnormal values are too common. Thus, in comparison to previously published reports, the requirement for a negative ESR was entirely eliminated and the panel eased the requirement for a negative ANA to negative or low titer ANA (e.g. 1:40 by indirect immunofluorescence)(6). In summary requests for definition of PRP are meeting 3 step criteria for diagnosis of RP: (i) normal capillaroscopy (e.g. clusters 1 and 2, which were described as “normal” and “perfect normal” by Ingegnoli *et al* (24)); (ii) negative physical examination for findings suggestive of secondary causes (e.g. ulcerations, tissue necrosis or gangrene, sclerodactily, calcinosis, or skin fibrosis); (iii) no history of connective tissue disease and (iv) negative or low titer ANA (e.g. 1:40 by indirect immunofluorescence) (6, 24).

In secondary RP there is an underlying disease. Table 2.1.1.

Connective tissue diseases:	
· Systemic sclerosis (SSc) and SSc spectrum disorders	Presence of clinical features of CTD First symptom of disease in many patients
· Inflammatory muscle disease	
· Systemic lupus erythematous	
· Sjögren's syndrome	
· Vasculitis	
Hand-arm-vibration syndrome ("vibration white finger")	Mimics Raynaud when presents with pain, cold bluish or pale extremity
Cervical rib/thoracic outlet syndrome	Unilateral with paraesthesia's pain and discoloration of the fingers
Other causes of large vessel disease:	
· Atherosclerosis	Extremity pain and ischemia
· Thromboangiitis obliterans (Buerger's disease)	
"Intravascular" disease:	
· Paraproteinemia	Physical obstruction of the microcirculation or immunologically
· Cryoglobulinemia	
· Cryofibrinogenemia	
· Malignancy	Occlusion of the microcirculation through malignant cells. Paraneoplasic effect. Production of cryoproteins and Antiphospholipid antibodies
Certain drugs or chemicals:	
· Beta blockers	Mimics Raynaud by peripheral vasoconstriction
· Clonidine	
· Ergotamine	
· Vinyl chloride	
Other causes/associations, including:	
· Hypothyroidism	As result of impaired thermoregulation, it is a potential cause of a exacerbating factor for RP
· Carpal tunnel syndrome	Painful dysthesia of the extremities. There is limited evidence base associating this two idiopathic conditions, but it has been reported to be as high as 60% Carpal tunnel syndrome is a well recognised complication of SSc (compression of median nerve during oedematous phase)
· Frostbite	Increased risk of frostbite in Raynaud's.
· Chillbains	Discrete acral lesions rather than digit discoloration
· Acrocyanosis	Non-paroxysmal Painless. Symmetrical discolorations
· Livedo reticularis	Resolves with warming
· Erythromelalgia	Pain and burning attacks after exposure to mild warmth. Alleviated by cooling.

Table 2.1.1: Main causes of secondary Raynaud phenomenon

The onset age is later, usually after the thirties, episodes are painful, intense and asymmetrical, ischemic skin lesions are frequently present, clinical features suggestive of a connective-tissue disease are common (e.g. arthritis, sicca symptoms, sclerodactylia, calcinosis, puffy fingers, telangiectasia), nailfold capillaroscopy shows evidence of microvascular disease and autoreactive antibodies are present.

Due to time constraints imposed on the Delphi exercise, the expert's did not attempt consensus criteria for secondary RP. However, the panel was undoubtedly in agreement that physical exam findings such as sclerodactily, calcinosis, fibrosis, ulcerations and an abnormal capillaroscopy examination

could be used to make a diagnosis of secondary RP in patients who meet the diagnostic criteria for RP. Autoreactive antibodies (specifically ANA, anti-centromere, and anti-RNA polymerase) were ranked as helpful and in some cases diagnostic for secondary RP (6). However, agreement could not be established for the usefulness of anti-SCL 70 antibodies. Although this is an excellent start, future exercises will be required to develop diagnostic criteria for SRP.

Systemic sclerosis as one of the main underlying secondary cause of RP has recently been discussed and new criteria have been reviewed. It was determined that skin thickening of the fingers extending proximal to the metacarpophalangeal joints is sufficient for the patient to be classified as having SSc; if that is not present, 7 additive items apply, with varying weights for each: (i) skin thickening of the fingers, (ii) fingertip lesions, (iii) telangiectasia, (iv) abnormal nailfold capillaries, (v) interstitial lung disease or pulmonary arterial hypertension, (vi) RP, (vii) SSc-related autoantibodies. Sensitivity and specificity in the validation sample were, respectively, 0.91 and 0.92 for the new classification criteria and 0.75 and 0.72 for the 1980 ACR classification criteria. All selected cases were classified in accordance with consensus- based expert opinion (25). These criteria will help to identify earlier patients with RP to be classified correctly as having the disease.

Assessment Methods for Raynaud Phenomenon

i) Capillaroscopy

Microvasculopathy with alterations in morphology and function can easily be detected at the nailfold bed with NVC. Capillaroscopy is a valuable, accessible, non-invasive, easy, safe and cheap tool with important diagnostic and prognostic value in patients with RP. Structural morphological and functional changes in capillaries allow differentiation between PRP and SRP, as structural abnormalities do not occur in primary Raynaud phenomenon.

Recently, three major “normal” morphologic capillaroscopic patterns were proposed by Ingegnoli et al: (i) “normal” pattern mainly with 2 to 5 U-shaped loops/mm and ≤2 tortuous loops/mm; (ii) “perfect "normal” pattern with ≥ 5U – shaped loops/mm and (iii) “unusual normal” with at least 1 meandering or bushy loop, or 1 microhemorrhages or with >4 crossed loops/mm (24).

Previous description of PRP NVC was 1 capillary within each single dermal papilla; morphological homogeneity of diameter and regular distribution of capillaries, hairpin or “U – shape” shaped capillaries, major axis of capillaries perpendicular to the distal row, ratio efferent: afferent limb<2:1, good level of skin transparency and no morphological abnormalities (18).

"Scleroderma pattern" is characterised by architectural capillary derangement, giant loops, enlarged loops, haemorrhage, angiogenesis and avascular areas. Nailfold capillaroscopy is the best technique for examining disease progression over time and the responsiveness to vasoactive treatment. Cutolo *et al* described an useful and reproducible qualitative NVC "Scleroderma patterns" with 3 different evolutive patterns: early, active and late (18). Several studies have been done in an attempt to identify NVC changes and its correlation to organ disease, in order to identify a possible predictive role of NVC in CTD. Main differences in NVC between PRP and SRP are summarized in Table 2.1.2.

NVC Pattern	Primary Raynaud Phenomenon	Secondary Raynaud Phenomenon
Morphological features	-Regular capillaries -Uniform distribution, shape and diameter -Hair pin or U-shape capillaries	-Architectural derangement is the morphological feature in SSc
Enlarged loops	-Absent	-Potential marker of microangiopathy -Scleroderma spectrum: Giant capillary - Valorise even single enlarged loop
Haemorrhages	- Microhemorrhages (punctate haemorrhage) may be present - present after traumatic injury or onychophagia	-Earliest finding of Scleroderma spectrum - Microhemorrhages;
Angiogenesis	-Absent	-Capillary neoformation: highly convoluted and branched capillary loop clusters -Meandering, ramified/bushy, bizarre capillaries
Capillary Density	-Normal	- Avascular areas - Desert-like appearance of nail fold - Less than 30 capillary per 5mm -Poor prognosis

Table 2.1.2: Main capillaroscopic findings in primary and secondary Raynaud phenomenon. *NVC: nailfold videocapillaroscopy*

Frequency of capillaroscopy examination in patients with RP in not consensual. If capillaroscopy has a "normal" pattern and patients have negative autoreactive antibodies the probability of developing SSc is 1.8% in long-term follow-up (26). In our clinical practice we repeat capillaroscopy and antibodies within 2 years and if normal characteristics persist we refer the patient to general practitioner. If either capillaroscopy or antibodies have abnormalities, follow-up every 6 months is recommended, due to 16% of patients might transit to a SRP within 5-year follow-up (27).

ii) Non-invasive methods

Non-invasive methods can help to differentiate between PRP and SRP, by assessing digital vascular structure and function objectively.

A lack of consensus on the utility of these methods still persists. We summarize the most useful clinical and research methods for micro-, macrovascular and function assessment in RP patients (Table 2.1.3). A number of new technologies are emerging and in the near future structural and dynamic evaluation of microvasculature might bring new insights to RP diagnosis.

TECNIQUE	OBJECTIVE	CLINICAL OUTPUT
Cold stress test (CST)	Assess sympathetic vasoconstriction to cold exposure	Microvascular Reactivity to cold exposure
Infrared thermography	Measures skin temperature as an indirect measure of skin perfusion (can include CST)	Microvascular Differentiation PRP/SRP Response to treatment
Laser Doppler flowmetry	Measures skin perfusion	Microvascular Differentiation PRP/SRP Disease progression Response to treatment
Arterial Doppler ultrasound Ankle Braquial Pressure Index	Measures vessel flow and size	Macrovascular Differentiation PRP/SRP
Flow-mediated dilatation	Measures endothelial-dependent dilatation in response to increased shear stress	Macrovascular Differentiation PRP/SRP Disease progression
Finger systolic pressure measurement	Measures finger systolic pressure as a response to temperatures variations	Macrovascular Differentiation PRP/SRP
Plethysmographgy	Relationship between arterial flow and microvascular perfusion Measures changes in venous blood flow /volume	Macrovascular Differentiation PRP/SRP

Table 2.1.3: Main investigations methods used in clinical and research approaches of Raynaud phenomenon patients.

iii) Other methods

Unilateral RP suggests large vessel disease such as thoracic outlet syndrome, atherosclerosis and trauma. When macrovascular disease is suspected in RP patients, it might be necessary further investigation with invasive methods such as digital subtraction angiography (DSA). Alternative methods may be magnetic resonance angiogram (MRA) or computed tomography angiogram (CTA). DSA has the advantage that, if indicated, endovascular intervention may be simultaneously performed.

Most of these techniques have been widely adopted in clinical practice and, at present, all of them remain primarily as research tools. There is considerable interest in application of these noninvasive techniques in the research setting,

alone or in combination, with the aim of improving diagnostic accuracy, measuring disease progression and treatment response (5).

MANAGEMENT

It is consensual that for PRP, conservative measures as warming, biofeedback, hand exercises, relaxation therapy, smoking cessation and avoiding triggers, as cold and stress are beneficial and sufficient most of the times to control the disease. Primrose oil has in our experience demonstrated good results in controlling number and duration of attacks. Nevertheless, given the adverse effects of smoking on vasculature and the exacerbation of symptoms in the cold, the non-pharmacologic lifestyle modifications should also be encouraged on all RP patients. However, SRP usually implies pharmacologic therapies.

After years of study, there is a lot of conflicting data and limited evidence for the effects of each drug. The absence of significant values are due to high placebo response, no standardization of outcome measures in RCT, lack of reliable biomarkers or surrogate end points, and the fact that the symptoms and course of this disease are highly influenced by context factors such as seasonal variations and personal lifestyle (28).

Calcium channel blockers (CCB) are widely used as first-line therapy, but adverse effects are not uncommon. We recommended starting with low and long acting doses. The most well studied CCB nifedipine has showed effects in reducing attack frequency and severity. When ineffective or not tolerated, Angiotensin II Receptor Blockers (ARB) and alpha blockers may be an alternative, as they also have demonstrated some good results in reducing Raynaud's frequency and severity. Phosphodiesterase (PDE) inhibitors are a recent addition to Raynaud's treatment, since they were proven to be safe and have shown some benefit. However, longer studies are required to lead to more consistent results. The new formulation of topical nitroglycerin can be useful, once it adds the nitrate vasodilatory effect with tolerable side effects. Selective Serotonin Re-uptake Inhibitors (SSRI) may have advantage when vasodilatory symptoms are not tolerated with the drugs mentioned above. Botulinium toxin, in all case series and case reports has showed overall improvement in patients' pain as well as a reduction in soft tissue ulceration, but its still lacking a randomized, placebo-controlled study. Evidence proved no effect for Angiotensin-Converting Enzyme (ACE) inhibitors neither for ginkgo biloba.

For severe Raynaud and active DU, IV prostanoids, in particular iloprost should be used first, as recommended by the EULAR agreement, in spite of a high

rate of adverse effects and no consensus between European and North American rheumatologists (29). Recently the use of continuous 5-day perfusion of iloprost through an elastomeric pump, the overall adverse events have been overcome and is now a more accessible, and tolerated useful treatment. Additionally this new approach can be done in ambulatory basis reducing hospitalization and costs. For DU prevention, the new endothelin-1 receptor antagonist, bosentan, has demonstrated to reduce the number of new digital ulcers in patients with SSc. If on-going disease, consider thoracic or digital sympathectomy, surgical and invasive treatments, but with potential to reduce recurrence of DU.

We show in Table 2.1.4 the conclusions of the latest studies published in the last 5 years. We found no reliable studies for antiplatelet therapy, mesoglycan, pentoxyfiline, Rho kinase inhibitor (fasudil), alpha-2C adrenoreceptors blockers or N-acetylcisteine.

Authors	Study Design	Year	Conclusions	Ref
Calcium channel blockers (Nifedipine, Nicardipine, Diltiazem)				
Act on vascular smooth muscle to cause arteriolar vasodilation and an increase in peripheral blood flow				
McMahan et al	Review	2010	Better results on PRP than SRP due to untolerated high doses of nifedipine needed in SRP. Long acting formulations are useful to minimize side effects and increase tolerability. Also effective in the treatment of DU.	(30)
Levien	Review	2010	Reduces the frequency and severity of attacks in both PRP and SRP. Remains the most widely used class of drugs in management of RP	(31)
Pope et al	Systematic review of PRP	2011	Nifedipine reduces the frequency and severity of Raynaud's attacks, but it is associated with adverse effects, such as tachycardia, headache and flushing. Nicardipine may also be used. No evidence for amlodipine or diltiazem.	(32)
Sinnathurai et al	Review of RP in SSc	2013	No sufficient data found to support the reduction of digital ulcers. Diltiazem only in PRP.	(33)
Seehusen et al	Evidence-based research	2014	Both reviewed 7 RCTs, with a total of 296 participants, analysing nifedipine and nicardipine in PRP.	(34)
Ennis et al	Cochrane review	2014	Both concluded that they are modestly effective in reducing the frequency of attacks and no clear evidence of reducing severity of RP attacks.	(35)
Alfa-adrenergic blockers (Prazosin)				
Alfa-2 receptors are present throughout much of the vascular system and play a significant role in cutaneous thermoregulation.				
Pope et al	Systematic review of PRP	2011	Might successfully treat PRP but there were no large enough studies to enable conclusions.	(32)
Huisstede et al	Meta-analysis of SRP	2011	Conflicting evidence for the effectiveness in the treatment of SRP Poor improvement percentage in the high quality RCT, with 1 of 5 patients improving in the experimental treatment and 0 of 5 in the placebo group.	(8)

Sinnathurai et al	Review of RP in SSc	2013	Prazosin was moderately effective in treating SSc SRP Significant decrease in frequency of attacks, but a small number of patients was studied (n=40).	(33)
Angiotensin II receptor antagonists (Losartan)				
Arterial vasodilators				
Herrick	Review	2011	Widely used when CCB are ineffective or not tolerated	(36)
Huisstede et al	Meta-analysis of SRP	2011	Reduces frequency and severity of Raynaud's attacks in both PRP and SRP, with no difference compared to nifedipine	(8)
Selective Serotonin Re-uptake Inhibitors (Fluoxetine and Ketanserin)				
Increases regional blood flow by blocking the uptake of the vasoconstrictor serotonin. May have an anti-fibrotic effect				
Stewart	Systematic review of oral vasodilators for PRP	2012	Systematic review of Ketanserin for PRP found significant reduction for the severity score. No statistically difference on the frequency or duration of attacks.	(37)
Sinnathurai et al	Review of RP in SSc	2013	Statistically significant decrease in severity of RP with fluoxetine when compared to nifedipine.	(33)
Prostaglandins (Iloprost, Cisaprost and Alprostadil)				
Act as a potent vasodilator and may also inhibit platelet aggregation and influence vascular remodelling				
Sinnathurai et al	Review of RP in SSc	2013	Endovenous iloprost reduces frequency and severity of attacks and development of DU. Rate of side effects may be as high as 92%, consisting in headache, flushing and nausea, but all reversible and controlled by the rate of infusion.	(33)
Huisstede et al	Meta-analysis of SRP	2011	Evidence for the effectiveness of oral and IV iloprost. Limited evidence was found for the infusion protocol of IV iloprost 10 times in 2 weeks instead of 20 times in 6 months. No evidence for the effectiveness of cisaprost. No evidence in favour of high doses of IV iloprost instead of regular doses in RP frequency and duration. No evidence for the effectiveness of IV alprostadil.	(8)
Levien	Review	2010	Conflicting data with oral iloprost. Daily infusion of alprostadil was no more effective than placebo.	(31)
McMahan et al	Review	2010	Cyclic iloprost can be used for severe Raynaud's and DU, with some benefit in digital ischemia prevention.	(30)
Oral endothelin receptor antagonist (Bosentan)				
Levels of endothelin are increased in the serum of patients with RP and SSc, acting as vasoconstrictor and contributing to the fibrotic and vasculopathic aspects of SSc				
Nguyen et al	RCT: 17 patients, 24 weeks Follow-Up	201	Bosentan did not improve frequency, duration, pain or severity of RP attacks. Had a significant decrease in functional scores. Bosentan is not effective in SSc-related RP without pre-existing DU	(38)
Arefiev et al	Review	2011	Effective in prevention of new DU.	(39)
Levien	Review	2010	Mention to 2 RCTs which documented significant reduction in the development of new ulcers but no difference in the healing of existing ulcers	(31)
McMahan et al	Review	2010		(30)
Matucci-Cerinic et al	RCT: 188 patients with SSc and DU, 24 weeks Follow-Up	2011	Bosentan is useful in prevention of new DU in patients with SSc. No healing improvement.	(7)
Phosphodiesterase type 5 inhibitors (Sildenafil, Taladafil and Vardenafil)				
Works by elevating levels of cGMP, causing intracellular calcium levels to fall, leading to vascular smooth muscle relaxation				
McMahan et al	Review	2010	A RCT associated sildenafil with decrease of frequency and duration of Raynaud's and decreased RCS.	(30)

Levien	Review	2010	Conflicting data with sildenafil and taladafil, with both drugs having a RCT each that found reduction of frequency and duration of Raynaud's attacks and another RCT that described no benefit at all.	(31)
Herrick et al	RCT: 57 patients with SRP, 6 months Follow-Up	2011	Tested modified-release sildenafil with significant reduction in attack frequency of RP. The decrease in Raynaud's Condition Score (RCS) duration of attacks and RP pain was not significantly different from placebo group.	(40)
Caglayan et al	RCT: 53 patients (6 primary, 47 secondary), 6 weeks Follow-Up	2012	Significant reduction in RCS and a decrease in number and cumulative duration of Raynaud attacks per day in patients on vardenafil compared to placebo. Better results in PRP and SRP patients with limited cutaneous systemic scleroderma.	(41)
McMahan et al	Review	2010	Both review 2 RCTs about oral taladafil: First RCT found no significant results, but with a large confounding placebo response; Second RCT(42) found it useful as add-on therapy for SRP resistant to vasodilators, with significant improvement in attacks frequency and duration and in healing and prevention of DU.	(30)
Sinnathurai et al	Review of RP in SSc	2013		(33)
Nitrates (MQX-503: New formulation of topical nitroglycerin) *Vasodilating properties*				
Levien	Review	2010	Improvement in blood flow RCS and severity of attacks No changes in the frequency or duration of attacks.	(31)
Sinnathurai et al	Review of RP in SSc	2013	Both reviewed a RCT that showed efficacy in terms of RCS. Better with RPR than in SRP.	(33)
McMahan et al	Review	2010	No changes in the attacks frequency or duration.	(30)
Angiotensin converting enzyme inhibitors (Captopril, Enalapril and Quinapril) *Vasodilating properties*				
McMahan et al	Review	2010	**No** benefit in treatment of SRP or digital ulcers as a first-line or monotherapy, as the larger study, with 2-3 years follow up, was unable to show any benefit.	(30)
Huisstede et al	Meta-analysis of SRP	2011	No evidence for the effectiveness of the use of quinapril on SRP.	(8)
Stewart	Systematic review of oral vasodilators for PRP	2012	Captopril and enalapril, and enalapril alone are associated with small **increase** in the frequency of attacks per week compared with placebo on PRP. For captopril alone, the difference between the treatment groups for frequency and duration of attacks was non-significant.	(37)
Sinnathurai et al	Review of RP in SSc	2013	Limited evidence for the efficacy of these agents	(33)
Botulinium Toxin *Efficacy is theoretically attributed to two potential mechanisms: modulation of abnormal adrenergic innervation and reduction of pain through antinociceptive pathways.*				
Levien	Review	2010	Decreased pain and numbness. Decreased frequency of vasospastic attacks, healing of digital ulcers.	(31)
Mannava et al	Review	2011	Decreasing pain and improving hand function.	(43)
Iorio et al	Review	2011	Both analysed 5 studies, including Neumeister, 2010 (44). All showed overall improvement in patient pain as well as a reduction in soft tissue ulceration.	(45)
Neumeister et al	Review	2014		(46)
Digital Sympathectomy *Adrenergic effect only at the site of adventitial stripping with limited decrease of vasoconstriction distally and no decrease in vasoconstriction of the proximal vasculature*				
Merrit	Review	2015	Reduce the frequency and severity of ischemic Raynaud attacks	(47)

			and decrease recurrence of ischemic ulcerations.	
Anti-oxidants				
Improves microcirculatory network, possibly modulating the structural microvascular modifications				
Bruni et al	RCT: **30** patients **6** months Follow-Up	2014	Compared treatment with nifedipine alone and nifedipine plus antioxidant complex in Primary RP. Significant improvement on RCS.	(48)
Ginkgo Biloba				
I*mproves peripheral blood circulation through decreased aggregation and enhanced deformability of erythrocytes, exhibits vasoregulatory activity via regulation of the balance between prostacyclin and thromboxane and protects against endothelial dysfunction through modulation of nitric oxide generation*				
Bredie et al	RCT: **41** patients **10** weeks Follow-Up	2012	An excellent safety profile but **no** significant effect in frequency, duration or severity of the RP attacks	(49)
Statins (Atorvastatin)				
Endothelial protective action				
Levien	Review	2010	Both referred a RCT with significant reduction of DU and a significant improvement on SHAQ-DI (Scleroderma Health Assessment Questionnaire) and on Visual Analogue Scale for RP and DU severity and pain scales.	(31)
Sinnathurai et al	Review of RP in SSc	2013		(33)
McMahan et al	Review	2010	Evidence of significantly decrease in the number of DU.	(30)
Huisstede et al	Meta-analysis of SRP	2011	Limited evidence for the effectiveness of the use of atorvastatin.	(8)

Table 2.1.4: Literature review on Raynaud's management PRP, *Primary Raynaud's Phenomenon; SRP, Secondary Raynaud's Phenomenon; DU, Digital Ulcers; SSc, Systemic Sclerosis; RCT, Randomized Controlled Trial; CCB, Calcium Channel Blockers; RCS, Raynaud's Condition Score*

Conflict of interest: none.

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2.2 MECHANISMS UNDERLYING PERIPHERAL VASCULOPATHY

2.2.1. ENDOTHELIUM DYSFUNCTION

Vascular endothelium is a highly specialized, complex and metabolically active organ that performs a number of essential biological functions. It provides a compatible interface to facilitate blood circulation, inhibits excessive platelet aggregation and leucocyte adhesion, produces a balance of vasoconstrictive and vasodilatory molecules that coordinate vascular tone and serve to inhibit extracellular matrix (ECM) deposition and prevents smooth muscle cell proliferation (1).

The prominent endocrine functions of the endothelium work to regulate vascular tone, through the production of vasoconstrictive and vasodilatory molecules, to maintain blood fluidity, to regulate platelet function and to control inflammation (1). Healthy functioning of the endothelium is critical for remodeling of blood vessels, through angiogenesis and vasculogenesis, during times of tissue growth and repair (1).

Nitric oxide (NO), prostanoids including prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2), and endothelium derived hyperpolarizing factor (EDHF) possess potent vasodilatory actions. Bradykinin, norepinephrine and serotonin are neurohumoral mediators that cause release of endothelium-derived relaxing factors, further contribute to the effect of vasodilation (2). Potent vasoconstrictors include endothelin, angiotensin-converting enzymes (ACE) which convert angiotensin I to angiotensin II, thromboxane A_2 and F_{2a}, endothelial derived constricting factors (EDCF) that are derived from cyclooxygenase, leukotriene's and free radicals. The endothelium responds to a wide range of physicochemical signals such as shear stress, wall stretching and hypoxia resulting in production and release of these vasoactive factors (3).

The term "endothelial dysfunction" has evolved over the years in the scientific literature. Endothelial dysfunction can be used to describe any pathological condition involving imbalance between relaxing and contracting vascular factors, pro-coagulant and anti-coagulant substances and between pro-inflammatory and anti-inflammatory mediators. Endothelial activation represents a spectrum of responses under both physiologic and pathophysiologic conditions (4).

Endothelial dysfunction is likely to result from endothelial cell injury triggered via a number of different mechanisms, including bacterial or viral infection, oxidative stress through abnormal regulation of reactive oxygen species, hypoxia, turbulent blood flow and shear stress, environmental irritants

such as tobacco and hyperlipidemia (1). These factors all lead to the generation of an inflammatory process and endothelial cell activation. However, if the stressors are too severe or excessive, endothelial activation can lead to a maladaptive and dysfunctional response with consequent permanent endothelial damage, leading to morbidity or mortality (5).

Endothelial dysfunction and free-radical damage are primary events throughout the course of the SSc disease, which result in vascular obliteration and diminished blood flow to the organs involved (6) and are prominent features of RP and DU. There are several serological biomarkers that reflect the vasculopathy of the disease, such as vasoconstrictor ET-1 (7), cell adhesion molecules (including selectin, anti-endothelial antibodies, and the controversial vasodilator nitric oxide (NO) (7) and the inhibitor of endothelial NO synthase (eNOS) ADMA.

The most prominent clinical manifestations of endothelial dysfunction are linked to deregulation of vascular tone leading to vascular spasm and reduced blood flow. Most of accumulated evidence suggests an imbalance between the levels of vasoconstrictor and vasodilator mediators. Moreover, there are defects in the mechanism of nitric oxide production by endothelial nitric oxide synthetase, an effect that impairs vSMCs survival and promotes apoptosis. Additionally, the potent vasodilator endothelin-1 (ET-1) is upregulated in SSc serum and tissues. The imbalance between vasodilator and vasoconstrictor signals leaves ECs vulnerable to apoptotic signals and promotes an environment of ischemia, hypoxia, and profibrosis through the release of cytokines and profibrotic growth factors, including TGF-β and ET-1(8). This complex array of molecular interactions involves a number of cell types including the endothelial cells and their attendant perivascular supporting cells (pericytes and vascular smooth muscle cells [vSMCs]), as well inflammatory cells, all profoundly influenced by the presence of growth factors, cytokines, chemokines and potent vasoactive factors. Together, this diverse range of factors is believed to initiate and drive the vascular pathogenesis that leads to severe vessel disease and occlusion (1).

The endothelial response to injury can be divided into two ‘levels’ of response: an initial rapid response and a slower phenotypic response. The initial rapid response involves, among other factors, changes in levels of nitric oxide, prostaglandins, ET-1, von Willebrand factor and tissue plasminogen activator. The slower response depends on fundamental changes in cell surface characteristics, and alterations in the underlying basement membrane and the vascular smooth muscle cells (vSMCs) that surround the endothelium. These changes are brought

about by molecules that are particularly potent growth factors involved in the deposition of ECM, and activation and proliferation of the vSMCs, pericytes and other mesenchymal cell types associated with the blood vessel wall (1). This ultimately results in vessel remodeling, with profound changes in cellular architecture.

In most of research the term "endothelial dysfunction" has been used specifically to refer to an impairment of endothelium-dependent vasodilation. This is also the definition of endothelial dysfunction adopted in this thesis.

As a response to increase in shear stress, several vasodilators are released such as NO, prostaglandins and endothelium-derived hyperpolarizing factor (9). This response is commonly known as flow-mediated dilatation (FMD), and has been largely used for endothelium-dependent dysfunction assessment. NO is probably the major mediator of vasodilation and reduced NO bioavailability has been broadly accepted as a marker of endothelium dysfunction (10).

Controversial results have been published regarding endothelial dysfunction assessment in SSc patients. In respect to FMD, there are studies favouring reduction of FMD in SSc compared to control groups (11-15) no reduction of FMD (16) and similar FMD (17, 18). These studies were not designed to analyse relationship of FMD and DU. A systematic review and meta-analysis (19) analyzed FMD assessment in SSc patients demonstrating that most of the studies (71%) assessing the FMD% found significantly lower brachial artery FMD% in SSc patients compared to controls. A low brachial artery FMD%, in particular, is an independent predictor of cardiovascular risk (19).

Calcification and fibrosis reduce vascular compliance and subsequently affect NO signaling by limiting vascular stretch (14), indicating that macrovascular disease and probably arterial stiffness are also important factors determining FMD induced by reactive hyperemia. Given that vascular changes leading to digital ulcers are histopathologically characterized by intimal fibrosis, the decrease in vascular compliance may also contribute to the reduction of FMD values by reducing NOS activity in SSc. Collectively, the decrease in endothelial NO production is a characteristic feature of SSc vasculopathy, which largely explains the reduced FMD values in SSc patients (14).

2.2.2. MICROVASCULAR DAMAGE

Microcirculation is a fundamental anatomical component of circulation made up of a network of small arterioles, metarterioles, capillaries and venules. From the structural point of view, capillaries are considered as unitary structures

consisting of an endothelial layer, a basement membrane and numerous branching contractile cells on external wall (Rouger's cells) (20). Microcirculation has two main tasks: carrying the blood to tissues and controlling its flow to capillaries. The vascular system supplying the skin has a regional heterogeneity vascular density, microvascular structure and organization of arterial supply (21).

At the microvascular level, arterioles and venules form two distinct but interconnected systems: first, an upper superficial network in the papillary dermis, with a higher microvascular density and the source of nutritional capillary loops that extend vertically into the dermal papilla (21) (in the nailfold, the capillary orientation changes from vertically to longitudinal inclination and is highly accessible to imaging and analysis with nailfold videocapillaroscopy (NVC)); second, a lower plexus in the dermal-subcutaneous interface that connects with the upper network and branches into lateral systems to supply sweat glands and hair follicles (21).

Glabrous skin contains a rick concentration of arteriovenous anastomoses (AVAs) that allow blood to bypass nutritional capillaries loops (21) and are key thermoregulatory structures that can rapidly distribute high volume of blood to the skin surface, enabling optimal regulation of core temperature (21). AVAs are located in both the lower and upper vascular plexuses (21).

As a consequence of repeated bouts of vasospasm, pathological changes occur in microcirculation characterized by decreased microvessel patency and blood supply. One of the earliest pathological events is the evidence of damage to endothelial cells, that induces a cascade of consequences to vessels and surrounding tissue, including platelet activation, fibrinolytic pathway activation, angiogenesis/vasculogenesis stimulation and release of vasoactive mediators (22). Vascular smooth muscle cells (vSMCs) proliferate resulting in intimal growth, matrix deposition and eventually stenosis or occlusion of vessel lumen (20).

On histological examination, microvascular changes can be seen in virtually all organ systems and include perturbation of endothelial cells, neointimal formation, increased numbers of myofibroblasts, pericyte activation, and perivascular lymphocyte infiltration. Small arteries and arterioles (50-500 |im in diameter) develop a fibrous, concentric intimal lesion that can obliterate the vessel lumen. Endothelial cell injury seems to occur at an early stage, with evidence of endothelial apoptosis, chronic platelet activation, and subsequent vascular thrombosis (23). Characteristic clinical findings include dropout of capillaries and distorted capillary structure, swelling of endothelial cells, reduplication of capillary basement membrane , large gaps between endothelial cells, vacuolization of endothelial cell cytoplasm, loss of membrane bound

storage vesicles in endothelial cells, capillary telangiectasia and intimal proliferation and accumulation of proteoglycans in arterioles and arteries (24).

Capillaroscopy is the only method for the evaluation of nutritional capillaries of the nailfold. The capillaries consist of an arterial and a venous limb and an apical loop. Normally nailfold capillaries are hairpin shaped, with parallel, regular arrangement; a single capillary loop is localized in a single dermal papilla (25).

Consistent with this, morphological changes in capillaries are detectable before or at disease onset, and enables the early differentiation between PRP and SRP using nailfold videocapillaroscopy (NVC). Besides identifying morphological patterns specific to various stages of SSc, NVC can also monitor disease severity and progression. Koenig et al (26) showed that microvascular changes were sequential in a 20-year follow-up study of 586SSc patients. Capillary enlargement was followed by capillary loss and then by capillary telangiectasia. This is succeeded by morphologic changes in the vessels as well as tissue fibrosis (26). In patients with recent-onset RP, these changes may be patchy, unilateral, or expressed in a single finger.

A patient with a PRP meets, among other criteria the following normal capillary pattern: capillaries in the distal row of the nailfold have an open hairpin shape, are homogeneously sized and regularly arranged in a parallel fashion and their number ranges in linear mm from 6 to 14 with a mean of 9 capillaries (27). In contrast, capillaroscopic image of an SSc patient with RP is characterized by pathognomonic microvessel structural damage consisting of the presence of giant capillaries, microhemorrhages, capillary loss and other morphologic anomalies such as progressive neoangiogenesis (27).

The presence of homogeneously and/or irregularly enlarged capillaries (circumscribed or homogeneous diameter >50μ) represents one of the earliest and most striking features of secondary RP and should be considered as a potential marker of microangiopathy. It has been suggested that microvascular dilation represents a local autoregulatory response to tissue hypoxia and a compensation of the morphofunctional changes in microcirculation such as decreased capillary density and hypoperfusion (28). In a recent study, enlarged capillaries were found in 100% of SSc patients, and in 56% of undifferentiated connective tissue diseases patients (29). As disease progresses architectural disarrangement of the nailfold microvascular network is observed. Local microhemorrhages are also associated with early vascular damage representing the ‘bridge’ between the presence of megacapillaries and the subsequent loss of capillaries (30). Loss of capillaries and/or avascular areas. Decreased number of loops (<30 over 5 mm in the distal row of the nailfold) with a “desert-like”

appearance should be considered highly specific for SRP (29). Progressive loss of capillaries has been associated with more extensive skin involvement and with a poor prognosis.

A wide range of morphologic features of capillary neoformation (angiogenesis) can be observed in patients with secondary RP. The main morphologic hallmark of angiogenesis is the clustering of tortuous capillaries with a pronounced shape heterogeneity, including thin or large, meandering, and bushy capillaries /ramified capillaries (29) often surrounded by a dropout of normal capillary loops.

Capillaroscopy has been described as the best predictor tool of transition of PRP to SRP (31). Qualitative, semi-quantitative and quantitative indexes/scores have been described. Scoring the capillaroscopic patterns changes during the follow-up of SSc patients (32) enables capillaroscopy as a mandatory tool to quantify microangiopathy. The usefulness of capillaroscopy in the follow-up of SSc patients and the possible prognostic role for the appearance of typical SSc vascular and visceral involvement, namely, digital ulcers and mortality, is suggested by many authors but still under debate (33).

Qualitative Evaluation Model

In clinical setting qualitative evaluation is the more accessible and easiest to perform. Architectural disorganization, enlarged loops, loss of capillaries, angiogenesis, and avascular areas characterize >95% of patients with overt SSc. Cutolo et al. (34) described a useful and reproducible qualitative NVC "Scleroderma pattern" with 3 different evolutive patterns: early, active and late.

Early pattern is characterized by the presence of a single or small number of giant capillaries and microhemorrhages, no avascular areas and a relatively well preserved capillary distribution; in active pattern there are present giant capillaries and microhemorrhages, moderate capillary loss (20-30%) and a mild disorganized capillary architecture, with rare branches capillaries; Late pattern is characterized by near-absence of giant capillaries and microhemorrhages, presence of extensive avascular areas (50-70% of capillary loss) and a presence of many branched and ramified bushy capillaries (neoangiogenesis) with a complete disorganization of capillary array (30, 34).

Semi-Quantitative Evaluation Model

To allow a longitudinal evaluation of SSc microangiopathy, a semi-quantitative analysis of microvascular alterations is required (33).

Sulli et al. (35) proposed a semi-quantitative score, Microangiopathy Evolution Score (MES). The sum of three scores regarding loss of capillaries, disorganization of the microvascular array and capillary ramifications were assessed to study the progression of the vascular damage. A rating scale to score each capillary abnormality was used (0 = no changes; 1= <33% capillary reduction / changes; 2= 33-66% capillary reduction / changes; 3= > 66% capillary reduction / changes) per linear millimetre (35). Four consecutive fields (one linear millimeter for each one) in the middle of the nailfold, in each 8 fingers were studied. The average score values from the eight digits were added together, and the final value divided by eight (35). The resulting value represents the evolution score of microangiopathy (MES):0-9 (15). We categorized MES scores in 3 subcategories with the following cut-off values: category 1 – from 0 to 3; category 2 – from 4 to 6 and category 3 – from 7 to 9, allowing a more detailed analysis between microangiopathy and endothelial dysfunction evaluation.

Smith et al has proposed a simple semi-quantitative NVC evaluation for day-to-day clinical use as simple prognostic index for digital trophic lesions for daily use in SSc. Capillary loss was counted over F32 (8 fingers, 4 fields per finger, fingers 2–5 of each hand). The described (semi)-quantitative rating scale (0, no changes; 1, <33% capillary reduction vs. normal (9/mm); 2, 33–66% capillary reduction; 3, >66% capillary reduction) was adopted to calculate the mean score of capillary loss. A mean score value of capillary loss of 1.67 was chosen as the cut-off value for the clinical prognostic index for present/future digital trophic lesions (36).

Quantitative Evaluation Model

Sebastini et al. (37) proposed a Capillaroscopic Skin Ulcer Risk Index (CSURI) as a predictor risk of the onset on new DU using NVC parameters. The CSURI index assessed the total number of capillaries in the distal row (N), maximum loop diameter (D), number of megacapillaries (M), and the ratio M: N. (CSURI = D x M: $N2^{)}$. A cut-off of 2.94 had a 73.3% positive predictive value for DU. An inverse correlation was found with high N or higher M: N ratio. The strong limitation of this index was the obligatory presence of megacapillaries, excluding patients with late scleroderma pattern and eventually more severe disease.

Presumably, capillaroscopy alone is unable to fully explore the complex pathophysiology of scleroderma microangiopathy, and other clinical, demographic, or serological parameters are essential in the characterization of prognostic value of the microvascular damage. Therefore, the evaluation of large scleroderma population and more complex predictive model, including

capillaroscopy and other clinical/ serological variables, are needed to identify new significant associations with SSc organ involvement and prognosis (33).

2.2.3. ANGIOGENESIS

Angiogenesis, the creation of new blood vessels from pre-existing ones, mainly depends on the activation, proliferation and migration of ECs, and is driven by angiogenic stimuli that also induce proteolytic enzymes cleaving extracellular matrix (ECM). The balance between pro-angiogenic and anti-angiogenic factors tightly regulates angiogenesis (38). This event is highly complex and requires a dynamic, temporally and spatially regulated interaction between ECs, soluble angiogenic growth factors and ECM molecules. On one hand, EC proliferation and new vessel formation is characteristic of several diseases such as cancer and macular degeneration, while on the other hand EC death is also a typical feature of diseases such as atherosclerosis, allograft vasculopathy, heart failure, diabetic retinopathy and SSc (39).

Endothelial cell damage results in ischemia-reperfusion injury due to the ongoing pathological process, which inevitably evolves towards chronic underperfusion. Ischemia-reperfusion injury is a complex inflammatory process that results from interaction between humoral and cellular components including the complement, vasoactive cytokines and the contact activation cascade within ischemic vascular beds. Soon after the start of reperfusion, EC dysfunction is perpetuated by superoxide radicals mainly produced by damaged ECs and neutrophils. Superoxide radicals inhibit the release of NO, prostacyclin and tissue plasminogen activator from ECs leading to the impairment of the vascular tone control and favoring thrombotic events with consequent chronic tissue hypoxia. A characteristic clinical finding is capillary dilation and atrophy noted in the skin by nailfold capillary microscopy. Similar attenuation of the microvessels is seen in the involved organs. In fact, the pathology suggests significant loss of the peripheral vascular network, with a defect in both the vascular repair and in the expected increase in vessel growth (angiogenesis, arteriogenesis, vasculogenesis); the net result is tissue ischemia, fibrosis, and organ failure (23).

Although persisting hypoxia should be a major stimulus for angiogenesis, sufficient angiogenesis does not occur in SSc patients despite severe tissue hypoxia (38, 40). Most likely, this is based on an insufficient response to the elevated levels of vascular endothelial growth factor (VEGF) in serum and tissue. VEGF controls several steps of angiogenesis, increases the vascular permeability, stimulates the migration and proliferation of ECs and induces tube formation

(41). Also, an extended exposure to VEGF may have also negative effects by fusing immature microvessels in an uncontrolled manner resulting in a chaotic vessel network with giant capillaries. Moreover, in experimental settings, isolated microvascular EC from SSc patients show an impaired response to VEGF and other growth factors (41).

An explanation for VEGF defective signaling, is that VEGF-A primary transcript can be alternatively spliced in its terminal exon, producing two distinct mRNA splice variants that are translated to the proangiogenic VEGF165 and antiangiogenic VEGF165b isoforms (42). This variant is selectively overexpressed at both the mRNA and protein levels in SSc skin. Thus, the increase in VEGF expression in SSc patients appears to be mostly due to increased levels of VEGF165b and not the pro- angiogenic VEGF165. This switch in SSc from proangiogenic to antiangiogenic VEGF isoforms may play a crucial role in explaining the insufficient angiogenic response to chronic ischemia (43). These two isoforms bind to the tyrosine kinase receptor VEGFR-2 with the same affinity, but binding of VEGF165b results in an insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient downstream signaling, which leads to an impaired angiogenic response (42). Manetti et al. (42) have recently reported that plasma levels of the VEGF165b, the antiangiogenic isoform, correlated with the severity of capillary architectural loss and derangement, suggesting that VEGF165b may directly or indirectly participate in loss of the microvessels in SSc.

Several antiangiogenic or angiostatic mediators have been described as being upregulated in SSc, including endostatin, angiostatin, endoglin, platelet factor 4, thrombospondin, and IL-4. However, results on angiostatic factors in SSc are conflicting or flawed by low patient numbers, poor clinical characterization and heterogeneity of the study population (44).

The role of vasculogenesis in SSc is less clear. Endothelial progenitor cells might be recruited for vascular repair during active disease However, conflicting results have been published concerning the presence and role of circulating endothelial progenitor cells in SSc (39). Avouac et al. (45) demonstrated increased circulating endothelial progenitor cell levels in SSc, supporting their mobilization from bone marrow. Furthermore, the subset of patients with digital vascular lesions and high severity disease score displayed lower endothelial progenitor cell counts, suggesting increased homing at this stage (45). On the other hand, there is data suggesting that vasculogenesis might be impaired in SSc (39).

Despite large research, a crucial question persists: why are the damaged microvessels not replaced by new capillaries via angiogenesis or vasculogenesis

in SSc (46). In this thesis we will discuss the role of angiogenesis in peripheral vasculopathy.

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2.3. VASCULAR BIOMARKERS OF DISEASE ACTIVITY ASSESSMENT

There is a significant amount of evidence from studies of peripheral blood in scleroderma that vascular perturbation is present, but still we know very little about the role of biomarkers in predicting vascular outcomes (1). In this chapter we will review current knowledge of the biomarkers of vascular disease included in our research.

Vascular biological biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes and of pharmacologic responses to a therapeutic intervention (2). They may be used either as a diagnostic instruments, disease staging tools, monitor of disease progress, indicators of disease prognosis or as predictors of new clinical outcomes (2).

Although a popular model to explain the pathogenesis of the underlying scleroderma vascular disease is not yet fully proven, it suggests that the endothelial cell layer is activated/injured early in the disease process leading to endothelial cell dysfunction, over-expression of adhesion molecules, enhanced leukocyte activation, proliferation of pericytes, adhesion and activation of platelets and an influx of a perivascular infiltrate (3). The downstream effect of blood vessel perturbation produces “biomarkers” of vascular damage that reflect disease and may predict clinical outcomes (3). Therefore, vascular biomarkers of SSc disease activity play a key role in the understanding of pathophysiology of disease progression, and should include mediators of angiogenesis, markers of tissue hypoxia, endothelial dysfunction and platelet activation (4). Cross-section of unselected scleroderma patients measuring the possible biomarkers at one point in time, correlation of these measures with clinical data or longitudinal measurement of these factors have been reported (1).

These studies have some limitations as the biological effects of many markers are pleiotropic and may change over time, modify in different stages of disease, with possible significant intra-patient variation over time, uncertainty of the marker’s relationship to vascular disease (relationship to vascular disease “activity” versus “damage”) and effects of medications. But despite these limitations, circulating factors that reflect ongoing vascular perturbation have the potential of serving as intermediate biological endpoints that can not only detect and characterize vascular disease activity but also predict ultimate disease outcome or treatment response (1). Ideally, a biomarker of vascular disease should be present in the preclinical state, involved directly in the pathogenic process, and correlate well with future vascular outcomes defined by gold-

standard testing (5). Validation of these biomarkers, however, require cross-sectional and cohort studies.

Endothelial dysfunction biomarkers

Endothelin-1 (ET-1)

The endothelin's are a family of three vasoactive peptides (ET-1, ET-2 and ET-3). The three 21-amino-acid endothelin isoforms bind to two endothelin receptors (endothelin receptor subtype A: ET-A and ET-B), with equal affinities for ET-B, but with a hierarchy of affinities for the ET-A receptor. ET-1 has the highest affinity for ET-A, followed by ET-2 and ET-3. The roles of the ET-A and ET-B receptors are becoming less clearly distinguished as the literature expands (6). In healthy arteries the production of ET-1 is small and the bioavailability of NO is preserved. This means that the balance of effects favors vasorelaxation through increased signaling of cyclic GMP. In endothelial dysfunction there is increased expression of ET-1 in smooth muscle cells and macrophages. There is also an increased expression of ET-B receptors on smooth muscle cells mediating vasoconstriction. ET-1 may also decrease endothelial NO synthase (eNOS) expression, thereby reducing NO production. Both the ET-A and the ET-B receptor on smooth muscle cells may mediate formation of superoxide (O_2) in endothelial dysfunction. Superoxide will decrease the biological activity of NO by forming peroxynitrate ($ONOO^-$). Collectively the balance of these effects is shifted towards more vasoconstriction, inflammation and oxidative stress in endothelial dysfunction (7).

Endothelin-1 plays a key role in vascular pathologies by exerting various deleterious effects including hypertrophy of the vascular smooth muscle cells, cellular proliferation and fibrosis, increased vascular permeability, activation of leucocytes, and induction of cytokine and adhesion molecule expression (8). It can also act in an autocrine manner on the vascular endothelium itself. ET-1 is also recognized as a potent mitogen, as there is some evidence that it contributes to the vascular remodelling and organ damage. Low oxygen tensions typical of ischemic phase of RP, were shown to rapidly increase ET-1 secretion from cultured human endothelial cells, 4 to 8 fold above the secretion rates at ambient oxygen tension (transcriptional gene-related) (9).

Endothelin-1 is elevated in the plasma of SSc patients compared to controls, and it increases during cold exposure in selected scleroderma patients (10). ET-1 levels have been reported to be increased in PRP and SRP (9, 11-16) and this might be explained by the fact that ET-1 is primarily involved in capillary

vasospasm, independently, of its association with underlying connective tissue disease (9). Elevated ET-1 is not only a biomarker of vascular disease, but may itself be a cause of abnormal vascular reactivity and mediate tissue fibrosis by its pro-fibrotic properties via activating TGF-β. Therefore, inhibiting ET-1 activity is considered an attractive target in treating scleroderma vascular disease (3).

Asymmetric dimethyarginine (ADMA)

The free radical nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) and is a potent endothelium-derived vasodilatory mediator. There are three main isoforms of NOS with a constitutive expression in neuronal NOS (nNOS or NOS 1), endothelial NOS (eNOS or NOS 3) and it is possible that an inducible NOS expression (iNOS or NOS 2) occurs in response to a variety of stimuli, with NO-mediated signaling apparent in the skin (17). However, it is considered that NO is a double-edged sword in physiological and pathological conditions, both beneficial and detrimental depending on the concentration and local environment (18). ADMA can inhibit the NOS resulting in decreased levels of NO. When NO overproduction occurs, S-nitrosylation of the ADMA regulating enzyme b diminishes the activity of DDAH, leading to an accumulation of ADMA (17). High level of NO may result in free-radical damage, and low level of NO may correlate with endothelial dysfunction, while the free-radical damage and endothelial dysfunction are both present in SSc patients.

NO is largely oxidized to nitrate (NO_3^-) and nitrite (NO_2^-) and measurements of total nitrate and nitrite production, as well as ADMA levels are described as a reflection of endothelial dysfunction in many diseases (17). Endothelial dysfunction in SSc has been considered to be among the primary events during the progression of the disease. Because the etiology of endothelial dysfunction is still unclear, free-radical-mediated damage and immunological insults remain the attractive proposals to mediate effects. ADMA represents a novel risk factor for endothelial dysfunction.

Doodley et al. (19) have described an increased formation of nitric oxide (NO) in patients with PRP or lcSSc, but nitration of proteins and ADMA are a particular feature of dcSSc and may reflect abnormal NO regulation and/or contribution to endothelial dysfunction in SSc (19).

Increased serum levels of ADMA in SSc patients have been reported (19-21). Conflicting results of ADMA and SSc disease subset may be probably due to differences in the degree of inflammatory disorder, disease subset and treatment of the patients (19).

Angiogenesis biomarkers

Vascular dysfunction and injury is a hallmark of SSc. Moreover, as the disease progress, 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 a hypoxic state. Tissue hypoxia is normally a trigger for vasculogenesis and angiogenesis; however, vascular recovery is impaired in SSc, and avascular areas are prominent (22). In SSc, several pro-angiogenic mediators have been shown to be upregulated, including angiogenic factors such as VEGF, interleukin-8 (IL-8/CXCL8), and basic fibroblast growth factor (bFGF) (22). Hence, although most studies point to a lack of compensatory blood vessel growth and deregulated aberrant angiogenesis in SSc, paradoxically, the balance of pro-angiogenic factors appears to well exceed the anti-angiogenic mediators.

Several anti-angiogenic or angiostatic mediators have been reported to be upregulated in SSc, including endostatin, platelet factor 4, thrombospondin, and IL-4 (23). Although the angiogenic properties of these factors are well-described in healthy and other disease settings, their role in SSc angiogenesis is mostly lacking (22), conflicting (24) or flawed by low patient numbers, poor clinical characterization and heterogeneity of the study population. In addition, the functional contribution of angiostatic factors to the disturbed angiogenesis in SSc has not been addressed.

Vascular Endotelial Growth Factor (VEGF)

VEGF is involved in many different steps of angiogenesis, including initial vasodilation, endothelial cell permeability, remodeling of the perivascular matrix, induction of proliferation and migration of endothelial cells (25) and precursor cells (26). It has biological effects extremely dose (24) and time (26) dependent. In SSc patients, platelets store and transport high levels of VEGF-A, and when activated in contact with injured endothelium, may be a source of circulating VEGF-A (27). Bielecki et al. (28) reported that peripheral blood mononuclear cells from SSc patients produce high amounts of VEGF-A in the early stage of the disease (28).

Tissue ischemia normally leads to the expression of angiogenic growth factor VEGF, which then initiate angiogenic sprouting by inducing vasodilation, proliferation and migration of ECs. This leads to stabilization of a new tubal structure and lumina that form new vessels. Interestingly, while both pro- and anti-angiogenic factors are overexpressed in SSc, there appears to be an imbalance in the ratio of these mediators, favoring inhibition of angiogenesis and progressive vascular disease. Surprisingly, several studies have demonstrated enhanced expression of the pro-angiogenic factor VEGF-A in both the skin and

the circulation of SSc patients (28, 29). The paradox is that VEGF levels, rather than being associated with evidence of angiogenesis, actually correlate with progressive microvascular loss and disease progression (30). Uncontrolled overexpression of VEGF might have deleterious rather than beneficial effects as it may result in the formation of irregular vessels with impaired blood flow (26).

VEGF-A exerts its biological functions by binding to the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) and the co-receptor neuropilin-1. In particular, the pro-angiogenic effects of VEGF-A are mediated principally by the activation of VEGFR-2. In fact, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), Akt, and activation of p38 mitogen-activated protein kinase (MAPK) by VEGFR-2 is necessary for the promotion of endothelial cell proliferation, resistance to apoptosis, migration through extracellular matrix, and formation of a vascular lumen. On the contrary, the specific role of VEGFR-1 in endothelial physiology is less well defined and somewhat controversial (25).

Manetti et al. (25) showed the first evidence that a switch from pro-angiogenic to anti-angiogenic VEGF-A isoforms may play a crucial role in the defective angiogenic and vascular repair processes that characterizes SSc vasculopathy. In this study increase of VEGF-A in SSc patients was the result of a significant increase in the anti-angiogenic VEGF165b isoform instead of VEGF165. Circulating levels of VEGF165b were raised in SSc, and were both early and persistent features of the disease. In addition, this study demonstrated that microvascular endothelial cells isolated from SSc skin constitutively expressed and released higher levels of VEGF165b than did endothelial cells from healthy individuals (29).

Angiogenic	**Angiostatic**
Vascular endothelial growth factor (VEGF)	Endostatin
Platelet growth factor (PlGF)	Angiostatin
Basic fibroblast growth factor	Interferon-inducible protein 10/CXCL10
Platelet-derived growth factor (PDGF)	Soluble endoglin
Hepatocyte growth factor (HGF)	Angiopoietin 2
Transforming growth factor beta (TGF-β)	Kallikrein 3
Fibroblast growth factor (FGF)	Pentraxin 3
Interleukin-8/CXCL8	Interleukin-4
Stromal cell-derived factor 1/CXCL12	Thrombospondin 1 (TSP-1) and TSP-2
Interleukin-6	Platelet factor 4
CD44 Kallikrein 9, 11, and 12	Monokine induced by interferon-/CXCL9
Urokinase plasminogen activator receptor	Tissue inhibitors of metalloproteinase
Matrix metalloproteinase 9 (MMP-9) and proMMP-1	

Table 2.3.1: Up-regulated vascular biomarkers in SSc (30, 31)

There was an attractive hypothesis that the lack of sufficient angiogenesis in SSc could be mediated by a downregulation of the VEGF-A/VEGFR system (32). Conversely, several studies have shown that VEGF-A expression is markedly increased in different cell types in the skin of patients with SSc (32, 33). Moreover, VEGFR-1 and VEGFR-2 were also found o be upregulated on dermal microvascular endothelial cells in SSc-affected skin (32). In addition, a number of studies have demonstrated that circulating levels of VEGF-A are significantly increased in SSc patients throughout different disease stages (5, 26, 34). A positive correlation was found between VEGF-A levels and the severity of nailfold capillary loss (24) as well as with the extent of skin sclerosis measured by Rodman skin thickness score. Kuryliszyn- Moskal et al. addressed the relationship between VEGF-A levels and organ systemic involvement in SSc (35) and described significant elevated concentrations of VEGF-A in the sera of SSc patients with organ systemic damage compared to those without systemic manifestations.

Although the upregulation of VEGF and other angiogenic factors might be a compensatory mechanism for the initial effect of unidentified angiostatic factors, the temporal kinetics of its expression appear to be critical in overcoming the inhibitory effects of angiostatic factors. In this regard, it has been shown that a brief upregulation of VEGF results in instability of newly formed vessels (23) . On the other hand, prolonged over-expression of VEGF, as is seen in SSc patients throughout various disease stages, also has deleterious effects because the vessels fuse in an uncontrolled manner and form a chaotic vessel network that is strikingly similar to the disturbed capillary network observed in SSc (23). In addition, isolated microvascular endothelial cells from patients exhibit an impaired response to VEGF in the Matrigel capillary morphogenesis assay (23) indicating that VEGF receptor signaling might be impaired in endothelial cells of SSc patients.

Also, when endothelial cells are exposed to angiogenic stimuli (VEGF), plasminogen activation is initiated through the binding of plasminogen and urokinase to their receptors. This leads to the formation of plasmin that activates pro-metalloproteases which degrades non-collagenous components of the matrix and releases peptides that can inhibit continued angiogenesis, these peptides are angiostatin and endostatin (36).

Endostatin

Endostatin is is a newly characterized angiogenesis inhibitor that is an endogenously produced, a C-terminal, 20 kDa C-terminal fragment of collagen XVIII (37) that inhibits angiogenesis and tumour growth strongly by reducing

endothelial cell proliferation and migration (37, 38) potentially by inhibiting membrane type 1 matrix metalloproteinase and matrix metalloproteinase 2 (26).

This action might be related to increased synthesis and proteolysis of type XVIII collagen in SSc (38). Endostatin specifically inhibits endothelial proliferation, migration and potently inhibits angiogenesis and tumor growth (37, 39). It has been reported that endostatin can inhibit VEGF expression in arthritic joints, suggesting that endostatin inhibits joint angiogenesis and arthritis progression in part by suppressing VEGF production (39). This could be an interesting view to explore in SSc patients. Endostatin could participate in the occurrence of ischemic manifestations in SSc, as it efficiently inhibits the assembly of human endothelial cells into complex vessels (36).

Moreover, endostatin has been shown to be contained in platelet alpha granules, and is released during platelet activation and aggregation. VEGF exerts its action on the release of different collagenases, which cleave endostatin from collagen XVIII in the endothelial cells. Endostatin exerts its anti-angiogenic effect by its interaction with several endothelial cell surface receptors as it competes for the receptors of VEGF (36).

Hebbar et al. (38) and Farouk et al. (36) described increased endostatin serum levels in SSc patient while Distler et al (24) described no difference in Endostatin levels in SSc patients compared to healthy controls, in dc/ lcSSc, in early /intermediate / late SSc disease stage, in the three capillaroscopic patterns or in patients with DU. with DU or scars compared to healthy control group.

Endoglin

Endoglin is a 180 kD homodimeric covalently disulphide-linked transmembrane glycosylated protein that acts as a co-receptor for TGF-β predominately expressed on cell surface of EC (40). A co-receptor is defined as a cell surface protein that is capable of interacting with ligand, but does not actively transmits signaling responses (41).

The vascular effect of TGF-β in angiogenesis results in activation of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) and has a critical function in the development of the vascular system, being required for efficient VEGF-induced angiogenesis (42). These effects are mainly mediated by endoglin (ENG, CD105). ENG plays a role in vascular integrity and endothelium functioning whereas soluble ENG (sENG) acts as an anti- angiogenic protein that interferes with the binding of TGF- β to its receptor (43). Serum levels of sENG have been reported as being increased in SSc patients compared to healthy controls (40, 43-45). Wipff et al. found showed association between higher sENG

levels and an SSc vascular phenotype that integrates the presence of digital ulcers (43).

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A PRISMA-Driven Systematic Review For Predictive Risk Factors of Digital Ulcers In Systemic Sclerosis Patients

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2.4. A PRISMA-DRIVEN SYSTEMATIC REVIEW FOR PREDICTIVE RISK FACTORS OF DIGITAL ULCERS IN SYSTEMIC SCLEROSIS PATIENTS

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Review

A PRISMA-driven systematic review for predictive risk factors of digital ulcers in systemic sclerosis patients

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ABSTRACT

Vasculopathy has a major role in the pathogenesis and tissue injury in systemic sclerosis (SSc). Raynaud's phenomenon (RP) is frequently the first clinical manifestation of SSc preceding by years other clinical manifestations. RP in SSc patients is frequent, often very severe and long lasting. The repeated bouts of RP lead to prolonged digital ischemia that may progress to digital ulceration or in extreme to critical digital ischemia with gangrene. Digital ulcers (DU) are a true burden for all patients. They are very painful, with a long and slow healing course, have high risk of infection and are extremely disabling. In adults, up to 40–50% of patients will experience at least one DU in the course of the disease and of these 31–71% will have recurrent ulcers.

In order to try to identify predictive risk factors for DU in SSc patients, an extensive literature review was conducted, according to the guidelines proposed at the PRISMA statement. MEDLINE database (PubMed) and Thomson Reuters Web of Knowledge platform were searched for articles published in peer-reviewed journals since 1990 with the last search run on June 2014 and published in English language. The keyword search terms included: digital ulcer/s, systemic sclerosis, scleroderma, digital scars, ischemic complications, autoantibodies, biomarkers, endothelium dysfunction, endothelin-1, vascular endothelial growth factor (VEGF), endostatin, ADMA, endoglin, angiostatin, and capillaroscopy.

The following criteria were included: (1) cohorts of SSc patients including patients with DU, (2) endothelium dysfunction and angiogenesis biomarkers compared with a healthy control group, (3) autoantibodies, capillary morphology and distribution, endothelium dysfunction and angiogenesis biomarkers compared between patients with and without digital ulcers, (4) detailed description of the statistical methods used to conclude for predictive factors, and (5) English language. Our search provided a total of 376 citations. Of these, 297 studies were discarded for not meeting the criteria proposed.

Abbreviations: α-IFN, interferon alpha; aβ$_2$GPI, anti-beta2 glycoprotein I antibodies; ACA, anti centromere autoantibody; aCL, anticardiolipin autoantibodies; ACR, American College of Rheumathology; ADMA, asymmetric dimethyarginine; AECA, antiendothelial cell autoantibodies; AFA, anti-fibrillarin autoantibodies; ANA, antinuclear autoantibodies; Ang-1, angiopoietin 1; Ang-2, angiopoietin 2; ANGPTL3, angiopoetin-like protein 3; Anti-PM, anti-exosome autoantibodies; CENP-A/B/F, centromeric proteins; CRP, C reactive protein; CSURI, Capillaroscopic Skin Ulcer Risk Index; dcSSc, diffuse systemic sclerosis; DLCO, diffusing capacity of the lung for carbon monoxide; DU, digital ulcers; EMA, European Medicines Agency; ENG, endoglin; EPC, endothelial progenitor cells; ESR, erythrosedimentation rate; ET-1, Endothelin-1; EULAR, European League against Rheumatism; EUSTAR, Eular scleroderma trials and research; FMD, flow mediated dilation; FVC, forced vital capacity; HAQ, health assessment questionnaire; icSSc, intermediate cutaneous scleroderma; LAC, lupus-like anticoagulant; IL, Interleucin; ILD, Interstitial lung disease; lcSSc, limited systemic sclerosis; Medgers DSS, Medgers disease severity score; MES, microangiopathy evolution score; NAN, North American native; NO, nitric oxide; NVC, nailfold videocapillaroscopy; PAH, pulmonary arterial hypertension; PDGF, platelet derived growth factor; PlGF, placental growth factor; pre-SSc, prescleroderma; PRP, primary Raynaud phenomenon; RNP, ribonucleoprotein; RP, Raynaud phenomenon; SRP, secondary Raynaud phenomenon; SSc, systemic sclerosis; ssSSc, systemic sclerosis sine scleroderma; sVCAM, Soluble Vascular Adhesion Molecules; TLC, total lung capacity; VEDOSS, vearly early disgnosis of systemic sclerosis; VEGF, vascular endothelial growth factor; yrs, years.

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Contents

1. Introduction

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by small vessel vasculopathy, inflammation, production of autoantibodies and fibroblast dysfunction leading to increased deposition of extracellular matrix [1] affecting skin and internal organs.

Vasculopathy has a major role in tissue injury pathogenesis in SSc. Injury of endothelial cell layer, infection, immune-mediated cytotoxicity, ischemia and reperfusion all contribute to microangiophaty. Evidence of vascular injury and endothelial damage are detected at the initial stage, resulting in tissue hypoxia with activation of both innate and adaptive immune systems [2]. Endothelial cell dysfunction leads to endothelial cell apoptosis, over-expression of adhesion molecules, leucocyte adhesion, activation and adhesion of platelets and influx of perivascular infiltrate [3]. Cellular growth factors, oxidative stress, and immune mediators stimulate the appearance of myofibroblasts in the vessel wall that synthesize a thickened intima layer [3], which are associated to smooth cell proliferation that leads to stenosis and/or occlusion of microvascular bed with stimulation of both angiogenesis and vasculogenesis [4] and in some patients macrovascular involvement is observed [5].

Injured endothelium leads to an imbalance of microvascular tone control, favoring vasoconstriction, due to overproduction of vasoconstrictors by endothelial cells or to reduced endothelium-dependent vasodilation [4]. Doubts persist about underproduction or impaired action of vasodilators produced by the endothelium, such as nitric oxide (NO) and prostacyclin [4].

Raynaud's phenomenon (RP) in SSc is usually very frequent, severe and long lasting. Frequently it's the first clinical manifestation of SSc proceeding by years other clinical manifestations. Initially RP has only functional implications, but the repeated bouts lead to structural abnormalities and digital ischemia that may progress to digital ulceration or in extreme cases to digital critical ischemia with gangrene. Microvascular structural changes are easily assessed by nailfold videocapillaroscopy (NVC), a simple, non-invasive, cheap and accessible tool. Capillaroscopic patterns are dynamic and probably reflect disease evolution and severity. Several qualitative, semi-quantitative and quantitative indexes have been proposed as predictors of organ involvement and in particular digital ulcers (DU).

Digital ulcers are a true burden for all patients. They are very painful, with a prolonged and slow healing, with a high risk of infection and are very disabling, impairing severely simple daily activities. In adults 40–50% [6,7] of patients will experience at least one ulcer in the course of the disease and of these 31–71% will have recurrent ulcers [8]. Patients may present with digital pitting scars, digital ulcers, calcinosis or in more severe state, gangrene with the need of amputation.

2. A PRISMA-driven systematic review for predictive risk factors of digital ulcers in systemic sclerosis patients

2.1. Objective

A PRISMA-driven systematic review was done to identify, epidemiological and clinical characteristics, capillaroscopic findings, autoantibodies and biomarkers of endothelium dysfunction and angiogenesis as putative predictive factors of digital ulcers.

2.2. Methods

An extensive literature review was conducted, according to the guidelines proposed at the PRISMA statement [9], to select predictive factors of digital ulcers in SSc patients. MEDLINE database (PubMed), Cochrane and Thomson Reuters Web of Knowledge platform were searched for articles published in peer-reviewed journals since 1990 with the last search run on June 2014 and published in English language. The keyword search terms included: digital ulcer/s; systemic sclerosis, scleroderma, digital scars, ischemic complications; autoantibodies, biomarkers, endothelium dysfunction, endothelin-1; vascular endothelial growth factor (VEGF), endostatin, ADMA, endoglin, angiostatin, capillaroscopy. The following criteria were assessed: (1) cohorts of SSc patients that included patients with digital ulcers, (2) endothelium dysfunction and angiogenesis biomarkers compared with the healthy control group, (3) antibodies, capillary morphology and distribution, endothelium dysfunction and angiogenesis biomarkers compared between patients with and without digital ulcers (4) detailed description of the statistical methods used to conclude for predictive factors.

For the identification of papers, the process included the following steps: screening of the identified records in cited databases; examination of potentially relevant papers; and application of the eligibility criteria to select the included papers. The first author was responsible for screening and assessing eligibility, the second author examined a small sample of them and the last author validated the screening.

2.3. Results

Our search provided a total of 378 citations. After reviewing the title and abstract 298 were discarded for not meeting the criteria proposed. A total of 80 papers were identified for review, as illustrated in PRISMA flow diagram (Fig. 1).

Due to rarity of disease many papers included studies with small samples, non-randomized controlled trials, retrospective or prospective cohort studies, cross-sectional and post-hoc analysis of registries. Clinical cases were excluded.

2.4. PRISMA 2009 flow diagram

3. Review of monocentric, multicentric, national and international SSC cohorts' published data

Systemic sclerosis epidemiology and knowledge of natural disease course is not completely understood due to rarity of the disease, difficulty in diagnosis, different disease classifications and to heterogeneity of clinical presentation among patients. Currently, more information on predictive and prognostic factors for organ involvement and survival in adult SSc patients is emerging. Several SSc cohort reports have been published regarding clinical, demographic and laboratory parameters.

3.1. Clinical findings/cohorts' review

In 2002 Ferri and al. [7] in a study designed to evaluate patient survival reported 48% DU incidence in 1012 patients followed for a mean of 5.1 years. DU were more common in intermediate cutaneous scleroderma (icSSc) — sclerosis of upper and lower limbs, neck, and face, without truncal involvement; and diffuse cutaneous scleroderma (dcSSc): distal and truncal skin sclerosis [7].

Walker et al. [10] in an extended report from the EULAR Scleroderma Trials And Research group database with 3656 patients identified more DU in dcSSc group and showed as strongest predictors factors of DU, early onset of Raynaud Phenomenon (<42.8 years -confirmed in uni and multivariate analysis) and positive anti-topoisomerase I autoantibodies [10].

Hacchula et al. [11] in an analysis from a single center registry in the French Nationwide screening program for pulmonary arterial hypertension (PAH), showed a DU prevalence of 43%, underestimated, as this study excluded patients with severe cardiac disease and severe pulmonary function abnormalities (forced vital capacity (FVC) <60%); most of these patients had dcSSc. In multivariate analysis, patients at younger age of SSc—first non-RP symptom onset, high Rodman skin score, patients not receiving vasodilator therapy and with dcSSc were at higher risk of developing DU. These typically occurred within 5 years after the first non-RP symptom. Recurrent ulcers in the first 2 years after the first episode had a higher risk for new recurrent DU. A great burden of DU is well documented as the healing time described was 105 ± 97 days (5 days to 2 years) [11].

In the registry of the German Network for Systemic Scleroderma with 1881 patients enrolled from 2003 to 2007, 24.1% had active ulcers when recruited. Sunderkotter et al. [12] reported that SSc patients with DU developed RP, skin sclerosis, and organ involvement 2–3 years earlier. A risk of probability for DU was proposed: 88%: male, early onset of RP (20 years age), erythrosedimentation rate (ESR) > 30 mm h^{-1}, positive anti-Scl70 antibodies and PAH; 63%: male, early onset of RP, ESR > 30 mm h^{-1}, positive ACA antibodies and PAH; and 78% risk if female, with an early onset of RP, ESR > 30 mm h^{-1}, positive anti-Scl70 antibodies and PAH. Univariate and multivariate analyses showed that diffuse skin sclerosis in combination with PAH is the most powerful predictor for the occurrence or DU, followed by male sex, anti-topoisomerase I autoantibodies, PAH and involvement of mouth and esophagus [12].

Tiev et al. [13] in a post-hoc analysis of a registry conducted to evaluate the prevalence of PAH in SSc patients in French ItinerAIR-sclerodermie registry reported a prevalence of 53% of DU in 599 patients enrolled in eleven months. This study had some limitations, as data of DU may be lacking and patients included were seen in tertiary referral centers for SSc, probably having more severe diseases. Patients with severe cardiac disease (left ventricular ejection fraction < 45%) and patients with severe pulmonary function abnormalities [defined as FVC, total lung capacity (TLC) or forced expiratory volume in 1 s < 60% of predicted] were excluded. DU were associated to positive anti-topoisomerase I autoantibodies and dcSSc. No association was found with smoking and exposure to occupational hazards. In a multivariate analysis, diffusing capacity of the lung for carbon monoxide (DLCO) impairment was strongly associated with current DU, independently of disease duration and may point to a pathophysiological link between systemic vasculopathy and DU [13].

Eighty five SSc patients cyclically treated with iloprost were followed by Caramaschi et al. [14] in order to evaluate the incidence and characteristics of patients with and without DU. An additive model was calculated with a score reflecting the sum of the following risk factors: younger age at SSc onset < 47 years, a delay in beginning iloprost therapy greater than 18 months, history of smoking and the presence of joint contractures. The prevalence of ischaemic DU increased progressively from the lowest (0 — no risk factor present) to the highest score (4 — all risk factors present) with a high statistical significance [14].

Steen et al. [15] described SSc patients attending two centers, the University of Pittsburgh (2080 patients with SSc, identified between 1972 and 1995, and prospectively followed up for a mean of 10 years) and the Royal Free Hospital in London (1168 patients with SSc). In the University of Pittsburg cohort DU prevalence was 58%, 32% were persistent/recurrent DU and 30% of these complicated, while Royal Free Hospital cohort had a 17% DU prevalence. Risk factors were also different in the 2 centers, DU were more frequent in women and in patients with positive ACA, and anti-topoisomerase I autoantibodies in University of Pittsburg cohort while in the Royal Free Hospital cohort DU were more frequent in women, limited SSc (lcSSc) subset and positive ACA and anti-topoisomerase I autoantibodies [15].

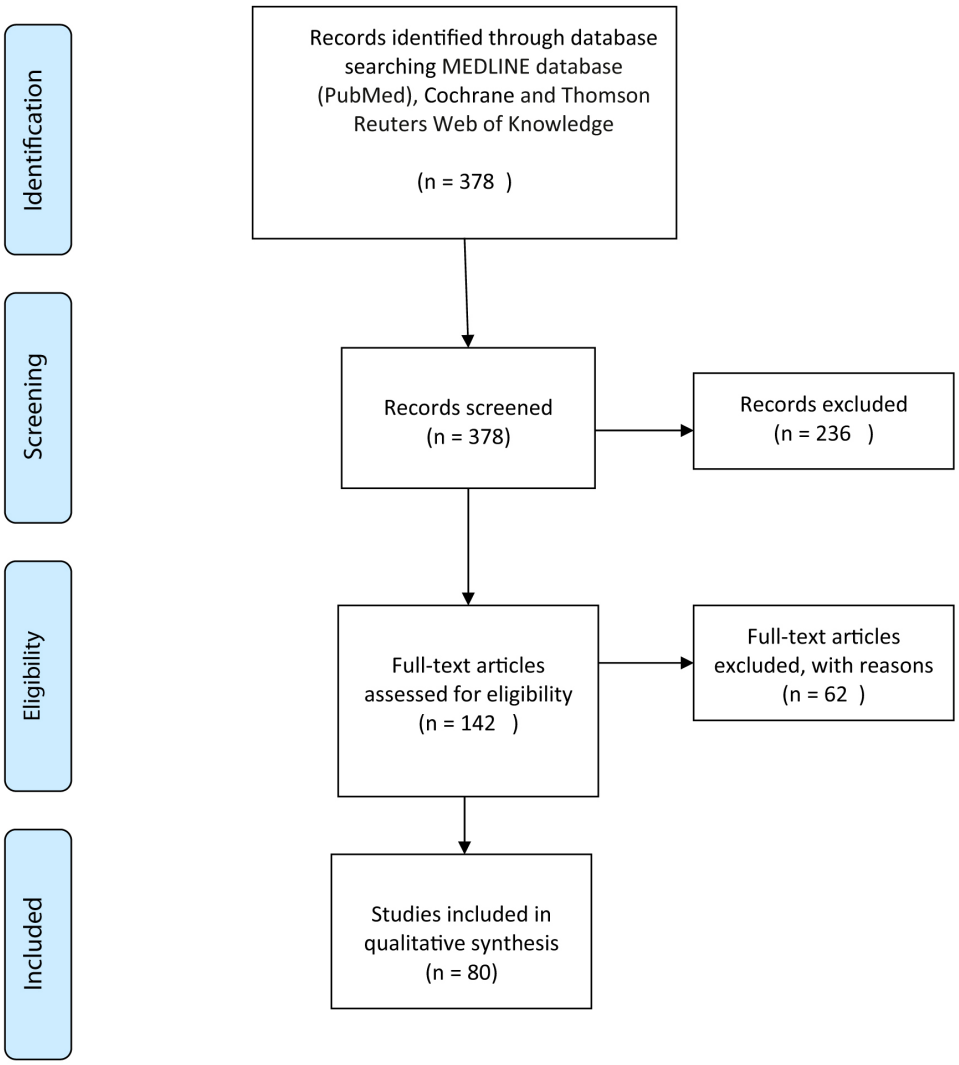

Fig. 1. PRISMA flow diagram.

Multi-site observational study of Canadian Scleroderma Research Group Registry with 938 patients was analyzed by Khimdas et al. [16] in order to define associations between DU and other SSc vascular complications and organ involvement. Eight percent had active DU at enrolment and 44% had past history of DU. This study had some potential pitfall as in cohorts where patients have long disease duration at time of entry to the study, the number of DU may be under- or overestimated, and relationship of both autoantibodies and skin involvement to DU may not be correctly related to the timing of the first DU. By univariate analysis, younger age at first visit, longer disease duration, younger age at onset of RP and first non-RP symptom and positive anti-topoisomerase I autoantibodies were associated with a history of DU. While in the multivariate analysis, the most important variables to predict digital ulcers were younger age of onset, interstitial lung disease (ILD), higher hand and finger skin score, and higher health assessment questionnaire (HAQ) score. Analyzing SSc subtypes, younger age of onset and hand and finger skin score were more common in both dcSSc and lcSSC, while ILD, oesophageal dilatation were also risk factors in dcSSc and higher HAQ score in lcSSC.

Manno et al. [17] ascertained prospectively in an on-going longitudinal database with a total of 2300 SSc patients attending John Hopkins Scleroderma Center between 1990 and 2009. Late-age onset SSc patients had a 36% reduction in risk for digital ischemia compared to those with younger age onset disease after adjustment for race, gender, SSc subtype, disease duration and smoking status.

DUO registry including 2439 patients with ongoing digital ulcer disease, irrespective of treatment regimen of 271 centers in 18 European countries, was designed to describe the clinical and autoantibody characteristics, disease course and outcomes of patients with DU. Some limitations of this observational study include data

lacking and different analysis methods. This study was initiated following European Medicines Agency (EMA) approval of Bosentan, and it is possible that patient entry into the registry was biased toward patients receiving Bosentan. Denton et al. [18] described that DU were associated with early disease onset and dcSSc. In anti-topoisomerase I positive patients, the first digital ulcer occurred approximately 6 years earlier than ACA-positive patients. Anti-topoisomerase I positive patients were younger at the onset of first RP symptoms and had shorter time periods from the onset of RP to the first digital ulcer.

A nationwide cross-sectional analysis of a large cohort of SSc patients from 14 Spanish Centers was reported by Simeón-Aznar et al. [19]. A total of 916 SSc patients were classified in 4 subsets using a modification of LeRoy and Medsger classification: "prescleroderma" (pre-SSc), lcSSc, dcSSc, and SSc sine scleroderma (ssSSc). DU were more frequent in dcSSc than in the lcSSc subset, and in both subsets than in the ssSSc patients. Patients who were anti-topoisomerase I positive had a higher prevalence of DU [19].

Gender, geographic localization, environmental determinants, ethnics and genetics may all play a role in ethology of vascular complications in SSc patients. Bacher et al. [20] published an interesting report of 1278 patients comparing 71 (6%) North American Native (NAN), 1038 (81%) white patients and 169 (13%) non-white/non-NAN [20]. Although not significant, a trend toward a greater burden of DU and more severe RP was observed in NAN compared to whites. In a multivariate regression analysis, although not statistically significant, NAN were 30% more likely to have DU than whites, after adjustment for socioeconomic status and smoking. Of note, smoking appeared to be an independent risk factor for worse RP severity and there was a strong trend for smoking to be associated with more DU. Assumed limitations of this report are definition of NAN, which incorporates various subgroups (Amerindian, Metis, Inuit) and sample size [20]. This study is a good contribute for research into genetic and environmental determinants of SSc.

Xu et al. [21] prospectively compared clinical characteristics in 267 Chinese SSc patients with and without DU, based on the Eular scleroderma trials and research (EUSTAR) database from Peking Union Medical College Hospital. There were no significant differences in the time of disease duration in patients with and without DU. Female sex, oesophageal dysmotility and anti-topoisomerase positivity were associated to DU. A multivariate analysis identified anti-topoisomerase I positivity, younger age at the time of SSc onset, and esophageal dysmotility as risk factors for DU in dcSSc patients, while no risk factor was identified in lcSSc patients [21].

Ennis et al. [22] reported the results of a prospective study of 148 SSc patients, with an overall digital tip and extensor surface ulcer prevalence of 10%. No differences in demographic and clinical variables assessed namely gender, age, disease and RP duration, disease subset, smoking status, and autoantibody profile were found between the DU and non-DU groups. The limitation of this study is that it was not adequately powered to look for associations between the presence of DU and different demographic and clinical features.

Poormoghim et al. [23] evaluated demographic, clinical and laboratory features associated with scleroderma-specific autoantibodies of 100 Iranian patients with SSC attending Firoozgar Hospital. In patients with significantly positive antinuclear autoantibodies (ANA) and higher skin score, RP and digital ulcer/gangrene were observed. In patients with positive anti-topoisomerase I there was an association with higher skin score, vascular and pulmonary features of the disease [23].

Ferri et al. [24] analyzing a large Italian SSc patient cohort evidenced a clear-cut evolution of disease pathomorphosis and prognosis during the last years with a lower rate of skin ulcers observed either at the beginning or at the end of follow-up, compared with the old series.

3.2. Autoantibodies

Autoantibodies are usually found in connective tissue diseases and can be useful for diagnosing, categorizing and predicting the likelihood of certain complications, such as DU associated with SSc. Several reports describe the potential association between autoantibodies and DU.

3.2.1. Anti-Scl-70 (anti-topoisomerase I autoantibodies)

Anti-topoisomerase I antibodies are one of the most studied autoantibodies in scleroderma.

In 2005, Steen et al. [25] in a retrospective study using the Pittsburgh Scleroderma Databank of 1432 patients found that digital tip ulcers and digital tuft resorption were seen more frequently in those with anti-topoisomerase I antibodies [25].

In a monocenter study, Hanke et al. [26] evaluated anti-topoisomerase I autoantibodies for the diagnosis and risk assessment of SSc in 280 patients. Patients with anti-topoisomerase I autoantibodies showed a higher burden of skin and lung fibrosis, contractures, electrocardiogram changes, as well as DU and had a more active disease. This study suggests that diagnosis and risk assessment in SSc patients can be supported by the detection of anti-topoisomerase I antibodies [26].

Sunderkötter et al. [12] using the German Network for Systemic Scleroderma identified anti-Scl70 antibodies as significantly associated to DU.

In a multicenter trial, Tiev et al. [13] also documented that the presence of anti-topoisomerase I antibodies is associated with prior or current digital ulcers [13].

In 2011, Müller et al. [27], in a monocentric study, concluded that the presence of anti-topoisomerase I autoantibodies related with dcSSc, disease activity, and DU [27]. In the same year, Denton et al. [18] described the clinical and antibody characteristics in DUO cohort and reported that almost all patients (95.7%) tested positive for ANA, 45.2% for anti-topoisomerase I and 43.6% for anti-centromere antibody (ACA). First DU episode in the anti-topoisomerase I positive patient cohort occurred approximately 5 years earlier than in the ACA-positive patient group and had RP earlier [28]. The early occurrence and high frequency of DU complications were especially seen in patients with dcSSc and/or anti-topoisomerase I antibodies [18].

Khimdas et al. [16] in a multicenter found that digital ulcers were associated with anti-topoisomerase I antibodies in lcSSc and dcSSc subsets [16]. Recently, Poormoghim et al. [23] also demonstrated that positive anti-topoisomerase I was significantly associated with dcSSc, higher skin score and digital ulcer/gangrene.

3.2.2. Anticentromere autoantibodies (ACA)

ACA were initially described in 1980 and are found in 20–30% of SSc patients. Six centromeric nucleoproteins (CENP) are known to be bound by sera from patients with SSc designated CENP-A through CENP-F.

In 1998, Jacobsen et al. [29] investigated the relationship between the presence of different types of ANA in patients with SSc and the presence of clinical features. ACA were found to be related to a high prevalence of calcinosis, telangiectasia, DU, acrosclerosis, primary biliary cirrhosis, isolated reduction of pulmonary diffusing capacity, and a low prevalence of radiological evidence of pulmonary fibrosis [29].

Steen et al. [25] found a higher frequency of ACA in patients with digital tip ulcers and in digital tuft resorption [25].

In a multicenter trial, Mierau et al. [30] analyzed ANA and their associations in SSc patients included in the German Network for Systemic Scleroderma Registry. DU in patients with ACA were less common compared to American patients and more similar to European and Japanese patients. Nevertheless, the presence of ACA does not by any means precluded digital ulcers [30].

More recently, Hudson et al. [31] in a multicenter trial with 802 SSc patients, studied the clinical phenotypes of centromeric proteins (CENP)-A- and CENP-B-positive and compared them ACA. Patients having ACA, CENP-A, and/or CENP-B resembled each other and were older chronologically and at disease onset, more commonly women, more likely to have limited disease and lower skin scores, less likely to have DU, digital tuft resorption, or finger contractures and more likely to have pulmonary hypertension [31].

Koenig et al. [32] suggested ACA as biomarkers for vascular phenotypes, such as peripheral vascular disease including DU, gangrene and acroosteolysis.

A lower incidence of digital ulcers among ACA positive SSc patients compared to positive Anti-topoisomerase I has been reported in several studies [28,31,33].

3.2.3. Anti-Fibrillarin autoantibodies (AFA)

Antibodies against fibrillarin-1 protein have been found to be highly associated with SSc in most ethnic groups.

In 2001, Reveille et al. [34] conducted a prospective study to determine whether ethnic factors influenced the presentation, serologic expression and immunogenetics of SSc. Hispanics and African Americans were more likely to have diffuse skin involvement, skin pigmentary changes, DU, pulmonary hypertension (African Americans), and an overall lower sociodemographic status than whites. The latter were likely to have more ACA and African Americans more anti-U1-ribonucleoprotein (RNP) and anti-U3-RNP (fibrillarin) autoantibodies [34].

Sharif et al. [35] examined the immunogenetic, clinical, and survival correlates of AFA in a large group of Afro-American SSc patients. After adjusting for disease duration, Afro-American SSc patients with AFA were 3.31 times more likely to have DU [35].

3.2.4. Antiendothelial cell autoantibodies (AECA)

Salojin et al. [36] in a study with 107 enrolled patients, investigated if AECA in patients with primary RP, lcSSc, and dcSSc could help to determine the long-term prognosis of the disease and documented that the presence of AECA were associated with digital scars and ulcers.

Pignone et al. [37] observed significant associations between AECA, DU and capillaroscopic abnormalities. These data suggest that in SSc the AECA antibodies are directly linked to vascular injury and could reflect endothelial damage [37].

Higher serum AECA levels have been reported as associated with the severity of peripheral vascular injury as assessed by NVC [38].

3.2.5. Less extensively studied autoantibodies in SSc

Hanke et al. [39] in a monocentric study with 280 patients found that Anti-PM/Scl-75/100 antibodies were associated with DU. Anti-PM/Scl-75 antibodies were detected more frequently in younger and more active patients with joint contractures [39]. Other studies have also demonstrated anti-PM/Scl positivity associated with an increased risk of DU [28,40]. In SSc patients, anti-Ku positivity may be protective against severe digital vasculopathies [28].

Marie et al. [41] studied anticardiolipin (aCL), anti-beta2 glycoprotein I autoantibodies (aβ_2GPI) and lupus-like anticoagulant (LAC). Anticardiolipin and aβ_2GPI antibodies and/or LAC were positive in 13 (19%) of 69 consecutive SSc patients while in the healthy control group, aCL antibody was found in only one (2%) subject. Pitting scars, pulmonary arterial hypertension, macrovascular involvement as well as severity of capillary impairment (using NVC) were more frequent in SSc patients with aCL/aβ_2GPI antibodies and/or LAC compared with those without [41].

Recently, Morrisroe et al. [42] in a study with 940 found an association between aCL IgG antibodies and digital ulcers and aCL-IgM with RP.

In 2010, Eloranta et al. [43] studied the presence of interferogenic autoantibodies SSc and their correlation with clinical manifestations, serum levels of interferon alpha (α-IFN) and chemokines involved in the disease process. DU including digital loss were associated with increased serum levels of α-IFN [43].

Mixed cryoglobulinemia (mixed cryoglobulins b type II IgG-IgMk monoclonal) in SSc patients with cryoglobulinemic vasculitis with positive ACA has been reported in association to skin ulcers [44].

3.3. Capillaroscopy

Microvasculopathy with alterations in morphology and function can easily be detected in the nailfold bed with NVC. Capillaroscopy has been described as the best predictor tool of transition of PRP to SRP [45]. Qualitative, semi-quantitative and quantitative indexes/scores have been described. Structural changes such as giant capillaries, microhemorrhages, avascular areas and neo-angiogenic capillaries are important markers of disease progression and are present in more than 90% of SSc patients [46].

Scoring the capillaroscopic pattern changes during the follow-up of SSc patients [47] enables capillaroscopy as a mandatory tool to quantify microangiopathy.

Several studies have been done in an attempt to identify association of NVC changes and clinical manifestations, in order to identify a possible predictive role of NVC. This is very important, as NVC, a very accessible tool, could be a putative biomarker of disease progression, allowing target preventive therapy to be initialized. Limitation in comparing conclusions is due to different clinical presentations and evolutions and to different ulcers being compared: hand and foot ulcers; digital or contracture ulcers or one of the 3 Medgers Disease Severity Scale — pitting scar, digital ulcer and gangrene.

3.3.1. Qualitative evaluation model

In clinical setting qualitative evaluation is the more accessible and easy to perform. Late NVC patterns have been mostly described as a risk for digital ulcer development.

Caramaschi et al. reports a strong correlation between patients with late pattern and DU in a cohort of 103 patients [48]. Furthermore the same author in a monocentric prospective study of 85 SSc patients treated with iloprost confirmed that late NVC pattern was more frequent in patients with ischemic lesions [14].

Smith et al. described a correlation between worsening of NVC patterns and peripheral vascular disease in 66 SSc patients with an 18 month follow-up. Risk of future severe peripheral vascular involvement rises according to worsening of NVC pattern: 13% for patients with a normal pattern, 20% in early pattern, 33% with an active pattern and 63% for the late pattern at baseline [49]. When adjusted for disease duration, LeRoy subset and vasoactive medication in a multiple logistic regression analysis, the future peripheral involvement was also stronger according to worsening NVC patterns [49].

3.3.2. Semi-quantitive evaluation model

Lambova et al. studied a group of 36 SSc patients evaluating semi-quantitative capillaroscopic patterns and its association with DU, aside from other parameters [50]. DU were strongly associated with active pattern, giant capillaries, hemorrhages and avascular areas. This group proposes that active pattern can be used as a predictor for trophic lesions in the future and can be an indication for preventive target therapy [50].

Sulli et al. in 2008 proposed a semi-quantitative microangiopathy evolution score (MES). In a longitudinal study his group followed 90 SSc patients for 72 months in average, and scored capillaroscopy findings in three parameters: loss of capillaries, disorganization of the microvascular array and capillary ramifications. This score paralleled the evolution of the SSc microangiopathy, as the score increases through

disease evolution. Giant capillaries and microhemorrhages did not enter the score for as disease progresses they decrease in number. In this study there is no reference to its association to DU, but it is an interesting microvascular injury surveillance score [51].

Alivernini et al. concluded from a prospective study of 130 SSc patients followed for 20 months that patients with active skin ulcers have more avascular areas and a lower capillary density [52].

Smith et al. has proposed a simple semi-quantitative NVC evaluation for day-to-day clinical use as a prognostic index of digital trophic lesions. Seventy one patients were evaluated and classified on mean score of capillary loss quantified over eight fingers (one field per finger). With a cut-off value of the mean score of capillary loss of 1.67 (based on the ROC analysis) this score had a specificity of 69.77% and sensitivity of 70.00% in predicting development of digital trophic lesions within 6 or 12 months after the first capillaroscopic visit. Limitation of the study is that it includes and compares severe digital pitting scars, digital tip ulcerations and gangrene without no differentiation of presentation and outcomes [53]. This study was further validated in a study of 18–24 month follow-up of 148 SSc patients in 2 Italian–Belgian Centers. Although with the same limitation of the previous study, higher prevalence's of novel future severe peripheral vascular involvement occurred according to more severe NVC patterns [54].

3.3.3. Quantitative evaluation model

In a monocenter prospective study evaluating 120 SSc patients with a 3 month follow-up, Sebastini et al. [55] proposed a Capillaroscopic Skin Ulcer Risk Index (CSURI) as a predictor risk of the onset on new DU using NVC parameters. The CSURI index assessed the total number of capillaries in the distal row (N), maximum loop diameter (D), number of megacapillaries (M), and the ratio M:N. ($CSURI = D \times M{:}N^{2)}$. A cut-off of 2.94 had a 73.3% positive predictive value for DU. An inverse correlation was found with high N or higher M:N ratio. The strong limitation of this index was the mandatory presence of megacapillaries, so late patterns were excluded [55]. This Index was then validated in a multicenter Italian Rheumatology Centres study, where 8229 SSc patients were enrolled and followed for 3 months. All patients with 12 or more capillaries in distal row had no DU and patients with 4 or less capillaries developed DU in all cases except two. CSURI has an important role in predicting DU in patients with 5–11 capillaries in the distal row. In particular, the comparison of the area under the ROC curve for N and CSURI showed a statistically significant difference between groups at the cut-off value of 2.96. The cumulative positive predictive value of CSURI was 62.3% and the negative predictive value was very high 97.2%. In addition, patients with DU presenting at baseline with CSURI > 2.96, had a negative predictive value of healing of 86.7% and when CSURI < 2.96, 77.8% showed regression [56]. Furthermore Sebastini validated the CSURI with three different videocapillaroscopy devices with distinct image widths, since the image length of 1.57 mm could represent a limitation factor for CSURI in clinical applicability. One hundred and seventy-six SSc patients were consecutively enrolled for the study during a six-month period, using three different capillaroscopy devices (image widths of 1.33, 1.57, and 1.70 mm). The results suggest that CSURI could be able to identify patients at higher risk of developing DU despite the use of different videocapillaroscopy devices. However, the lower positive predictive value observed with the image width of 1.33 mm suggests that a wider picture length, possibly in the range of 1.57–1.70 mm, should appreciably increase both specificity and sensitivity of the CSURI [57].

Manfredi et al. [58] recently reported a multicenter study to predict DU through a predictive risk chart taking into account capillaroscopic, demographic, and clinical–serological parameters. Multivariate logistic regression analysis showed a significant positive association between DU and male gender, past DU, CSURI with a cut-off of 2.96 and ERS rate ≥ 20 mm h^{-1} [58].

Kim et al. described a quantitative analysis of NVC in 6 digits. Number of capillaries, number of capillaries loss (deletions in 3 mm of central part of digits), apical limb width, capillary width and Endothelin-1 (ET-1) were analyzed [59]. The major limitation of this study was that dilated capillaries were mandatory. The authors concluded that capillary dimension and loss of capillaries were strongly associated with DU. Apical limb width, capillary width, capillary hemorrhage and dimension of capillary loop were also significant. Capillary dimension negatively correlates with the number of capillaries and showed positive correlations to apical and capillary widths, namely in patients with DU. ET-1 levels in plasma were increased in DU SSc group and correlated to capillary dimension in NVC [59]. NVC and ET-1 plasma levels could be an early marker of disease activity. ET-1 closely related to disease progression and severity of SSc [59]. It must be highlighted that this data is applicable for early diagnosis only.

Recently Ennis et al. [60] reported a quantitative evaluation of NVC and its correlation with the presence of current DU. Patients with most marked microvascular abnormalities are more likely to develop DU, and lends further support for NVC as a clinical biomarker. They reported that intercapillary distance was greater in patients with active ulcers, capillary density was lower although not significant and no differences between patients with or without DU regarding capillary width, tortuosity or derangement [60].

CAP multicenter observational, longitudinal prospective study of SSc patients with or without history of DU and with a six month follow-up reached the conclusion that the final model for prediction of new DU in SSc patients with a past history of DU has a reduced mean number of capillaries in the middle finger of the dominant hand, an increased number of current DU and the presence of current critical digital ischemia [61].

3.4. Endothelium dysfunction and angiogenesis biomarkers

Several studies have been conducted to identify possible biomarkers of peripheral microangiopathy. A validation of a putative biomarker would be very helpful in identification and stratification of risk, in screening protocols and implement early preventive target therapy. Difficulty in identifying a biomarker relates to the multifactorial vascular etiology and its relationship with the different disease stages, early damage, active and on-going disease. Most of the studies are cross sectional and compare SSc patients with healthy controls but more prospective studies are needed to validate these biomarkers.

3.5. Endothelium dysfunction biomarkers

3.5.1. Endothelin-1 (ET-1)

Cozzani et al. [62] analyzed retrospectively the sera of SSc patients, collected at the time of diagnosis and 6 years later if not on bosentan therapy, or, before bosentan therapy and 3 months later. They concluded that the levels of ET-1 were higher than the normal range in SSc patients and correlated with the severity of the disease. No correlation was found with new DU development. Patients on therapy with bosentan had a reduction of ET-1 levels confirming the efficacy of this molecule on both treatment and prevention of digital ulcers [62].

In a cross-sectional analytical study of Aghaei et al. [63] compared ET-1 levels in SSc patients with and without DU and found a statistically significant difference in these two groups of patients, with higher levels of ET-1 in DU group [63].

Sulli et al. [64] determined ET-1 levels in SSc patients with PRP or SRP, and in the latter group correlated with capillaroscopy microangiopaphty pattern. ET-1 levels were higher in the more advanced stages of the SSc microangiopathy, namely in the late NVC pattern. Although the study was not primarily designed to investigate correlations between single organ involvement and ET-1 levels, this

study confirmed higher mean ET-1 plasma levels in patients with active or previous DU [64].

Kim et al. [59] identified capillary dimension as a parameter for prediction of early diagnosis of microangiopathy and for regular follow-up assessments of treatment. They found a strong correlation between the levels of plasma ET-1 and the capillary dimension in NVC. Comparison of mean plasma ET-1 levels in SSc patient group showed notably higher values in the group with DU [59].

3.5.2. Asymmetric dimethyarginine (ADMA)/endoglin (ENG)

Wipff et al. [65] analyzed serum levels of ENG in SSc patients and found higher levels in patients with DU, confirmed in multiple linear regression. Disease phenotype associations remained significant between higher levels of ENG, taken as the dependent variable, for the presence of DU. Higher levels of ENG in sera of SSc patients might highlight a possible contribution of this antiangiogenic protein in the SSc vasculopathy. The authors hypothesized decoupling between enhanced angiogenesis and endothelial dysfunction in SSc and that endothelial dysfunction in SSc is not related to ADMA levels. The negative correlation between levels of ENG and ADMA in SSc patients may be a consequence of early endothelial cell damage although no association between ENG and disease duration was encountered [65].

Doodley et al. [66] have described an increased formation of nitric oxide (NO) in patients with PRP or lcSSc, but nitration of proteins and ADMA are a particular feature of dcSSc and may reflect abnormal NO regulation and/or contribution to endothelial dysfunction in SSc [66].

Our group has identified increased ET-1 and ADMA serum levels in SSc patients with DU compared to SSc patients without DU, particularly in late NVC pattern. This might point to an excessive vasoconstrictor tone in association with occlusion of distal digital circulation conducting to the reduced flow mediated dilation (FMD) in patients with DU [67].

Conflicting results of ADMA and SSc disease subset may be probably due to differences in disease subset and duration, the degree of inflammatory disorder and treatment of the patients [66].

3.5.3. Von Willebrand factor

Von Willebrand factor (vWF), a circulating glycoprotein synthesized by endothelial cells and megakaryocytes, acts as a carrier protein for the coagulation factor VIII. It promotes adhesion of platelets to the subendothelium following vascular injury [68] and is involved in hemostasis, fibrinolysis, synthesis of growth factors and in regulation of vessel tone and permeability [69]. vWf levels have been proposed as a possible indicator of endothelial dysfunction as it's levels are increased in endothelium damage [69]. There is great discussion on the usefulness of this marker of endothelial activation as it is limited by the great variability in normal values [70] and may be influenced by factors such as exercise and ABO blood group [69].

Barnes et al. found no relationship between baseline vWF and baseline concurrent/recent DU nor was it a significant predictor of outcome for new ulcer formation [69].

3.6. Angiogenesis and vasculogenesis biomarkers

Angiogenesis biomarkers have been extensively studied in SSc patients and investigated as possible putative biomarkers of organ involvement. Early microvascular morphological changes such as megacapillaries, ramified or bushy capillaries, reduction and dropout of capillaries suggest an important role of angiogenesis in peripheral microvasculopathy. This might be a consequence of imbalance of angiogenic factors and angiostatic factors [71]. It has been postulated that avascular areas are consequence of chronic hypoxia and that enlarged and bushy/ramified capillaries are a pro-angiogenic response not related to hypoxia but to the overexpression of vascular endothelial growth factor (VEGF) driven by key cytokines platelet derived growth factor (PDGF) and Interleucin (IL-1) throughout different disease stages [72].

We will analyze key pro-angiogenic biomarker VEGF, and angiostatic biomarkers such as angiostatin, endostatin and endoglin as possible predictors of digital ulcers in SSc patients.

3.6.1. Vascular endothelial growth factor (VEGF)

VEGF is involved in several steps of angiogenesis, proliferation, survival and migration of endothelial cells, with biological effects extremely dose dependent [71]. In SSc patients, platelets store and transport high levels of VEGF-A, and when activated in contact with injured endothelium, may be a source of circulating VEGF-A [73]. Bielecki et al. [74] reported that peripheral blood mononuclear cells from SSc patients produce high amounts of VEGF-A in the early stage of the disease [74].

Distler et al. [71] demonstrated an increase in VEGF in SSc patients when compared to healthy controls and significantly higher levels of VEGF in dcSSc compared to lcSSc. These were already increased in early phases of disease with no significant difference when compared to intermediate/late SScl. VEGF was increased in all capillaroscopic patterns when compared with healthy controls with no significant differences between the three NVC SSc patterns. In patients with DU, VEGF levels were lower when compared to patients without DU, pointing that high levels of VEGF may be protective against DU development. This difference was more pronounced in dcSSc [71].

Farouk et al. [75] also demonstrated a significantly higher serum VEGF level in early disease stage in SSc patients without digital ischemic manifestations compared to those with DU.

An interesting paper of Manetti et al. [76] regarding proangiogenic VEGF165 and antiangiogenic VEGF165b isoforms showed the first evidence that a switch from proangiogenic to antiangiogenic VEGF-A isoforms may play a crucial role in the defective angiogenic and vascular repair processes that characterize SSc vasculopathy. In this study, the increase of VEGF-A in SSc patients was the result of a significant increase in the antiangiogenic VEGF165b isoform instead of VEGF165. Circulating levels of VEGF165b were raised in SSc, and were both early and persistent features of the disease. In addition, this study demonstrated that microvascular endothelial cells isolated from SSc skin constitutively expressed and released higher levels of VEGF165b than did endothelial cells from healthy individuals [77]. No association to DU was described.

3.6.2. Endostatin

Endostatin is a C-terminal, 20 kDa fragment of the basement protein collagen type XVIII that inhibits angiogenesis and tumor growth strongly by reducing endothelial cell proliferation and migration [78]. This action might be related to increased synthesis and proteolysis of type XVIII collagen in SSc [79]. Conflicting reported results regarding endostatin may be due to different clinical SSc disease profiles in the patients enrolled in studies.

Distler et al. [71] described no difference in Endostatin levels in SSc patients compared to healthy controls, in dc/lcSSc, in early/intermediate/late SSc disease stage, in the three capillaroscopic patterns or in patients with DU. But patients with giant capillaries showed significantly lower levels of endostatin.

Hebbar et al. [79] described increased endostatin serum levels in SSc patients with DU or scars compared to the healthy control group.

Farouk et al. [75] also described higher levels of serum endostatin levels in SSc patients with ischemic DU and significantly higher serum levels of VEGF in those patients without ischemic manifestations. The author concludes that endostatin could participate in the occurrence of ischemic manifestations in SSc, as it efficiently inhibits the assembly of human endothelial cells into complex vessels [75].

3.6.3. Endoglin

Endoglin an angiostatic biomarker has been measured in SSc patients' serum and correlated mainly with pulmonary disease. Wipff et al. found higher levels in patients with DU, confirmed in multiple linear regression as referred above [65].

3.6.4. Placental growth factor (PlGF) and endothelial progenitor cells (EPC)

Placental growth factor (PlGF), a secreted dimeric glycoprotein, is chemotactic and mitogenic for endothelial cells in vitro and proangiogenic in vivo.

Avouac et al. [80] identified high PlGF serum levels and low circulating endothelial progenitor cell (EPC) counts as predictors of new DU in SSc patients, pointing to an insufficient vasculogenesis to counterbalance vascular damage. In multivariate analysis high PlGF serum levels and a history of DU were identified as independent predictors of new DU. Also when excluded patients with a history DU at baseline, high PlGF serum levels and low EPC counts were found to be predictors of new DU [80].

3.6.5. Angiopoietins

Angiopoietins are known to be involved in the development, remodeling and stability of blood vessels [81]. Altered expression of angiopoietin 1 (Ang-1), angiopoietin 2 (Ang-2) [81] and angiopoetin-like protein 3 (ANGPTL3) [82] has been studied as biomarkers of SSc-related microangiopathy.

Ang-1 is an endothelium protective factor, has vasoprotective and anti-inflammatory actions, mediates vessel maturation and keeps vessel integrity by the recruitment of peri-endothelial cells [81].

Ang-2 promotes endothelial activation, apoptosis and destabilization of blood vessels having an essential role in vascular remodeling. It has a contextual paper, promoting angiogenesis in the presence of VEGF, but inducing vessel regression in the absence of proangiogenic activity [81]. Ang-2 is considered as a biomarker of endothelial injury/activation as it can be rapidly released from endothelial cells upon stimulation.

In humans, serum ANGPTL3 levels correlate positively with the intima–media thickness of carotid and femoral arteries in healthy subjects, and showed a positive association with arterial wall thickness [82].

Michalska-Jakubus et al. [81] described a mean significantly lower concentration of Ang-1 in SSc patients while Ang-2 was significantly higher in patients with intermediate/late SSc comparing to patients with early disease stage and in patients with active DU. In multivariate analysis, Ang-2 significantly correlated with the presence of DU and tended to correlate with disease duration. The authors concluded that Ang-2 might be a new biomarker of disease activity.

Ichimura et al. [82] described the increase in serum ANGPTL3 levels linked to the development of DU suggesting a role in the pathogenesis of SSc vasculopathy, especially proliferative obliterative vasculopathy.

4. Discussion

Systemic sclerosis is a rare heterogeneous connective tissue disease characterized by small vessel vasculopathy, inflammation, production of autoantibodies and fibroblast dysfunction leading to increased deposition of extracellular matrix [1]. While RP and skin thickening are present in majority of patients, a variety of different clinical manifestations and presentations affecting skin and internal organs are described.

Different clinical classifications, disparity of inclusion criteria and an absence of a validated diagnostic test difficult diagnosis of SSc. Several classification criteria for SSc have been proposed: Leroy and Medsger [83], EUSTAR criteria for the very early diagnosis of systemic sclerosis (VEDOSS) [83] and ACR-EULAR classification criteria [84]. All have limitations and may exclude potential SSc patients. Even though with limitations (RP, positive autoantibodies and abnormal capillaroscopy would not be classified as SSc) ACR-EULAR is a great advance toward the early diagnosis of SSc.

Several nationwide multicenter and single center registry reports with a considerable number of patients have brought some new knowledge to the epidemiology and natural history of SSc disease course. Clinical, demographic and laboratory parameters and their association with different organ involvements have been studied.

But a lack of uniformed criteria in clinical practice, research, inclusion of patients in national and international databases and trials, difficult the task of a better knowledge of SSc disease, natural course, organ involvement and outcomes and compromises comparison of different cohort study results.

This review identified several disparities of results between cohorts, probably due to different SSc patient populations, different disease stages and/or clinical presentations, different organ involvements non-uniform DU classification and small number of SSc patients in most of the clinical and investigational research cohorts being compared. Another bias is that most studies are from tertiary center's surely including SSc patients with more severe disease.

Regarding DU, several classifications, and different types of ulcers, different locations and a non-uniform complication and outcome definition, make the comparison between different reports very difficult. Peripheral vascular involvement in SSc Medsger disease severity scale [85] ranging from moderate to end stage (2–4 scale) includes digital pitting scars, digital tip ulcerations and digital gangrene. These 3 different clinical presentations are all included in some studies [49,53,54], while most of studies refer DU as outcome with no other specific characterization.

Another limitation is that most of studies were not designed to outlook for predictable risk factors for DU on its own although important information and lessons may be learned from these studies (Table 1).

Digital ulcer prevalence ranged from 10% (22) to 58% (15). Even though different disease subset classifications were used, dcSSc was a risk factor for digital ulcers in most of the large cohort registries [7,10,11,13,16,18,21,24] as early onset of RP [10,12,16,18,21]. Early first non-RP symptom was a relevant risk factor in six registries [11,13,14,16,17,21]. A great extent of skin fibrosis (high Rodman's skin score) was also associated to DU [11,13,16,21].

Anti-topoisomerase I positive antibodies have shown a strong association with DU [12,15,16,19,21,23,33].

Despite associations of autoantibodies with SSc clinical phenotypes, these are not 100% sensitive or specific for certain end organ involvement, therefore it's mandatory to monitor for major organ complications, even if their autoantibody status confers relative protection from a specific organ disease or an overall good prognosis [28].

Gender was not an important risk factor in most studies. In the German SSc population being a male was the most powerful independent predictor of DU. Two reports showed a higher prevalence of DU in males [12,13].

Smoking habit and its association with DU was reported of in an Italian registration [14]. The same study showed an association between the delay in beginning iloprost therapy and DU presentation. Low socioeconomic status was not associated with an increase in digital ulcer [86,87]. Recently environmental exposures have been suggested to play a role in SSc pathogenesis [88] but no specific agent with higher DU incidence has been reported.

The German Network found a strong association between pulmonary arterial hypertension (PAH), ESR > 30 mm and DU. Impaired diffusing capacity for carbon monoxide (DLCO < 60%) had a statistical significance of association with DU in the post-hoc analysis of nationwide multicenter cohort (ItinerAIR-Sclerodermie) [13] and in the Canadian Scleroderma Research Group Registry [16].

Nailfold videocapillaroscopy capillaroscopy is an accessible, non-invasive, easy, safe and cheap tool with important diagnostic and prognostic values in patients with SSc. Capillaroscopy has been described as

Table 1
Most frequent predictor risk factors for DU in SSc patients. SSc: systemic sclerosis; RP: Raynaud phenomenon; Anti-Scl 70: anti topoisomerase 70 antibody.

Study year	Diffuse SScl	Early RP onset	Early non-RP first symptom	High Rodmann score	Scl70+	Male	Reference
Ferri et al., 2002	+						[6]
Walker et al., 2007	+	+			+		[9]
Hachulla et al., 2007	+		+	+			[10]
Sunderkotteret al., 2009	+	+		+	+	+	[11]
Tiev et al., 2009	+		+	+	+	+	[12]
Caramaschi et al., 2009			+				[13]
Steen et al., 2009					+		[14]
Khimdas et al., 2011	+	+	+	+	+	+	[15]
Manno et al., 2011			+				[16]
Denton et al., 2012	+	+			+		[17]
Simeon-Aznar et al., 2012	+				+		[18]
Xu et al., 2013	+	+	+	+	+	+	[12,20]
Poormoghim et al., 2013					+		[22]

the best predictor tool of transition of primary to secondary RP [45]. Structural changes such as giant capillaries, microhemorrhages, avascular areas, and neo-angiogenic capillaries are important markers of disease progression and are present in more than 90% of SSc patients [46]. Qualitative, semi-quantitative and quantitative indexes/scores have been described (Table 2).

Qualitative parameters associated with DU are late NVC Scleroderma pattern [14,48,54], active pattern [50], active/late pattern [64] and worsening of capillary scleroderma NVC pattern [49]. Smith et al. [53] suggested a semi-quantitative parameter as a simple prognostic index for digital trophic lesions for daily use in SSc patients, calculating the mean score of capillary loss in eight fields (8 fingers, 1 field per finger). A cut-off value of 1.67 has a sensitivity and specificity of about 70% in predicting future DU. Sebastini et al. proposed a quantitave predictor index, CSURI, as a prognostic tool, with the great limitation being that giant capillaries are mandatory, but as so, it can be used in earlier stages of disease. The index has been validated on different studies and with different length image devices and a cut-off of 2.96 will predict DU. Its greater advantage is in patients in gray zone of more than 4 but less than 11 capillaries in the distal row. Avascular areas and lower capillary density [52], capillary dimension, apical limb width, capillary width and dimension of capillary loop [59] are other parameters studied and associated to DU.

In pathophysiology of DU, endothelium dysfunction and impaired angiogenesis probably have the main roles. Early detection of endothelium lesion and inefficient angiogenesis/vasculogenesis could help in identifying patients at high risk for DU for target therapy. Conflicting results have been reported (Table 3) but this might be due to SSc patient cohort in different disease stages. Also not all studies have DU prediction as primary endpoint and probably used different biomarker assays.

Endothelin-1 is endothelium dysfunction most studied biomarker. One study [62] reports no association between ET-1 serum levels and DU, while 3 studies reported higher serum ET-1 levels in patients with DU [59,63,64]. vWf levels have been proposed as a possible indicator of endothelial dysfunction as its levels are increased in endothelium damage [69]. But a report found no relationship between baseline vWF and baseline concurrent/recent ulcers nor was it a significant predictor

Table 2
Capillaroscopic finding associated wit digital ulcers. CSURI: Capillaroscopic Skin Ulcer Risk Index; NVC: nailfold videocapillaroscopy.

Author/year	Qualitative parameters	Semi-quantitative parameters	Quantitative parameters	References
Caramaschi et al., 2007	Late NVC pattern			[47]
Sulli et al., 2008		Score with 3 parameters: loss of capillaries, disorganization of the microvascular array and capillary ramifications		
Alivernini et al., 2009		Avascular areas and a lower capillary density		[51]
Sebastini et al., 2009			Capillaroscopic Skin Ulcer Risk Index — (CSURI — D X M:N$^{2)}$: cut-off > 2.94 73.33% positive predictive value	
Caramaschi et al., 2009	Late NVC pattern			[13]
Kim et al., 2009			Capillary dimension, loss of capillaries, Apical limb width, capillary width, capillary hemorrhage and dimension of capillary loop	[58]
Sulli et al., 2009	Active and late patterns			[63]
Smith et al., 2010		Mean score of capillary loss of 1.67 in eight fingers		[52]
Sebastini et al., 2011			Avascular areas associated with DU cut-off CSURI > 2.96	
Lambova et al., 2011		Active pattern: giant capillaries, hemorrhages and avascular areas		[49]
Smith et al., 2012	Increased risk with worsening of NVC pattern			[48]
Smith et al., 2013	Late pattern NVC			[53]
Ennis et al., 2013			Intercapillary distance was greater in patients with active ulcers ($p = 0.03$) — patients with most marked microvascular	
Manfredi et al., 2014			Predictive value of CSURI	
Cutolo et al.			Reduced number of capillaries in the middle finger of dominant hand	

Table 3
Biomarkers as putative predictive factors for digital ulcers. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF).

Biomarker	Author/year	Relationship with DU	references
ET-1	Aghaei et al., 2012	ET-1 levels were higher in DU patients Relationship between plasma level of ET-1 and the number of ulcers and scars in hands and feet	[62]
ET-1	Sulli et al., 2009	Higher ET-1 plasma levels in patients with digital ulcers (active or previous)	[63]
ET-1	Kim et al., 2010	Higher values in the patients with DU	[58]
Endoglin ADMA	Wipff, 2008	Higher sENG levels and SSc vascular phenotype with digital ulcers.	[64]
ET-1 ADMA	Silva, 2012	Higher levels of Endothelin-1 and ADMA in patients with DU	[66]
vWF	Barnes et al., 2012	Baseline vWF:Ag was not a significant predictor new ulcer formation	[67]
VEGF ENDOSTATIN bFGF	Distler et al., 2002	Patients without fingertip ulcers showed significantly higher levels of VEGF no significant differences in the levels of endostatin between patients without fingertip ulcers and those with fingertip ulcers no association of bFGF levels with the presence of fingertip ulcers.	[70]
VEGF ENDOSTATIN	Farouk et al., 2013	Higher levels of serum endostatin in SSc patients with ischemic manifestations, while serum levels of VEGF were significantly higher in those patients without ischemic manifestations.	[74]
ENDOSTATIN	Hebbar et al., 2000	Mean circulating endostatin concentration was significantly higher in patients with cutaneous scars or ulcers	[78]
EPC- endothelial progenitor cells CEC - Circulating Endothelial Cells PlGF placental growth factor sVCAM -Soluble Vascular Adhesion Molecules VEGF vascular endothelial growth factor	Avouac et al., 2011	PlGF and EPC levels are independent predictors of the development of digital ulcers sVCAM serum levels were identified as predictive biomarkers of the occurrence of at least one new digital ulcer.	[79]
Angiopoietin-1 (Ang-1) Angiopoietin-2 (Ang-2)	Michalska-Jakubus, 2011	Ang-2 significantly correlated with the presence of digital ulcers	[80]
Angiopoetin-like protein 3 levels	Ichimura et al., 2013	Increase in serum ANGPTL3 levels was linked to the development of digital ulcers	[81]

of outcome for new ulcer formation [69]. Regarding ADMA, conflicting results of ADMA and SSc disease subset may be probably due to differences in the degree of inflammatory disorder, disease subset and patient treatments [66].

Angiogenesis biomarkers have been extensively studied in SSc patients and investigated as possible putative biomarkers of organ involvement. Chronic hypoxia due to reduced blood flow is not compensated by efficient angiogenesis; even though elevated angiogenic biomarker VEGF in SSc patients may be an attempt to induce neoangiogenesis and capillary neoformation, increased serum levels of angiostatic markers, such as endoglin, angiostatin or endostatin, may counteract this activity.

Pro-angiogenic high levels of VEGF may be protective against the development of DU reported in SSc patients with DU. Lower VEGF levels in SSc patients with DU have been reported [71] as significantly higher serum VEGF value in early stages of disease in SSc patients without digital ischemic manifestations [75]. Angiostatic biomarkers have despair results. Higher levels of ENG in SSc patients may highlight a possible contribution of this antiangiogenic protein in the SSc vascular disturbances [65,75,79]. No association between endostatin levels and fingertip ulcers were found [71].

Vasculogenesis biomarkers are not so well known as predictors of DU. High placental growth factor serum levels and low EPC counts might predict new digital ulcers in SSc pointing to an insufficient vasculogenesis to counterbalance vascular damage [80].

5. Conclusions

The different clinical classifications, disparity of inclusion criteria and an absence of a validated diagnostic test difficult diagnosis of SSc. Several nationwide multicenter and single center registry reports with a considerable number of patients have brought some new knowledge to the epidemiology and natural history of SSc disease course. Clinical, demographic and laboratory parameters and their association with different organ involvements have been studied. A lack of uniformed criteria in clinical practice, research, inclusion of patients in national and international databases and trials, difficult the task of a better knowledge of SSc disease, natural course, organ involvement and outcomes and compromises comparison of different cohort study results.

This review identified several disparities of results between cohorts, probably due to different SSc patient populations, different disease stages and/or clinical presentations, different organ involvements, non-uniform DU classification and a small number of SSc patients in most of the clinical and investigational research cohorts being compared. Another bias is that most studies are from tertiary centers with SSc patients with more severe disease. Furthermore most of the studies were not designed to outlook for predictable risk factors for DU on it's own although important information and lessons may be learned from these publications. Ferri et al. [24] in a recent report of the evolution of clinical SSc spectrum of an Italian cohort in the last years compared to other published studies identified a significant reduction of skin ulcers from 54 to 16.5% and a favorable evolution of SSc pathomorphosis. The authors agree with this group as they concluded that a greater awareness of disease, the availability of diagnostic tools and early diagnosis with improved therapeutic strategies are fundamental for a new positive evolution of the peripheral microangiopathy in SSc patients.

Take-home messages

- Diffuse systemic sclerosis and early onset of RP are the most frequent risk factors for digital ulcers (DU).
- Early first non-RP symptom and a great extent of skin fibrosis (high Rodman's skin score) are strong predictors of DU.
- Gender and smoking are not important risk factor in most studies.
- Anti-topoisomerase I positive autoantibodies have shown a strong association with DU.
- Late nailfold videocapillaroscopy (NVC) scleroderma pattern and worsening of NVC SSc patterns are strong predictors for DU.
- Patients with DU have higher endothelin-1 serum levels.
- VEGF levels are reduced in SSc patients with DU and significantly higher serum VEGF value are found in early stages of disease in SSc patients without digital ischemic manifestations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.autrev.2014.10.009.

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CHAPTER 3
PATIENTS AND METHODS

A observational cohort study with a 3-year follow-up was conducted to evaluate 109 enrolled patients attending the Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal.

Patients included:

- Thirty-two patients with primary Raynaud phenomenon.
- Seventy-seven patients with secondary Raynaud phenomenon.
- Based on 2013 classification criteria for SSc of American College of Rheumatology (1), all SRP patients had SSc.
- Systemic sclerosis patients were divided into two subgroups: DU group - 38 patients with an active ulcer at time of study, with or without a past history of DU and naïve-DU group - 39 patients with no DU in the course of disease until enrolment.
- Thirty-four controls (sex and age matched) healthy non-obese, without self-reported cardiovascular risk factors such as smoking, hypertension, diabetes or hyperlipidaemia participated. No control subject was on any vasoactive medication.

Exclusion criteria:

- Patients with factors that could potentially interfere with FMD (smokers, diabetic, hypertension, hyperlipidaemia and a past history of myocardial infarction).
- Patients on bosentan were excluded from the study due to possible interference with ET-1serum levels.
- Patients that had ECG and echocardiograph measurements out the normal range
- Previous history of liver or renal disease.

We lost no patients to follow-up.

The institutional review board approved this study (including Ethical for Health Committee of Centro Hospitalar do Porto). All subjects and parents of the two children signed the informed consent. Data was collected by analysis of clinical file data and by clinical interview.

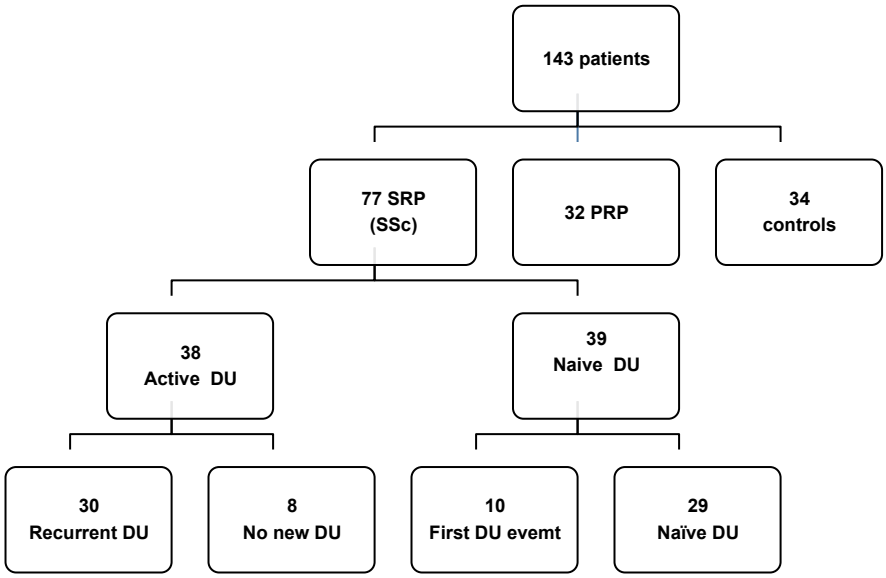

Figure 3.1: Evolution of our cohort in the 3-year follow-up. *PRP: primary Raynaud phenomenon; SRP: secondary Raynaud phenomenon; SSc: systemic sclerosis; DU: digital ulcer.*

Definition of digital ulcer

Ischemic digital fingertip ulcerations were defined as painful area of 2 mm or greater in diameter with visible depth and loss of dermis, amenable to healing and localized to the fingertip. Digital ulcers caused by conditions other than SSc were not considered.

Methods

At baseline all patients and controls were studied with all the described methods in this chapter (Allen test, Flow-mediated dilatation, nailfold capillaroscopy, autoantibody and vascular biomarkers detection).

In the 3-year clinical follow-up sequential NVC were performed to SRP patients.

1) Allen test

Allen test was performed as follows: 1. instructing patient to clench his/her fist; 2. applying occlusive pressure to both ulnar and radial arteries by finger pressure; 3. confirm palm and finger blanching with the patient´s hand relaxed; 4. release the occlusive pressure on ulnar artery; 5. Positive test if the hand flushes within 5-15 seconds it indicates that the ulnar artery has good blood flow and palmar arch is complete; Negative test if the hand does not flush within 5-15 seconds, it indicates that ulnar circulation is inadequate with an incomplete palmar arch.

2) Ultrasound examination and FMD

Ultrasounds scans were performed using a two-dimensional ultrasonography General Electric Logic 7 with a 9 MHz Linear wideband multihertz imaging probe. The same operator performed all exams.

For spectral waveform measurement two cursors were placed on sonographic image of braquial artery examined; sample gate cursor and alignment angle correct cursor. Three contiguous spectral waveforms were recorded for determination of peak systolic velocity (PSV), end diastolic velocity (EDV) and Resistive Index (RI). The latter was calculated as (PSV-EDV)/PSV. All these parameters were measured for each waveform and obtained as the average of three measurements.

Flow mediated dilatation of the braquial artery in the lower arm was evaluated following International Brachial Artery Reactivity Task Force guidelines (2) for the ultrasound assessment of brachial artery endothelial-dependent flow-mediated vasodilatation. Patients and healthy subjects were on overnight fasting for twelve hours before the study. The exams were performed in the morning, after patients being kept in a quiet temperature controlled room (22-24ºC) for a 20-minute rest. Vasoactive drugs were withheld for 10 half-lives. Patients did not exercise or ingest substances that could affect the response to ischemia like caffeine, vitamin C, tobacco or high-fat foods for 24 hours.

The braquial artery was scanned in a longitudinal section below the antecubital fossa in 2 to 3 cm section. Mean baseline braquial artery diameter was calculated as the result of the mean of 3 measurements. A sphygmomanometric blood pressure cuff localized above the antecubital fossa was inflated 50mmHg above patient's systolic blood pressure (measured in left upper arm) and kept inflated for five minutes. No exam had to be discontinued due to pain or discomfort. Before cuff deflation, images were recorded continuously from 15s before deflation to 180s after cuff deflation. Ultrasound images were analysed for 3 consecutive end diastolic frames (onset of R wave) at 45 to 60s after cuff deflation. The intraoperator variability was 3.6%. FMD measurements were blindly performed with respect to the NVC evaluation.

FMD was calculated as the percentage of change of the peak diameter in response to reactive hyperaemia in *(FMD%= (peak diameter – baseline diameter/baseline diameter) × 100) (2)*.

3) Nailfold Videocapillaroscopy

Nailfold Videocapillaroscopy (NVC) was performed with KK technology videocapillaroscopy with a 200x magnification lens. The same operator

performed all capillaroscopies, with blind information regarding FMD. In the end all images were scored for each patient at baseline and at 3-year follow-up and were controlled by a capillaroscopy expert. The inter observer variability was 2.5%.

All subjects were in a quiet room with controlled temperature (21-24ºC). Nailfold distal row capillaries of 4 fingers, 2nd, 3rd, 4th and 5th of both hands were examined.

Two classifications were used to describe capillaroscopic findings: a qualitative classification of scleroderma microangiopathy damaged described by Cutolo (3) in 3 patterns early, active and late. Early pattern was characterized by the presence of small number of giant capillaries and microhemorrhages, no avascular areas and a relatively well preserved capillary distribution; Active pattern was classified according to the presence of numerous giant capillaries and microhemorrhages, moderate capillary loss (20-30%) and a mild disorganized capillary architecture, with rare branches capillaries; Late pattern is characterized by near-absence of giant capillaries and microhemorrhages, presence of extensive avascular areas (50-70% of capillary loss) and a presence of many branched and ramified bushy capillaries (neoangiogenesis) and a complete disorganization of capillary array (3).

A semi-quantitative score Microangiopathy Evolution Score (MES) was adapted from Sulli et al (4). The sum of three scores regarding loss of capillaries, disorganization of the microvascular array and capillary ramifications were assessed to study the progression of the vascular damage. A rating scale to score each capillary abnormality was used (0 = no changes; 1= <33% capillary reduction / changes; 2= 33-66% capillary reduction / changes; 3= > 66% capillary reduction / changes) per linear millimeter (4). Four consecutive fields (one linear millimeter for each one) in the middle of the nailfold, in each 8 fingers were studied. The average score values from the eight digits were added together, and the final value divided for eight (4). The resulting value represents the evolution score of microangiopathy (MES): 0-9 (5). Following, we categorized MES scores in 3 subcategories with the following cut-off values: subcategory 1 – from 0 to 3; subcategory 2 – from 4 to 6 and subcategory 3 – from 7 to 9, allowing a more detailed analysis between microangiopathy and endothelial dysfunction evaluation.

4) Autoantibody detection

Antinuclear antibodies (ANA) were accessed by indirect Immunofluorescence on Hep-2 cells (NOVAlite ANA, Inova Diagnostics, Inc., San Diego, CA, USA).

Samples with a titer greater than or equal to 1:80 were considered positive. Autoantibodies anti-Scl-70, anti-centromere (ACA), anti-Ro52, anti-PM-Scl, anti-RNA-polymerase, anti-fibrillarin (AFA), and anti-NOR 90 were detected by immunoblotting using a Euroline Myositis Profile antibody test syst (Euroimmun, Lübeck, Germany). Quantification of anti-U1RNP, antibodies was carried out using a Fluoro Enzyme Immuno Assay (EliA™ U1RNP70; Phadia, Uppsala, Sweden)

5) Vascular Biomarkers

Fasting venous blood samples were collected into a serum tube and a tube containing sodium heparin (Vacuette, Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC until analysis for ADMA, endoglin, endostatin, VEGF-A.

ET-1 assessment: Plasma was centrifuged immediately in a refrigerated centrifuge and stored at -70ºC until analysis for endothelin. Plasma endothelin was measured using a RIA assay, (Euro-Diagnostics AG, Sweden). The resulting values are reported as pmol/ml.

ADMA assessment: Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent assay, (Immunodiagnostik AG, Germany). The resulting values are reported as umol/l.

VEGF assessment: Serum VEGF-A was measured using enzyme-linked immunosorbent assay, (IBL International GMBH, Germany). The resulting values were reported as pg/ml.

Endoglin and Endostatin assessment: Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay, (Uscn, Life Science Inc., Wuhan). The resulting values were reported as ng/ml.

6) Follow-up

When included in the study cohort, patients were seen on a regular basis of 3-6 months intervals as indicated by disease severity. Patients were instructed to come to the hospital clinics whenever a new digital lesion developed. Final observation was in forth trimester of 2014.

7) Outcomes measures

Primary outcome was the occurrence of at least one or more new ischemic fingertip DU in the 3-year follow-up period.

In addition we applied survival analysis to new occurrence of DU during the study period, regarding endothelium dysfunction (FMD, ET-1 and ADMA) and microvascular damage (MES). We also propose a clinical model for day-to-day use for prediction of DU in SSc patients.

8) Statistical analysis

For comparison of normally distributed scale variables, we used unpaired or paired two-sided student's t-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD) followed by the minimal and the maximal values (range). Normal distribution was tested by Q-Q plots. In cases of non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_2;), where Q_1 represents the first quartile (corresponding to 25% of data) and Q_3 represents the third quartile (corresponding to 75% of data). In Anova test, when the homogeneity of variance it is not satisfied, we used the Welch test. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. In cases of multiple testing, a Bonferroni correction or a Games-Howell test (for equal variances not assumed) was used. Predictors of digital ulcers were evaluated by univariate and multivariate logistic regression and summarised as HR and 95%CI. We applied survival analysis to determine the probability of freedom from new DU during the study period and evaluated the effects of FMD, MES, ET-1, ADMA, VEGF, endostatin and endoglin in that probability using the Kaplan-Meier method and the Cox regression multivariate analysis. A receiver operating characteristic (ROC) curve analysis was performed to obtain the predictive accuracy of FMD, MES score, ET-1, ADMA, VEGF, endostatin, endoglin and 2 risk clinical score model with regards to new ulcer development. We considered p values <0.05 as significant. Data were analysed using the SPSS software (v.22.0, SPSS, Chicago, IL).

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CHAPTER 4
RESULTS

The results of our investigational study are presented in 6 sections following STROBE Statement recommendations (Strengthening the Reporting of Observational Studies in Epidemiology) (1).

Section 1. Peripheral vasculopathy in Raynaud phenomenon: vascular disease biomarkers

The aim of this study was to assess the differences between primary RP (PRP) and secondary RP (SRP) regarding macrovascular disease parameters, endothelial dysfunction and angiogenesis biomarkers.

Section 2. Microvascular damage, endothelial dysfunction and ischemic peripheral vasculopathy in secondary Raynaud phenomenon

In this study we evaluated endothelial dysfunction and microvascular damage in secondary Raynaud Phenomenon (SRP) patients as predictors of ischemic fingertip digital ulcers (DU) in a 3-year clinical follow-up.

Section 3. Digital ulcers in Systemic Sclerosis: role of flow-mediated dilatation and capillaroscopy as risk assessment tools

The objective was to evaluate macrovascular disease and microvascular damage as risk factors for digital ulcers in SRP SSc-associated patients.

Section 4. Impaired angiogenesis as a feature of digital ulcers in systemic sclerosis

We investigated if angiogenic biomarkers (vascular endothelial growth factor (VEGF), endoglin and endostatin) are related to microvascular damage and determined their prediction value for new digital ulcer episode.

Section 5. Predictive value of vascular disease biomarkers for digital ulcers in systemic sclerosis patients

In this publication we analysed the role of endothelial dysfunction and angiogenesis vascular biomarkers as risk factors and their predictive value for digital ulcers in systemic sclerosis patients.

Section 6. Endothelial dysfunction and nailfold videocapillaroscopy pattern as predictors of digital ulcers in systemic sclerosis: a cohort study and review of the literature

Our aim was to investigate the diagnostic and predictive value for DU the full set of parameters investigated in our work (endothelial dysfunction biomarkers (flow-mediated dilatation (FMD), serum levels of Endothelin-1 (ET-1) and ADMA), angiogenic/angiostatic biomarkers (vascular endothelial growth factor (VEGF), endoglin and endostatin) and nailfold videocapillaroscopy (NVC).

Manuscript submitted

Peripheral vasculopathy in raynaud phenomenon: vascular disease biomarkers

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4.1 PERIPHERAL VASCULOPATHY IN RAYNAUD PHENOMENON: VASCULAR DISEASE BIOMARKERS

ABSTRACT

BACKGROUND:

Introduction: Raynaud's phenomenon (RP) is a well-defined clinical syndrome. Systemic sclerosis (SSc) is the most frequent associated disease to RP (96%). The aim of this study was to assess the differences between primary RP (PRP) and secondary RP (SRP) regarding macrovascular disease parameters, endothelial dysfunction and angiogenesis biomarkers.

MATERIALS AND METHODS:

Flow-mediated dilatation (FMD), endothelin-1 (ET-1), asymmetric dimethylarginine (ADMA) vascular endothelial growth factor (VEGF), endoglin and endostatin were analysed in a cohort study of 32 PRP patients and 77 SRP all with SSc. 38 of the SRP SSc-associated patients had severe digital ulcer (DU).

RESULTS:

Patients with PRP had significantly longer history of RP compared to SRP SSc-sssociated patients ($p = 0.028$).

FMD was significantly lower in SRP patients 10.85±11.0% ($p<0.001$), more evidenced in SRP SSc-associated DU patients 5.34±7.49 ($p<0.001$). ET-1 plasma levels were significantly increased in both PRP 7.53 (0.16-11.73) and SRP patients 11.85 (7.42-17.23) ($p<0.001$). Significant increased serum levels of ADMA 0.52 (0.45-0.63) umol/L ($p<0.001$) and endoglin 3.01(1.46-7.02) mg/ml ($p<0.001$) were found in the SRP SSc-associated group with DU. VEGF was significantly decreased in the DU group 245.06 (158.68-347.33) pg/ml compared to other ($p<0.001$). No significant differences were found between groups regarding endostatin ($p=0.118$).

Comparing PRP and SRP SSc-associated patients without DU no statistically significant difference regarding FMD, ET-1, ADMA, VEGF, plasma levels were observed.

CONCLUSION

Overproduction of ET-1 and VEGF is present in PRP patients. Macrovascular disease and an impaired response to shear stress are more characteristic of SRP with a grater expression in patients with peripheral ischemic lesions.

Keywords: Raynaud disease, endothelial dysfunction, digital ulcers, angiogenesis.

INTRODUCTION

Raynaud's Phenomenon (RP) was first described by Maurice Raynaud in 1862 and is defined as bouts of reversible vasospastic ischemia of the extremities (1, 2). Episodic color changes of the fingers classically turn into white (ischemia), then blue (cyanosis) and red (reperfusion). In a recent Delphi exercise round, 12 invited experts agreed recently in three-step outline for a newly proposed diagnostic method. Consensus was achieved in that at least biphasic color changes are required to make the diagnosis of RP. They also agreed that white/pallor and blue/cyanosis were the two most important colors and that patients must report cold temperatures as one of the triggers for their RP attacks (3).

Primary RP (PRP), also known as Raynaud's disease, is a functional vascular disorder that occurs isolated as an exaggerated response to cold and emotional stress, not progressing to irreversible tissue injury (3, 4). The requests for definition of PRP defined in Delphi exercise round were: (i) normal capillaroscopy (ii) negative physical examination for findings suggestive of secondary causes (e.g. ulcerations, tissue necrosis or gangrene, sclerodactily, calcinosis, or skin fibrosis); (iii) no history of existing connective tissue disease and (iv) negative or low titer ANA (3, 5).

Secondary RP (SRP), also known as Raynaud's Syndrome, appears in response to those triggers too, however, it occurs in the setting of underlying structural vascular disease and is often associated with digital ulceration, scarring or gangrene (6). Recent advances in the diagnosis of RP has have recognized that abnormalities in nailfold capillary pattern and specific autoantibodies are independent risk factors for connective tissue disease(4). Autoreactive antibodies specifically ANA, anti-centromere and anti-SCL 70 antibodies are helpful as diagnostic for secondary RP.

PRP is a common condition, which has a prevalence of 3-5% in the general population (2). The onset age is below 40 years and there could be a history of PRP in family but the entire clinical course is benign (6). By contrast, SRP is a much rarer condition, but frequent in the patients with connective diseases such as Systemic Sclerosis (SSc) (90%), systemic lupus erythematous (30%), rheumatoid arthritis (20%), Sjögren’s syndrome and polymiositis.

Endothelial dysfunction and free-radical damage are primary events throughout the course of the RP disease, which result in vascular obliteration and diminished blood flow to the organs involved (7) and are prominent features of RP and ischemic peripheral digital ulcers. There are several serological biomarkers that reflect the vasculopathy of the disease, such as vasoconstrictor

ET-1(8), the controversial vasodilator nitric oxide(NO) (8) and the inhibitor of endothelial NO synthase(eNOS) ADMA.

Endothelial cell damage results in ischemia-reperfusion injury due to the ongoing pathological process, which inevitably evolves towards chronic underperfusion. A characteristic clinical finding is capillary dilation and atrophy diagnosed by nailfold capillary microscopy. Thse findings suggest significant loss of the peripheral vascular network with a defect in both the vascular repair and in the expected increase in vessel growth (angiogenesis, arteriogenesis, vasculogenesis); the net result is tissue ischemia, fibrosis, and organ failure (9).

The aim of the current study was to evaluate macrovascular disease parameters, endothelial dysfunction and angiogenic vascular biomarkers in a cohort of RP patients, in an attempt to define the boundaries between PRP and SRP allowing early identification of PRP patients who are at risk of developing an underling secondary disease.

Materials and Methods

An observational cohort study was conducted to evaluate 109 RP patients (32 PRP and 77 SRP) attending our Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal. We excluded from our study all patients with risk factors that could potentially interfere with flow-mediated dilatation (FMD): smokers, diabetics, with hyperlipidaemia, and with past history of myocardial infarction, as well as patients on bosentan treatment, due to possible interference with endothelin-1 levels (ET-1).

Controls and PRP patients were followed for 3-years to ensure no underlying secondary disease. All 77 SRP included patients had SSc based on 2013 classification criteria for SSc of American College of Rheumatology (10). A washout of the vasodilator drugs was done before inclusion in the study. Thirty-four healthy, sex/age matched, non-obese, without self-reported cardiovascular risk factors controls were invited to participate. No control subject was on any vasoactive medication.

SRP SSc-associated patients were divided into two groups: DU group, that included 38 patients having an active ischemic ulcer at inclusion (34 women; mean age 52.7 ± 14.8 years; range 14-75); and a 39 patients group with no history of DU until enrolment (38 women; mean age 53.2 ± 10.3 years; range 30-79).

The institutional ethical review board of Centro Hospitalar do Porto approved this study. All subjects signed informed consent before inclusion in the study. Data were collected by analysis of clinical file data and by clinical interview.

2.1 Methods

2.1.1. Allen test

Allen test was performed as follows: 1. instruct patient to clench his/her fist; 2. apply occlusive pressure to both ulnar and radial arteries by finger pressure; 3. confirm palm and finger blanching with the patient´s hand relaxed; 4. release the occlusive pressure on ulnar artery; 5. Positive test: If the hand flushes within 5-15 seconds, this indicates that the ulnar artery has good blood flow and palmar arch is complete; Negative test: If the hand does not flush within 5-15 seconds, this indicates that ulnar circulation is inadequate with an incomplete palmar arch.

2.1.2. Flow-mediated dilatation (FMD)

Ultrasounds scans were performed using a two-dimensional ultrasonography General Electric Logic 7 with a 9 MHz Linear wideband multihertz imaging probe. Ultrasound images were recorded and analysed for 3 consecutive end diastolic frames (onset of R wave) at 45 to 60s after cuff deflation. The inter-operator variability was 3.6%.

Flow mediated dilatation of the brachial artery in the lower arm was evaluated following International Brachial Artery Reactivity Task Force Guidelines (11) for the ultrasound assessment of brachial artery endothelial-dependent flow-mediated vasodilatation. Patients and controls (healthy subjects) were on overnight fasting for twelve hours before the ultrasound study was performed. The exams were performed in the morning, with patients being kept in a quiet temperature controlled room (22-24ºC) for a preliminary 20-minute rest. Vasoactive drugs were withheld for 10 half-lives. It was assured that patients did not exercise or ingest substances that could affect the response to ischemia like caffeine, vitamin C, tobacco or high-fat foods for 24 hours.

FMD was calculated as the percentage of change of the peak diameter in response to reactive hyperaemia in (FMD%= (peak diameter – baseline diameter/baseline diameter) × 100) (11).

2.1.3. Vascular Biomarkers

Venous blood samples from fasting individuals were collected into a serum tube, and another tube containing sodium heparin (Vacuette, Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from cells within 60 minutes, and stored at -70 ºC until analysis for asymmetric dimethylarginine (ADMA), endoglin, endostatin, vascular endothelial growth factor (VEGF-A).

ET-1 assessment: Plasma was centrifuged immediately in a refrigerated centrifuge and stored at -70ºC until analysis for endothelin. Plasma endothelin was measured using a RIA assay, (Euro-Diagnostics AG, Sweden). The resulting values are reported as pmol/ml.

ADMA assessment: Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent assay, (Immunodiagnostik AG, Germany). The resulting values are reported as umol/l.

VEGF assessment: Serum VEGF-A was measured using enzyme-linked immunosorbent assay, (IBL International GMBH, Germany). The resulting values were reported as pg/ml.

Endoglin and Endostatin assessment: Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay, (Uscn, Life Science Inc., Wuhan). The resulting values were reported as ng/ml.

2.2.4 Statistical analysis

For comparison of normally distributed scale variables, we used unpaired two-sided Student's t-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD) followed by the minimal and the maximal values (range). Normal distribution was tested by Q-Q plots. In cases of non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_3;), where Q_1 represents the first quartile (corresponding to 25% of data) and Q_3 represents the third quartile (corresponding to 75% of data). In Anova test, when the homogeneity of variance was not satisfied, we used the Welch test. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. A receiver operating characteristic (ROC) curve analysis was performed to obtain the predictive accuracy of FMD, MES score, ET-1, ADMA, VEGF, endostatin and endoglin. We considered p values <0.05 as significant. Data were analysed using the SPSS software (v.22.0, SPSS, Chicago, IL).

RESULTS

The demographic and clinical characteristics of the 143 subjects are described in Table 4.1.1 and 4.1.2. No major differences were observed between SSc patients, PRP patients and control group regarding age, gender, mean arterial pressure and total cholesterol.

Disease duration was significantly longer in PRP patients (median value: 15 years) compared to SRP SSc patients (median value: 10 years) (*p = 0.028).* All PRP patients and controls had negative ANA and normal capillaroscopy.

Variables	PRP	SRP- SSc associated		Control	p-value
		DU	naive-DU		
Subjects, n	32	38	39	34	NA
Age (years), *mean ± SD*	49.9 ± 12.5	52.7 ± 14.8	53.2 ± 10.3	47.1 ± 10.96	0.137[a]
Gender Women, n (%)	25 (78.1)	34 (89.5)	38 (97.4)	29 (85.3)	0.067[b]
Disease duration (years), *median (Q_1-Q_3)*	15	10	10	NA	0.028*[,c]
Mean arterial pressure (mmHg), *mean ± SD*	87.6 ± 5.6	87.9 ± 6.04	88.3 ± 6.4	86.7 ± 6.8	0.75[a]
Total cholesterol (mg/dl), *mean ± SD*	188.8 ± 8.8	191.3 ± 9.0	187.1 ± 12.1	190.6 ± 7.6	0.242[a]
Allen test, positive (%)	2 (6.3%)	27 (71.1)	7 (17.9)	0 (0)	<0.001*

Table 4.1.1: The demographic and clinical characteristics of the 143 subjects. *RP: Raynaud phenomenon; PRP: primary SRP: secondary RP; SSc: systemic sclerosis; DU: digital ulcer; NA: non applicable; SD: standard deviation; Q: quartile.* [a] *: Chi-Square test.* [b] *: Fisher's Exact test.* [c] *: Mann-Whitney test.* [*]*: Statistical significance for a level of 5%.*

Variables	SSc DU	SSc naïve DU	p-value
Subjects, n	38	39	
Disease subset			0.001[*,a]
Limited, n (%)	26 (68.4)	38 (97.4)	
Diffuse, n (%)	12 (31.6)	1 (2.6)	
Onset of 1st ulcer (years), Median (Q_1-Q_2)	5 (3-13.25)	NA	NA
Telangiectasias, Positive, n(%)	38 (100)	27 (69.2)	<0.001[*,a]
Allen Test, Positive, n (%)	27 (71.1)	7 (17.9)	<0.001[*,a]
Autoantibodies			
ACA, Positive, n (%)	22 (57.9)	27 (69.2)	0.301
Scl-70, Positive, n (%)	12 (31.6)	6 (15,4)	0.093[a]
Anti-PM. Scl ,Positive, n(%)	2 (5.3)	5 (12.8)	0.431[b]
Anti-RO 52, Positive, n (%)	20 (52.6)	12 (30.8)	0.052[a]
Anti-NOR, Positive, n (%)	0 (0)	3 (7.7)	0.240[b]
Anti-fibrilarin, Positive, n (%)	0	1 (2.6)	1.000[b]
Anti U1 RNP, Positive, n(%)	2 (5.3)	2 (5.1)	1.000[b]
NVC Pattern			
Early, n (%)	0 (0)	13 (33.3)	
Active, n (%)	11 (28.9)	22 (56.4)	< 0.001[a]
Late, n (%)	27 (71.1)	4 (10.3)	

Table 4.1.2: Comparison between SRP SSc-DU and SSc naïve DU groups. *SSc: systemic sclerosis; DU: digital ulcer; dcSSc: diffuse systemic sclerosis subset lcSSc: limited systemic sclerosis subset; SRP: secondary Raynaud phenomenon; ACA: autoantibody anti-centromere; NVC: nailfold videocapillarosocopy; MES: microangiopathy evolution score; NA: no applicable.* [a] *: Chi-Square test.* [b] *: Fisher's Exact test.* [*]*: statistical significance for a level of 5%*

Macrovascular disease

Macrovascular disease was evaluated by clinical and hemodynamic parameters. Only 6.3% of PRP patients had a positive Allen test. However it was positive in 71% of SRP SSc-associated patients with DU whilst only 18% in patients without DU (*p < 0.001*).

Macrovascular ultrasound examination showed no difference in braquial artery diameter between groups (*p=0.620*). Primary RP (*p<0.001*), SRP naive-DU (*p=0.001*) and SRP DU (*p=0.002*) had significantly decreased basal state PSV compared to control group. No differences were found between SRP patients with and without DU (*p=0.989*). Table 4.1.3.

Flow-mediated dilatation at 60 seconds after deflation was significantly lower in SRP patients (p<0.001). Patients with DU had significantly reduced FMD% (p<0.001) when compared to all other groups. No statistical differences were found between PRP and control groups (p=0.999) and between PRP and SRP SSc-associated naive-DU (p=0.07). Figure 1. No correlation was found between FMD and disease duration (*R = 0.41)*.

After 5 minutes braquial artery occlusion, PRP and SRP had significant differences regarding EDV (p<0.001) and RI (p=0.007). PSV and EDV were significantly decreased in SRP group SSc-associated DU group (p<0.001). Table 4.1.3.

Vascular disease biomarkers Table.4.1.3 and 4.1.4; Figure 4.1.1

Endothelin-1

ET-1 plasma levels were found to be significantly higher (p<0.001) in patients with both PRP and SRP SSc-associated compared with controls. A statistically significant difference for ET-1 plasma levels was observed between patients with PRP and SRP SSc-associated patients (p<0.001).

Among patients with SSc, ET-1 plasma levels were significantly higher (p <0.001) in patients with DU. No statistically significant difference for ET-1 plasma levels was observed between the PRP and SRP SSc patients without DU.

ADMA

ADMA serum levels were significantly higher in the SRP SSc-associated DU group (*p<0.001)*. No significant differences were found between SRP SSc naive-DU and PRP (p=0.757) and between PRP and controls (p=0.204).

Variables	PRP (n=32)	SRP – SSc (n=77)	Control (n=40)	p-value
FMD %, *mean ± SD*	17.96 ± 12.78	10.85±11.0	20.17 ± 8.86	<0.001[*,b]
PSV 60 sec after cuff deflation (cm/s), *mean ± SD*	177.69±26.69	165.35 ±53	199.77±32.93	<0.001[*,b]
EDV 60 sec after cuff deflation (cm/s), *mean ± SD*	92.95 ± 35.05	67.28 ±24.37	93.70 ± 20.01	<0.001[*,b]
RI, *mean ± SD*	0.47 ± 0.23	0.51± 0.18	0.43 ± 0.08	=0.034[b]
ET-1 pmol/ml, *Median (Q_1-Q_3)*	7.53 (0.16-11.73)	11.85 (7.42-17.23)	2.48 (0.00-5.60)	<0.001[*,a]
ADMA, umol/l, *Median (Q_1-Q_3)*	0.40 (0.37-0.49)	0.49 (0.41-0.54)	0.38 (0.32-0.43)	<0.001[*,a]
Endoglin ng/ml, *Median (Q_1-Q_3)*	0.52 (0.28-0.88)	2.17 (1.27 -4.21)	0.28 (0.15-0.71)	<0.001[*,a]
Endostatin ng/ml, *Median (Q_1-Q_3)*	0.90 (0.38-1.43)	0.51 (0.19-1.24)	0.565 (0.35-0.77)	0.268[a]
VEGF pg/ml, *Median (Q_1-Q_3)*	438.50 (269.26854.00)	290 (166.71-361.78)	178.03 (101.27-222.10)	<0.001[*,a]

Table 4.1.3: Comparison of variables investigated between SRP, PRP and controls at baseline. *SSc: systemic sclerosis; DU: digital ulcer; SRP: secondary Raynaud phenomenon, ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor. FMD: flow mediated dilatation; Q; quartile; SD: standard deviation; [a] : Kruskal Wallis; [*] [b]:Anova test: Statistical significance for a level of 5%.*

VEGF

Significant differences were found in VEGF between PRP and SRP patients (p<0.001) and between PRP and control group patients (p<0.001). Lower plasma levels of VEGF were found in patients with fingertip digital ulcers (p<0.001). We found no difference when PRP was compared to SRP SSc naive-DU group (p=0.099).

Endoglin

Angiostatic serum endoglin levels were increased in SRP patients with active DU (*p<0.001*) and no significant difference was found between other groups.

Endostatin

No significant differences were found between groups (*p=0.118).* Comparing SRP with PRP no significant difference was found (p=0.302).

Variables	PRP	SRP- SS		Control	p-value
		DU	Non-DU		
Baseline artery (mm), *mean ± SD*	3.51 ± 0.69	3.34 ± 0.51	3.44 ± 0.52	3.37 ± 0.55	0.620[a]
Absolute difference (mm), *mean ± SD*	0.57 ± 0.29	0.17 ± 0.23	0.52 ± 0.29	0.65 ± 0.23	<0.001[*,a]
FMD (%), *mean ± SD*	17.96 ± 12.78	5.34 ± 7.49	16.21 ± 11.31	20.17 ± 8.86	<0.001[*,a]
Baseline PSV (cm/s), *mean ± SD*	65.77 ± 11.23	76.65 ± 19.72	75.26 ± 18.91	94.99 ± 21.57	<0.001[*,a]
PSV 60 sec after cuff deflation (cm/s), *mean ± SD*	177.69±26.69	148.78±34.10	181.51 ± 38.10	199.77±32.93	<0.001[*,a]
EDV 60 sec after cuff deflation (cm/s), *mean ± SD*	92.95 ± 35.05	58.03 ± 16.89	78.43 ± 24.37	93.70 ± 20.01	<0.001[*,a]
RI, *mean ± SD*	0.47 ± 0.23	0.68 ± 0.23	0.57 ± 0.09	0.43 ± 0.08	0.024[*,a]
ET-1 pmol/ml, *median (Q_1-Q_3)*	7.53 (0.16-11.73)	16.13 (10.97-21.17)	8.8 (5.89-12.68)	2.48 (0.00-5.60)	<0.001[b,*]
ADMA umol/L, *median (Q_1-Q_3)*	0.40 (0.37-0.49)	0.52 (0.45-0.63)	0.45 (0.41-0.51)	0.38 (0.32-0.43)	<0.001[b,*]
Endoglin ng/ml, *median (Q_1-Q_3)*	0.52 (0.28-0.88)	3.01 (1.46-7.02)	1.88 (0.84-3.28)	0.28 (0.15-0.71)	<0.001[b,*]
Endostatin ng/ml, *median (Q_1-Q_3)*	0.90 (0.38-1.43)	0.70 (0.26-1.73)	0.43 (0.16-0.80)	0.565 (0.35-0.77)	0.118[b]
VEGF pg/ml, *median (Q_1-Q_3)*	438.50 (269.26-854.00)	245.06 (158.68-347.33)	422.47 (269.26-847.97)	178.03 (101.27-222.10)	<0.001[b,*]

Table 4.1.4: Comparison between PRP, SRP (with and without digital ulcers) and controls. *PRP: primary Raynaud phenomenon; SRP: secondary Raynaud phenomenon; SSc: systemic sclerosis; DU: digital ulcer; FMD: flow mediated dilatation; PSV: peak systolic velocity; EDV: end diastolic velocity; RI: resistive index. ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor. FMD: flow mediated dilatation; Q; quartile; [a]: Anova test; [b] : Kruskal Wallis; [*] statistical significance for a level of 5%*

The AU-ROC ($CI_{95\%}$) of the macrovascular parameters and vascular biomarkers investigated associated to SRP were: FMD (AUC: 0.737 95%CI: 0.655-0.819); post-occlusion PSV (AUC: 0.681 95%CI: 0.593-0.768); post-occlusion EDV (AUC: 0.766 95%CI: 0.689-0.844); RI (AUC: 0.634 95%CI: 0.544-0.725), ET-1 (AUC: 0.826 95%CI: 0.758-0.895); ADMA (AUC: 0.754 95%CI: 0.675-0.832); VEGF (AUC: 0.508 95%CI: 0.410-0.606); endoglin (AUC: 0.914 95%CI: 0.870-0.959) and endostatin (AUC: 0.591 95%CI: 0.463-0.720).

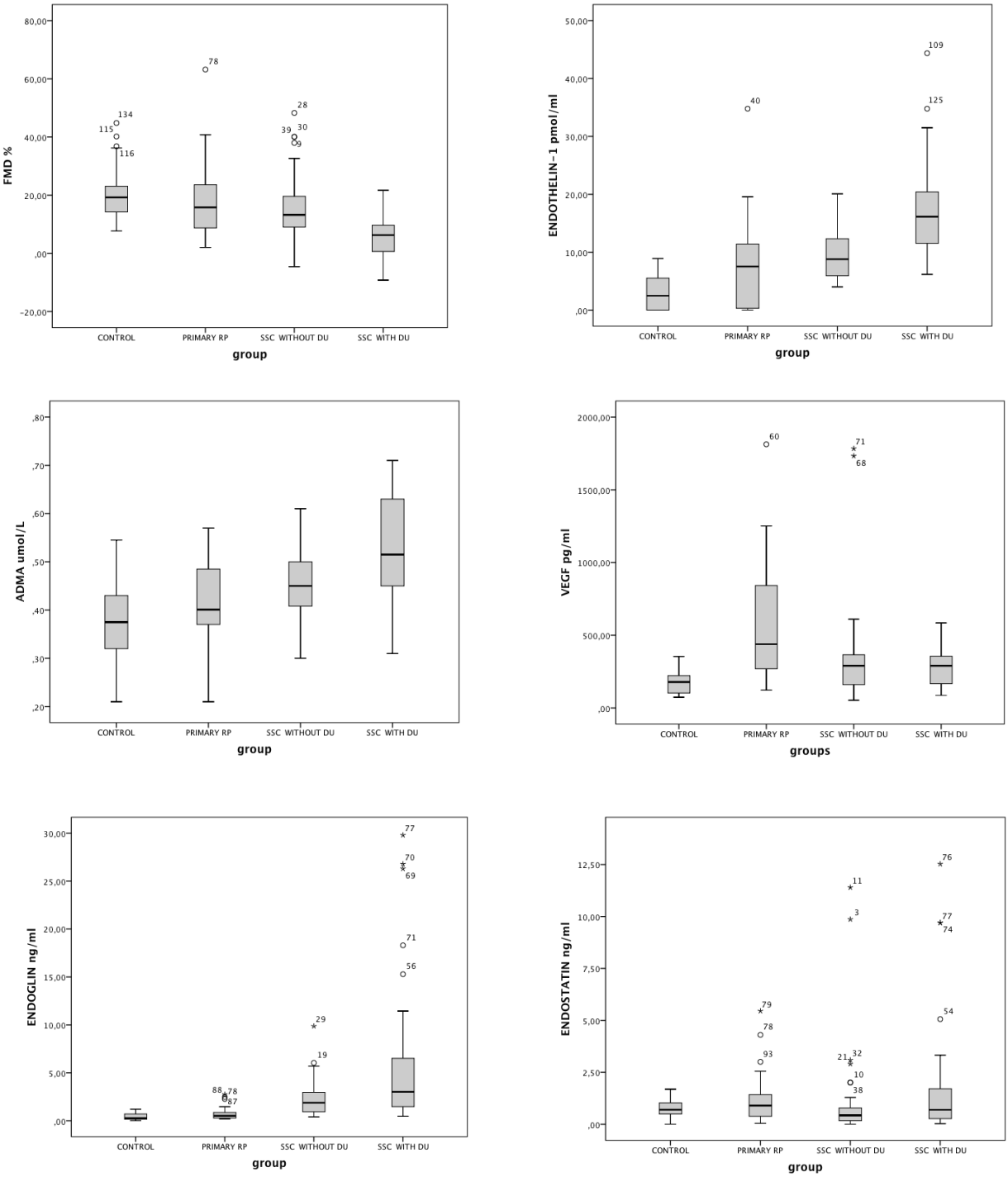

Figure 4.1.1: Graphic representation of variables investigated. FMD (*p<0.001*), ET-1 (*p<0.001*), ADMA (*p<0.001*), VEGF (*p<0.001*), Endoglin (*p<0.001*) and Endostatin p=0.266. *ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor FMD: flow mediated dilatation*

DISCUSSION

We report here on the clinical and laboratory data regarding a large group of patients with diagnosis Raynaud's phenomenon. This is an observational cohort

study of 109 RP patients that were divided into two subpopulations PRP and SRP, the latter in 2 groups (with or without previous ischemic peripheral lesions).

Clearly, our findings suggest that endothelial dysfunction suggested by increased serum levels of ET-1 as well a pro-angiogenic state due to increased serum levels of VEGF are already present in PRP and when comparing these patients with SRP SSc- associated without DU no major difference were found regarding the vascular biomarkers investigated. Thus, a new and useful information coming out of this investigation is that severe obliterative peripheral vasculopathy is present only in SRP patients with DU as expressed by the increased peripheral resistance, low FMD response to shear stress, decreased PSV and EDV and high RI mostly consequent of the EC injury with endothelial dysfunction associated to an impaired angiogenesis.

RP occurs when the balance of vascular tone is disturbed, favoring vasoconstriction. This endothelial activation and/or damage leads to reduce efficacy of vasodilators and/or overproduction of vasoconstrictors (4). Doubts persist whether there is overproduction of endothelium vasoconstrictor endothelin-1 (ET-1), underproduction of vasodilators such as nitric oxide (NO) and prostacyclin, or whether they are impaired in RP (4). Further complicating the role of NO, patients with SRP and SSc, have increased the plasma levels of an endogenous inhibitor of endothelial NOS—asymmetric dimethyl arginine—(ADMA) leading to reduced NO production (12).

As a response to increase in shear stress, several vasodilators are released such as NO, prostaglandins and endothelium-derived hyperpolarizing factor (13). This response is commonly known as flow-mediated dilatation (FMD), and has been largely used for endothelium-dependent dysfunction assessment. NO is probably the major mediator of vasodilation and reduced NO bioavailability has been broadly accepted as a marker of endothelium dysfunction (14).

In our cohort increased serum levels of ET-1 were present in PRP and SRP but only SRP SSc-associated patients with DU had significantly increased plasma levels of an endogenous inhibitor of endothelial NOS – ADMA. This favors early endothelial dysfunction with overproduction of vasoconstrictors (ET-1) even in PRP but only in severe SRP with peripheral vasculopathy is there an impaired inhibition of endothelial NOS. Furthermore endothelial dependent FMD was impaired in SRP, whilst PRP and control groups had similar response to shear stress.

Controversial results have been published regarding endothelial dysfunction assessment in SRP SSc patients. A systematic review and meta-analysis (15) analyzed FMD assessment in SSc patients demonstrating that most of the studies

(71%) assessing the FMD% found significantly lower brachial artery FMD% in SSc patients compared to controls. The lack of compensatory increase in blood flow to the ischemic stimulus may be due to endothelial dysfunction, reduced compliance, impaired distensability or increased arterial stiffness (16-18).

Positive Allen test has been associated to RP and SSc (19). Occlusion of ulnar artery in SSc patients as a predictor of DU has been reported (20) probably due to lack of compensatory flow of radial/ulnar artery and incomplete palmar arch. In this study patients with DU had more positive Allen tests compared to other groups favouring macrovascular disease in these patients.

Endothelial cell damage results in ischemia-reperfusion injury due to the ongoing pathological process, which inevitably evolves towards chronic underperfusion. Chronic hypoxia due to reduced blood flow is not compensated by efficient angiogenesis; even though elevated angiogenic biomarkers VEGF in SSc patients may be an attempt to induce neoangiogenesis and capillary neoformation. Yet, increased serum levels of angiostatic markers, such as endoglin, angiostatin or endostatin, may counteract this activity (12).

SRP SSc-associated with DU patients expressed lower VEGF and increased angiostatic endoglin serum levels suggesting impaired vascular remodelling in response to the chronic ischemia. No significant differences were found when PRP and SRP SSc-associated patients with no peripheral lesions were compared.

In conclusion endothelial dysfunction and a pro-angiogenic stimulus are already present in patients with PRP. Macrovascular disease, increased peripheral resistance due to structural lesions and an impaired response to shear stress are characteristic of SRP, particularly in patients with peripheral ischemic lesions. SRP SSc-associated patients with DU overproduce endothelial dysfunction (ET-1 and ADMA) and angiostatic (endoglin) vascular biomarkers.

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Microvascular damage, endothelial dysfunction and ischemic peripheral vasculopathy in secondary Raynaud phenomenon

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4.2 MICROVASCULAR DAMAGE, ENDOTHELIAL DYSFUNCTION AND ISCHEMIC PERIPHERAL VASCULOPATHY IN SECONDARY RAYNAUD PHENOMENON

ABSTRACT

Objective: To evaluate endothelial dysfunction and microvascular damage in secondary Raynaud Phenomenon (SRP) Systemic sclerosis (SSc) associated patients as possible predictors of ischemic fingertip digital ulcers (DU) in a 3-year clinical follow-up.

Methods: Flow-mediated dilatation (FMD), nailfold videocapillaroscopy (NVC), endothelin-1 (ET-1) and asymmetric dimethylarginine (ADMA) were analysed in a 3-year observational cohort study of 77 SRP with systemic sclerosis. Primary outcome was the occurrence of a new DU.

Results: Risk factors for DU at baseline were low FMD% ($p<0.001$), NVC pattern ($p<0.001$), high microangiopathy evolution score (MES) ($p<0.001$), increasedET-1 ($p<0.001$) and ADMA serum levels ($p=0.001$). Median time to the occurrence of a new DU was 4.50 (1.25-16.25) months. History of at least one DU before enrolment ($p<0.001$), autoantibody anti-scleroderma-70 ($p=0.012$), NVC late pattern ($p<0.001$), high MES score ($p<0.001$), low FMD% ($p<0.001$) and increased ET-1 serum levels ($p<0.001$) were identified as risk factors for the occurrence of at least one new DU episode in follow-up.

By univariate Cox regression analysis, FMD>9.41% (HR: 0.37 95%CI: 0.14-0.99) and ET-1 >11.85 *pmol/L* (HR: 3.81 95%CI: 1.41-10.26) and NVC (HR: 2.29 95%CI: 0.97-5.38) were predictors of DU recurrence, whereas for first DU event in naïve DU patients at baseline late NVC pattern (HR: 12.66 95%CI: 2.06-77.89) and MES score (HR: 1.693 95%CI: 1.257–2.279) were independent predictors.

Conclusion: This study identified endothelium dysfunction biomarkers (FMD and ET-1) and severe microvascular damage in NVC as strong predictors of new DU in SSc patients.

Keywords:

Systemic Sclerosis, digital ulcers, flow-mediated dilatation, capillaroscopy, Allen test, ET-1, ADMA, microangiopathy evolution score.

INTRODUCTION

Intimal hyperplasia, endothelial dysfunction and occlusive vasculopathy are ubiquitous features of systemic sclerosis (SSc) (1).

Endothelial cells (ECs) are the primary targets in systemic sclerosis (SSc). Underlying its pathogenesis is the interaction of ECs with other cells of the innate and adaptive immune system, platelets, coagulation factors, smooth muscle cells and fibroblasts (2).

Manifestations of vasculopathy in SSc patients are frequent. Raynaud phenomenon is frequently the first symptom and may precede other clinical manifestations of organ involvement by years. An estimated 30- 50% of all patients with SSc will experience a DU at some stage in their disease course (3-7). Prevalence rates reported vary between studies ranging from 8% to 31% (3, 8, 9). Differences in reported DU frequencies reflect not only the difficulties in defining them but also inconsistencies between studies as to which types are considered (6). Digital ulcers develop in an average of 30% pear year in patients with SSc (10, 11). These are a true burden, as a source of pain, have frequently severe complications such as infection, tissue loss and amputation, lead to severe hand impairment with a high impact in quality of life. Telangiectasia is a clinical marker of widespread aberrant microvascular disease (10) and are frequently seen in SSc patients due to the dilatation of the postcapillary venules located in the papillary and superficial reticular dermis (12).

As a response to the increase in shear stress, several vasodilators are released such as nitric oxide (NO), prostaglandins and endothelium-derived hyperpolarizing factor. This response is commonly known as flow-mediated dilatation (FMD) and has been largely used for endothelium dysfunction assessment and as functional bioassay of NO bioavailability (13).

Endothelin-1 (ET-1) has been identified as a key player of endothelial dysfunction in connective tissue diseases. It has been reported to be over-expressed in biological fluids, tissue sections and in multiple organs that are affected by SSc, notably blood vessels, lung, kidney and skin (14). Increased expression of ET-1 and increased receptor binding sites are found in pre-sclerotic and early diffuse skin lesions in SSc patients (15).

It has been suggested that decreased NO may contribute to endothelial dysfunction (16). Recently, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO-synthase, has emerged as a promising biomarker of endothelial dysfunction in cardiovascular diseases (17).

Microvasculopathy with alterations in morphology and function can easily be detected in the nailfold bed with capillaroscopy (18). Structural changes such as

giant capillaries, microhemorrhages, avascular areas and neo-angiogenic capillaries are important markers of disease progression and are present in more than 90% of SSc patients (19). Scoring the capillaroscopic patterns changes during the follow-up of SSc patients (20) enables capillaroscopy as a mandatory tool to quantify microangiopathy.

In this study, we hypothesized that endothelial dysfunction assessment (FMD, ET-1 and ADMA) and microvascular damage (NVC) in SSc patients could predict new ischemic fingertip DU in a 3-year clinical follow-up. Our primary outcome was the occurrence of a new DU in the 3-year follow-up.

PATIENTS AND METHODS

Patients

We describe a prospective observational study with 3-year follow-up of 77 SRP patients (72 women; mean age 52.95 ± 12.6 years; range 14-79 years) that attended the multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal from October 2011 to December 2014. We excluded patients with factors that could potentially interfere with FMD (smokers, hypertension, hyperlipidaemia and a past history of myocardial infarction) and patients on bosentan.

All patients fulfilled 2013 classification criteria for SSc of American College of Rheumatology (21) and had ECG and echocardiographic measurements within normal range. According to Leroy classification (22), 13 patients (16.9%) had diffuse SSc (dcSSc) and 64 (83.1%) had limited SSc (lcSSc).

Secondary RP SSc patients were divided into two groups: DU group, that included 38 patients having an active ulcer at the beginning of our follow-up study, with or without a past history of DU, (34 women; mean age 52.7 ± 14.8 years; range 14-75 years); and DU naïve group with no history of DU, that included 39 patients with no history of DU in the course of disease until enrolment (38 women; mean age 53.2 ± 10.3 years; range 30-79 years). Onset of disease was defined at the time of first RP episode.

The local institutional Health and Ethical Committee approved this study and all subjects signed informed consent before the study. Data was collected by analysis of clinical file data and by clinical interview.

Allen test

Allen test was performed as follows: 1. instructing patient to clench his/her fist; 2. applying occlusive pressure to both ulnar and radial arteries by finger pressure; 3. Confirm palm and finger blanching with the patient´s hand relaxed;

4. release the occlusive pressure on ulnar artery; 5. Positive test: If the hand flushes within 5-15 seconds it indicates that the ulnar artery has good blood flow and palmar arch is complete; Negative test: If the hand does not flush within 5-15 seconds, it indicates that ulnar circulation is inadequate with an incomplete palmar arch.

Flow-mediated dilatation

Ultrasounds scans were performed using a two-dimensional ultrasonography General Electric Logic 7 with a 9 MHz Linear wideband multihertz imaging probe. The same operator performed all exams. Before cuff deflation, images were recorded continuously from 15s before deflation to 180s after cuff deflation. Ultrasound images were analysed offline manually for 3 consecutive end diastolic frames (onset of R wave) at 45 to 60s after cuff deflation and averaging the arterial diameter along 10 mm segments from 3 consecutive end-diastolic frames. Ultrasound images were analyzed offline manually, averaging the arterial diameter during respective experimental stages along a 10-mm segment. The inter-operator variability was 3.6%. FMD measurements were blindly performed with respect to the NVC evaluation.

Flow mediated dilatation of the braquial artery in the lower arm was evaluated following International Brachial Artery Reactivity Task Force guidelines (23) for the ultrasound assessment of brachial artery endothelial-dependent flow-mediated vasodilatation. Patients and healthy subjects were on overnight fasting for twelve hours before the study. The exams were performed in the morning, after patients being kept in a quiet temperature controlled room (22-24ºC) for a 20-minute rest. Vasoactive drugs were withheld for 10 half-lives. Patients did not exercise or ingest substances that could affect the response to ischemia like caffeine, vitamin C or high-fat foods for 24 hours.

The braquial artery was scanned in a longitudinal section below the antecubital fossa in 2 to 3 cm section and the ultrasound probe was positioned using a mechanical probe stabilizer.

A sphygmomanometric blood pressure cuff localized above the antecubital fossa was inflated 50mmHg above patients systolic blood pressure (measured in left upper arm) and kept inflated for five minutes. No exam had to be discontinued due to pain or discomfort.

FMD was calculated as the percentage of change of the peak diameter in response to reactive hyperaemia in *(FMD%= (peak diameter – baseline diameter/baseline diameter) × 100) (23).*

Nailfold Videocapillaroscopy

Nailfold videocapillaroscopy (NVC) was performed with KK technology videocapillaroscopy with an x200 magnification lens. The same operator performed all capillaroscopies, with blind information regarding FMD. Images were recorded and kept anonyms in a KK image analysis software. In the end all images were scored for each patient at baseline and at 3-year follow-up and were controlled by a capillaroscopy expert. The inter observer variability was 2.5%.

All subjects were in a quiet room with controlled temperature (21-24ºC). Nailfold distal row capillaries of 4 fingers, 2nd, 3rd, 4th and 5th of both hands were examined.

Two classifications were used to describe capillaroscopic findings: a qualitative classification described by Cutolo in 3 patterns early, active and late (12). As SSc is recognized to be a progressive, obliterative microvascular disease, the borders between the consecutive NVC patterns are delineated, in between others, by gradually more severe capillary loss (24). A semi-quantitative score microangiopathy evolution score (MES) was adapted from Sulli et al (25). The sum of three scores regarding loss of capillaries, disorganization of the microvascular array and capillary ramifications were assessed to study the progression of the vascular damage.

Autoantibody detection

Antinuclear antibodies (ANA) were accessed by indirect Immunofluorescence on Hep-2 cells (NOVAlite ANA, Inova Diagnostics, Inc., San Diego, CA, USA). Autoantibodies anti-Scl-70, anti-centromere (ACA) was detected by immunoblotting using a Euroline Myositis Profile antibody test system (Euroimmun, Lübeck, Germany).

Biomarkers: ET-1 and ADMA

Fasting venous blood samples were collected into a serum tube and a tubecontaining sodium heparin (Vacuette, Greiner-Bio-One, Austria).

ET-1 assessment: Plasma was centrifuged immediately in a refrigerated centrifuge and stored at -70ºC until analysis for endothelin. Plasma endothelin was measured using a RIA assay, (Euro-Diagnostics AG, Sweden). The resulting values are reported as pmol/ml.

ADMA assessment: Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent

assay, (Immunodiagnostik AG, Germany). The resulting values are reported as umol/l.

Follow-up

Ischemic digital fingertip ulcerations were defined as painful area of 2 mm or greater in diameter with visible depth and loss of dermis, amenable to healing and localized to the fingertip. Digital ulcers caused by conditions other than SSc were not considered. Patients were instructed to come to clinics whenever a new digital tropic lesion occurred. If no DU developed, patients were seen on a regular basis at 3-6 months intervals as indicated by disease severity. Final observation was in last trimester of 2014.

Outcomes measures

Primary outcome was the occurrence of one or more new ischemic DU in the 3-year follow-up period. We applied survival analysis to new DU occurrence during the 3-year clinical follow-up, regarding endothelium dysfunction (FMD, ET-1 and ADMA) and microvascular damage (MES).

Statistical analysis

For comparison of normally distributed scale variables, we used unpaired two-sided student's t-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD). Normal distribution was tested by Q-Q plots. In cases of non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_3), where Q_1 represents the first quartile (corresponding to 25% of data) and Q_3 represents the third quartile (corresponding to 75% of data). In Anova test, when the homogeneity of variance is not satisfied, we used the Welch test. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. Predictors of digital ulcers were evaluated by univariate and multivariate logistic regression and summarised as OR and 95%CI. We applied survival analysis to determine the probability of freedom from new DU during the study period and evaluated the effects of FMD, MES, ET-1 and ADMA in that probability using the Kaplan-Meier method and the Cox regression. Values of $p \leq 0.05$ were considered as significant. Receiver-operating characteristic (ROC) analysis was performed for the selection of the optimal cut-off value of the FMD, MES, ET-1 and ADMA serum levels for the prediction of new DU. Data were analysed using the SPSS software (v.22.0, SPSS, Chicago, IL).

The association between baseline characteristics and the DU reoccurrence or new DU event was first analysed in univariate Cox regression followed by multivariate Cox regression including all variables with p<0.2 in univariate analysis.

RESULTS

A: Baseline characteristics

The demographic and clinical baseline characteristics of the 77 SSc patients are described in Table 1. Diffuse subset SSc patients had significantly more active DU at enrolment (31.6%, *p<0.001*). Sclerodactily (55.3%, *p < 0.001),* telangiectasia (100%, *p < 0.001),* digital pitting scars (84.2%, *p = 0.001)* and calcinosis (47.4%, *p < 0.001*) were also significantly associated to the presence of active DU. Endothelial dysfunction biomarkers (decreased FMD% (*p<0.001*) and enhanced increased serum levels of ET-1 (*p<0.001)* and ADMA (*p=0.001*)) as well as microvascular damage evidenced by worse NVC patterns (*p<0.001*), and higher MES score (*p<0.001)* were also significantly associated with the presence of active DU. Naïve DU patients exhibited more puffy hands (87.2%*, p = 0.008*).

B. Primary outcome: occurrence of a DU event in 3-year follow-up (Table 4.2.2)

In the 3-year follow-up, 40 (51.95%) patients developed new ischaemic digital ulcers (Table 4.2.2). Diffuse subset disease had proportionally more DU in follow-up (76.9%) compared to limited SSc (46.9%, *p=0.048*). No differences regarding disease duration were found between groups (*p=0.101*). Median time to the occurrence of a new DU was 4.50 (1.25-16.25) months. History of at least one DU before enrolment (*p<0.001*), autoantibody anti-scleroderma-70 (*p=0.012*), presence of telangiectasia (*p=0.042*), NVC late pattern (*p<0.001*), high MES score (*p<0.001*), low FMD% (*p<0.001*), positive Allen test (*p<0.001*) and increased ET-1 serum levels (*p<0.001*) were identified as risk factors for the occurrence of at least one new DU episode in follow-up. The occurrence of at least one new DU episode in follow-up.

Variables	DU (n=38)	Naïve DU (n=39)	p-value
Age (years), mean ± *SD (min-max)*	52.7 ± 14.8 (14-75)	53.2 ± 10.3 (30-79)	0.137[a]
Gender, *Women, n (%)*	34 (89.5)	38 (97.4)	0.067[b]
Disease duration (years), *median (Q_1-Q_3)*	10 (5-23)	10 (7-20)	0.028[c,*]
Total cholesterol (mg/dl), *mean ± SD (min-max)*	191.3 ± 9.0 (169-216)	187.1 ± 12.1 (156-202)	0.242[a]
Disease subset			0.001[a,*]
Limited, n (%)	26 (68.4)	38 (97.4)	
Diffuse, n (%)	12 (31.6)	1 (2.6)	
Disease duration, *Median (Q_1-Q_3)*	10.00 (5.00-23.00	10.00 (7.00-20.00)	0.602[c]
Onset of 1st ulcer (years), *Median (Q_1-Q_3)*	5 (3-13.25)	NA	NA
Puffy hands, *Presence, n(%)*	23 (60.5)	34 (87.2)	0.008[a,*]
Sclerodactily, *Presence, n (%)*	21 (55.3)	5 (12.8)	<0.001[a,*]
Telangiectasia, *Presence, n (%)*	38 (100)	27 (69.2)	<0.001[a,*]
Digital Pitting scars, *Presence n (%)*	32 (84.2)	19 (48.7)	0.001[a,*]
Calcinosis, *Presence, n (%)*	18 (47.4)	3 (7.7)	<0.001[a,*]
Hand / arm contractures, *Presence, n (%)*	19 (50)	3 (7.7)	<0.001[a,*]
Allen Test, *Positive, n (%)*	27 (71.1)	7 (17.9)	<0.001[a,*]
Autoantibodies			
ACA, Positive, n (%)	22 (57.9)	27 (69.2)	0.301[a]
Anti-scleroderma-70, Positive, n (%)	12 (31.6)	6 (15,4)	0.093[a]
FMD (%), *Median (Q_1-Q_3)*	5.3 (2.88-7.80)	16.21 (12.55-19.88)	<0.001[c,*]
ET-1 (pmol/mi), *Median (Q_1-Q_3)*	16.13 (10.97-21.17)	8.8 (5.89-12.68)	<0.001[c,*]
ADMA(umol/i), *Median (Q_1-Q_3)*	0.52 (0.45-0.63)	0.45 (0.41-0.51)	0.001[c,*]
NVC Pattern			
Early, n(%)	0 (0)	13 (33.3)	
Active, n(%)	11 (28.9)	22 (56.4)	< 0.001[a,*]
Late, n(%)	27 (71.1)	4 (10.3)	
MES score, *Median (Q_1-Q_3)*	5.00 (2.75-6.00)	1.00 (1.00-2.00)	<0.001[c,*]

Table 4.2.1: Comparison between DU and naïve-DU groups at baseline. *SSc: systemic sclerosis; DU: digital ulcer; dcSSc: diffuse systemic sclerosis subset; lcSSc: limited systemic sclerosis subset; ACA: autoantibody anti-centromere; NVC: nailfold Videocapillaroscopy; MES: microangiopathy evolution score; FMD: flow-mediated dilatation; ET-1: endothelin-1; ADMA: asymmetric dimethylarginine.; NA: non applicable; SD: standard deviation; Q: quartile. [a]: Chi-Square test. [b]: Fisher's Exact test. [c]: Mann-Whitney test. [*]: Statistical significance for a level of 5%.*

Variables	New DU in follow-up (n=40)	No- DU in follow-up (n=37)	p-value
dcSSc/lcSSc			0.048[a,*]
Limited, n(%)	30 (75.0)	34 (91.9)	
Diffuse, n(%)	10 (25.0)	3 (8.1)	
Disease duration, *Median (Q_1-Q_3)*	9.50 (4.25-20.00)	13.00 (7.50-22.00)	0.101[d]
History of DU, *n(%)*	30 (75.0)	8 (21.6)	<0.001[a,]
Time to new DU occurrence, *Median (Q_1-Q_3)*	4.50 (1.25-16.25)	NA	NA
Telangiectasias, *Presence, n(%)*	37 (92.5)	28 (75.7)	0.042[a,*]
Allen Test, *Positive, n(%)*			
Autoantibodies			
ACA , Positive, n(%)	27 (67.5)	22 (59.5)	0.464[a]
Anti-scleroderma-70 Positive, n(%)	14 (35.0)	4 (10.8)	0.012[a,*]
NVC Pattern baseline			
Early, n(%)	2 (5.0)	11 (29.7)	<0.001[a,*]
Active, n(%)	12 (30.0)	21 (56.8)	
Late, n(%)	26(65.0)	5 (13.5)	
MES score baseline, *Mean ± SD*	4.55±1.986	1.89±1.646	<0.001[c,*]
FMD (%), *Mean ± SD*	6.42±7.45	15.64±12.26	<0.001[c,*]
ET1 (pmol/ml), *Median (Q_1-Q_3)*	15.05 (11.12-20.27)	7.63 (5.98-12.74)	<0.001[d,*]
ADMA (umol/l), *Mean ± SD*	0.498±0.112	0.48±0.078	0.419[c]

Table 4.2.2: Primary outcome: new digital ulcer occurrence in the 3-year follow-up. *DU: digital ulcer; dcSSc: diffuse systemic sclerosis; lcSSc: limited systemic sclerosis; ACA: anti-centromere autoantibody; NVC: nailfold Videocapillaroscopy; MES: microangiopathy evolution score; FMD: flow-mediated dilatation; ET-1: endothelin-1; ADMA: asymmetric dimethylarginine. [a]: Chi-Square test; [b]: Fisher's Exact test; [c]: Student's t test; [d]: Mann-Whitney test; [*]: Statistical significance for a level of 5%.*

ROC analysis was performed in order to define positive diagnostic value of the different parameters studied as potential risk factors for DU events, reflected by an area under the curve: ET-1 (AUC: 0.758 95%CI: 0.649-0.866); FMD (AUC: 0.754 95%CI: 0.643-0.864) and for NVC scleroderma pattern (AUC: 0.846 95%CI: 0.760-0.932).

B.1 Recurrence of digital ulcers

Thirty (79%) of the 38 SSc patients with active DU at baseline had DU recurrence (Supplementary Table 1). By univariate Cox regression analysis, FMD<9.41% (HR: 0.37 95%CI: 0.14-0.99) and ET-1 >11.85 *pmol/L* (HR: 3.81 95%CI: 1.41-10.26*)* and NVC (HR: 2.29 95%CI: 0.97-5.38) were predictors of DU

recurrence (Table 4.2.3). Kaplan Meyer analyses of freedom from the DU recurrence showed that the median values of FMD (*p=0.035*), ET-1 (*p=0.004*) and qualitative scleroderma NVC (*p=0.047*) were significantly associated with the recurrence of DU (Figure 4.2.1: A-C).

Baseline characteristics	Univariate Cox regression	
	p-value	HR (95% CI)
Age	0.324	0.99 (0.97-1.01)
Male gender	0.155	2.17 (0.75-6.33)
Disease duration	0.235	0.98 (0.93-1.02)
Diffuse cutaneous subset	0.418	1.37 (0.64-2.93)
Positive Allen Test	0.471	1.37 (0.59-3.20)
Positive Anti-scleroderma-70	0.339	1.44 (0.68-3.04)
Positive Anti-centromere	0.518	0.79(0.38-1.63)
FMD > 9.41%	0.047	0.37 (0.14-0.99)
ET-1 > 11.85 *pmol/L*	0.008	3.81 (1.41-10.26)
ADMA > 0.49 *μmol/L*	0.897	0.95 (0.46-1.97)
Late NVC Pattern baseline	0.050	2.29 (0.97-5.38)
MES score	0.064	2.49(0.95-6.56)
Vasodilator therapy	0.462	0.58 (0.14-2.47)

Table 4.2.3: Univariate Cox Regression evaluating the predictive value for digital ulcer recurrence. *FMD: flow-mediated dilatation; ET-1: endothelin1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor; NVC: nailfold Videocapillaroscopy; NA: non applicable.*

B.2 First DU event in 3-year follow-up

In univariate analysis predictive factors for the first DU event in naïve DU patients at baseline only late NVC pattern (HR: 12.66 95%CI: 2.06-77.89) and MES score (HR: 1.69 95%CI: 1.26–2.28) were independent predictors of first DU event (Table 4.2.4).

Kaplan Meyer analyses of freedom from first DU event strengthen the results of Cox analysis with NVC qualitative scleroderma pattern (*p<0.001*) and MES score (*p<0.001*) being significantly associated with the occurrence of the first DU in follow-up. (Figure 4.2.2: A-B).

C. Clinical model for prediction of new DU

We considered as potential predictors of DU the following variables: MES score, FMD, ET-1, ADMA, presence of telangiectasia, NVC pattern (as markers of endothelial dysfunction and microvascular damage), Allen test (macrovascular disease) and disease subset. Univariate analysis identified as risk factors for new DU higher MES score, increased serum levels of ET-1, positive Allen Test, telangiectasia, late capillaroscopic pattern and dcSSc. Higher FMD % values were found to be protective from new DU.

When all these variables are included in a enter logistic regression, only MES score (2<MES≤6, HR 428.44, 95% CI 4.36 to 4.21E4), FMD (5.7%<FMD≤9.5% HR 0.005, 95% CI 0.00 to 0.59) and positive Allen test (HR 71.64 95% CI 2.75 to 1.87E3) remained significantly associated with DU. We then analysed the combined effect of these 3 variables in an attempt to build a clinical model for prediction of DU. With this aim, we created a score reflecting the sum of these risk variables present for each patient (MES>2, FMD≤5.7% and positive Allen test). When considering the 3 variables, all patients with 2 or more risk factors had DU. when considering only simple day-to-day office tools (MES and Allen test) patients with 1 risk factor had 75 % new DU risk and all patients with the 2 variables had new DU.

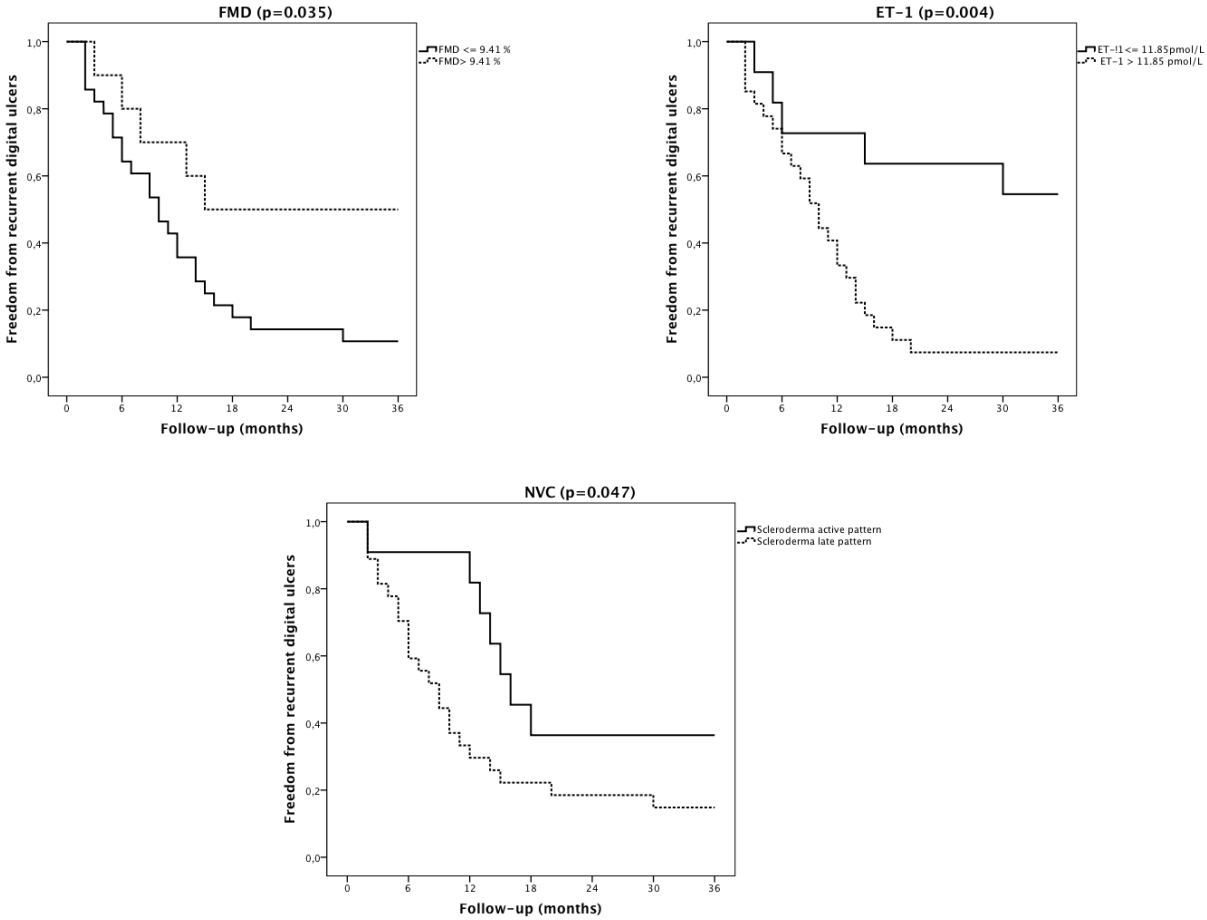

Figure 4.2.1: (A-C): Kaplan-Meier analyses of freedom from digital ulcer recurrence in 36 months follow-up of 38 SSc patients with active ulcer at baseline. *Curves are shown for: A – patients who had FMD levels ≤ 9.5% or > than 9.5% (p=0.001); B - patients who had ET-1 serum levels ≤ 11.9 pmol/ml or > than 11.9 pmol/ml (p<0.001); C – NVC patterns (p=0.047). SSc: systemic sclerosis; NVC: nailfold videocapillaroscopy; FMD: flow-mediated dilatation; ET-1: endothelin-1.*

Variables	DU at Baseline			Naïve DU at Baseline			p-value
	Follow-up 3 years		p-value	Follow-up 3 years		p-value	
	New DU (n=30)	Non-new DU (n=8)		First DU (n=10)	Naïve DU (n=29)		
Age (years), ***mean ± SD***	50.67±15.64	60.13±7.94	0.027[d, *]	53.10±11.04	53.28±10.27	0.964[d]	0.141[a]
Gender ***Women, n (%)***	26 (86.7)	8 (100)	0.560[b]	10 (100)	28 (96.6)	1.000[b]	0.368[b]
Disease duration (years), ***Median (Q_1-Q_3)***	7.00 (4.00-20.75)	20.50 (13.00-24.50)	0.050[e, *]	15.00 (5.25-20.75)	10.00 (7.00-20.00)	0.974[e]	0.160[c]
dcSSc/lcSSc			1.000[b]			1.000[b]	0.005[b,*]
Limited, n (%)	20 (66.7)	6 (75.0)		10 (100)	28 (96.6)		
Diffuse, n (%)	10 (33.3)	2 (25.0)		0 (0)	1 (3.4)		
Sclerodactily ***Presence, n (%)***	17 (56.7)	4 (50.0)	1.000[b]	0 (0)	5 (17.2)	0.302[b]	<0.001[b,*]
Puffy hands ***Presence, n (%)***	18(60.0%)	5(62.5)	1.000[b]	10(100)	24(82.8)	0.302[b]	0.030[b,*]
Telangiectasia ***Presence, n (%)***	30 (100)	8 (100)	NA	7 (70.0)	20 (69.0)	1.000[b]	0.001[b,*]
Digital Pitting scars ***Presence, n (%)***	24 (80.0)	8 (100.0)	0.309[b]	4 (40.0)	15 (51.7)	0.716[b]	0.005[b, *]
Digital amputation ***Presence, n (%)***	8 (26.7)	2 (25.0)	1.000[b]	0 (0)	0 (0)	NA	0.004[b,*]
Calcinosis ***Presence, n (%)***	15 (50.0)	3 (37.5)	0.697[b]	1 (10.0)	2 (6.9)	1.000[b]	0.001[b,*]
Hand / arm contractures ***Presence, n (%)***	15 (50.0)	4 (50.0)	1.000[b]	1 (10.0)	2 (6.9)	1.000[b]	<0.001[b,*]
Allen Test ***Positive, n (%)***	23 (76.7)	4 (50.0)	0.195[b]	3 (30.0)	4 (13.8)	0.344[b]	<0.001[b,*]
Autoantibodies							
ACA ***Positive, n (%)***	17 (56.7)	5 (62.5)	1.000[b]	10 (100)	17 (58.6)	0.017[b,*]	0.051[b]
Anti-scleroderma-70 ***Positive, n (%)***	11 (36.7)	1 (12.5)	0.393[b]	3 (30.0)	3 (10.3)	0.163[b]	0.080[b]
FMD ***Mean ± SD / median (Q_1-Q_3)***	3.96±6.06	10.55±10.26	0.025[d,*]	11.49 (9.41-19.86)	13.52 (8.36-21.83)	0.700[e]	<0.001[c,*]
ET-1 ***Median (Q_1-Q_3)***	17.24 (12.89 -23.72)	7.62 (6.31-14.39)	0.002[e,*]	10.34 (7.14-12.95)	8.06 (5.78-12.74)	0.421[e]	<0.001[c,*]
ADMA ***Median (Q_1-Q_3)***	0.51 (0.44-0.63)	0.56 (0.48-0.62)	0.654[e]	0.41 (0.35-0.51)	0.45 (0.41-0.50)	0.233[e]	0.006[c,*]
NVC Pattern baseline			0.195[b]			0.060[b]	<0.001[b,*]
Early, n (%)	0(0)	0 (0)		2 (20)	11 (37.9)		
Active, n (%)	7(23.3)	4 (50.0)		5 (50.0)	17 (58.6)		
Late, n (%)	23(76.7)	4 (50.0)		3 (30.0)	1 (3.4)		
MES score baseline ***Median (Q_1-Q_3)***	5.50 (4.50-6.0)	3.50 (2.00-5.75)	0.070[e]	2.50 (1.00-5.25)	1.00 (1.00-2.00)	0.010[e,*]	<0.001[c,*]

Table 4.2.4. Supplementary Table 1: Three-year follow-up of 77 SSc patients according to inclusion at baseline. *DU: digital ulcer; dcSSc: diffuse systemic sclerosis; lcSSc: limited systemic sclerosis; ACA: anti-centromere autoantibody; NVC: nailfold Videocapillaroscopy; MES: microangiopathy evolution score; FMD: flow-mediated dilatation; ET-1: endothelin-1; ADMA: asymmetric dimethylarginine. [a]:Anova test. [b]: Fisher's Exact test. [c]: Kruskal-Wallis test. [d]: Student's t test. [e]: Mann-Whitney test [*]: statistical significance for a level of 5%.*

Baseline characteristics	Univariate Cox regression	
	p-value	HR (95% CI)
Age	0.879	0.99 (0.94-1.06)
Disease duration	0.821	0.99 (0.92-1.07)
Telangiectasia	0.969	0.97 (0.25-3.77)
Positive Allen Test	0.270	2.14 (0.55-8.29)
Positive Anti-scleroderma-70	0.112	3.01 (0.77-11.71)
FMD > 9.41%	0.523	1.66 (0.35-7.82)
ET-1 > 11.85 *pmol/L*	0-708	0.74 (0.16-3.50)
ADMA > 0.49 *μmol/L*	0.721	1.28 (0.33-4.95)
Late NVC Pattern baseline		
Active pattern	0.661	1.44 (0.28-7.44)
Late pattern	0.006	12.66 (2.06-77.89)
MES score	0.001	1.69 (1.26 – 2.28)
Vasodilator therapy	0.326	2.82 (0.36-22.23)

Table 4.2.5: Univariate Cox Regression evaluating the predictive value for first digital ulcer event. *FMD: flow-mediated dilatation; ET-1: endothelin1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor; NVC: nailfold Videocapillaroscopy; NA: non applicable.*

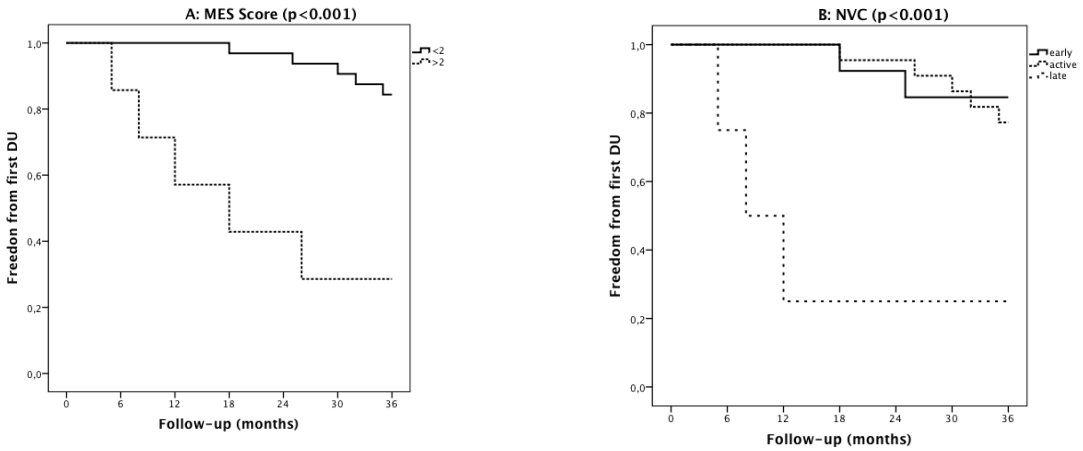

Figure 4.2.2: (A-B): Kaplan-Meier analyses of freedom from first digital ulcer event in 36 months follow-up of 39 naïve DU SSc patients at baseline. *Curves are shown for: A – patients who had MES score ≤ 2 or > than 2 (p=0.001); B – NVC patterns (p=0.001). SSc: systemic sclerosis; NVC: nailfold videocapillaroscopy; MES: microangiopathy evolution score.*

DISCUSSION

Several reports have identified different risk factors associated to DU. Different disease subsets cohorts, variable disease duration, sample size, risk factors analysed might contribute to different results reported. In a recent review, dcSSc, early onset of RP, early first non-RP symptom, high Rodman's skin score and anti-scleroderma-70 positive autoantibodies were considered strong predictors of DU (18).

In our study DU incidence was similar to placebo group of RAPIDS-1 study (26) (55.8%/61%) respectively. Diffuse subset, positive Allen test, presence of telangiectasia, late NVC pattern, high MES score, positive anti-scleroderma 70 autoantibody and impaired FMD were associated to the presence of new DU. Additionally past history of DU was identified as a risk factor for new DU. These factors favour microvascular damage and simultaneously macrovascular disease as potential contributors for the impaired vascular response to ischemic bouts of Raynaud phenomenon. The present study suggests that a positive Allen test and NVC findings are as a simple and easy office tests, valuable in early identification of patients at risk for DU. Further studies are needed to validate this model as a screening tool. Further investigation with pressure measurement of digits is needed and palmar and digital arteries ultrasound evaluation and cutaneous blood flow was measured using laser Doppler flowmetry.

Controversial results have been published regarding endothelial dysfunction assessment in SSc patients. In respect to FMD, there are studies favouring reduction of FMD in SSc compared to control groups (27-31) no reduction of FMD (32) and similar FMD (33, 34). These studies were not designed to analyse relationship of FMD and DU. Even though not always available, FMD may be in the near future a screening prognostic tool for vasculopathy in SSc patients. In our study patients with DU had a reduced vasodilatation response to the increased shear stress. Values of FMD greater than 9.5% were found to be protective from DU, suggesting that impaired FMD might be an alert trigger for peripheral vasculopathy.

Three recent studies reported increased serum ET-1 levels in patients with DU (18, 35-37) and one (38) describes no association between ET-1 serum levels and DU. Regarding ADMA, conflicting results of ADMA and SSc also have been described. Increased levels have been reported by Doodley et al (39) in dcSSc and by Blaise et al (17) in SSc reflecting abnormal NO regulation and/or contribution to endothelial dysfunction in SSc.

Our results have identified high serum levels of ET- as independent predictor of the development of a new DU episode in patients with or without a past history of DU. Additionally when freedom from digital ulcer was analysed, ET-1 serum levels > than 11.9 pmol/ml was found to be a risk for new DU in the 3-year follow-up. These findings are consistent with previous reports that suggest that ET-1 plays a major role in vascular dysfunction and vascular disease, through vasoconstrictive effects and as an important mediator in vessel remodelling, which ultimately results in major changes in cellular and tissue architecture (40).

Our patients with SSc have increased serum levels of ADMA. These results are in line with previous reports (39, 41). Although its an independent risk factor for DU it had no predictable value for new DU. Systemic sclerosis serum significantly reduces NO synthase activity, paralleled by decreases in intracellular cGMP and NO production in the cell medium, suggesting the presence of a factor that inhibits NOS (42). Nitric oxide has vasodilation effects on vessels and inhibits the thrombogenicity and the proliferation of vascular muscle cells, and is important for the regulation of blood flow and the maintenance of normal vascular wall structure (42).

As a research tool, qualitative and semi-quantitative capillaroscopic scoring has an indisputable value, but it has not yet found its way into day-to-day clinical practice (43). Several scores have been described. Smith et al (43) proposed as prognostic index for digital trophic lesions, Sulli et al reported a semi-quantitative microvascular injury surveillance score, MES, that paralleled the evolution of microangiopathy in SSc, although there is no reference to its association to DU (25). Sebastini et al suggested the Capillaroscopic Skin Ulcer Risk Index (CSURI) as a predictor risk of the onset on new DU (44).

In the present study capillaroscopy has great value in identifing high-risk patients for new DU events. Late capillaroscopy pattern was an independent predictor for DU recurrence and for the first DU episode in the 3-year follow-up. Qualitative and semi-quantitative assessments demonstrated that late NVC pattern (12) and disorganization of microvascular array with loss of capillaries, and angiogenesis were risk factors for DU occurrence. Additionally in 3-year freedom from DU analysis, MES score >2 was associated to a higher incidence of new DU. Late scleroderma pattern evidenced increased serum level of endothelial dysfunction biomarkers favouring the hypothesis that increased ET-1 concentrations may contribute to the pathogenesis of both RP and progressive microvascular/fibrotic damage in SSc (36).

Predictive clinical scores or models would be of great value for daily clinical practice (45). We propose a simple day-to-day clinical model for early identification of patients at high risk for DU. As FMD is not always accessible and reproducible, we suggest a simple clinical model for clinical practice: A 2-risk factor score (Allen test and MES) that identified SSc patients with 1 factor as having a 75% risk of new DU while all patients with both factors had new DU episodes. It would be interesting to validate this score in large sample studies, allowing the application of this simple day-to-day clinical tool.

Our study has some limitations, such as small patient sample limited by number of patients with active DU at time of enrolment. Small number of dcSSc

patients might have interfered in risk factors. Additional studies with larger cohorts are needed to validate the score and predictive risk factors enabling further understanding of the progression of vascular damage and endothelium dysfunction in the aetiology of DU.

CONCLUSIONS

Our study identified flow-mediated dilatation, microangiopathy score and Allen test as risk factors for DU. Additionally endothelium dysfunction biomarkers, FMD and ET-1 and severe microvascular damage in NVC as strong predictors of new DU in SSc patients. Further studies are needed to validate these data and identify patients who are candidate for early preventive target therapy.

Key messages:

1. Elevated serum ET-1 concentrations and impaired endothelial dependent flow-mediated dilatation are associated with an increased risk of developing new digital ulcers.
2. Scleroderma patients with late videocapillaroscopic pattern show an increased risk to have an active ulcer disease and more a severe microvascular damage is predictor for new digital ulcer episodes.
3. Allen test and Nailfold Videocapillaroscopy are simple clinical prognostic tools for digital ulcers.

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Digital ulcers in Systemic Sclerosis: role of flow-mediated dilatation and capillaroscopy as risk assessment tools

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4.3 DIGITAL ULCERS IN SYSTEMIC SCLEROSIS: ROLE OF FLOW-MEDIATED DILATATION AND CAPILLAROSCOPY AS RISK ASSESSMENT TOOLS

ABSTRACT

Aim: The aim of this study was to evaluate macrovascular endothelial dysfunction and microvascular damage as clinical markers of peripheral microangiopathy in patients with Raynaud's phenomenon (RP).

Patients and methods: Seventy-seven secondary RP with systemic sclerosis, 32 primary RP and 34 healthy controls were included in our study. Secondary RP patients were divided into two subgroups: 39 with digital ulcers (DU) and 38 without digital ulcers (non-DU).

Results: Patients with DU had significantly lower flow-mediated dilatation values (5.34 ± 7.49%) compared to non-DU patients (16.21 ± 11.31%), primary RP (17.96 ± 12.78%) and controls (20.17 ± 8.86%), $p<0.001$, favouring macrovascular endothelium dysfunction.

Regarding microvascular damage, DU group had predominately capillaroscopic late pattern (71.1%) whereas non-DU patients had active pattern (56.4%). Microangiopathy evolution score was significantly higher in DU group compared to non-DU group (4.79±1.82 vs. 1.79±1.56, $p < 0.001$). Flow-mediated dilation was significantly lower in late pattern (6.13±7.09%) compared to active (12.58±10.66%) and early pattern (17.72±14.90%), $p= 0.016$ and $p= 0.044$ respectively.

Conclusions: Low flow-mediated dilatation and microvascular damage in capillaroscopy are early clinical markers of DU risk in RP patients.

Keywords: Raynaud phenomenon, systemic sclerosis, digital ulcer, endothelial dysfunction, flow-mediated dilatation, capillaroscopy.

Highlights

1. Patients with systemic sclerosis and severe vasculopathy have:
   a. lower endothelial dependent flow-mediated dilatation.
   b. higher capillary dropout and impaired angiogenesis in capillaroscopy.
   c. increased microangiopathy evolution score
2. FMD and NVC are important tools in early identification of patients at risk of developing DU.

INTRODUCTION

Raynaud phenomenon (RP) is the earliest clinical presentation of some connective tissue disease and in systemic sclerosis (SSc), is for most authors, considered the first clinical manifestation of disease. Although microvessel involvement has largely been described, it is important to highlight that macrovascular disease my also affect more than half of the SSc patients [1].

Endothelium is a dynamic endocrine organ that plays a critical role in vascular homeostasis by secreting substances that regulate vascular tone, platelet activity and coagulation factors. It participates in vascular inflammation, cell migration and proliferation [2]. An increased apoptotic state is involved in endothelium denudation and dysfunction [2], leading to impaired vascular tone, pro-inflammation and pro-thrombotic state [2], increased expression of adhesion molecules and oxidative stress [3]. Structural integrity of endothelium is fundamental for a normal function. An impaired release and bioavailability of endothelium derived relaxing factor nitric oxide (NO) is observed in endothelial dysfunction [2]. NO is probably the major mediator of vasodilation and reduced NO bioavailability has been broadly accepted as a marker of endothelium dysfunction. [4]

Endothelial dependent flow-mediated dilatation (FMD) measurement is a functional bioassay of a NO bioavailability [4], a combination of the endothelium NO production and destruction by reactive oxygen species [5]. FMD is simple, safe, cheap, non-invasive, sensitive, reproducible method, and has been largely used for endothelium dysfunction assessment.

Microvasculopathy with alterations in morphology and function can easily be detected at the nailfold bed with nailfold Videocapillaroscopy (NVC). Capillaroscopy is a valuable, accessible, non-invasive, easy, safe and cheap tool with important diagnostic and prognostic value in patients with SSc. Qualitative, semi-quantitative and quantitative indexes/scores may be performed. Capillaroscopy has been described as the best predictor tool of transition of primary to secondary RP [6]. Structural changes are present in more than 90% of SSc patients [7]. Structural morphological and functional changes in capillaries allows differentiation between PRP and SRP identification of different Scleroderma evolution patterns with a close monitoring of disease evolution.

In clinical setting qualitative evaluation is the most accessible and easy to perform an enables diagnosis of different stages of disease activity and progression. Architectural disorganization as a result of microvascular injury includes giant capillaries, microhemorrhages, capillary loss and avascular areas and morphological changes with branched/ramified/bushy capillaries suggesting

angiogenesis. Cutolo et al. described an useful and reproducible qualitative NVC "Scleroderma patterns" with 3 different evolutive patterns: early, active and late [8]. Several studies have been done in an attempt to identify NVC changes and correlation to organ disease, in order to identify a possible predictive role of NVC.

The aim of this study was to evaluate and compare macrovascular endothelial dysfunction and microvascular damage as potential clinical markers of peripheral microangiopathy in patients with Raynaud's phenomenon. Additionally we determined the relationship of these clinical markers with severity of vasculopathy, namely the presence of digital ulcers.

PATIENTS AND METHODS

Patients

All hundred and nine enrolled patients, 97 women; mean age 50.9 ± 12.4 years; range 14-79, attended the Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal.

Thirty two patients had primary Raynaud phenomenon (PRP), 25 women; mean age 49.9 ± 12.5 years; range 22-73, with negative autoantibodies (ANA), no digital ulcers and had a bi or triphasic colour change of fingers when exposed to cold.

Seventy-seven patients had secondary Raynaud phenomenon (SRP), 72 women; mean age 52.95 ± 12.6 years; range 14-79, and based on 2013 classification criteria for SSc of American College of Rheumatology [9], all SRP patients had SSc. According to Leroy [10] classification, 13 (16.9%) had diffuse SSc (dcSSc) and 64 (83.1%) had limited SSc (lcSSc). SSc patients were divided into two subgroups: DU group - 38 patients with an active ulcer at time of study (painful area of 2 mm or greater in diameter with visible depth and loss of dermis, amenable to healing and localized to the fingertip), with or without a past history of DU, (34 women; mean age 52.7 ± 14.8 years; range 14-75) and non-DU group - 39 patients with no DU in the course of disease (38 women; mean age 53.2 ± 10.3 years; range 30-79). Onset of disease was defined at time of first episode of RP.

A group of 34 (sex, age matched) healthy non-obese, without self-reported cardiovascular risk factors: smoking, hypertension, diabetes or hyperlipidaemia were invited to participate as controls (29 women; mean age 47.1 ± 10.96 years; range 23-66). No control subject was on any vasoactive medication.

The study was done in the winter months from November to February. Pre-menstrual women were assessed in day 1 to 7 in the menstrual cycle. Three patients with factors that could potentially interfere with FMD were excluded at the beginning of the study (smoking, diabetes, hypertension, hyperlipidaemia and a history of myocardial infarction). All subjects studied had ECG and

echocardiographic measurements within normal range. Allen test was performed in all subjects for macrovascular impairment screening.

This study was approved by the Local institutional Ethical Committee and all subjects signed informed consent before the study.

Allen test

Allen test was performed as follows: 1. instructing patient to clench his/her fist; 2. Applying occlusive pressure to both ulnar and radial arteries by finger pressure; 3. Confirm palm and finger blanching with the patient´s hand relaxed; 4. Release the occlusive pressure on ulnar artery; 5. Positive test: If the hand flushes within 5-15 seconds it indicates that the ulnar artery has good blood flow and palmar arch is complete; Negative test: If the hand does not flush within 5-15 seconds, it indicates that ulnar circulation is inadequate with an incomplete palmar arch.

Ultrasound examination and FMD

Ultrasounds scans were performed using a two-dimensional ultrasonography General Electric Logic 7 with a 9 MHz Linear wideband multihertz imaging probe. All exams were performed by the same operator and recorded in the image analysis software.

For spectral waveform measurement two cursors were placed on sonographic image of braquial artery examined; sample gate cursor and alignment angle correct cursor. Three contiguous spectral waveforms were recorded for determination of peak systolic velocity (PSV), end diastolic velocity (EDV) and Resistive Index (IR). The latter was calculated as (PSV-EDV)/PSV. All these parameters were measured for each waveform and obtained as the average of three measurements in each waveform.

Flow mediated dilatation of the braquial artery in the lower arm was evaluated following International Brachial Artery Reactivity Task Force guidelines [11] for the ultrasound assessment of brachial artery endothelial-dependent flow-mediated vasodilatation. Patients and healthy subjects were on overnight fasting for twelve hours before the study. The exams were performed in the morning, after patients being kept in a quiet temperature controlled room (22-24ºC) for a 20 minute rest. Vasoactive drugs were withheld for 10 half-lives. Patients did not exercise or ingest substances that could affect the response to ischemia like caffeine, vitamin C, tobacco or high-fat foods for 24 hours.

The braquial artery was scanned in a longitudinal section below the antecubital fossa in 2 to 3 cm section. Mean baseline braquial artery diameter

was calculated as the result of the mean of 3 baseline measurements. A sphygmomanometric blood pressure cuff localized above the antecubital fossa was inflated 50mmHg above patients systolic blood pressure (measured in left upper arm) and kept inflated for five minutes. No exam had to be discontinued due to pain or discomfort. Before cuff deflation, images were recorded continuously from 15s before deflation to 180s after cuff deflation. Ultrasound images were analysed for 3 consecutive end diastolic frames (onset of R wave) at 45 to 60s after cuff deflation. The intraoperator variability was 3.6%. FMD measurements were blindly performed with respect to the NVC evaluation.

FMD was calculated as the percentage of change of the peak diameter in response to reactive hyperaemia in *(FMD%= (peak diameter – baseline diameter/baseline diameter) × 100) [11].*

Nailfold Videocapillaroscopy

Nailfold videocapillaroscopy (NVC) was performed with KK technology videocapillaroscopy with a 200x magnification lens. The same operator performed all capillaroscopies, with blind information regarding FMD. Images were recorded and kept anonyms in a KK image analysis software. The inter observer variability was 2.5%.

All subjects were in a quiet room with controlled temperature (21-24ºC). Nailfold distal row capillaries of 4 fingers, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} of both hands were examined.

Two classifications were used to describe capillaroscopic findings: a qualitative classification of scleroderma microangiopathy damaged described by Cutolo [12] in 3 patterns early, active and late. Early pattern was characterized by the presence of small number of giant capillaries and microhemorrhages, no avascular areas and a relatively well preserved capillary distribution; Active pattern was classified according to the presence of numerous giant capillaries and microhemorrhages, moderate capillary loss (20-30%) and a mild disorganized capillary architecture, with rare branches capillaries; Late pattern is characterized by near-absence of giant capillaries and microhemorrhages, presence of extensive avascular areas (50-70% of capillary loss) and a presence of many branched and ramified bushy capillaries (neoangiogenesis) and a complete disorganization of capillary array [12].

A semi-quantitative score Microangiopathy Evolution Score (MES) was adapted from Sulli et al [13]. The sum of three scores regarding loss of capillaries, disorganization of the microvascular array and capillary ramifications were assessed to study the progression of the vascular damage. A rating scale to

score each capillary abnormality was used (0 = no changes; 1= <33% capillary reduction / changes; 2= 33-66% capillary reduction / changes; 3=> 66% capillary reduction / changes) per linear millimeter [13]. The average score values from the eight digits were added together, and the final value divided for eight [13]. The resulting value represents the evolution score of microangiopathy (MES): 0-9 [14]. Following, we categorized MES scores in 3 subcategories with the following cut-off values: subcategory 1 – from 0 to 3; subcategory 2 – from 4 to 6 and subcategory 3 – from 7 to 9, allowing a more detailed analysis between microangiopathy and endothelial dysfunction evaluation.

Autoantibody detection

Antinuclear antibodies (ANA) were accessed by indirect Immunofluorescence on Hep-2 cells (NOVAlite ANA, Inova Diagnostics, Inc., San Diego, CA, USA). Samples with a titer greater than or equal to 1:80 were considered positive. Autoantibodies anti-Scl-70, anti-centromere (ACA), anti-Ro52, anti-PM-Scl, anti-RNA-polymerase, anti-fibrillarin (AFA), and anti-NOR 90 were detected by immunoblotting using a Euroline Myositis Profile antibody test syst (Euroimmun, Lübeck, Germany). Quantification of anti-U1RNP, antibodies was carried out using a Fluoro Enzyme Immuno Assay (EliA™ U1RNP70; Phadia, Uppsala, Sweden)

Statistical analysis

For comparison of normally distributed scale variables, we used unpaired or paired two-sided student's t-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD) followed by the minimal and the maximal values (range). Normal distribution was tested by Q-Q plots. In cases of non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_2;), where Q_1 represents the first quartile (corresponding to 25% of data) and Q_3 represents the third quartile (corresponding to 75% of data). In Anova test, when the homogeneity of variance it is not satisfied, we used the Welch test. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. In cases of multiple testing, a Bonferroni correction or a Games-Howell test (for equal variances not assumed) was used. We considered p values <0.05 as significant. Data were analysed using the SPSS software (v.22.0,SPSS,Chicago,IL).

RESULTS

Clinical assessment

The demographic and clinical characteristics of the 143 subjects are described in Table 4.3.1. No major differences were observed between SSc patients, PRP patients and control group regarding age, gender, mean arterial pressure and total cholesterol.

Variables	PRP	SRPSS		Control	p-value
		DU	Non-DU		
Subjects, n	32	38	39	34	NA
Age (years),	49.9 ± 12.5	52.7 ± 14.8	53.2 ± 10.3	47.1 ± 10.96	0.137[a]
mean ± SD (min-max)	(22-73)	(14-75)	(30-79)	(23-66)	
Gender					
Women, n(%)	25 (78.1)	34 (89.5)	38 (97.4)	29 (85.3)	0.067[b]
Disease duration (years),	15	10	10	NA	0.028[*,c]
median (Q_1-Q_3)	(11.25-30)	(5-23)	(7-20)		
Mean arterial pressure (mmHg),	87.6 ± 5.6	87.9 ± 6.04	88.3 ± 6.4	86.7 ± 6.8	0.75[a]
mean ± SD (min-max)	(76-102)	(79-102)	(77-103)	(70-101)	
Total cholesterol (mg/dl),	188.8 ± 8.8	191.3 ± 9.0	187.1 ± 12.1	190.6 ± 7.6	0.242[a]
mean ± SD (min-max)	(169-201)	(169-216)	(156-202)	(170-201)	

Table 4.3.1: Demographic and clinical data of subjects included. *PRP: primary Raynaud phenomenon; SRP: secondary Raynaud phenomenon; SSc: systemic sclerosis; NA: no applicable.* [a]*:Anova test.* [b]*: Fisher's Exact test.* [c]*: Kruskal-Wallis test.* [*]*: Statistical significance for a level of 5%.*

Disease duration was significantly different between groups (*p = 0.028).* Patients with PRP had significantly longer history of RP (median value: 15 years) compared to SSc patients (median value: 10 years). The DU group had a median of 5 years of DU history.

The characteristics of the SSc patients with or without ischemic digital ulcer are summarized in Table 4.3.2.

Allen test was positive in 71% of patients with DU and in 18% in SSc non-DU patients (*p < 0.001*). Patients with SSc-DU had significantly more sclerodactily (55.3%, *p < 0.001),* telangictasia (100%, *p < 0.001),* digital pitting scars (84.2%, *p = 0.001)* and calcinosis (47.4%, *p < 0.001*). Patients without DU had significantly more puffy hands (87.2%, *p = 0.008*).

Variables	SRPSS-DU	SRPSS non-DU	p-value
Subjects, n	38	39	
dcSSc/lcSSc			0.001[*,a]
Limitada, n(%)	26 (68.4)	38 (97.4)	
Difusa, n(%)	12 (31.6)	1 (2.6)	
Onset of 1st ulcer (years) ***Median (Q_1-Q_2)***	5 (3-13.25)	NA	NA
Puffy hands ***Positive, n(%)***	23 (60.5)	34 (87.2)	0.008[*,a]
Sclerodactily ***Positive, n(%)***	21 (55.3)	5 (12.8)	<0.001[*,a]
Telangiectasia's ***Positive, n(%)***	38 (100)	27 (69.2)	<0.001[*,a]
Digital Pitting scars ***Positive, n(%)***	32 (84.2)	19 (48.7)	0.001[*,a]
Periungeal haemorrhages ***Positive, n(%)***	29 (76.3)	24 (61.5)	0.162[a]
Digital amputation ***Positive, n(%)***	10 (26.3)	0 (0)	0.001[*,b]
Calcinosis ***Positive, n(%)***	18 (47.4)	3 (7.7)	<0.001[*,a]
Hand / arm contractures ***Positive, n(%)***	19 (50)	3 (7.7)	<0.001[*,a]
Allen Test ***Positive, n(%)***	27 (71.1)	7 (17.9)	<0.001[*,a]
Autoantibodies			
ACA ***Positive, n(%)***	22 (57.9)	27 (69.2)	0.301
Scl-70 ***Positive, n(%)***	12 (31.6)	6 (15,4)	0.093[a]
Anti-PM. Scl ***Positive, n(%)***	2 (5.3)	5 (12.8)	0.431[b]
Anti-RO 52 ***Positive, n(%)***	20 (52.6)	12 (30.8)	0.052[a]
Anti-NOR ***Positive, n(%)***	0 (0)	3 (7.7)	0.240[b]
Anti-fibrilarin ***Positive, n(%)***	0	1 (2.6)	1.000[b]
Anti U1 RNP ***Positive, n(%)***	2 (5.3)	2 (5.1)	1.000[b]
NVC Pattern			
Early, n(%)	0 (0)	13 (33.3)	
Active, n(%)	11 (28.9)	22 (56.4)	< 0.001[a]
Late, n(%)	27 (71.1)	4 (10.3)	
Normal, n(%)	0 (0)	0 (0)	
MES			
from 0 to 3	11 (28.9)	35(89.7)	<0.001[*,b]
from 4 to 6	21 (55.3)	4 (10.3)	
from 7 to 9	6 (15.8)	0 (0)	

Table 4.3.2: Comparison between SSc-DU and SSc non-DU groups. *Limited systemic sclerosis subset; SRP: secondary Raynaud phenomenon; ACA: autoantibody anti-centromere; NVC: nailfold videocapillarosocopy; MES: microangiopathy evolution score;NA: no applicable. a : Chi- Square test. b : Fisher's Exact test. *: Statistical significance for a level of 5%.*

Autoantibodies

There were no significant differences between SSc-DU and SSc non-DU patients regarding to ACA (*p = 0.301*), anti-Scl-70 (*p = 0.093*), AFA (*p = 1.000*), anti-PM-Scl (*p = 0.431*), anti-NOR 90(*p = 0.240*), and anti-RNA-polymerase

(*p=1.000*). Although without statistical significance, anti-Ro52 showed a higher clinical prevalence in the SSc- DU group (52.6%) comparatively with the SSc non-DU group (30.8%) (*p = 0.052*).

Videocapillaroscopy

All SSc patients had scleroderma pattern at NVC examination. Significantly differences were found between SSc DU group and SSc non-DU group regarding qualitative evaluation (*p < 0.001*): late pattern was the most frequent pattern in DU patients (71.1%) whereas early and active patterns were the most representative patterns in non-DU patients (33.3% and 56.4%, respectively). Significant differences of qualitative evaluation were found relatively to disease subset (*p = 0.010*). Patients with diffuse disease had predominately late pattern (76.9%) while limited disease had more active pattern (46.9%). Concerning semi-quantitative MES score significantly differences were observed: MES score was higher in DU group compared to non-DU group (4.79±1.82 vs. 1.79±1.56, *p < 0.001*). Relatively to MES subcategories, we found significantly differences (*p < 0.001*): subcategory 2 is the most frequent in SSc-DU patients (55.3%) while subcategory 1 is the most representative in SSc non-DU group (89.7%).

Macrovascular ultrasound examination and FMD

Ultrasound patterns and FMD are described in Table 4.3.3.

Flow-mediated dilatation at 60 seconds after deflation was significantly lower in patients with DU (mean 5.34±7.49%; range -9.22 - 21.67) when compared to SSc non-DU (mean 16.21±11.31%; range -4.60 - 48.27; *p < 0.001*), primary RP (mean 17.96±12.78%; range 2.04 - 63.21; *p < 0.001*) and control (mean 20.17±8.86%; range 7.66 - 44.81; *p < 0.001*). No statistical differences were found between SSc non-DU, PRP and control groups. When comparing the values of FMD in limited (n = 64) and diffuse (n = 13) SSc subjects, no statistical differences were founded (mean 11.91±11.35% vs. 5.63±7.45%, *p = 0.06*). No linear regression was found between FMD and disease duration (*p = 0.542*).

Baseline artery diameter was similar between groups (*p=0.620*). Absolute difference between mean baseline and post occlusive hyperemia dilatation at 60 seconds showed significant differences between SSc DU patients and all the other groups (*p<0.001*), see Table 4.3.3.

Primary RP (*p< 0.001*), SSc non-DU (*p = 0.001*) and control group (*p = 0.002*) had significantly decreased basal state PSV compared to SS DU group (mean 94.99±21.57cm/s; range 44.6-125.3). No differences were found between patients with and without DU (*p = 0.989*). After 5 minutes braquial artery

occlusion PSV and EDV were significantly reduced in SSc naïve DU patients (PSV: mean 151.51 ± 38.10cm/s; EDV: mean 58.03±16.89cm/s) and in PRP (PSV: *p=0.003*; EDV: *p < 0.001*) and in controls (PSV and EDV: *p < 0.001*) compared to SSc DU (PSV and EDV: *p<0.001*) and controls (PSV and EDV: *p < 0.001*). Resistance Index (RI) had significant differences between groups (*p = 0.024*).

Variables1	PRP	SRPSS		Control	p-value
		DU	Non-DU		
Baseline artery (mm)	3.51 ± 0.69	3.34 ± 0.51	3.44 ± 0.52	3.37 ± 0.55	0.620[a]
mean ± SD (min-max)	(1.48-4.51)	(1.95-4.72)	(2.31-4.17)	(2.31-4.69)	
Absolute difference (mm)	0.57 ± 0.29	0.17 ± 0.23	0.52 ± 0.29	0.65 ± 0.23	<0.001[*,a]
mean ± SD (min-max)	(0.08-1.08)	(-0.31-0.60)	(-0.17-1.24)	(0.28-1.09)	
FMD(%)	17.96 ± 12.78	5.34 ± 7.49	16.21 ± 11.31	20.17 ± 8.86	<0.001[*,a]
mean ± SD (min-max)	(2.04-63.21)	(-9.22-21.67)	(-4.60-48.27)	(7.66-44.81)	
Baseline PSV (cm/s)	65.77 ± 11.23	176.65 ± 19.72	75.26 ± 18.91	64.99 ± 21.57	<0.001[*,a]
mean ± SD (min-max)	(49.10-94.80)	(39.80-213.90)	(37.10-113.50)	(44.60-125.3)	
PSV 60 sec after cuff deflation(cm/s)	77.69±26.69	158.78±34.10	111.51 ± 38.10	39.77±12.93	<0.001[*,a]
mean ± SD (min-max)	(16.9-122,0)	(51.7-207.8)	(45.4-168.5)	12.7-112.8)	
EDV sec after cuff deflation(cm/s)	29.95 ± 15.05	56.70 ± 20.01	48.43 ± 24.37	18.03 ± 16.89	<0.001[*,a]
mean ± SD (min-max)	(11.8-29.3)	(40.5-131.9)	(23.9-131.4)	(14.6-31.3)	
RI	0.47 ± 0.23	0.78 ± 0.23	0.57 ± 0.09	0.53 ± 0.08	0.024[*,a]
mean ± SD (min-max)	(0.38-0.94)	(0.60-0.95)	(0.29-0.82)	(0.33-0.70)	

Table 4.3.3: Macrovascular ultrasound and Flow mediated dilation in study population. *PRP: primary Raynaud phenomenon; SRP: secondary Raynaud phenomenon; SSc: systemic sclerosis; DU: digital ulcer; FMD: flow mediated dilatation; PSV: peak systolic velocity; EDV: end diastolic velocity; RI: resistive index. [a]:Anova test. [*]: Statistical significance for a level of 5%.*

FMD and NVC

Regarding qualitative NVC patterns statistically differences of FMD were found between groups (*p=0.005,* Figure 4.3.1). FMD was significantly lower in the late pattern (mean 6.13±7.09%; range -9.22 - 21.67) compared to active pattern (mean 12.58±1.07%; range -8.49 - 40.04, *p = 0.016*) and early pattern (mean 17.72±1.49%; range -4.60 - 48.27, *p = 0.044*). No differences found between active and early patterns (*p = 0.506*).

FMD and MES

Flow-mediated dilatation was statistically different in MES subcategories (*p=0.006,* Figure 4.3.2): lower in the subcategory 3 (mean 4.37±5.55% range -1.49 - 12.98) compared to subcategory 1 (mean 14.03±1.21% range -8.49 - 48.27) and subcategory 2 (mean 6.55±7.44%; range -9.22; 21.67).

DISCUSSION

Vascular dysfunction is a key element of SSc pathogenesis [14]. Functional and structural changes involve microvessels, digital arteries and small elastic conduit arteries, such as radial and braquial artery. Vascular changes in SSc can be classified in two subgroups: destructive vasculopathy with progressive loss of capillaries early in disease course and proliferative obliterative vasculopathy characterized by proliferation of vascular cells, with luminal narrowing due to intimal hyperplasia and intimal fibrosis [15]. Although microvessel involvement has largely been described, macrovascular disease may affect more than half of the SSc patients [1] but there are few studies regarding small elastic conduits such as brachial, ulnar and radial arteries [16-18].

Comparing results of FMD across studies is troublesome. The absolute mean FMD varies among studies between -1,9 to 19,2% [19]. These differences may be due to different study populations, risk profiles and measurement procedures or non-compliance to FMD recommendations and guidelines. Celemajer et al (1992) [20] described a non-invasive technique to measure endothelial function and defined guidelines and recommendations [4 11 21].

Controversial results have been published regarding FMD in SSc patients. Favouring reduction of FMD and non endothelial dependent nitroglycerine mediated dilatation (NMD) [22-24], only reduction of FMD [14 15], reduction of FMD and preserved NMD[25] and similar FMD and NMD [26 27].

In SSc patients, ultrasonography of digital arteries show small artery lumen, reduced pulsation, thickened artery walls, low vessel compliance [18] and increased RI [16]. Vessel fibrosis and calcification reduce vascular compliance, and affect NO signalling by limiting vascular stretch due to arterial stiffness [15]. Different disease profile and mostly disease duration may reflect different responses to shear stress stimulus.

We assessed endothelial-dependent macrovascular functional impairment in PRP and SRP patients. Baseline artery diameter was similar between groups. FMD and absolute difference in baseline diameter/post-ischemic artery diameter were significantly lower in patients with SSc patients with active DU. (Figure 4.3.1). Patients without DU still had a preserved response to shear stress as no

significant differences were found when compared to PRP and control group. Late pattern had significantly reduced FMD. Our SSc patients were mostly lcSSc just as other reports with similar values of FMD in SSc patients [26 27]. Lekakis et al [22] ,and Cypiene et al [23] patients have demonstrated that dcSSC patients have lower FMD value. In our study, most of dcSSc patients had DU, and no significant difference was found when comparing lcSSc and dcSSc patients regarding FMD. More investigation with larger series of patients is needed to analyse FMD in SSc disease subset.

Basal PSV was decreased in RP patients compared to control group. Post-occlusive PSV and EDV were significantly reduced in SSc DU patients while RI was considerably increased in SSc DU patient group. We found no relationship of PSV, EDV and RI with progression of microvascular damage in NVC. The lack of compensatory increase in blood flow to the ischemic stimulus may be due to endothelial dysfunction, reduced compliance, impaired distensability or increased arterial stiffness [28-30]. Increased arterial stiffness has been related to vascular fibrosis leading to decreased elastic proprieties of large conduit arteries [31] and to diffuse inflammation involving arterioles and elastic arteries [26]. An increase in peripheral resistance due to microvascular damage might also contribute to impaired FMD.

In accordance to Takahashi et al [15] our study suggests that FMD might be a useful marker of severity of obliterative vasculopathy in SSc patients with DU, as shown by the lower FMD value, decreased PSV and EDV and higher RI.

Few studies evaluating endothelial dysfunction and microangiopathy as clinical markers for increased risk for DU have been reported. In our study, the main highlights were: 1) DU SSc patients had more avascular area, disorganization of capillary array and neoangiogenesis capillaries than the non-DU group; 2) high number of giant capillaries and haemorrhages with moderate capillary drop-off was the most frequent pattern in the non-DU group; 3) no patients with DU had early scleroderma pattern; 4) MES had lower scores in non-DU group (89.7% had scores<3) while in the DU group severe alterations in capillary morphology and distribution were present; 5) patients with late pattern had significantly lower FMD in accordance with Le et al [32] and Rollando et al [14]. Our findings point to a trend towards association between macrovascular endothelial dysfunctions disease and damage of microvascular structure in patients with DU. In agreement with Le et al [32], micro and macrovascular function might still be partially preserved in early phase of vascular disease.

Positive Allen has been associated to RP and scleroderma [33]. Occlusion of ulnar artery in SSc patients as predictive of DU has been reported [34] probably

due to lack of compensatory flow of radial/ulnar artery and incomplete palmar arch. In our study patients with DU had high prevalence of positive Allen test compared to other groups suggesting macrovascular disease in these patients This simple test might be a useful tool in evaluation of patients with increased risk for DU.

We failed to identify association of ACA and anti-Scl70 with DU. A trend towards association of positive Anti-Ro52/TRIM21 and DU was verified. These antibodies can be detected across a number of autoimmune diseases with significant prevalence, but it clinical relevance remains controversial. They are often detected in patients with scleroderma but are not diagnostically specific [35 36]. They may be overlapped with other main scleroderma related antibodies, like anti-centromere, anti-topoisomerase I, anti-RNA polymerase III, or anti-Pm/Scl antibodies and there is some evidence of its association with interstitial lung disease, and overlap syndrome [37]. More studies of larger patients SSc with DU cohorts are needed to identify possible autoantibodies as a possible risk for DU.

CONCLUSIONS

In our study, findings favour FMD and NVC as important tools in early identification of patients at risk of developing DU. Lower values of FMD, late NVC scleroderma pattern and worsening of capillaroscopic scleroderma pattern are red flags towards DU occurrence.

More studies, mainly longitudinal, are needed to investigate these vascular markers as long-term risk predictors, possible role in evaluation of disease evolution and markers for target therapy.

Acknowledgments

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Conflict of interests

None.

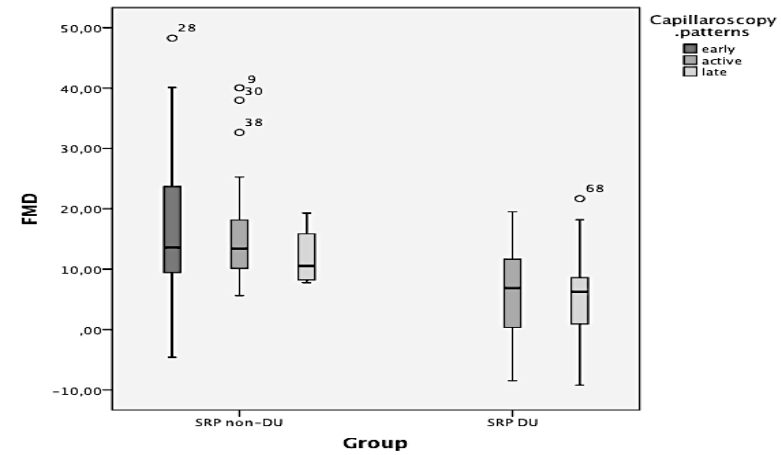

Figure 4.3.1: I. Silva. Comparison of FMD in different qualitative capillaroscopy patterns in patients with SRP non-DU and DU patients. *FMD: flow mediated dilation; SRP: Secondary Raynaud Phenomenon patients; DU: digital ulcers.*

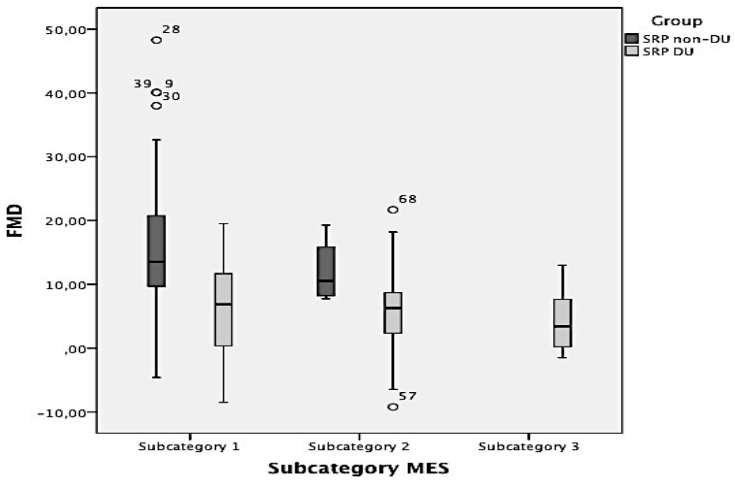

Figure 4.3.2: I. Silva FMD and MES sub-categories in SSc patients with and without digital ulcers. MES Sub-category 1 (*n= 46)*; Sub-category 2 (*n= 25)* and Sub-category 3 (*n=6). FMD: flow mediated dilation; MES:Microangiopathy evolution score; SSc Systemic sclerosis.*

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Impaired angiogenesis as a feature of digital ulcers in systemic sclerosis

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4.4 IMPAIRED ANGIOGENESIS AS A FEATURE OF DIGITAL ULCERS IN SYSTEMIC SCLEROSIS

ABSTRACT

Introduction: Impaired angiogenesis in systemic sclerosis has a major role in tissue injury pathogenesis. Our objective was to determine whether angiogenic biomarkers (vascular endothelial growth factor [VEGF], endoglin, and endostatin) are related to microvascular damage and to determine their predictive value for new digital ulcers (DU. The main outcome of the study was the occurrence of a new digital ulcer during 3-year follow-up.

Methods: This prospective longitudinal study was performed between October 2011 and December 2014. Seventy-seven patients definitely diagnosed with systemic sclerosis where divided into two groups: those with active DU at baseline and those with no DU until enrollment. Patients were matched by sex and age with healthy controls. Serum levels of VEGF, endoglin, and endostatin were measured at enrollment, and several nailfold videocapillaroscopies were performed during the 3-year follow-up.

Results: Serum levels of VEGF were lower (245.06: 158.68-347.33;p<0.001) and those of endoglin were higher (3.013: 1.463-7.023;p<0.001) in patients with active DU than those with no DU history (339.49: 202.00-730.93/ 1.879: 0.840-3.280), and they were higher than those found in controls (178.030: 101.267-222.102)/0.277:0.154-0.713) respectively. No differences in endostatin levels were found between groups (p=0.450). Endoglin was the only biomarker significantly different (p=0.031) between patients with diffuse versus limited systemic sclerosis and between early, active, and late patterns (p=0.020). VEGF was identified as an independent predictor for the development of new DU.

Conclusion: Our study confirmed the relationship between angiogenic vascular biomarkers and the occurrence of DU. Endoglin and VEGF serum levels are potential risk factors, and VEGF has a predictive value for the occurrence of new DU.

Keywords: systemic sclerosis, digital ulcers, VEGF, endoglin, endostatin, capillaroscopy.

INTRODUCTION

Microvascular alterations are key features of systemic sclerosis (SSc). Disease outcome depends on extent and severity of vasculopathy, and frequently the earliest clinical symptoms are related to the peripheral vascular system (1).

Raynaud phenomenon (RP) is the earliest clinical finding of SSc, and for many it is considered the first manifestation of disease. Initially RP has only functional implications, but repeated bouts lead to structural abnormalities and digital ischemia, which may progress to digital ulceration or, in extreme cases, to digital critical ischemia (2).

Digital ulcers are a true burden for patients because they are very painful, heal slowly, carry high risks of infection, and are extremely disabling, leading to severe impairment of simple daily activities. In adults, 40% to 50% (3, 4) of patients experience at least one ulcer in the course of disease and of these, 31% to 71% will have recurrent ulcers (5).

Endothelial cell (EC) injury results in disorganization of the EC layer favoring an early disorganized capillary architecture with loss of capillaries (1). In early disease stages enlarged, giant, meandering, ramified, and bushy capillaries occur in part as a result of EC injury and subsequent vascular remodelling (6). With disease progression, capillary loss might result from an uncompensated endothelial repair through angiogenesis and vasculogenesis, leading to reduced peripheral blood flow with consequent tissue hypoxia and formation of new blood vessels from the pre-existing microvasculature (6-8).

Microvascular structural changes are easily assessed using nailfold videocapillaroscopy (NVC), a simple, non-invasive, inexpensive, and accessible tool. Capillaroscopic patterns are dynamic and probably reflect disease evolution and severity. Several qualitative, semi-quantitative, and quantitative indices have been proposed as predictors of organ involvement and, in particular, digital ulcers (DUs) (2).

Angiogenesis, the creation of new blood vessels from pre-existing ones, depends primarily on the activation, proliferation, and migration of ECs and is driven by angiogenic stimuli that also induce proteolytic enzymes that cleave the extracellular matrix (9). It is tightly regulated by the balance between pro-angiogenic and anti-angiogenic factors (8). This event is highly complex and requires a dynamic, temporal, and spatially regulated interaction between ECs, soluble angiogenic growth factors, and extracellular matrix molecules (9).

Several studies have reported impaired angiogenesis in SSc, suggesting immune reactions to viral or environmental factors, reperfusion injury, or to anti-

endothelial antibodies (10). Alternatively, it might be a consequence of an imbalance between angiogenic factors and angiostatic factors.

Angiogenesis biomarkers have been extensively studied in SSc patients and investigated as possible putative biomarkers of organ involvement. Vascular endothelial growth factor (VEGF) is the most widely researched and understood angiogenic mediator; it is a potent angiogenic factor that stimulates migration, proliferation, and survival of ECs and endothelial pre-cursor cells (11).

Distler et al. (8) found increased levels of VEGF in SSc patients without fingertip ulcers, suggesting it has a protective effect. Farouk et al. (12) also demonstrated a significantly higher serum VEGF level in patients in the early phases of SSc without digital ischemic manifestations when compared to those with ischemic manifestations. Avouac et al. (1) reported increased levels of VEGF in late phases of the disease, suggesting that its upregulation might be an insufficient compensatory mechanism to stimulate angiogenesis and an inverse correlation between capillary density and VEGF.

Endoglin (CD105 or ENG) is a co-receptor for TGF-β predominantly expressed on cell surfaces of ECs. In SSc patients, this angiostatic biomarker has been measured and correlated primarily with pulmonary arterial hypertension. Wipff et al. (13) analyzed serum levels of sENG in SSc patients and found that they were higher in those with SSc with a vascular phenotype that integrates the presence of DUs.

Endostatin is a C-terminal 20-kDa fragment of the basement protein collagen type XVIII that strongly inhibits angiogenesis and tumour growth by reducing EC proliferation and migration (14). Hebbar et al. (15) demonstrated greater endostatin serum levels in SSc patients than in healthy controls and greater mean endostatin concentrations in SSc patients with cutaneous ulcers or scars than in those without cutaneous ulcerations. Farouk et al. (12) found significantly higher levels of serum endostatin in the late stages of SSc in patients with ischemic manifestations. Distler et al. (8) found no association between endostatin levels and fingertip ulcers, but did find an association between endostatin levels and the presence of giant capillaries.

We hypothesize that angiogenic biomarkers (VEGF, endoglin, and endostatin) reflecting disturbances of angiogenesis are related to microvascular damage objectified in NVC. Decreased angiogenic factors and/or increased angiostatic factors may be associated with the presence and development of new ischemic fingertip DUs during a 3-year follow-up of SSc patients. In this study, our primary outcome was the occurrence of a new DU during the 3-year follow-up.

PATIENTS AND METHODS

Patients

A prospective, longitudinal observational study was carried out between October 2011 and December 2014. Seventy-seven SSc patients (72 women; mean age 52.95 ± 12.6 years; range, 14-79) attending the Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal were followed. All patients fulfilled the 2013 classification criteria for SSc of American College of Rheumatology (16). According to the Leroy classification (17), 13 (16.9%) had diffuse SSc (dcSSc), and 64 (83.1%) had limited SSc (lcSSc). A group of 34 (sex and age matched) healthy controls were invited (29 women; mean age 47.1 ± 10.96 years; range, 23-66). They were non-obese and without self-reported cardiovascular risk factors (smoking, hypertension, diabetes, or hyperlipidaemia). No control was on vasoactive medications. Three patients died during the study period and were excluded.

At enrolment, patients were divided into two groups. The DU group comprised 38 patients with an active ulcer at baseline, with or without a past history of DUs, (34 women; mean age 52.7 ± 14.8 years; range, 14-75). The naïve-DU group comprised 39 patients with no DU in the course of disease until inclusion (38 women; mean age 53.2 ± 10.3 years; range, 30-79). Onset of disease was defined as the first episode of RP. All patients were on vasodilators, either the calcium channel blocker nifedipine or the angiotensin II receptor antagonist losartan, and a washout of these drugs was done before inclusion in the study.

This study was approved by the institutional review board (including Ethical for Health Committee of Centro Hospitalar do Porto). All patients signed informed consents before inclusion in the study. Data were collected by analysis of clinical file data and by clinical interview.

Nailfold videocapillaroscopy

Nailfold videocapillaroscopy was performed using a KK technology videocapillaroscope with a 200x magnification lens. The same operator performed all exams.

With the patient in a quiet room at a controlled temperature (21ºC to 24ºC), the nailfold distal row capillaries of 8 fingers (2nd, 3rd, 4th, and 5th of both hands) were examined.

Capillaroscopic findings were described according to the qualitative classification of scleroderma microangiopathy damage as proposed by Cutolo (18): early, active, or late pattern. The early pattern was characterized by the

presence of a small number of giant capillaries and microhemorrhages, no avascular areas, and a relatively well preserved capillary distribution. The active pattern was characterized by the presence of numerous giant capillaries and microhemorrhages, moderate capillary loss (20%-30%), and a mildly disorganized capillary architecture with few branched capillaries. The late pattern was characterized by a near-absence of giant capillaries and microhemorrhages, presence of extensive avascular areas (50%-70% capillary loss), presence of many branched and ramified bushy capillaries (neoangiogenesis), and a complete disorganization of the capillary array (18).

Biomarkers

Fasting venous blood samples were collected at enrollment into a serum tube and a tube containing sodium heparin (Vacuette; Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from the cells within 60 minutes and stored at -70ºC until analysis for endoglin, endostatin, and VEGF-A.

VEGF assessment: Serum VEGF-A was measured using an enzyme-linked immunosorbent assay, (IBL International GMBH, Germany). The resulting values were reported in pg/mL.

Endoglin and endostatin assessment: Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay, (Uscn; Life Science Inc., Wuhan). The resulting values were reported in ng/mL.

Follow-up

Ischemic digital fingertip ulcerations in the Stage 3 peripheral vascular category according to the Medsger Disease Severity scale (19) were considered to be DUs.

When included in the study cohort, patients were seen on a regular basis at 3 to 6 months intervals, as indicated by disease severity. The final observation was made in the fourth quarter of 2014.

Outcome measures

Our main objectives were to analyze the roles of angiogenic and angiostatic factors, to determine their associations with active ulcers at enrollment, and to establish whether they are predictors of developing new DUs during the 3-year follow-up period.

Statistical analysis

For comparisons of normally distributed scale variables, we used unpaired or paired two-sided Student *t* tests or analysis of variance (ANOVA). In these cases, data were described using mean ± standard deviation. Normal distribution was tested using Q-Q plots. In cases of non-normally distributed variables, we used the non-parametric Mann-Whitney and Kruskal-Wallis tests, and data were described using median and interquartile interval (Q_1-Q_3), where Q_1 represents the first quartile (corresponding to 25% of data) and Q_3 represents the third quartile (corresponding to 75% of data). When using ANOVA, if homogeneity of variance was not satisfied, we used the Welch test. For comparing categorical variables, we used chi-square or Fisher exact probability tests. Predictors of digital ulcers were evaluated using univariate and multivariate logistic regression. We applied survival analysis to determine the probability of freedom from new DUs during the study period and evaluated the effects of VEGF, endoglin, and endostatin in that probability using the Kaplan-Meier method and the Cox regression. When $p \leq 0.05$, significance was recognized. Data were analysed using SPSS software (v.22.0, SPSS, Chicago, IL).

RESULTS

Study population

Demographic and clinical characteristics are described in Table 4.4.1. Significant differences in the disease subset ($p < 0.001$) and capillaroscopic patterns ($p < 0.001$) were found between the DU and naïve-DU groups. The former predominantly showed the late pattern while the early and active patterns were more frequent in the latter. No differences were found in age, sex, disease duration (RP onset), or presence of auto-antibodies.

The DU group had lower VEGF serum levels 245.06 (158.68-347.33) pg/ml($p < 0.001$) and higher endoglin serum levels 3.013 (1.463-7.023) ng/ml ($p < 0.001$) compared to naïve-DU group 339.49(202.00-730.93)pg/ml/ 1.879(0.840-3.280ng/ml). See Table 4.4.2. In contrast, patients with active DUs had higher serum levels of VEGF and endoglin when compared to the control group 178.030(101.267-222.102)pg/ml/0.277(0.154-0.713ng/ml) respectively ($p < 0.001$). No significant differences were found in endostatin levels between the groups ($p = 0.450$). See Fig. 4.4.1.

Variables	DU (n=38)	naïve-DU (n=39)	p-value
Age (years), mean ± SD (min-max)	52.7 ± 14.8 (14-75)	53.2 ± 10.3(30-79)	0.845[d]
Gender *women n(%)*	34 (89.5)	38 (97.4)	0.2[b]
dcSSc/lcSSc			0.001[a,*]
Limited, n(%)	26 (68.4)	38 (97.4)	
Diffuse, n(%)	12 (31.6)	1 (2.6)	
Disease duration (years), *Median (Q_1-Q_3)*	10.00 (5.00-23.00)	10.00 (7.00-20.00)	0.602[c]
Onset of 1st ulcer (years), *Median (Q_1-Q_3)*	5 (3-13.25)	NA	NA
Autoantibodies			
ACA Positive, n(%)	22 (57.9)	27 (69.2)	0.301 [a]
Anti-scleroderma-70 Positive, n(%)	12 (31.6)	6 (15,4)	0.093[a]
Endoglin ng/ml, *Median (Q_1-Q_3)*	3.013, (1.463-7.023)	1.879 , (0.84-3.28)	0.017[c,*]
Endostatin ng/ml, *Median (Q_1-Q_3)*	0.695, (0.26-1.73)	0.429, (0.16-0.8)	0.129[c]
VEGF pg/ml , *Median (Q_1-Q_3)*	245.06, (158.68-347.33)	339.49, (202.00-730.93)	0.009[c,*]
NVC Pattern			
Early, n(%)	0 (0)	13 (33.3)	< 0.001[a,*]
Active, n(%)	11 (28.9)	22 (56.4)	
Late, n(%)	27 (71.1)	4 (10.3)	

Table 4.4.1: Comparison between DU and naïve-DU groups at enrolment. *SSc: systemic sclerosis; DU: digital ulcer; dcSSc: diffuse systemic sclerosis subset; lcSSc: limited systemic sclerosis subset; ACA: autoantibody anti-centromere; NVC: nailfold Videocapillaroscopy; VEGF: vascular endothelial growth factor. NA: non applicable; SD: standard deviation; Q: quartile. [a] : Chi-Square test. [b] : Fisher's Exact test. [c] : Mann-Whitney test. [d] : Student's t test. [*]: Statistical significance for a level of 5%.*

	Control	naïve-DU group	DU group	p
Endoglin ng/ml, *Median (Q_1-Q_3)*	0.277, 0.154-0.713	1.879, 0.840-3.280	3.012, 1.462-7.022	<0.001[a*]
Endostatin ng/ml, *Median (Q_1-Q_3)*	0.565, 0.350-0.770	0.429, 0.160-0.770	0.695, 0.260-1.727	=0.450
VEGF pg/ml, *Median (Q_1-Q_3)*	178.030, 101.267-222.102	339.49202.00-730.93,	245.060, 158.675-347.327	<0.001[a*]

Table 4.4.2: Comparison of pro-angiogenic VEGF and angiostatic endostatin and endoglin between groups at enrollment. *DU: digital ulcer VEGF: vascular endothelial growth factor. Q: quartile. [a] : Mann-Whitney test. [*]: Statistical significance for a level of 5%.*

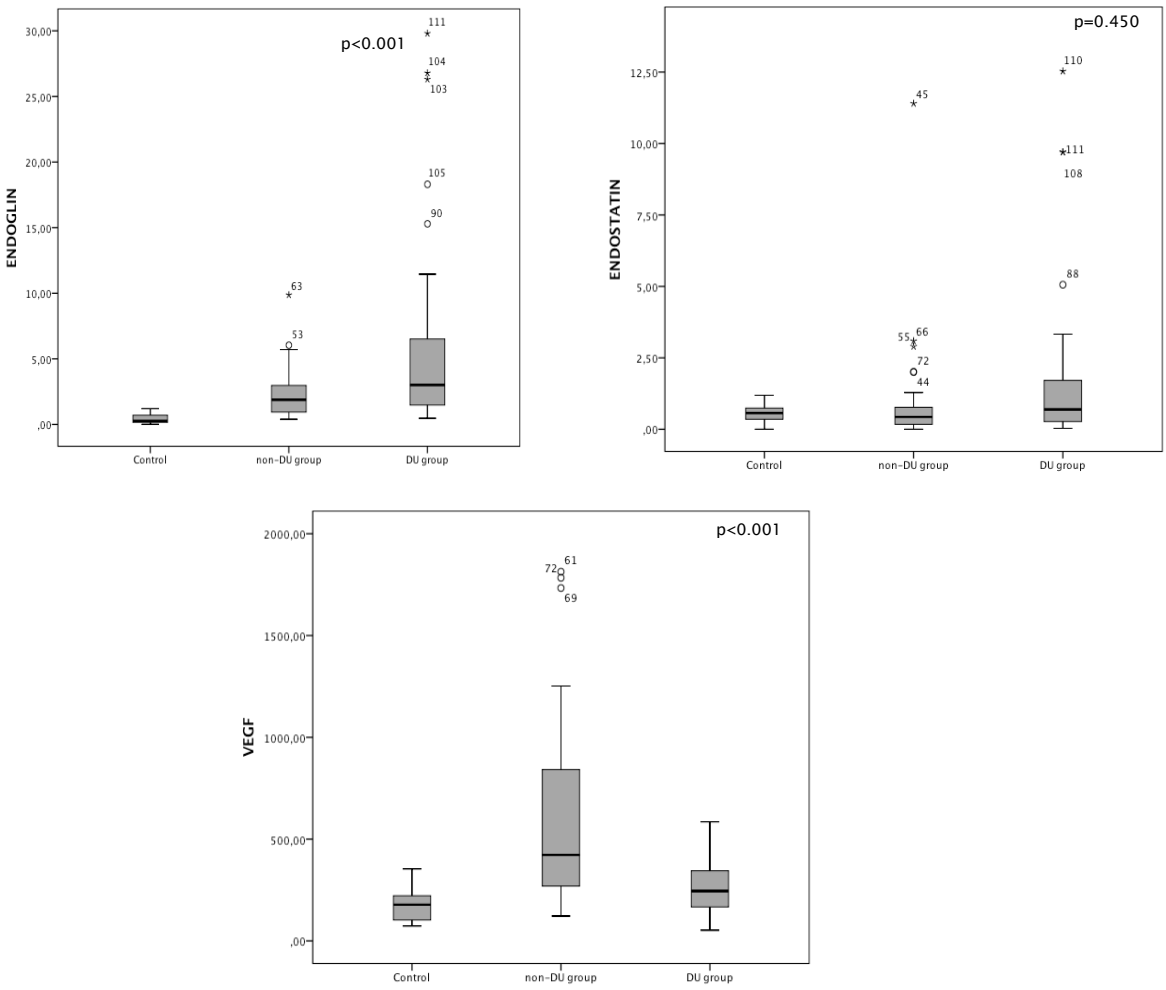


Figure 4.4.1: Serum levels of vascular endothelial growth factor (VEGF), endostatin and endoglin in 77 SSc patients with and without digital ulcers (DU) compared to 34 healthy controls. *Serum levels of Endoglin were increased in patients with DU 3.012(1.462-7.022) compared to controls 0.277(0.154-0.713) and patients without DU 1.879(0.840-3.280) p<0.001. VEGF serum levels were increased in patients without DU 339.49 (202.00-730.93) p<0.001 suggesting that VEGF increased levels are protective for new DU development. No significative difference between groups regarding endostatin p=0.450.*

No significant differences were found between those classified with dcSSc and lcSSc in terms of VEGF (344,50: 267.54- 379.56/ 269.26: 192,74- 453,82 pg/ml; p = 0.492) or endostatin (0.46: 0.28.1.70 / 0.53: 0.17- 1.07ng/ml; p = 0.355). Endoglin serum levels were higher among those with dcSSc (3.21: 2.20-17.51/ 2.06: 1.06- 3.96 ng/ml; p = 0.031).

Endoglin levels were significantly different between those with early, active, or late pattern NVC (p = 0.020), but VEGF and endostatin levels were not (p = 0.318 and p = 0.074, respectively).

Using univariate logistic regression, baseline VEGF (p = 0.007) and endoglin (p = 0.028) were identified as risk factors for digital ulcers. Multivariate regression analysis confirmed both as independent risk factors for DU (p = 0.006 and p = 0.028, respectively).

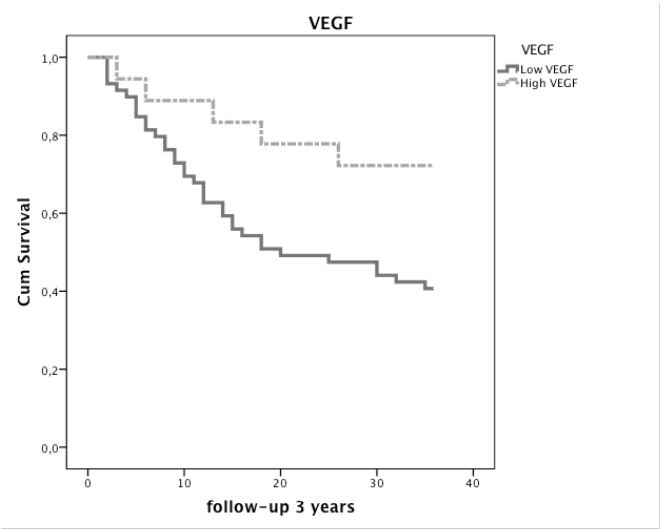
Occurrence of a new DU during the 3-year follow-up

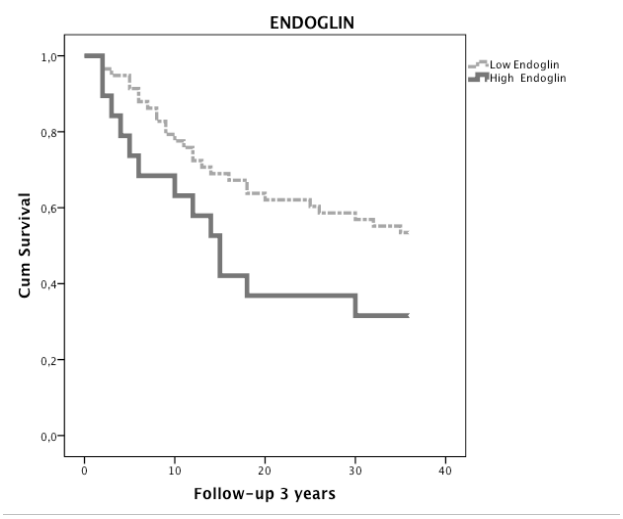
In the 3-year clinical follow-up, 40 (51.95%) patients developed new ischaemic digital ulcers (Table 4.4.3). Those with dcSSc had significantly more new DUs (76.92%) than those with lcSSc (46.88%), p = 0.048. No differences in disease duration were found (p = 0.101). Median time to the occurrence of a new DU was 4.50 months (range, 1.25-16.25).

Variables		**DU (n=40)**	**naïve-DU (n=37)**	**p-value**
dcSSc/lcSSc				0.048[a,*]
	Limited, n(%)	30 (75.0)	34 (91.9)	
	Diffuse, n(%)	10 (25.0)	3 (8.1)	
Disease duration		9.50	13.00	0.101[d]
Median (Q_1-Q_3)		(4.25-20.00)	(7.50-22.00)	
History of DU, *n(%)*		30(75.0)	8(21.6)	<0.001[a,*]
Time to new DU occurrence		4.50	NA	NA
Median (Q_1-Q_3)		(1.25-16.25)		
Autoantibodies				
ACA				0.464[a]
	Positive, n(%)	26 (65.0)	22 (59.5)	
Anti-scleroderma-70				0.012[a,*]
	Positive, n(%)	14 (35.0)	4 (10.8)	
NVC Pattern baseline				
	Early, n(%)	2 (5.0)	11 (29.7)	
	Active, n(%)	12 (30.0)	21 (56.8)	<0.001[a,*]
	Late, n(%)	26(65.0)	5 (13.5)	
Endoglin		2.57	2.14	0.153[d]
Median (Q_1-Q_3)		(1.39-4.73)	(1.03-3.33)	
Endostatin		0.695	0.429	0.142[d]
Median (Q_1-Q_3)		(0.273-1.755)	(0.16-0.77)	
VEGF		279.94	335.17	0.018[d,*]
Median (Q_1-Q_3)		(142.37-360.29)	(207.53-717.01)	

Table 4.4.3: Primary outcome: new digital ulcer occurrence during the 3-year follow-up. *DU: digital ulcer; dcSSc: diffuse systemic sclerosis; lcSSc: limited systemic sclerosis; ACA: anti-centromere autoantibody; NVC: nailfold Videocapillaroscopy; VEGF: vascular endothelial growth factor.* [a] *: Chi-Square test;* [b] *: Fisher's Exact test;* [c]*: Student's t test;* [d]*: Mann-Whitney test;* [*]*: Statistical significance for a level of 5%.*

Using univariate analysis, VEGF was the only predictive risk factor identified for the occurrence of at least one new DU during the three-year follow-up (p = 0.018). The Kaplan-Meyer analysis of freedom of DU for VEGF, endostatin, and endoglin is shown in Figs. 4.4.2 A-C. Those with low VEGF serum levels (< 422.47 pg/mL) had significantly more DUs (p = 0.028) in the 3-year follow-up period. While not significant, a trend toward increased serum levels of endoglin (>4.215ng/mL) was associated with a new DU (p = 0.053). No predictive value was found for endostatin (p = 0.130). Multivariate Cox analysis confirmed low VEGF as an independent predictor for the development of new DUs (p = 0.002).





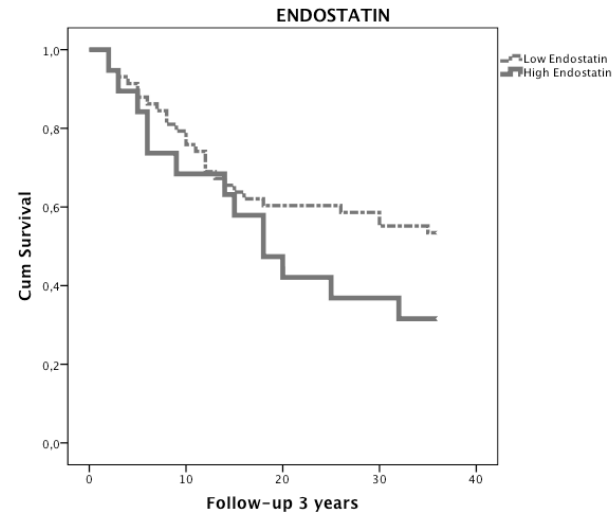
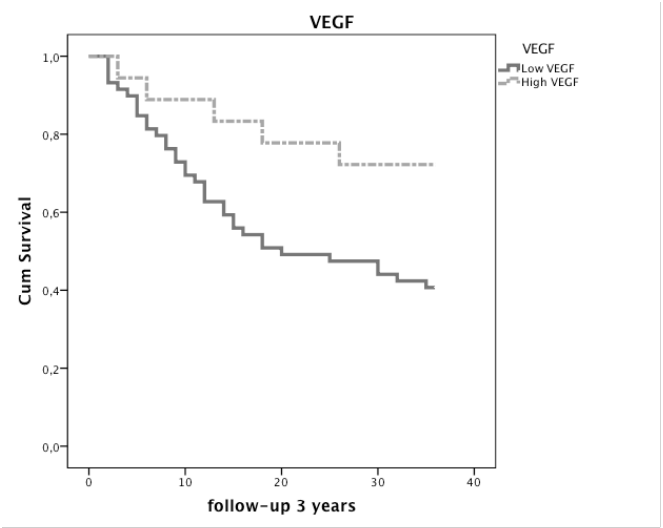
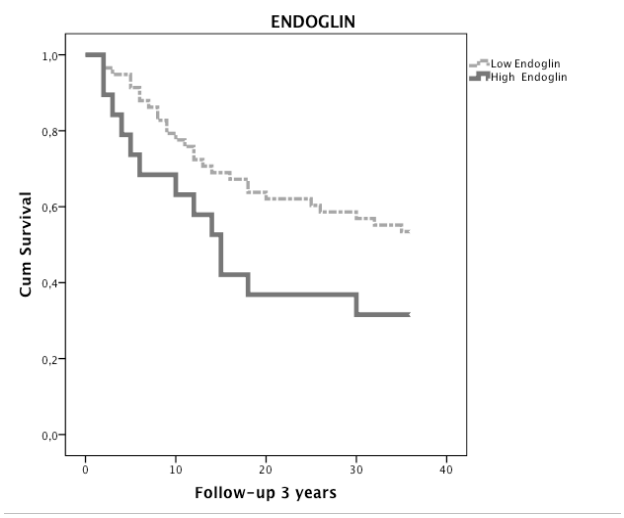


Figure 4.4.2 (A-C): Kaplan-Meier analyses of freedom from new digital ulcers in 36 months follow-up of 77 systemic sclerosis patients. Curves are shown for: A - patients who had Endoglin serum levels ≥ 4.215 ng/ml or < than 4.215 ng/ml (*p=0.05*); B - patients who had Endostatin serum levels ≤ 1.246 ng/ml or > than 1.246 ng/ml (*p=0.130*); C - patients who had VEGF serum levels ≤ 422.47 pg/ml or > than 422.47 pg/ml (*p=0.028*).

The VEGF (p = 0.024) and endoglin (p = 0.020) levels were significantly different between those with early or active patterns and those with late pattern NVCs. No difference was found in endostatin levels (p = 0.151). See Fig. 4.4.3.

Only VEGF levels were significantly different (p = 0.027) between the DU and naïve-DU groups 267(134.570-357.302 vs 415.230(207.525-863.165) in terms of developing a new DU during the follow-up period.





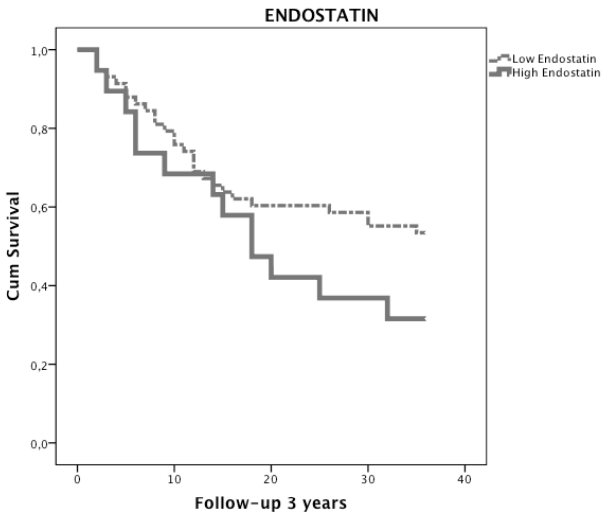


Figure 4.4.2 (A-C): Kaplan-Meier analyses of freedom from new digital ulcers in 36 months follow-up of 77 systemic sclerosis patients. Curves are shown for: A - patients who had Endoglin serum levels ≥ 4.215 ng/ml or < than 4.215 ng/ml (*p=0.05*); B - patients who had Endostatin serum levels ≤ 1.246 ng/ml or > than 1.246 ng/ml (*p=0.130*); C - patients who had VEGF serum levels ≤ 422.47 pg/ml or > than 422.47 pg/ml (*p=0.028*).

Analysing angiogenic and angiostatic serum baseline levels biomarkers and the progression of the capillaroscopy pattern during 3-year follow-up VEGF

(*p=0.024*) and endoglin (*p=0.020*) had significant differences when comparing early/active to late pattern in NVC. No difference was found respecting endostatin (*p=0.151*) Figure 4.4.3.

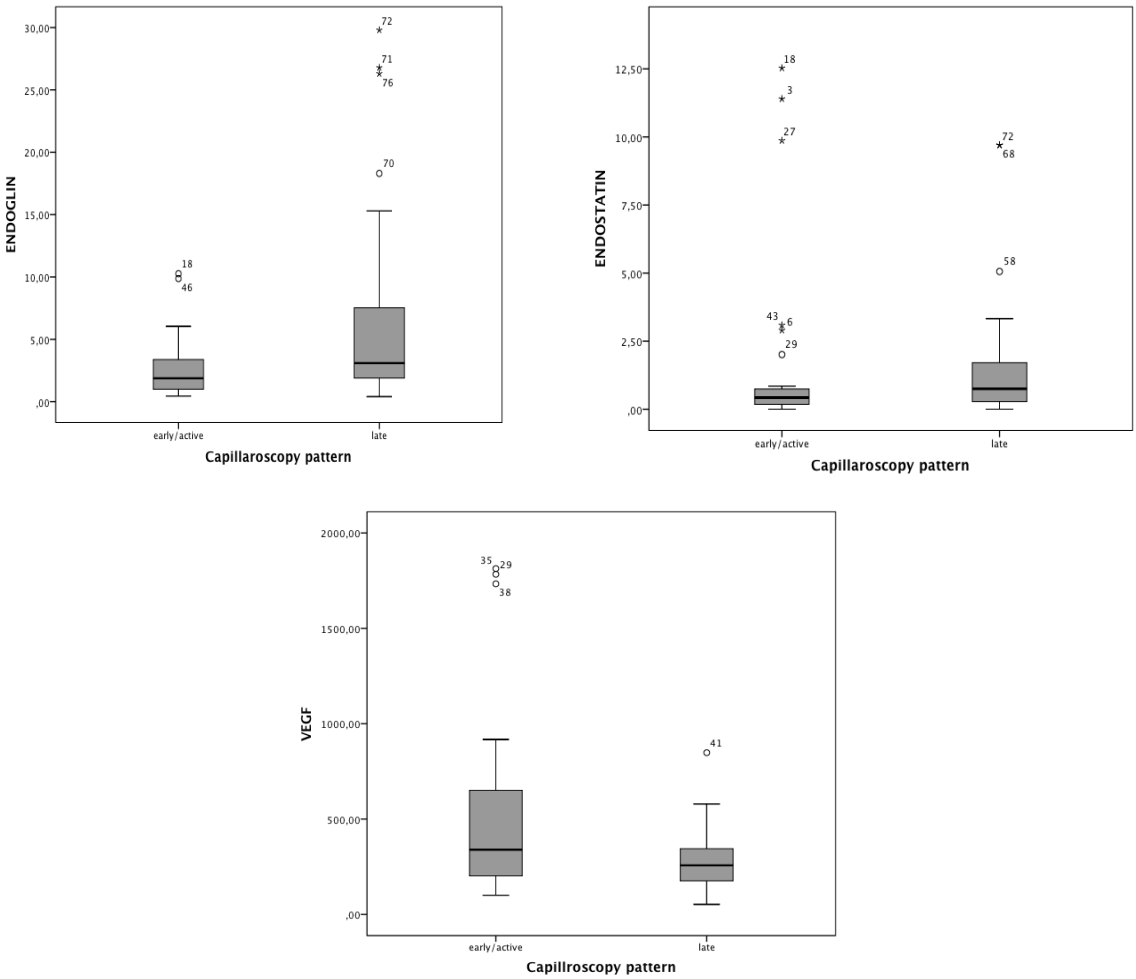


Figure 4.4.3: Serum levels of vascular endothelial growth factor (VEGF), endoglin and endostatin according to progression of nailfold capillaroscopic pattern in 3-year follow-up. VEGF *(p=0.024)* and endoglin *(p=0.020)* had significant differences when comparing early/active with late pattern in NVC. No differences were found relative to endostatin *(p=0.151)*.

When comparing patients with/without DU at enrolment with reference to a new DU development in the 3-year follow-up, only VEGF showed significant differences (*p=0.027*) in patients with an active ulcer at baseline 267 (134.570-357.302 compared to patients with no DU until inclusion 415.230 (207.525-863.165).

DISCUSSION

Disparities seen in the results of various studies are likely the result of different SSc patient populations, disease stages, or clinical presentations as well as diverse organ involvement, non-uniform DU classification, and a small number of SSc patients in most clinical and investigational research cohorts. Furthermore, most of the studies were not designed to find predictable risk factors for DU on their own, but information and lessons may be learned from these publications.

Our study shows that circulating levels of VEGF are increased in SSc patients with early or active NVC patterns and without DUs, suggesting it has a protective role against digital ulcers. Reduced VEGF levels in late NVC patterns suggest that ineffective angiogenesis may contribute to the avascular areas observed in this pattern, largely responsible for the ischemic territory underlying digital ulcers. This insufficient angiogenesis in the late pattern also might be related to increased levels of angiostatic factors, which are reportedly increased in the late stages of SSc (20). Another explanation is that a prolonged, uncontrolled, and chronic overexpression of VEGF in SSc may have a deleterious effect on the vascular network, resulting in a chaotic vascular morphology with reduced blood flow in the newly-formed vessels (1, 20). Our study was not designed to analyze any association between VEGF and capillary morphology, but our results confirmed those found by Distler et al. (8) and Farouk et al. (12), finding significantly higher serum VEGF levels in the early phases of the disease when digital ischemic manifestations are lacking. In contrast, Avouac et al. (1) reported increased levels of VEGF in the late pattern as well, suggesting that this upregulation might be an insufficient compensatory mechanism to stimulate angiogenesis and an inverse correlation between capillary density and VEGF level.

We found similar levels of endoglin as that reported by Wipff et al. (13). Greater serum levels in patients with the SSc vascular phenotype were associated with the presence of digital ulcers. High levels of sENG in the sera of SSc patients highlight a possible contribution of this antiangiogenic protein in tSSc vascular disturbances. Greater serum levels of endoglin in the subset with dcSSc are expected, given the important profibrotic role of endoglin in SSc fibroblasts (21, 22).

Conflicting results have been reported regarding endostatin in SSc patients. Our results confirm those of Distler et al. (8), finding no association between endostatin levels and DU, yet this contrasts with results reported by Hebbar et al. (15) and Farouk et al. (12), who reported associations between endostatin and the presence of DU. Additionally, we identified greater endostatin serum levels in SSc patients than in the healthy controls, as did Hebbar et al. (15). Endostatin plays

an important role in microvascular changes and balance in SSc patients, and its actions are more probably mediated by several other angiostatic factors that remain to be identified. Larger observational studies are required to define the relathionships between angiostatic biomarkers, DUs, and other clinical manifestatins of vasculopathy in SSc patients.

Limitations of this study are its small patient sample, all recruited from the same centre, and the number of patients with active DU at the time of enrollment. Additional longitudinal studies with larger cohorts are needed to validate predictive risk factors, thus enabling a better understanding of the progression of vascular damage and angiogenesis in the aetiology of DU. It would be interesting to study angiopoietins and VEGF-A isoforms and analyze their predictive roles. Angiopoietins are known to be involved in the development, remodelling, and stability of blood vessels (23). Altered expressions of Angiopoietin 1 (Ang-1), Angiopoietin 2 (Ang-2) (23), and Angiopoetin-like Protein 3 (ANGPTL3) (24) have been studied as biomarkers of SSc-related microangiopathy. Manetti et al. (25) described proangiogenic VEGF165 and antiangiogenic VEGF165b isoforms, finding the first evidence of a switch from proangiogenic to antiangiogenic VEGF-A isoforms and their crucial roles in the defective angiogenic and vascular repair processes that characterize SSc.

CONCLUSION

Our study confirms the relationship between some angiogenic vascular biomarkers and the occurrence of DU and determines which of them could be predictors of these disabling complications of the disease. In SSc patients, reduced levels of VEGF, after an initial increase in the early stages of disease, independently predict development of new DUs. We identified a trend toward high serum endoglin levels as a predictor of DUs. Increased endoglin is present in dcSSc and the late NVC scleroderma pattern. We found that endostatin is not an independent risk factor for active DUs nor a predictor of the occurrence of new DUs. The molecular analysis of the vascular mediators associated with SSc, particularly with peripheral microangiopathy, may identify new therapeutic targets that lead to the prevention of further vascular injury or the improvement of SSc disease.

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Conflict of interests:

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José Oliveira: none

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Predictive value of vascular disease biomarkers for digital ulcers in systemic sclerosis patients

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4.5 PREDICTIVE VALUE OF VASCULAR DISEASE BIOMARKERS FOR DIGITAL ULCERS IN SYSTEMIC SCLEROSIS PATIENTS

Predictive value of vascular disease biomarkers for digital ulcers in systemic sclerosis patients

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Key words: systemic sclerosis, digital ulcers, endothelin-1, vascular endothelial growth factor, asymmetric dimethylarginine, endoglin

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ABSTRACT

Objective. *To investigate the role of endothelial dysfunction and angiogenesis vascular biomarkers as risk factors and their predictive value for digital ulcers in systemic sclerosis patients.*

Methods. *Endothelin-1 (ET-1), asymmetric dimethylarginine (ADMA), vascular endothelial growth factor (VEGF), endostatin and endoglin were measured in an observational prospective cohort of 77 SSc patients. The primary outcome was the occurrence of one or more new ischaemic digital ulcers during a planned 3-year follow-up.*

Results. *After the 3-year follow-up, 40 patients developed new digital ulcers. Logistic regression confirmed VEGF (HR 1.128, 95% CI 1.010–1.260, p=0.033) and ADMA (HR 0.995, 95% CI 0.991–0.998, p=0.006) as independent predictors of new digital ulcers. Patients with serum levels of ET-1>11.9pmol/ml (p<0.001) and VEGF<422.47 pg/ml (p=0.028) had significantly more DU in the 3-year follow-up. Although not significant, a trend towards increased serum levels of endoglin>4.215ng/ml (p=0.053) was associated to a new DU episode. No predictive serum value was found for ADMA (p=0.075) and endostatin (p=0.130).*

Conclusions. *Endothelial dysfunction and angiogenic vascular biomarkers have an important role in the underlying and in the progression of microvascular disease in systemic sclerosis. Increased serum levels of ET-1, ADMA and VEGF are strong predictors of severe microangiopathy complications, namely ischaemic digital ulcers.*

Introduction

Vascular disease is of fundamental importance in the pathogenesis of scleroderma from very early onset of the disease through late clinical complications (1). Vascular involvement is widespread with an extremely heterogeneous clinical expression, from Raynaud phenomenon to severe digital ulcers (DU) up to life threatening pulmonary arterial hypertension (2). Key issue is to understand the pathophysiology underling vascular dysfunction in order to explain why some patients with systemic sclerosis (SSc) progress to severe digital ischemia while others have no DU in the disease course.

Endothelial cell (EC) dysfunction has been postulated as a key and early inciting event in the disease process. Injured endothelium leads to an imbalance of microvascular tone control, favouring vasoconstriction, due to overproduction of vasoconstrictors by endothelial cells (such as potent vasoconstrictor ET-1) or to reduced endothelium-dependent vasodilation (3). Doubts persist about underproduction or impaired action of vasodilators produced by the endothelium, such as nitric oxide (NO) and prostacyclin (3).

Vascular remodelling following microvascular injury with intimal and medial thickening and adventitial fibrosis leads to progressive stenosis and vascular occlusion (4). It has been postulated that avascular areas are consequence of chronic hypoxia and that enlarged and bushy/ramified capillaries are a pro-angiogenic response not related to hypoxia but to the overexpression of VEGF (5) or as a consequence of an imbalance in angiogenic factors/angiostatic factors. Angiogenesis biomarkers have been extensively studied in SSc patients and investigated as possible putative biomarkers of organ involvement.

Our main objective was to analyse the role of vascular biomarkers of endothelial dysfunction and angiogenesis as risk factors for active DU or as predictors for new DU episodes in a 3-year follow-up.

Materials and methods

Patients

Seventy-seven SSc patients (72 women; mean age 52.95±12.6 years; range

14-79) attending Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal were followed in a prospective, longitudinal observational study between October 2011 and December 2014. All patients fullfield 2013 classification criteria for SSc of ACR/EULAR (6). Thirteen patients (16.9%) had diffuse SSc (dcSSc) and 64 (83.1%) had limited SSc (lcSSc) According to Leroy classification (7). Thirty-four healthy, sex/age matched, non-obese, without self-reported cardiovascular risk factors controls were invited to participate. No control subject was on any vasoactive medication.

Patients were divided into two groups: SSc-DU group - 38 patients with an active ulcer at baseline, with or without a past history of DU (34 women; mean age 52.7±14.8 years; range 14–75) and SSc-non-DU group - 39 patients with no DU in the course of disease until inclusion (38 women; mean age 53.2±10.3 years; range 30–79).

The local institutional health ethics committee approved this study and consent forms were signed by all participants, in accordance with the ethical standards of Helsinki Declaration.

Table I. Comparison of vascular biomarkers between groups at baseline.

Variables	DU (n=38)	Non-DU (n=39)	Control (n=40)	*p*-value
Endoglin ng/ml Median (Q_1-Q_3)	3.01 (1.46-7.02)	1.88 (0.84-3.28)	0.28 (0.15-0.71)	<0.001*,a
Endostatin ng/ml Median (Q_1-Q_3)	0.695 (0.26-1.73)	0.429 (0.16-0.77)	0.565 (0.35-0.77)	0.164a
VEGF pg/ml Median (Q_1-Q_3)	245.06 (158.68-347.33)	422.47 (269.26-847.97)	178.03 (101.27-222.10)	<0.001*,a
ET-1 pmol/ml Median (Q_1-Q_3)	16.13 (10.97-21.17)	8.8 (5.89-12.68)	2.48 (0.00-5.60)	<0.001*,a
ADMA umol/L Median (Q_1-Q_3)	0.515 (0.45-0.63)	0.45 (0.41-0.51)	0.38 (0.32-0.43)	<0.001*,a

DU: digital ulcer; VEGF: vascular endothelial growth factor.
[a]Kruskal Wallis; *Statistical significance for a level of 5%.

Biomarkers

Fasting venous blood samples were collected into serum and sodium heparin tubes (Vacuette, Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70 ºC until analysis.

ET-1 assessment (pmol/ml): Plasma was centrifuged immediately in a refrigerated centrifuge and stored at -70ºC until analysis for endothelin. Plasma endothelin was measured using a RIA assay, (Euro-Diagnostics AG, Sweden).

ADMA assessment (umol/L): Serum was allowed to clot at room temperature and then separated from cells within 60 minutes and stored at -70ºC before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent assay, (Immunodiagnostik AG, Germany).

VEGF assessment (pg/ml): Serum VEGF was measured using enzyme-linked immunosorbent assay, (IBL International GMBH, Germany).

Endoglin and Endostatin assessment (ng/ml): Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay, (Uscn, Life Science Inc., Wuhan).

Table II. Logistic regression of vascular biomarkers as baseline.

	Simple				Adjusted			
	B	HR	CI	*p*-value	B	HR	CI	*p*-value
VEGF pg/ml	-0.004	0.996	0.994-0.999	0.007*	-0.006	0.994	0.989-0.998	0.009*
ET1 pmol/ml	0.194	1.214	1.096-1.344	<0.001*	0.153	1.165	1.041-1.304	0.008*
ADMA umol/L	9.533	1.381E4	41.229-4.625E6	0.001	10.378	3.216E4	10.109-1.023E8	0.012*
Endoglin ng/ml	0.197	1.218	1.021-1.452	0.028*	0.098	1.103	0.959-1.270	0.170
Endostatin ng/ml	0.089	1.093	0.909-1.315	0.342	0.205	1.228	0.9251.631	0.156

ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; HR: hazard ratio; CI: confidence interval. *statistical significance for a level of 5%.

Table III. Logistic regression of vascular biomarkers as predictive mediators for new digital ulcers episode in the 3-year follow-up.

	Simple				Adjusted			
	B	HR	CI	*p*-value	B	HR	CI	*p*-value
VEGF pg/ml	0.169	1.184	1.067-1.313	0.001*	0.120	1.128	1.010-1.260	0.033*
ET1 pmol/ml	5.155	173.334	0.91-3.301E4	0.054	4.558	95.436	0.079-1.154E5	0.079
ADMA umol/L	0.004	0.996	0.993-0.999	0.002*	0.005	0.995	0.991-0.998	0.006*
Endoglin ng/ml	0.152	1.164	0.98-1.381	0.083	0.061	1.063	0.929-1.216	0.929
Endostatin ng/ml	0.155	1.168	0.913-1.493	0.216	0.293	1.341	0.972-1.851	0.972

ET-1: endothelin-1; ADMA: asymmetric dimethylarginine; HR: hazard ratio; CI: confidence interval. *statistical significance for a level of 5%.

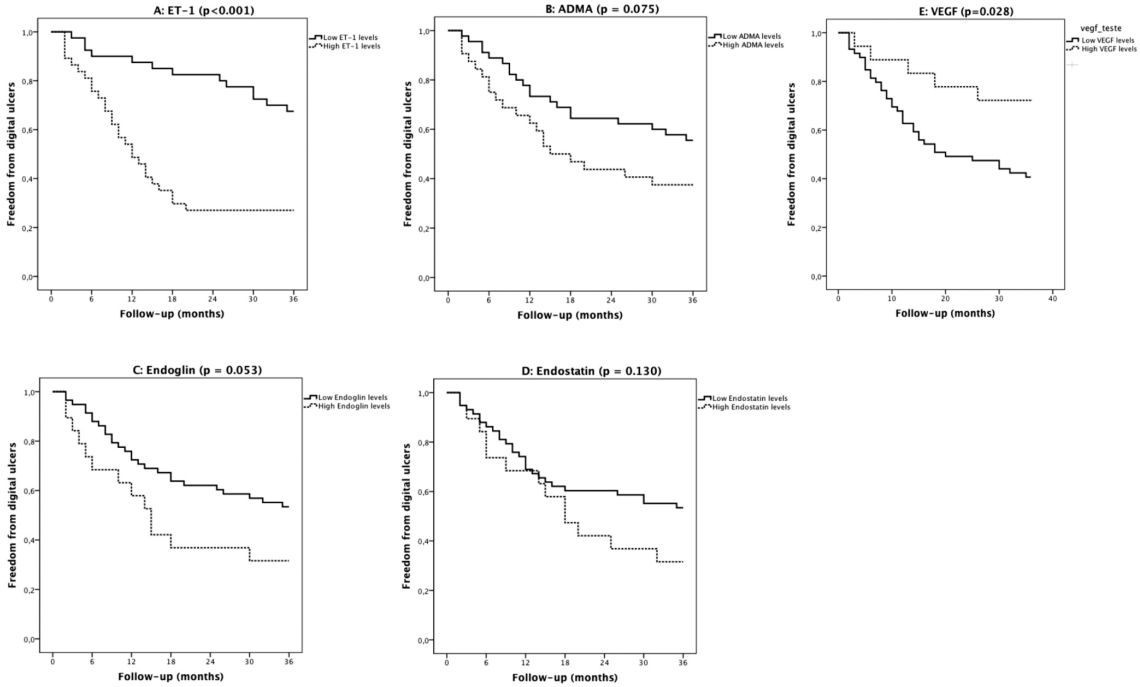

Fig. 1 (A-E). Kaplan-Meier analyses of freedom from new digital ulcers in 36 months follow-up of 77 systemic sclerosis patients. Curves are shown for: **A**: Low ET-1 serum levels (≤11.9 pmol/ml) or high levels (>11.9 pmol/ml); **B**. ADMA low serum levels (≤0.49 umol/l) or high levels (>0.49 umol/l); **C**: low Endoglin serum levels (≤4.215 ng/ml) or high levels (>4.215 ng/ml); **D:** low Endostatin serum levels (≤1.246 ng/ml) or high levels (>1.246 ng/ml); **E:** low VEGF serum levels (≤ 422.47 pg/ml) or high levels (>422.47 pg/ml).
ET-1; endothelin-1; ADMA: asymmetric dimethylarginine; VEGF: vascular endothelial growth factor. Statistical significance for a level of p<0.05.

Statistical analysis

For comparison of normally distributed variables, we used unpaired or paired two-sided student's *t*-test or analysis of variance (Anova). In these cases, data were described by mean ± standard deviation (SD). Normal distribution was tested by Q-Q plots. In non-normally distributed variables, we used non-parametric tests: Mann-Whitney and Kruskal Wallis tests and data were described by median followed by the interquartile interval (Q_1-Q_3), where Q_1 and Q_3 represent the first and the third quartiles, respectively. For comparison of categorical variables, we used Chi-square or Fisher's exact probability test. Predictors of digital ulcers were evaluated by univariate and multivariate logistic regression. We applied survival analysis to determine the probability of freedom from new DU and evaluated the effects of the biomarkers in that probability using the Kaplan-Meier method and the Cox regression. Values of $p \leq 0.05$ were considered as significant. Data were analysed using the SPSS software (v. 22.0, SPSS, Chicago, IL).

Results

Baseline digital ulcers

The demographic and clinical baseline characteristics of the 77 SSc patients are described in supplementary data. (Available online - supplementary data: Table I). All biomarkers had significant differences between groups and controls (Table I).

By univariate logistic regression analysis there were significant differences between DU and non-DU groups regarding disease subset dcSSc ($p<0.001$), increased serum levels of ET-1 (HR 1.214, 95% CI 1.096–1.344, $p<0.001$), ADMA (HR 1.381E4, 95% CI 41.229–4.625E4, $p=0.001$) and endoglin (HR 1.218, 95% CI 1.021–1.452, $p=0.028$) as well as reduced levels of VEGF (HR 0.996, 95% CI 0.994–0.999, $p=0.007$). Multivariate logistic regression analyses confirmed ET-1 (HR 1.165, 95% CI 1.041–1.304, $p=0.008$), ADMA (HR 3.216E4, 95% CI 10.109–1.023E8, $p=0.012$), and VEGF (HR 0.994, 95% CI 0.989–0.998, $p=0.009$) as independent risk factors for active ulcers (Table II).

Main outcome: new digital ulcer episode in 3-year follow-up

In the 3-year follow-up, 40(51.95%) patients presented new ischaemic digital ulcers. By univariate analysis, history of at least one DU before enrolment ($p<0.001$), dcSSc ($p=0.048$), increased ADMA (HR 0.996, 95% CI 0.993-0.999, $p=0.002$) and low serum VEGF levels (HR 1.184, 95% CI 1.067–1.313, $p<0.001$) were identified as risk factors for the occurrence of at least one new DU. Although not significant increased serum ET-1 levels (HR 173.334, 95% CI 0.91–3.301E4, $p=0.054$) were also associated to new DU episodes. Multivariate logistic regression confirmed VEGF (HR 1.128, 95% CI 1.010–1.260,

p=0.033) and ADMA (HR 0.995, 95% CI 0.991–0.998, p=0.006) as independent predictors of new DU (Table III).

We determined serum cut-off levels (Q_2) of the vascular biomarkers to further evaluate their prediction value for new DU in the 3-year follow-up. Kaplan-Meyer analysis of freedom of DU are shown in Figure 1. Patients with serum levels of ET-1 >11.9pmol/ml (p<0.001) and VEGF <422.47 pg/ml (p=0.028) had significantly more DU. Although not significant, a trend towards increased serum levels of Endoglin >4.215ng/ml (p=0.053 was associated to a new DU episode. No predictive value was found for ADMA >0.49umol/l (p=0.075) and Endostatin >1.246ng/ml (p=0.130).

Discussion

The present study demonstrated that increased circulating serum levels of ET-1 and ADMA as biomarkers of endothelium dysfunction as well as low angiogenic mediator VEGF were independent risk factors of active digital ulcers. Analysing the prediction value of these vascular biomarkers only ADMA and VEGF were identified as independent predictors of new DU episodes.

Endothelin-1, apart from being one of the strongest endogenous vasoconstrictor mediators, has also been recognised as a potent mitogen, and there is experimental evidence to suggest that ET-1 contributes to the vascular remodeling and organ damage in different clinical conditions (8).

Endogenous inhibitor of endothelial NO-synthase, ADMA, has emerged as a promising biomarker of endothelial dysfunction in cardiovascular diseases (9). A reduction in NO amplifies vasoconstrictor episodes and promotes pathological changes in vascular system such as thrombotic and inflammatory signalling and vascular remodelling (10). Our results suggest increased serum levels of ADMA as a risk factor for active ulcers as well as predictive biomarker for new DU.

Reduced VEGF levels suggest that ineffective angiogenesis may contribute to the avascular areas largely responsible for the ischemic territory underlying digital ulcers. Other explanation is that a prolonged, uncontrolled and chronic overexpression of VEGF in SSc may have a deleterious effect on the vascular network resulting in a chaotic vascular morphology with reduced blood flow in the newly formed vessels (5, 11). The present data confirm previous reports regarding serum ET-1 (8, 12) and VEGF (4, 13, 14) and their association to DU.

Regarding angiostatic biomarkers, we found an association between active ulcers and increased levels of endoglin, similar to Wipff *et al*. (15) but no predictive value for new DU episode. Conflicting results have been reported regarding endostatin in SSc patients (4, 13, 16). We failed to show any connection or predictive value of endostatin and DU.

Our study has some limitations, small patient sample all recruited from the same centre and limited by number of patients with active DU at time of enrolment. Additional studies, with larger cohorts are needed to validate these predictive factors, enabling further understanding of the progression of vascular damage and angiogenesis in the aetiology of DU.

Conclusion

Our study confirmed the relationship and predictive value of endothelial dysfunction and angiogenic biomarkers with DU.

Overproduction of the potent vasoconstrictor ET-1, increased ADMA concentration down-regulating the production of NO-synthase with consequent abnormal regulation of NO production and impaired angiogenesis due to low VEGF values underlie microvascular disease pathways. Angiostatic factors although increased in patients with DU had no predictor value for new fingertip ulcers.

The analysis of these vascular mediators associated to SSc vasculopathy are particularly interesting as they might help to identify new therapeutic targets in order to prevent further vascular injury.

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Endothelial dysfunction and nailfold videocapillaroscopy pattern as predictors of digital ulcers in systemic sclerosis: a cohort study and review of the literature

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4.6 ENDOTHELIAL DYSFUNCTION AND NAILFOLD VIDEOCAPILLAROSCOPY PATTERN AS PREDICTORS OF DIGITAL ULCERS IN SYSTEMIC SCLEROSIS: A COHORT STUDY AND REVIEW OF THE LITERATURE

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Endothelial Dysfunction and Nailfold Videocapillaroscopy Pattern as Predictors of Digital Ulcers in Systemic Sclerosis: a Cohort Study and Review of the Literature

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Abstract Raynaud's phenomenon and digital ulcers (DUs) are frequent among systemic sclerosis (SSc) patients. Our aim was to investigate the diagnostic and predictive value for DU of endothelial dysfunction biomarkers (flow-mediated dilatation (FMD), serum levels of endothelin-1 (ET-1), and ADMA), angiogenic/angiostatic biomarkers (vascular endothelial growth factor (VEGF), endoglin, and endostatin), and nailfold videocapillaroscopy (NVC). We compared our results with a literature review. In a cohort study of 77 SSc patients, we followed two groups of patients: (i) naïve DU patients (39) and (ii) active DU at baseline (38 patients) for 3 years. Telangiectasia ($p<0.001$) and diffuse disease subset ($p=0.001$) were significantly more frequent in patients with active DU at enrolment. Additionally, NVC late scleroderma pattern (AUC 0.846, 95%CI 0.760–0.932), lower values of FMD (AUC 0.754, 95%CI 0.643–0.864), increased serum levels of ET-1 (AUC 0.758, 95%CI 0.649–0.866), ADMA (AUC 0.634, 95%CI 0.511–0.757), and endoglin as well as low VEGF serum levels (AUC 0.705, 95%CI 0.579–0.830) were significantly associated to new DU events in the 3-year follow-up. Cox regression analysis showed that FMD> 9.41 % (HR 0.37, 95%CI 0.14–0.99); ET-1 >11.85 pmol/L (HR 3.81, 95%CI 1.41–10.26) and late NVC pattern (HR 2.29, 95%CI 0.97–5.38) were independent predictors of DU recurrence. When estimating the probability of occurrence of first DU in naïve DU patients, only late NVC pattern (HR 12.66, 95%CI 2.06–77.89) was an independent predictor factor. In conclusion, late scleroderma patterns in NVC are the best independent predictors of SSc patients who are at risk of developing DU. Endothelial dysfunction assessed by FMD and ET-1 was also found to be an independent predictor of DU recurrence in a 3-year follow-up.

Keywords Digital ulcer · Endothelin-1 · Capillaroscopy · Flow-mediated dilatation · Endothelin-1 · VEGF

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Abbreviations

ADMA	Symmetric dimethylarginine
ANA	Antinuclear antibodies
DU	Digital ulcer
ET-1	Endothelin-1
RP	Raynaud's phenomenon
FMD	Flow-mediated dilatation
VEGF	Vascular endothelial growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NVC	Nailfold videocapillaroscopy
SSc	Systemic sclerosis

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Introduction

Systemic sclerosis (SSc) is a severe autoimmune connective tissue disease that has at its core a triad of abnormalities: vasculopathy of small arterial vessels, elevated production of autoantibodies, and disjunction of fibroblasts with enhanced synthesis of extracellular matrix. The ethology and pathogenesis of SSc are still not completely understood. Important components of the clinical features of SSc are mainly derived from vascular damage [1]. Amongst them are endothelial dysfunction with abnormal vascular reactivity, hypoxia, insufficient neoangiogenesis, and oxidative stress with direct damage of vascular and perivascular cells. Vascular involvement plays a decisive role in early stages of the disease, with the first episodes of Raynaud's phenomenon (RP) [2]. As a result of the progression of their severe vascular disease, up to 30–50 % of SSc patients will suffer from at least one ischemic digital ulcer (DU) [3, 4], and this type of lesion may become recurrent throughout the natural history of a SSc patient.

Vascular insufficiency resulting from progressive obliteration of both small arteries and microvessels in SSc is associated with a state of chronic tissue hypoxia [3]. Recurrent episodes of ischemia-reperfusion injury may contribute to tissue fibrosis, organ dysfunction, and significant morbidity and mortality in SSc patients. Currently, there are no biological measurements (biomarkers) to assess the subclinical vascular activity in patients with SSc, and therefore, the clinician is not able to detect disease until a late and irreversible stage [4].

The pathogenesis of DU is multifactorial and may differ with regard to DU localization. DUs may develop both on fingers or toes and can occur over the extensor surface of the joint, on the finger creases, under the nails, and in most cases, on the fingertips. Fingertip DUs are derived not only from the presence of the underlying vasculopathy but also from the persistent and repeated vasospasm bouts of the Raynaud phenomenon (RP) [5]. In contrast, DUs located on the dorsal side of the fingers are largely due to epidermal thinning and cutaneous retraction that lead to cracks on the skin overlying the joints [6]. DU may also develop from a preexisting calcinosis and sometimes from digital pitting scars [6]. DUs are painful, slow to heal, and are frequently complicated by secondary infections, with disabling effects, particularly regarding grip, feeding, dressing, and hand hygiene[7]. Severe DU episodes may necessitate hospitalization and require time away from the workplace [8].

Although new insights to management of DU have emerged, it is still challenging to identify patients at risk of developing DU episodes. Several reports have identified endothelial dysfunction and angiogenic vascular biomarkers, as well as capillaroscopic data, as potential parameters to predict new DU. However, these tools are expensive and difficult to be applied in routine clinical medicine. Even so, using these potential biomarkers of DU are pertinent to be performed, as the molecular analysis of the vascular mediators associated with SSc and its complications, peripheral microangiopathy in particular, may help in the early identification of patients at risk and in the search for new therapeutic that will prevent the progression of vascular injury in SSc patients.

The aim of the current study was to investigate the role of endothelial dysfunction, angiogenic factors, and microvascular abnormalities detected by nailfold videocapillaroscopy (NVC) as possible parameters to evaluate the risk of SSc patients to develop a first episode of DU or to have a recurrence of DU during a 3-year long follow-up period.

Materials and Methods

A prospective observational cohort study with a 3 years long clinical follow-up was conducted to evaluate 77 SSc patients, attending our Multidisciplinary Raynaud Clinics of the Clinical Immunology Unit at Centro Hospitalar do Porto in Portugal. We excluded from our study all patients with risk factors that could potentially interfere with flow-mediated dilatation (FMD): smokers, diabetics, with hyperlipidemia, and with past history of myocardial infarction, as well as patients on bosentan treatment, due to possible interference with endothelin-1 levels (ET-1). We had no patients lost during the entire course of our follow-up period.

All 77 included patients had SSc based on 2013 classification criteria for SSc of American College of Rheumatology [9]. Onset of the disease was defined as the time of first RP episode. All patients were on vasodilators calcium channel blockers (nifedipine) or angiotensin II receptor antagonist (losartan), and a washout of the vasodilator drugs was done before inclusion in the study. Thirty-four healthy, sex/age-matched, nonobese, without self-reported cardiovascular risk factors controls were invited to participate. No control subject was on any vasoactive medication.

SSc patients were divided into two groups: DU group that included 38 patients having an active ulcer at the beginning of our follow-up study, with or without a past history of DU (34 women; mean age 52.7±14.8 years; range 14–75), and a group with no history of DU that included 39 patients with no history of DU until enrolment (38 women; mean age 53.2± 10.3 years; range 30–79).

The institutional ethical review board of Centro Hospitalar do Porto approved this study. All subjects signed informed consent before inclusion in the study. Data were collected by analysis of clinical file data and by clinical interview.

Definition of DU

Ischemic digital fingertip ulceration was defined as a painful area of 2 mm or greater in diameter, with visible depth and loss of dermis, amenable to healing and localized at a fingertip.

DUs caused by conditions other than SSc were not considered.

Methods

Allen Test

Allen test was performed as follows: (1) instruct patient to clench his/her fist, (2) apply occlusive pressure to both ulnar and radial arteries by finger pressure, (3) confirm palm and finger blanching with the patient's hand relaxed, (4) release the occlusive pressure on ulnar artery, and (5) positive test—if the hand flushes within 5–15 s, this indicates that the ulnar artery has good blood flow and palmar arch is complete—and negative test—if the hand does not flush within 5–15 s, this indicates that ulnar circulation is inadequate with an incomplete palmar arch.

FMD

Ultrasounds scans were performed using a two-dimensional ultrasonography General Electric Logic 7 with a 9-MHz Linear wideband multihertz imaging probe. Ultrasound images were recorded and analyzed for three consecutive end diastolic frames (onset of R wave) at 45 to 60 s after cuff deflation. The interoperator variability was 3.6 %.

Flow-mediated dilatation of the brachial artery in the lower arm was evaluated following International Brachial Artery Reactivity Task Force Guidelines [10] for the ultrasound assessment of brachial artery endothelial-dependent flow-mediated vasodilatation. Patients and controls (healthy subjects) were on overnight fasting for 12 h before the ultrasound study was performed. The exams were performed in the morning, with patients being kept in a quiet temperature-controlled room (22–24 °C) for a preliminary 20-min rest. Vasoactive drugs were withheld for ten half-lives. It was assured that patients did not exercise or ingest substances that could affect the response to ischemia like caffeine, vitamin C, tobacco, or high-fat foods for 24 h.

FMD was calculated as the percentage of change of the peak diameter in response to reactive hyperemia in *(FMD%= (peak diameter− baseline diameter/baseline diameter)× 100)* [10].

NVC

Nailfold Videocapillaroscopy was performed with KK technology Videocapillaroscopy with a ×200 magnification lens. In the end, all images were scored for each patient at the beginning of the follow-up study and 3 years later and were controlled by a capillaroscopy expert. The interoperator variability was 2.5 %.

Capillaroscopic findings were described following qualitative classification of scleroderma microangiopathy damaged described by Cutolo [11] in three patterns early, active, and late.

Autoantibody Detection

Antinuclear antibodies (ANA) were accessed by indirect immunofluorescence on Hep-2 cells (NOVAlite ANA, Inova Diagnostics, Inc., San Diego, CA, USA). Samples with a titer greater than or equal to 1:80 were considered positive. Autoantibodies anti-Scl-70, anti-centromere (ACA), anti-Ro52, anti-PM-Scl, anti-RNA-polymerase, anti-fibrillarin (AFA), and anti-NOR 90 were detected by immunoblotting using a Euroline Myositis Profile antibody test syst (Euroimmun, Lübeck, Germany). Quantification of anti-U1RNP and antibodies was carried out using a Fluoro Enzyme Immuno Assay (EliA™ U1RNP70; Phadia, Uppsala, Sweden).

Vascular Biomarkers

Venous blood samples from fasting individuals were collected into a serum tube and another tube containing sodium heparin (Vacuette, Greiner-Bio-One, Austria). Serum was allowed to clot at room temperature and then separated from cells within 60 min, and stored at −70 °C until analysis for asymmetric dimethylarginine (ADMA), endoglin, endostatin, and vascular endothelial growth factor (VEGF-A).

ET-1 assessment: Plasma was centrifuged immediately in a refrigerated centrifuge and stored at −70 °C until analysis for endothelin. Plasma endothelin was measured using a RIA assay (Euro-Diagnostics AG, Sweden). The resulting values are reported as picomole per milliliter.

ADMA assessment: Serum was allowed to clot at room temperature and then separated from cells within 60 min and stored at −70 °C before analysis for ADMA. Serum ADMA was measured using enzyme-linked immunosorbent assay (Immunodiagnostik AG, Germany). The resulting values are reported as micromole per liter.

VEGF assessment: Serum VEGF-A was measured using enzyme-linked immunosorbent assay (IBL International GMBH, Germany). The resulting values were reported as picogram per milliliter.

Endoglin and endostatin assessment: Serum endoglin and endostatin were measured using enzyme-linked immunosorbent assay (Uscn, Life Science Inc., Wuhan). The resulting values were reported as nanogram per milliliter.

Follow-up

When included in the study cohort, patients were followed for 3 years. Patients were instructed to come to the hospital clinics whenever a new digital tropic lesion occurred. If no DU developed, patients were seen on a regular basis at 3–6-month intervals, as indicated by disease severity.

Major Clinical Outcomes

Our primary outcome was the occurrence of at least one new ischemic fingertip DU in the 3-year clinical follow-up period. In addition, we applied survival analysis to investigate freedom from DU recurrence in the study period, regarding (i) endothelium dysfunction (FMD, ET-1 and ADMA), (ii) microvascular abnormalities in qualitative scleroderma pattern NVC, and (iii) angiogenic VEGF/angiostatic (endostatin, endoglin) factors.

Statistical Analysis

For comparison of normally distributed scale variables, we used unpaired two-sided Student's t test or analysis of variance (ANOVA). In these cases, data were described by mean± standard deviation (SD) followed by the minimal and the maximal values (range). Normal distribution was tested by Q–Q plots. In the cases of nonnormally distributed variables, we used nonparametric tests: Mann–Whitney and Kruskal–Wallis tests and data were described by median followed by the interquartile interval (Q_1–Q_3), where Q_1 represents the first quartile (corresponding to 25 % of data) and Q_3 represents the third quartile (corresponding to 75 % of data). In ANOVA test, when the homogeneity of variance was not satisfied, we used the Welch test. For comparison of categorical variables, we used chi-square or Fisher's exact probability test. We applied survival analysis to determine the probability of freedom from new DU during the study period and evaluated the effects of FMD, ET-1, ADMA, VEGF, endostatin, endoglin, and NVC scleroderma patterns in that probability using the Kaplan–Meier method and the Cox regression. To evaluate the predictive effect of these variables, we considered them as categorical variables, and associations were assessed according to the median value of each variable. The association between initial characteristics and the DU reoccurrence or new DU event was first analyzed in univariate Cox regression followed by multivariate Cox regression including all variables with $p<0.2$ in univariate analysis. A receiver operating characteristic (ROC) curve analysis was performed to obtain the predictive accuracy of FMD, MES score, ET-1, ADMA, VEGF, endostatin, and endoglin. We considered p values <0.05 as significant. Data were analyzed using the SPSS software (v.22.0, SPSS, Chicago, IL).

Results

Study Population

The mean age of the 77 SSc patients (72 women, 93.5 %) was 52.95±12.65 years, and the median disease duration was 10.00 years (6.00–20.5) (Table 1). At enrolment, two subpopulations were distinguished among these 77 SSc patients: (i) with active DU and (ii) with no history of DU. Active DU was present in 38 patients, with or without a past history of DU (34 women; mean age 52.7±14.8 years; range 14–75), and 39 of the SSc patients had no history of DU (38 women; mean age 53.2±10.3 years; range 30–79). The median time for the first DU occurrence was 4.00 years (2.02–11.75) since disease onset.

Table 1 Baseline characteristics of study population

Characteristics	Patients (n=77)
Age (years), mean±SD	52.95±12.65
Gender, women, *n* (%)	72 (93.5)
Disease duration (years), median (Q_1–Q_3)	10.00 (6.00–20.5)
Current and/or past digital ulcers, *n* (%)	38 (49.4)
Median time for first DU occurrence (years), median (Q_1–Q_3)	4.00 (2.02–11.75)
Limited/diffuse cutaneous subset, *n* (%)	64 (83.1)/13 (16.9)
Telangiectasia, *n* (%)	65 (84.4)
Sclerodactily, *n* (%)	26 (33.8)
Puffy hands, *n* (%)	57 (74.0)
Digital Pitting scars, *n* (%)	51 (66.2)
Digital amputation, *n* (%)	10 (13.0)
Calcinosis, *n* (%)	21 (27.3)
Hand/arm contractures, *n* (%)	22 (28.6)
Positive Allen Test, *n* (%)	34 (44.2)
Positive anticentromere antibodies, *n* (%)	49 (63.6)
Positive anti-scleroderma 70, *n* (%)	18 (23.4)
FMD %, median (Q_1–Q_3)	9.41 (5.66–14.78)
ET-1 (pmol/L), median (Q_1–Q_3)	11.85 (7.42–17.24)
ADMA (μmol/L), median (Q_1–Q_3)	0.49 (0.41–0.54)
Endoglin (ng/ml), median (Q_1–Q_3)	2.17 (1.28–4.22)
Endostatin (ng/ml), median (Q_1–Q_3)	0.51 (0.19–1.25)
VEGF (pg/ml), median (Q_1–Q_3)	299.13 (199.49–422.47)
NVC pattern	
Early pattern, *n* (%)	13 (16.9)
Active pattern, *n* (%)	33 (42.9)
Late pattern, *n* (%)	31 (40.3)
MES score, median (Q_1–Q_3)	2.00 (1.00–6.00)

FMD flow-mediated dilatation, *ET-1* endothelin1, *ADMA* asymmetric dimethylarginine, *VEGF* vascular endothelial growth factor, *NVC* nailfold videocapillaroscopy, *MES* microangiopathy evolution score, *SD* standard deviation, Q_1 first quartile, Q_3 third quartile

Clinical features such as telangiectasia ($p<0.001$), sclerodactily ($p<0.001$), calcinosis ($p<0.001$), digital pitting scars ($p=0.001$), and positive Allen test ($p<0.001$) were significantly more frequent in patients in the subpopulation of SSc patients with active DU. Additionally, diffuse disease subset ($p=0.001$), NVC scleroderma pattern ($p<0.001$), lower values of FMD ($p<0.001$), increased serum levels of ET-1 ($p<0.001$), ADMA ($p=0.049$), and endoglin($p=0.017$) as well as low VEGF serum levels ($p=0.009$) were significantly associated to active DU. No significant differences were found concerning age, gender, disease duration ($p=0.0602$) or in angiostatic biomarker endostatin ($p=0.129$) (Table 2).

ROC analysis was performed in order to define the serum levels that were the best predictors for new DU events (Table 3). Positive diagnostic value of the different parameters studied as potential risk factors for DU events reflected by an area under the curve: ET-1 (AUC 0.758, 95%CI 0.649–0.866), FMD (AUC 0.754, 95%CI 0.643–0.864), VEGF (AUC 0.705, 95%CI 0.579–0.830), ADMA (AUC 0.634, 95%CI 0.511–0.757), endoglin (AUC 0.606, 95%CI 0.480–0.733) and endostatin (AUC 0.591, 95%CI 0.463–0.720), and for NVC scleroderma pattern (AUC 0.846, 95%CI 0.760–0.932).

Primary Outcome: New Ischemic Digital Ulcer Episode in 3-Year Follow-up

During the 3-year clinical follow-up of this study, 40 of the 77 SSc patients (51.9 %) developed at least one new ischemic fingertip. Most of these 40 patients (30, i.e., 75 %) belonged to the subpopulation with active DU at enrolment. The median time of occurrence of these new DU was 4.5 months (1.25–16.25), which is significantly shorter than the data available at the Eustar registry [12] (Table 4). Also, patients with active DU at enrolment had a more severe progression of their capillaroscopic patterns ($p<0.001$) when initial and final (3 years) results were compared (Supplementary Table 1).

Reccurence of DU

Of the 38 SSc patients with active DU at beginning of the study, 30 of them (79 %) had DU recurrence during the 3-year course of this investigation (Table 4). By the use of univariate Cox regression analysis, we were able to identify the following predictor factors of DU recurrence: FMD>9.41 % (HR 0.37, 95%CI 0.14–0.99), ET-1>11.85 pmol/L (HR 3.81, 95%CI 1.41–10.26), and late NVC pattern (HR 2.29, 95%CI 0.97–5.38) (Supplementary Table 2). Kaplan–Meyer analyses of freedom from the DU recurrence showed that the median values of FMD ($p=0.035$), ET-1 ($p=0.004$), and qualitative scleroderma NVC ($p=0.047$) were significantly associated with the new episodes of DU (Fig. 1a–c). Multivariate Cox regression confirmed ET-1 (HR 4.23, 95%CI 1.51–11.83) and late NVC pattern (HR 2.49, 95%CI 1.03–6.04) as independent predictors of DU recurrence (Supplementary Table 3).

First DU Event in SSc Patients with No History of DU

We have also searched for predictive factors for the first DU event among the patients with no history of DU at enrollment in this study (Table 4). From univariate analysis, we are able to conclude that only late NVC pattern (HR 12.66, 95%CI 2.06–77.89) is an independent predictor for a first DU event. Kaplan–Meyer analyses of freedom from the occurrence a DU event at any time of disease course strengthen the results of Cox analysis with NVC qualitative late scleroderma pattern ($p<0.001$) as a significant factor (Fig. 1d). Multivariate Cox regression confirmed late NVC (HR 13.38, 95%CI 2.10–85.38) as an independent predictor of the first DU (Supplementary Table 4).

SSc Patients with No DU at Enrolment and in the 3-Year Follow-up

Median SSc duration in patients with no previous history of DU was 10.00 years (5.25–22.5) with no significant differences in comparison with disease duration in the subpopulation of SSc patients with DU. These naïve DU SSc patients were largely lcSSc (96.6 %; $p=0.025$) and anti-scleroderma 70 negative ($p=0.036$). They also presented less peripheral vascular impairment, as evidenced by encompassing a smaller number of patient with telangiectasia ($p=0.007$) and the predominance of negative Allen test ($p< 0.001$); this was strengthened by a good response to shear stress FMD% ($p< 0.001$). They exhibited lower serum levels of vasoconstrictive ET-1 ($p<0.001$), and increased ADMA ($p=0.049$) and VEGF ($p=0.003$) serum levels. Late scleroderma pattern was only present in one of the patients (3.4 %) (Table 5).

Discussion

We report here on the clinical and laboratory data regarding a large group of patients with diagnosis of SSc patients, all with Raynaud's phenomenon, with the goal of identifying predictor factors for the development of DU. This study is based in 3-year long follow-up of 77 SSc patients that were divided into two subpopulations (with or without previous history of DU).

As far as we know, this is the first study that demonstrates that both endothelial dysfunction and impaired angiogenesis are risk factors for DU and have a predictive role for new DU events. We were also able to conclude that late scleroderma pattern is the strongest independent predictive risk factor for the occurrence of DU in SSc patients. Clearly, our findings suggest that endothelial dysfunction underlies recurrence of digital ulcers, which might explain why endothelin receptor

Table 2 Comparison between DU and DU naïve groups at baseline

Variables	DU (n=38)	DU naïve (n=39)	p value
Age (years), mean±SD (min–max)	52.7±14.8 (14–75)	53.2±10.3 (30–79)	0.137[a]
Gender, women, n (%)	34 (89.5)	38 (97.4)	0.067[b]
Disease duration (years) median (Q_1–Q_3)	10 (5–23)	10 (7–20)	0.028[c,*]
Mean arterial pressure (mmHg) mean±SD (min–max)	87.9±6.04 (79–102)	88.3±6.4 (77–103)	0.75[a]
Total cholesterol (mg/dl), mean±SD (min–max)	191.3±9.0 (169–216)	187.1±12.1 (156–202)	0.242[a]
Disease subset			0.001[a,*]
Limited, n (%)	26 (68.4)	38 (97.4)	
Diffuse, n (%)	12 (31.6)	1 (2.6)	
Disease duration, median (Q_1–Q_3)	10.00 (5.00–23.00	10.00 (7.00–20.00)	0.602[c]
Onset of 1st ulcer (years), median (Q_1–Q_3)	5 (3–13.25)	NA	NA
Puffy hands, presence, n (%)	23 (60.5)	34 (87.2)	0.008*[,a]
Sclerodactily, presence, n (%)	21 (55.3)	5 (12.8)	<0.001[a,*]
Telangiectasia's, presence, n (%)	38 (100)	27 (69.2)	<0.001[a,*]
Digital pitting scars, presence, n (%)	32 (84.2)	19 (48.7)	0.001[a,*]
Digital amputation, presence, n (%)	10 (26.3)	0 (0)	0.001[b,*]
Calcinosis, presence, n (%)	18 (47.4)	3 (7.7)	<0.001[a,*]
Hand/arm contractures, presence, n (%)	19 (50)	3 (7.7)	<0.001[a,*]
Allen Test, positive, n (%)	27 (71.1)	7 (17.9)	<0.001[a,*]
Autoantibodies			
ACA, positive, n (%)	22 (57.9)	27 (69.2)	0.301[a]
Anti-scleroderma-70, positive, n (%)	12 (31.6)	6 (15, 4)	0.093[a]
FMD (%), median (Q_1–Q_3)	5.3 (2.88–7.80)	16.21 (12.55–19.88)	<0.001[c,*]
ET-1 (pmol/ml), median (Q_1–Q_3)	16.13 (10.97–21.17)	8.8 (5.89–12.68)	<0.001[c,*]
ADMA (umol/L), median (Q_1–Q_3)	0.52 (0.45–0.63)	0.45 (0.41–0.51)	0.001[c,*]
Endoglin (ng/ml), median (Q_1–Q_3)	3.013 (1.463–7.023)	1.879 (0.84–3.28)	0.017[c,*]
Endostatin (ng/ml), median (Q_1–Q_3)	0.695 (0.26–1.73)	0.429 (0.16–0.8)	0.129[c]
VEGF, median (Q_1–Q_3)	245.06 (158.68–347.33)	339.49 (202.00–730.93)	0.009[c,*]
NVC pattern			
Early, n (%)	0 (0)	13 (33.3)	<0.001[a,*]
Active, n (%)	11 (28.9)	22 (56.4)	
Late, n (%)	27 (71.1)	4 (10.3)	
MES score, median (Q_1–Q_3)	5.00 (2.75–6.00)	1.00 (1.00–2.00)	<0.001[c,*]

SSc systemic sclerosis, *DU* digital ulcer, *dcSSc* diffuse systemic sclerosis subset, *lcSSc* limited systemic sclerosis subset, *ACA* autoantibody anti-centromere, *NVC* nailfold Videocapillaroscopy, *MES* microangiopathy evolution score, *FMD* flow-mediated dilatation, *ET-1* endothelin-1, *ADMA* asymmetric dimethylarginine, *NA* non applicable, *SD* standard deviation, *Q* quartile

*Statistical significance for a level of 5 %

[a] Chi-Square test

[b] Fisher's Exact test

[c] Mann–Whitney test

[d] Student's t test

antagonist treatment (bosentan) reduces the recurrence of DU [13]. Another new and useful information coming out of this investigation is that the capillaroscopic findings make up the only independent predictor factor for a first DU episode in SSc patients.

Association between previous history of DU and its recurrence is a commonly accepted concept [14], but this correlation has not been scientifically demonstrated. Manfredi et al. [15] have recently reported that DUs in the previous year were associated with new DU in the next 6 months. Our data do confirm previous DU as strong predictors for new DU episodes.

NVC is routinely used as a clinical marker in early and even preclinical stages of SSc [16] since it captures impaired capillary architecture and capillary loss that is a result of endothelial cell injury with disorganization of the inner layer of

Table 3 AUC–ROC analysis of investigated variables

Variable	AUC (95%CI)	*p* value	Cutoff	Sensibility	Specificity
FMD	0.754 (0.6430.864)	<0.001	≤13.12	81.3 %	55.2 %
ET-1 (pmol/L)	0.758 (0.6490.866)	<0.001	≥7.49	87.5 %	51.7 %
ADMA (μmol/L)	0.634 (0.5110.757)	0.049	≥0.465	66.7 %	55.2 %
VEGF (pg/ml)	0.705 (0.5790.830)	0.003	≤408.33	85.4 %	56,2 %
Endostatin (ng/ml)	0.591 (0.4630.720)	0.182	NA	NA	NA
Endoglin (ng/ml)	0.606 (0.4800.733)	0.120	NA	NA	NA
NVC pattern	0.846 (0.760–0.932)	<0.001	NA	NA	NA

AUC area under the curve, *CI* confidence interval, *FMD* flow-mediated dilatation, *ET-1* endothelin-1, *ADMA* asymmetric dimethylarginine, *NA* non applicable

arterial vessels. As a very accessible tool, NVC could be a putative biomarker of disease progression, allowing target preventive therapy to be initialized [17].

Structural changes such as giant capillaries, microhemorrhages, avascular areas, and neo-angiogenic capillaries are important markers of disease progression and are present in more than 90 % of SSc patients [18]. Scoring the capillaroscopic patterns changes during the follow-up of SSc patients [19] enables capillaroscopy as a mandatory tool to quantify microangiopathy.

In clinical setting, qualitative evaluation is the more accessible and easy to perform. Late NVC patterns have been mostly described as an increased risk for DU development [17]. Caramaschi et al. reports a strong correlation between patients with late pattern and DU [20], Smith et al. described a correlation between worsening of NVC patterns and peripheral vascular lesions [21], Lambova et al. reported that DUs were strongly associated with active pattern, giant capillaries, hemorrhages, and avascular areas, proposing that active pattern could be used as a predictor for trophic lesions in the future and an indication for preventive target therapy [22], Alivernini et al. reported that patients with active skin ulcers have more avascular areas and a lower capillary density [23], and Ghizzoni et al. [2] described a correlation of DU with progression of NVC features.

Several scores have been proposed with prognostic purposes. Sulli et al. [24] proposed a semiquantitative microangiopathy evolution score (MES) that paralleled the evolution of the SSc microangiopathy but with no reference to association to DU, but it is an interesting microvascular injury surveillance score [17]. Smith et al. has proposed a simple semiquantitative NVC evaluation for day-to-day clinical use as a prognostic index of digital trophic lesions. Limitation of this study is that it includes and compares severe digital pitting scars, digital tip ulcerations, and gangrene without no differentiation between presentation and outcomes [25]. Sebastini et al. [26] proposed a Capillaroscopic Skin Ulcer Risk Index (CSURI) as a predictor risk of new onset of DU using NVC parameters. The strong limitation of this index was the mandatory presence of megacapillaries, so late patterns were excluded [26].

Manfredi et al. [15] recently suggested predictive risk chart of DU, taking into account capillaroscopic, demographic, and clinical–serological parameters. A significant positive association between DU and male gender, past DU, CSURI with a cutoff of 2.96, and ERS rate ≥20 mm h^{-1}[15] was found.

Kim et al. described a quantitative analysis of NVC in six digits. Number of capillaries, number of capillaries loss, apical limb width, capillary width, and endothelin-1 (ET-1) were analyzed [27]. The major limitation of this study was that dilated capillaries were mandatory. The authors concluded that capillary dimension and loss of capillaries were strongly associated with DU. Recently, Ennis et al. [28] reported that intercapillary distance was greater in patients with active ulcers and capillary density was lower although not significant and no differences between patients with or without DU regarding capillary width, tortuosity, or derangement [28].

CAP multicenter observational, longitudinal prospective study of SSc patients with or without history of DU and with a 6 months follow-up reached the conclusion that final model for prediction of new DU in SSc patients with a past history of DU was a reduced mean number of capillaries in the middle finger of the dominant hand, increased number of current DU, and presence of current critical digital ischemia [29].

Our cohort investigation confirms results from previous studies that suggest a direct correlation between DU and NVC patterns [21, 25, 26, 30–32]. In addition to strengthening previous data on NVC and DU in SSc, we conclude that NVC is a useful method to predict which of the SSc patients are more likely to develop DU.

Endothelial dysfunction and free-radical damage are primary events throughout the course of the SSc disease, which result in vascular obliteration and diminished blood flow to the organs involved [33] and are prominently features of RP and DU. Among serological biomarkers of vasculopathy are vasoconstrictor ET-1 [34], cell adhesion molecules, including selectin, anti-endothelial antibodies, and the controversial

Table 4 Comparison of evolution of 77 patients enrolled in the study

Baseline	DU			Naïve Du			*p* value
Variables	Follow-up 3 years		*p* value	Follow-up 3 years		*p* value	
	DU (*n*=30)	Non-DU (*n*=8)		DU (*n*=10)	Non-DU (*n*=29)		
Age (years), mean±SD	50.67±15.64	60.13±7.94	0.027[d,*]	53.10±11.04	53.28±10.27	0.964[d]	0.141[a]
Gender, women, *n* (%)	26 (86.7)	8 (100)	0.560[b]	10 (100)	28 (96.6)	1.000[b]	0.368[b]
Disease duration (years), median (Q_1–Q_3)	7.00 (4.00–20.75)	20.50 (13.00–24.50)	0.050[c,*]	15.00 (5.25–20.75)	10.00 (7.00–20.00)	0.974[e]	0.160[c]
Disease subset			1.000[b]			1.000[b]	0.005[b,*]
Limited, *n* (%)	20 (66.7)	6 (75.0)		10 (100)	28 (96.6)		
Diffuse, *n* (%)	10 (33.3)	2 (25.0)		0 (0)	1 (3.4)		
Sclerodactily, Presence, *n* (%)	17 (56.7)	4 (50.0)	1.000[b]	0 (0)	5 (17.2)	0.302[b]	<0.001[b,*]
Puffy hands, Presence, *n* (%)	18 (60.0 %)	5 (62.5)	1.000[b]	10 (100)	24 (82.8)	0.302[b]	0.030[b,*]
Telangiectasia, Presence, *n* (%)	30 (100)	8 (100)	NA	7 (70.0)	20 (69.0)	1.000[b]	0.001[b,*]
Digital Pitting scars, presence, *n* (%)	24 (80.0)	8 (100.0)	0.309[b]	4 (40.0)	15 (51.7)	0.716[b]	0.005[b,*]
Digital amputation, presence, *n* (%)	8 (26.7)	2 (25.0)	1.000[b]	0 (0)	0 (0)	NA	0.004[b,*]
Allen Test, positive, *n* (%)	23 (76.7)	4 (50.0)	0.195[b]	3 (30.0)	4 (13.8)	0.344[b]	<0.001[b,*]
Autoantibodies							
ACA, positive, *n* (%)	17 (56.7)	5 (62.5)	1.000[b]	10 (100)	17 (58.6)	0.017[b,*]	0.051[b]
Anti-scleroderma-70, positive, *n* (%)	11 (36.7)	1 (12.5)	0.393[b]	3 (30.0)	3 (10.3)	0.163[b]	0.080[b]
FMD, mean±SD/median (Q_1–Q_3)	3.96±6.06	10.55±10.26	0.025[d,*]	11.49 (9.41–19.86)	13.52 (8.36–21.83)	0.700 [e]	<0.001[c,*]
ET-1 (pmol/L), median (Q_1–Q_3)	17.24 (12.89–23.72)	7.62 (6.31–14.39)	0.002[c,*]	10.34 (7.14–12.95)	8.06 (5.78–12.74)	0.421[e]	<0.001[c,*]
ADMA (μmol/L), median (Q_1–Q_3)	0.51 (0.44–0.63)	0.56 (0.48–0.62)	0.654[e]	0.41 (0.35–0.51)	0.45 (0.41–0.50)	0.233[e]	0.006[c,*]
VEGF (pg/ml), median (Q_1–Q_3)	267,84 (134,57–357,30)	240.19 (186.36–333.76)	0–971[e]	286.66 (154.93–431.91)	415.23 (207.53–863.17)	0.115[e]	0–027[c,*]
Endoglin (ng/ml), median (Q_1–Q_3)	3,10 (1.77–7.02)	0.47 (1.10–8.70)	0.474[e]	1.40 (0.77–3.86)	2.14 (0.96–2.97)	0.596[e]	0.093[c]
Endostatin (ng/ml), median (Q_1–Q_3)	0.70 (0.28–1.81)	0.46 (0.15–1.48)	0.390[e]	0.54 (0.03–1.69)	0.43 (0.17–0.74)	0.822[e]	0.374[c]
NVC pattern baseline			0.195[b]			0.060[b]	<0.001[b,*]
Early, *n* (%)	0 (0)	0 (0)		2 (20)	11 (37.9)		
Active, *n* (%)	7 (23.3)	4 (50.0)		5 (50.0)	17 (58.6)		
Late, *n* (%)	23 (76.7)	4 (50.0)		3 (30.0)	1 (3.4)		

DU digital ulcer, *dcSSc* diffuse systemic sclerosis, *lcSSc* limited systemic sclerosis, *RP* Raynaud phenomenon, *ACA* anti-centromere autoantibody, *NVC* nailfold Videocapillaroscopy, *MES* microangiopathy evolution score, *FMD* flow-mediated dilatation, *ET-1* endothelin-1, *ADMA* asymmetric dimethylarginine, *VEGF* vascular endothelial growth factor, *SD* standard deviation, Q_1 first quartile, Q_3 third quartile, *NA* no applicable

[a] ANOVA test

[b] Fisher's Exact test

[c] Kruskal-Wallis test

[d] Student's *t* test

[e] Mann–Whitney test

*Statistical significance for a level of 5 %

vasodilator nitric oxide (NO), as well as ADMA, inhibitor of endothelial NO synthase (eNOS) [34].

ET-1 is known to play a key role in vascular pathology by various deleterious effects including hypertrophy of the vascular smooth muscle cells, cellular proliferation and fibrosis, increased vascular permeability, activation of leucocytes, and induction of cytokine and adhesion molecule expression [35]. Elevated ET-1 serum levels have been reported in

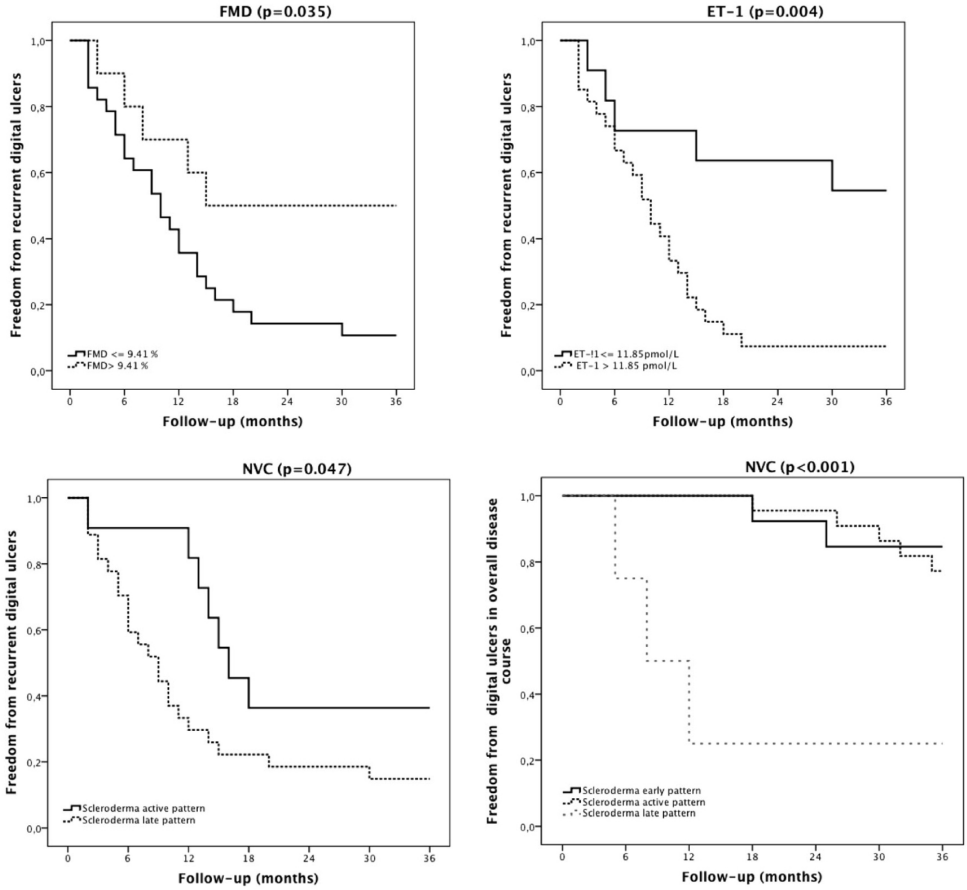

Fig. 1 **a-d** Kaplan–Meier analyses of freedom from new digital ulcers in 36 months follow-up of 77 SSc patients. Recurrence DU: curves A–C are shown for **a** patients who had FMD levels ≤9.41 % or >9.41 % (p= 0.035), **b** patients who had ET-1 serum levels ≤11.85 pmol/ml or >than 11.85 pmol/ml (p=0.004), and **c** patients who had active/late scleroderma pattern (p=0.047). Naïve DU patients: curve **d** shows freedom of digital ulcers at any time of the disease course/patients who had early/active/late pattern (p<0.001). *FMD* flow-mediated dilatation, *ET-1* endothelin-1, *NVC* nailfold videocapillaroscopy

primary RP, secondary RP, and SSc patients [36, 37], as well in the "late" scleroderma variant. ET-1 serum levels are correlated with a more severe clinical involvement, namely the presence of DU, either active or in the past [27, 38, 39]. Nevertheless, it must be referred that other studies found no correlation between ET-1 and DU [37, 40]. Sulli et al. [38] described significantly lower plasma levels of ET-1 in patients with "early" pattern compared to patients with late NVC pattern, thus supporting the involvement of ET-1 in the progression of microvascular/fibrotic damage in SSc. In our cohort of SSc patients with DU, we found increased serum levels of ET-1, compared with values for SSc patients with no previous episode of DU. In addition, we found that ET-1 levels less than 11.85 pmol/ml were strong predictors for a new DU episode in our 3-year long follow-up study. This finding supports the use of an endothelin receptor antagonist (bosentan) for preventive therapy of DU recurrence.

Elevated ADMA concentrations in patients with secondary Raynaud [41] and in diffuse cutaneous disease [42] are considered to be a reflection of the endothelial insult through either reduced and enhanced NO production. Besides the inhibition of endothelial NOS, deranged NO regulation due to overexpression of iNOS occurs in patients with SSc [43] and may contributes to accumulation of ADMA by eliminating dimethylarginine dimethylamonohydrolase (DDAH) activity [41]. In our study, SSc patients with active DU had increased ADMA serum levels compared to values from SSc patients with no previous DU; however, we could not demonstrate a predictive value for ADMA in DU.

Table 5 Comparison between DU and naïve DU SSc patients in overall disease course

Characteristics	Naïve Du (n=29)	Patients with DU (n=48)	p value
Age (years), mean±SD	53.28±10.27	52.75±13.99	0.861[c]
Gender, women, n(%)	28 (96.6)	44 (91.7)	0.645[b]
Disease duration (years), median (Q_1–Q_3)	10.00 (7.00–20.00)	10.00 (5.25–22.5)	0.617[d]
Disease subset			0.025[b,*]
Limitada, n(%)	28 (96.6)	36 (75.0)	
Difusa, n(%)	1 (3.4)	12 (25.0)	
Sclerodactily, presence, n(%)	5 (17.2)	21 (43.8)	0.017[a,*]
Puffy hands, presence , n(%)	24 (82.8)	33 (68.8 %)	0.174[a]
Telangiectasias, presence, n(%)	20 (69.0)	45 (93.8)	0.007[b,*]
Calcinosis, presence , n(%)	2 (6.9)	19 (39.6)	0.002[a,*]
Hand/arm contractures, presence , n(%)	2 (6.9)	20 (41.7)	0.001[a,*]
Allen Test, positive, n(%)	4 (13.8)	30 (62.5)	<0.001[a,*]
Autoantibodies			
ACA, positive, n (%)	17 (58.6)	32 (66.7)	0.477[a]
Anti-scleroderma-70, positive, n(%)	3 (10.3)	15 (31.2)	0.036[a,*]
FMD %, median (Q_1–Q_3)	13.52 (8.36–21.83)	7.01 (2.55–10.95)	<0.001[d,*]
ET-1 (pmol/L), median (Q_1–Q_3)	8.06 (5.78–12.74)	14.01 (9.04–19.61)	<0.001[d,*]
ADMA (μmol/L), median (Q_1–Q_3)	0.45 (0.41–0.50)	0.51 (0.42–0.59)	0.049[d,*]
Endoglin (ng/ml), median (Q_1–Q_3)	2.14 (0.95–2.97)	2.57 (1.39–4.73)	0.120[d]
Endostatin (ng/ml), median (Q_1–Q_3)	0.43 (0.17–0.74)	0.70 (0.23–1.70)	0.182[d]
VEGF (pg/ml), median (Q_1–Q_3)	415.23 (207.5–863.16)	257.16 (162.6–352.98)	0.003[d,*]
NVC Pattern baseline			<0.001[a,*]
Early, n (%)	11 (37.9)	2 (4.2)	
Active, n (%)	17 (58.6)	16 (33.3)	
Late, n (%)	1 (3.4)	30 (62.5)	

FMD flow-mediated dilatation, *ET-1* endothelin-1, *ADMA* asymmetric dimethylarginin, *VEGF* vascular endothelial growth factor, *NVC* nailfold Videocapillaroscopy, *MES* microangiopathy evolution score, *SD* standard deviation, Q_1 first quartile, Q_3 third quartile

[a] Chi-square test

[b] Fisher's Exact test

[c] Student's t test

[d] Mann–Whitney test

*Statistical significance for a level of 5 %

As a response to increase in shear stress observed in SSc, patients release several vasodilators such as NO, prostaglandins, and endothelium-derived hyperpolarizing factor [44]. This response is commonly known as FMD and has been largely used for endothelium dysfunction assessment. NO is probably the major mediator of vasodilation, and reduced NO bioavailability has been broadly accepted as a marker of endothelium dysfunction [45]. A systematic review and meta-analysis of FMD [46] showed that in 71 % of the analyzed studies, brachial artery FMD% was significantly lower in SSc patients, as compared with controls. Also, low brachial artery FMD% has been reported as an independent predictor of cardiovascular risk [46]. Moreover, calcification and fibrosis both reduce vascular compliance and subsequently affect NO signaling by limiting vascular stretch [47], favoring macrovascular disease and arterial stiffness. Given that vascular changes leading to DU are characterized microscopically by intimal fibrosis, the decrease in vascular compliance may also contribute to the reduction of FMD values by reducing NOS activity in SSc patients. Clearly, the decrease in endothelial NO production is a characteristic feature of SSc vasculopathy, which largely explains the reduced FMD values in these patients [47]. Our study showed reduced FMD in SSc patients with active DU. Also, FMD<9.41 % had a predictive role for new DU episodes. Strengthening macrovascular disease in DU pathogenesis is the predominance of positive Allen test in DU patients.

The severe capillary loss observed in the late-NVC pattern might also be related to impaired angiogenesis due to an imbalance of angiogenic factors. Our results are in accordance to those of other research groups that have reported significantly lower VEGF levels in SSc patients with active DU [48–50]. In

contrast with the findings of Avouac et al. [51], we found lower serum levels of VEGF in patients with late NVC patterns compared to those with early/active NVC pattern, supporting those authors that have proposed before an inverse correlation between serum VEGF levels and capillary density [49, 52]. A prolonged overexpression of VEGF may have deleterious effects on the vascular network, as it may result in a chaotic vascular morphology with reduced blood flow in the newly formed vessels. A chronic and uncontrolled overexpression of VEGF does occur in SSc, and it may be significantly implicated in the altered vessel morphology observed in patients with the late NVC pattern [48, 51]. Previous reports have hypothesized that the insufficient angiogenesis observed in patients presenting the late-NVC pattern might be a result of increased angiostatic factors levels, which have been reported to be predominant in the late stages of SSc [51, 53–55]. In our cohort of SSc patients, enhanced serum levels of endoglin were a risk factor for DU but had with no significant predictive role. Further evaluation of other angiostatic markers should be assessed to confirm these findings.

Our study has several limitations that deserve consideration. First, our study is limited by its observational design, and only ischemic digital ulcers as marker of vasculopathy were assessed. Additionally, further studies are required to confirm our findings. However, our results show that qualitative capillaroscopic features can be used as an early identification tool of the SSc patients who are at the greatest risk of developing DU. Moreover, our NVC findings demonstrate its predictive value for new DU events in SSc patients. Endothelial dysfunction assessed by FMD and ET-1 levels were also predictor factors for DU recurrence, as detected in our 3-year long follow-up investigation. We propose that these findings are useful to identify high-risk patients since they will signal those patients that are to be selected for a closer clinical follow-up. Further studies assessing further clinical usefulness of our data are needed to validate their application in routine use, preventive or early therapeutic strategies. Finally, we should note that scleroderma remains amongst the most enigmatic of the connective tissue diseases, including not only its classification but also its associated conditions such as primary biliary cirrhosis, introduction of new therapies, development of new biomarkers, and genetic and epigenetic phenomenon [56–69].

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Conflict of Interest The authors declare that they have no conflict of interest.

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CHAPTER 5
DISCUSSION

With this observational cohort study, it was our main aim to answer the following research questions:

i. Is endothelium-dependent flow-mediated dilatation an early diagnostic tool for severe peripheral vascular complications in patients with RP?
ii. Can a qualitative "scleroderma pattern" or semi-quantitative "microangiopathy evolution score" identify patients at risk for DU?
iii. Can serum levels of endothelial dysfunction (*ET-1 and ADMA*) and angiogenesis (*VEGF, endostatin, and endoglin*) biomarkers identify patients at risk for DUs?
iv. What are the predictive roles of all these variables?

We will discuss our results in 3 sections:

5.1 Comparison between Primary and Secondary Raynaud patients
5.2 Peripheral ischemic vasculopathy in patients with SSc-associated SRP
    a. Risk factors
    b. Predictive factors for new DU events
        i. DU Recurrence
        ii. First DU episode in the 3-year clinical follow-up
    c. Protective factors
5.3 Overall considerations

The presence of RP and the loss of normal regulation of cutaneous vascular tone predict the underlying disease in RP patients (1). RP occurs in almost all SSc patients and is usually the first sign of the disease. Compelling clinical and biological evidence suggests that the primary target for both initiating and propagating the disease is the blood vessel, from the very early onset of the disease through late clinical complications (2). Indeed, RP and morphological changes in nailfold capillaroscopy may occur months or even years before the onset of disease, suggesting a crucial role of the microangiopathy in the overall disease process (3).

It has been suggested that RP in SRP patients may be triggered by endothelial injury. The exact mechanism for the initial endothelial injury is unknown, but apoptosis induced by infection, immune-mediated cytotoxicity, anti-endothelial cell antibodies, and ischemia-reperfusion injury have all been implicated (2).

Vascular insufficiency from a progressive obliteration of both small arteries and microvessels is associated with a state of chronic tissue hypoxia (4). Recurrent RP episodes of ischemia-reperfusion injury to skin and other organ systems may contribute to tissue fibrosis, organ dysfunction, and significant morbidity and mortality. A number of patients with SSc exhibit the severe consequences of this vascular disease with catastrophic events, such as digital ischemia with ulceration, gangrene and amputation, scleroderma renal crisis, or pulmonary arterial hypertension; these jeopardize patients' quality of life (5, 6).

The nutritional skin flow of fingers is linked to the integrity of the microvasculature, which consists of a superficial subpapillary plexus, a profound cutaneous plexus, and interconnections between these 2 plexuses. These so-called arteriovenous anastomoses (AVA) enable a fast increase or decrease of blood flow through the skin to regulate the body temperature. The AVA are innervated by the autonomous nervous system (*a*2c adrenergic receptors). In the fingers of patients with SRP, the absence of a correlation between skin perfusion and digital arterial flow is due to microvascular damage (giant capillaries, disorganization of the normal capillary array, avascular areas) and AVA shunts. In SSc, microvascular damage is responsible for the baseline digital blood flow reduction, while the AVA may be the main, but not exclusive, factor in controlling the digital blood flow during a cold stimulus. Therefore, local factors are also involved in the regulation of digital blood flow during a cold stimulus (7).

The development of DUs is a frequent and early complication in SSc. Surveys shows that up to 75% of patients will have their first DU episode within 5 years after their first non-RP symptom, and 30% of patients will experience DU complications each year. In extreme cases, DUs lead to the loss of a digit as a consequence of infection and/or associated macrovascular disease. DUs have a significant impact on the quality of life and the cost of patient care (2).

Several reports have identified different risk factors associated with DUs. Different disease subset cohorts, variable disease duration, sample size, and the risk factors analysed all might contribute to the different reported results. In our PRISMA-driven review of predictable factors for DUs, dcSSc, early onset of RP, early first non-RP symptom, high Rodman's skin scores, and anti-scleroderma-70 positive autoantibodies were considered strong predictors of DUs.

Currently, there are no biological measurements (biomarkers) to assess subclinical vascular activity in patients with SSc, and therefore the clinician often does not detect the disease until a late and irreversible stage (8). Additionally, until now, no therapy has been able to modify vasculopathy disease evolution and progression in SSc-associated SRP patients (9).

5.1 COMPARISON BETWEEN PRIMARY AND SECONDARY RAYNAUD PATIENTS

This is the first study that demonstrates that both endothelial dysfunction and an angiogenic stimulus are both already present in PRP. Increased serum levels of ET-1 and vascular endothelial growth factor (VEGF) might be a response to the repeated bouts of vasospasm. Our investigation revealed some new and useful information: i) an increased vasoconstrictor status due to increased serum levels of ET-1 is observed in PRP, although endothelial NOS inhibitor (ADMA) is not affected, suggesting overproduction of vasoconstrictors. No differences in endothelial nitric oxide (NO)-dependent FMD were observed between PRP and controls; ii) increased peripheral resistance, low FMD response to shear stress, and a high RI are features of SRP, mostly likely as a consequence of irreversible EC injury; iii) severe obliterative peripheral vasculopathy is present only in SRP patients with DU; and iv) patients with SRP and DU have an impaired angiogenic response to chronic ischaemia.

Different mechanisms induce and perpetuate endothelial dysfunction and progressive vasculopathy in SSc-associated SRP patients. Among these, dysregulation of vascular tone (a consequence of an imbalance between vasoconstrictor and vasodilator mediators), defective angiogenesis, endothelial injury/activation elicited by the activation of innate and adaptive immune response, and functional defects of progenitor endothelial cells have been advocated as the main pathogenic mechanisms underlying endothelial damage in SSc (10). Moreover, chronic endothelial cell perturbation and activation induced by ischemia and reperfusion lead to dysfunction and an irreversible loss of integrity, with cell detachment and tissue injury, which constitute a major stimulus for increased expression of VEGF and abnormal angiogenesis (10). A recent study has shown that the anti-angiogenic VEGF isoform VEGF165b is overexpressed in SSc (9). This finding could explain, at least in part, the marked attrition of microvessels (11).

Angiogenic biomarkers have not been extensively investigated in PRP. As in the studies of Distler et al. (12) and Farouk et al. (13), our study confirms increased VEGF in SSc-associated SRP patients, but increased VEGF serum levels in PRP have not been reported. Conflicting results and the lack of a sufficient response to hypoxia and the consequent vascular changes in SSc might be explained by impairment of mechanisms that are differently mediated by angiogenic or angiostatic factors (14).

5.2 PERIPHERAL ISCHEMIC VASCULOPATHY IN PATIENTS WITH SYSTEMIC SCLEROSIS-ASSOCIATED SECONDARY RAYNAUD PHENOMENON

a. Risk factors

No major differences were observed between SSc-associated SRP patients, PRP patients, and the control group regarding age, gender, mean arterial pressure, and total cholesterol.

Concerning macrovascular disease evaluation, the Allen test was negative in all control patients and in 94.4% of PRP patients. SRP patient groups were different: patients with DU tested positive 75% of the time, whereas only 19.4% of DU-naïve SSc patients tested positive. This suggests an important role for macrovascular disease in the pathogenesis of the ischemic peripheral vascular lesions, and we propose the Allen test as a simple screening tool for DU risk assessment.

Endothelium-dependent responses to various pharmacological (acetylcholine/bradykinin) and physical (shear stress) (15) stimuli have been described as important research tools to evaluate endothelial function.

Several vasodilators (NO, PG2, ET-1 (16), and EDHF (17)) are released in response to increased shear stress, but their physiological importance is unknown. NO has been widely used as a clinical marker of endothelium function, determined by measuring the vasomotor response. The magnitude of the shear stress created with reactive hyperaemia is influenced by several factors, such as baseline diameter (18), age (19), sex (19, 20), cuff position (21), occlusion duration (22), and addition of ischemic exercise (23). Several reports with guidelines have been published, but the rationales of the critical conditions to induce a reliable NO-dependent response are still undefined (24).

Comparing the results of FMD across studies is troublesome. The absolute mean FMD varies among studies, from between -1.9 to 19.2% (25). These differences may be due to different study populations, risk profiles, measurement techniques, and probable non-compliance with FMD recommendations and guidelines.

In our study, baseline artery diameter was similar between groups. FMD% was significantly lower in patients with active DU than in all other groups. No correlation was found between FMD and disease duration (since the first RP episode). Regarding qualitative nailfold videocapillaroscopy (NVC) patterns, FMD was significantly lower in the late pattern than in the active and early patterns; no differences were found between the active and early patterns. Our data reveal

that severe vascular disease with EC injury and capillary dropout are strong contributors for the impaired endothelium-dependent FMD response.

All RP patients had significantly decreased basal state PSV compared to the control group, with no differences between patients with and without DU. After 5 minutes of brachial artery occlusion, PSV, and EDV were significantly reduced in active DU patients compared to all other groups. The Resistance Index (RI) was increased in active DU patients but was not significantly different in DU-naïve patients.

PSV and EDV capillaroscopic patterns were significantly different from each other. We describe a successive decrease of PSV and EDV from early to late patterns. Vascular resistance, measured by RI, increased progressively with the worsening of microvascular damage and progression of the capillaroscopic pattern. This is the first study that analyzed FMD and MES. A decrease of FMD with progression of MES was observed; MES $\geq$ 7 had significantly lower FMD values.

These data support overall microvascular and macrovascular dysfunction in RP patients and are in accordance with several previous studies. Patients with DU had a worse response to a shear stress stimulus than other groups. In proper palmar digital arteries (PPDA), blood flow is reduced and the RIs increase with progression of structural alterations of microvasculature (26); skin perfusion is maintained through compensatory mechanisms of microcirculation, both functional and structural (autonomous nervous system, arteriovenous anastomoses, neoangiogenesis) in the active pattern but not in the late pattern (7). In SSc patients, vessel fibrosis and calcification may reduce vascular compliance and affect NO signalling by limiting vascular stretch due to arterial stiffness (27). Anderson et al. (28) described endothelium dysfunction in SSc patients as a result of inflammation with a correlation between plasmatic markers of endothelium inflammation and brachial artery wall stiffness.

Several studies previously reported macrovascular disease in SSc patients: reduced FMD and increased carotid IMT (29), reduced FMD and normal IMT (30), reduced FMD and increased artery stiffness (31), and lower FMD in dcSSc patients (29, 31). Controversial results were also published regarding FMD and non-endothelium-dependent nitro-glycerine mediated dilatation (NMD) in SSc patients: reduction of FMD and NMD (29, 31, 32); only reduction of FMD (27, 33); reduction of FMD and preserved NMD (30); and similar FMD and NMD (28, 34).

As in other studies, we conclude that structural macrovascular damage is present in our SRP group and that it progresses with worsening of SSc microangiopathy.

Vascular biomarkers of endothelial dysfunction have been extensively studied in SSc patients. Increased levels of ET-1 are associated with endothelial dysfunction. ET-A receptor antagonists improve FMD in patients with endothelial dysfunction and in patients with elevated ET-1. Small or absent responses of FMD may be due to high levels of ET-1 with consequent endothelial dysfunction (16).

Our study has identified high serum levels of ET-1 and ADMA as independent risk factors of DU in patients, consistent with previous reports that reported increased serum ET-1 levels in patients with DU (35-38) and one (39) described no association between ET-1 serum levels and DU, suggesting major roles for ET-1 in vascular dysfunction and disease through vasoconstrictive effects and in vessel remodeling, ultimately leading to major changes in cellular and tissue architecture (40).

SSc serum significantly reduces NO synthase activity *in vitro*, paralleled by decreases in intracellular cGMP and NO production in the cell medium, suggesting the presence of a factor that inhibits NOS (41). NO has vasodilating effects on vessels, inhibits the thrombogenicity and proliferation of vascular muscle cells, and has an important role in regulation of blood flow and maintenance of normal vascular wall structure (41). The loss of NO, the hallmark of endothelial dysfunction, is an attractive possibility as an overall mechanism for the pathogenesis of RP, since perturbations in the NO pathway may have pleiotropic effects on multiple effector pathways that may be relevant in RP, including ET-1 (42). Increased levels of the circulating endogenous inhibitor of NOS, ADMA, have been reported by Doodley et al. (43) in dcSSc and by Blaise et al. (44) in SSc, reflecting abnormal NO regulation and/or contribution to endothelial dysfunction in SSc. A novel finding in this study was elevated ADMA in patients with SRP and DU, compared to all other groups.

As a research tool, qualitative and semi-quantitative capillaroscopic scoring has an indisputable value, but it has not yet found its way into day-to-day clinical practice (45). Several scores have been described. In our research, we adopted the qualitative classification of "scleroderma pattern" proposed by Cutolo (46) and a semi-quantitative score validated by Sulli et al. that paralleled the evolution of microangiopathy in SSc, although there is no reference to its association with DU (47).

Significant differences were found between the SSc DU and SSc non-DU groups regarding qualitative evaluation: the late pattern was the most frequent pattern in DU patients (71.1%), whereas early and active patterns were the most representative patterns in non-DU patients (33.3% and 56.4%, respectively). Significant differences were related to the disease subset: patients with diffuse

disease predominately had late pattern disease (76.9%), whereas those with limited disease predominately had active pattern disease (46.9%). Concerning semi-quantitative MES scores, significant differences were observed, as the MES score was higher in the DU group than the DU-naïve group.

We conclude that capillaroscopy has great value in identifying high-risk patients, as demonstrated by qualitative and semi-quantitative assessments. These results are in accordance with other studies that report late NVC pattern, disorganization of microvascular array with loss of capillaries, and worsening of capillaroscopic pattern as risk factors for DU occurrence (48-50).

We considered the MES score, FMD, ET-1, ADMA, telangiectasia presence, NVC pattern (markers of endothelial dysfunction and microvascular damage), the Allen test (macrovascular disease), and disease subset to be potential predictors of DU. Univariate analysis identified higher MES scores, increased serum levels of ET-1, positive Allen Tests, telangiectasia, late capillaroscopic patterns, and dcSSc as risk factors for new DUs. Higher FMD% values indicated protection from new DUs.

When all of these variables were included in a logistic regression, only the MES score ($2<MES\leq6$, HR 428.44, 95% CI 4.36 to 4.21E4), FMD ($5.7\%<FMD\leq9.5\%$ HR 0.005, 95% CI 0.00 to 0.59) and positive Allen test (HR 71.64 95% CI 2.75 to 1.87E3) remained significantly associated with DU. We then analysed the combined effect of these 3 variables to build a clinical model that predicted DU. Thus, we created a score reflecting the sum of these risk variables that were present in each patient ($MES>2$, $FMD\leq5.7\%$, and positive Allen test). When considering the 3 variables, all patients with 2 or more risk factors had DU. When considering only simple day-to-day office tools (MES and Allen test), patients with 1 risk factor had a 75% chance of a new DU; all patients with 2 variables had a new DU.

Tissue ischemia and hypoxia are usually the main triggers for angiogenesis via the upregulation of pro-angiogenic factors, which then initiate angiogenic sprouting from pre-existing microvessels by: i) inducing vasodilation, proliferation, and migration of endothelial cells; ii) invasion of the surrounding extracellular matrix by endothelial cells; and iii) formation and stabilization of the vascular lumen of newly formed capillary vessels. However, despite the loss of microvessels in multiple vascular beds, compensatory angiogenesis is deregulated, insufficient, and does not allow vascular recovery in the disease course (9).

The functional role of angiogenic mediators in DU pathogenesis is poorly understood. Among the pro-angiogenic factors, VEGF-A (also referred to as VEGF)

is considered one of the most potent regulators of physiologic and pathologic angiogenesis and is overexpressed in most angiogenic conditions (9). Surprisingly, several studies published during the past decade have shown that, despite the lack of angiogenesis, VEGF-A expression is markedly increased both in the skin and in the circulation of patients with SSc (12, 51, 52).

Our cohort study found no significant differences regarding VEGF biomarkers between the control, PRP, and DU-naïve SSc groups. Our results are in accordance with those of Distler et al. (52) and Farouk et al. (13), whose data suggest a functional deficit of VEGF in SSc patients, which can be overcome if the levels of VEGF exceed an individual threshold.

Increased VEGF serum levels were present in SSc patients with early/active NVC pattern and without DUs, suggesting a protective role of increased serum values of VEGF with respect to DUs. Reduced VEGF levels in the late pattern suggest that ineffective angiogenesis may contribute to the avascular areas observed in this pattern, largely responsible for the ischemic territory underlying DUs. This insufficient angiogenesis might be related to increased levels of angiostatic factors that were reportedly increased in the late stages of SSc (53). Another explanation is that a prolonged, uncontrolled, and chronic overexpression of VEGF in SSc may have a deleterious effect on the vascular network, resulting in a chaotic vascular morphology with reduced blood flow in the newly formed vessels (53, 54). Avouac et al. (54) reported increased levels of VEGF in the late pattern, suggesting that this upregulation might be an insufficient compensatory mechanism to stimulate angiogenesis and demonstrated an inverse correlation between capillary density and VEGF.

Regarding angiostatic biomarkers, our research had similar results as those reported by Wipff et al. (55). Serum etonogestrel levels were higher in patients with the SSc vascular phenotype associated with the presence of DU, highlighting a possible contribution of this antiangiogenic protein in SSc vascular disturbances. We failed to identify increased serum levels of endostatin in SSc patients, similar to Hebbar et al. (56) and Farouk. et al. (13). Endostatin plays an important role in microvascular changes and balance in SSc patients, and its actions are likely mediated by other several angiostatic factors that remain to be identified. Larger observational studies are required to define the relationship between angiostatic biomarkers and DUs or other manifestations of vasculopathy in SSc patients.

In summary, low FMD%, increased serum levels of ET-1, ADMA, and endoglin, and low VEGF serum levels were risk factors for DU episodes.

We failed to identify an association between DU and ACA autoantibodies, anti-Scl70, AFA, anti-PM-Scl, anti-NOR 90, and anti-RNA-polymerase. A trend towards an association between positive Anti-Ro52/TRIM21 and DU was verified. These antibodies can be detected across a number of autoimmune diseases with significant prevalence, but their clinical relevance remains controversial. Additional studies with more SSc patients with DUs are needed to identify specific autoantibodies as possible risk factors for DU.

b. Predictive factors for new DU events

In our study, new DU incidence was similar to the placebo group of the RAPIDS-1 study (57) (55.8%/61%). The diffuse disease subset had proportionally more new DUs (76.92%) compared to the limited SSc subset (46.88%) (*p=0.048*). No differences were found regarding disease duration between the new DU and DU-naïve groups. The median time to the occurrence of a new DU event in study period was 4.50 (1.25-16.25) months.

The risk factors for the development of a new DU in the study period were a previous history of DU, anti-scleroderma-70 autoantibody, telangiectasia, NVC late pattern, high MES score, low FMD%, a positive Allen test, and increased ET-1, VEGF, and endoglin serum levels. Multivariate Cox analysis confirmed MES score, FMD, and ET-1, ADMA, and VEGF serum levels as independent predictors of the development of a new DU.

Because the two groups studied were very different (active DU vs. DU-naïve) in a variable that could affect the main outcome (occurrence of DU during 3-year clinical follow-up) and was, by definition, a source of potential bias, we checked the incidence of ulcer recurrence and, in ulcer-naïve patients, the first DU episode in the study period, analysing their predictive value.

i) DU Recurrence

Of the 38 SSc patients with active DU at beginning of the study, 30 (79%) had at least one DU recurrence during the 3-year course of this investigation. Predictor factors of DU recurrence were: FMD>9.41%, ET-1 >11.85 *pmol/L,* and late NVC pattern. Kaplan-Meier analyses of freedom from the DU recurrence showed that the median values of FMD, ET-1, and qualitative scleroderma patterns in NVC were significantly associated with new episodes of DU. Multivariate Cox regression confirmed ET-1 and late NVC pattern as independent predictors of DU recurrence.

Endothelial dysfunction assessment in SSc patients has had controversial results in the literature. Although not always available, FMD may be, in the near

future, a screening prognostic tool for vasculopathy in SSc patients. Most of the studies were not designed to analyse the relationship of FMD with DU. In our study, patients with DU recurrence had a reduced vasodilatation response to the increased shear stress. Values of FMD lower than 9.5% predicted DU recurrence, suggesting that impaired FMD might be an alert trigger for peripheral vasculopathy.

Additionally, ET-1 serum levels > than 11.9 pmol/ml were a risk for new DU in the 3-year follow-up and an independent predictor of DU recurrence. These findings are consistent with previous reports that suggest that ET-1 plays a major role in vascular dysfunction and vascular disease through vasoconstrictive effects and is an important mediator in vessel remodeling, which ultimately results in major changes in cellular and tissue architecture (40). Capillaroscopy may be the most useful tool for targeting patients at risk for DU episodes. Patients with late pattern disease are closely monitored for alert signs for peripheral ischemic lesions.

ii) First DU episode in the 3-year clinical follow-up

Our research study identified late NVC pattern as the main alert sign for first DU occurrence. Kaplan-Meier analyses of freedom from the occurrence of DU event strengthen these results.

VEGF serum levels in these patients were half of the corresponding values of SSc patients without a DU in overall disease course, although not significant. This might be due to a small number of patients, ten, that developed a first DU event in the study period. Considering that only a small increase in VEGF protein results in efficacious new vessel formation in a variety of animal models and that angiogenesis is regulated by a balance of angiogenesis inducers and inhibitors, possible explanations for insufficient angiogenesis in SSc include a blockade of the biologic effects of VEGF by one or more angiostatic factors (52). Although other reasons, such as signaling defects or lack of VEGF receptors, cannot be excluded, this hypothesis is strongly supported by the fact that patients without fingertip ulcers showed highly elevated serum VEGF concentrations. A further increase of VEGF might be a therapeutic option for SSc patients with fingertip ulcers. In fact, encouraging animal studies led to clinical trials using recombinant VEGF or gene therapy in patients with different ischemic diseases. In a phase I study with recombinant VEGF165 in patients with coronary ischemia, the therapy was safely tolerated and resulted in improved perfusion and collateralization in a subset of patients (52).

The process of angiogenesis appears to be largely impaired in SSc, following the profound disarrangement of the microcirculation. The damage of the vessels evolves progressively from the early to late stages and is characterized by different morphological aspects (52). The fact that baseline NVC patterns are associated with future severe disease may be one of the first steps in positioning capillaroscopy as a welcome biomarker for the disease (50).

c) Protective factors

Median SSc disease duration in DU-naïve patients (n=29) was similar to the subpopulation of SSc patients that had at least one DU (n=48) in the study period. These DU-naïve SSc patients were largely lcSSc and anti-scleroderma 70 negative. They also presented less peripheral vascular impairment; fewer patients had telangiectasia and most were Allen test negative. These results were strengthened by a good response to shear stress with higher FMD%. They exhibited lower serum levels of vasoconstrictive ET-1, increased ADMA, and VEGF serum levels. The late scleroderma pattern was only present in one of the patients (3.4%).

Values of FMD greater than 9.5% associated with protection from DU, suggesting that impaired FMD might be an alert trigger for peripheral vasculopathy.

Vascular biomarkers, if not expensive and unavailable in daily clinical practice, could be a good predictor tool for new DU occurrence.

5.3 OVERALL CONSIDERATIONS

There are some epidemiological, cross-sectional, and cohort studies that investigated potential predictors of DU in SSc patients, but additional studies are needed to ascertain the existing evidence in order to make the currently-available information more profitable.

To our knowledge, this is the first study that simultaneously analysed macro- and microvascular disease, endothelial dysfunction, and angiogenesis markers as risk factors for DU and identified their predictive roles for new ischemic events. The prospective design of the study, with a clear definition of the protocol, a thorough data collection by a single, trained doctor, no missing data per item, and a complete follow-up of all patients in the 3-year period, minimized information bias.

Although it's a single-centre study with a restricted number of patients in each group, limited by the number of patients with active DU at time of enrolment, the variables we found were clinically significant and relevant, and some can be performed in daily clinical practice. This research enriches previous reports through its prospective design, with robust behaviour (AUC) in most of the vascular biomarkers and in the morphological and functional parameters analysed.

There are some limitations that should be identified:

1. We did not perform endothelium-independent shear stress evaluations.
2. We did not study morphology and blood flow of the proper palmar digital arteries (PPDA) and their relationship with NVC.
3. We did not study skin blood perfusion and digital arteries' pulsatility by Laser Doppler Perfusion Imaging (LDPI) and photoplethysmography (PPG).
4. Carotid artery intimal / medial thickness as an arterial stiffness parameter was not evaluated, as these tools have been extensively studied as a prognostic indicator of accelerated atherosclerosis, which was not our aim.

The principal biases of our study were that a small number of men and patients with dcSSc were included.

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5.4 CONCLUSIONS AND FURTHER RESEARCH

The results of the this cohort study allowed the following conclusions as answers to research questions hypothesized in the beginning of the investigation:

1. Macrovascular disease is a feature of severe peripheral ischemic lesions in patients with secondary RP and SSc as shown by patients with FMD levels ≤ 9.5% had significantly more new DU in 3-year clinical follow-up as well as a predominance of positive Allen test in patients with DU.
2. Microangiopathy is a dynamic process in SSc patients. Patients with DU had predominantly late pattern whereas early and active patterns were the most representative patterns in naïve-DU patients. Concerning semi-quantitative MES score significantly differences were observed: MES score was higher in DU group compared to naive-DU group at baseline. A MES score greater than 2 in the beginning of the study was a risk factor for new digital ulcers occurrence in the 3-year follow-up.
3. Vascular biomarkers play an important role as diagnostic and disease staging tools as well as prognostic indicators. In our study increased serum levels of ET-1, ADMA and low serum levels of VEGF were identified as independent risk factors for active ulcers. Patients with serum levels of ET-1>11.9pmol/ml and VEGF<422.47 pg/ml had significantly more DU in the 3-year follow-up. Although not significant, a trend towards increased serum levels of endoglin>4.215ng/ml was associated to a new DU episode. No predictive serum value was found for ADMA and endostatin.
4. Endothelial dysfunction and microvascular damage identified in NCV are the best predictors of DU recurrence.
5. Late NVC pattern was the only independent predictor for a first DU event.
6. Capillaroscopy is the most useful tool in targeting patients at risk for DU episodes. Patients with late pattern need to be closely monitored for alert signs for peripheral ischemic lesions.

Recommendations for future areas of research should focus on:

1. It would be interesting to study skin blood perfusion and correlate with present findings.
2. Propose an investigational observational study of consecutive very early/ early SSc disease patients to identify predictive vascular biomarkers of peripheral vasculopathy.
3. Correlate all these finding with other organ involvement in SSc patients.

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