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**Microangiopatia e lesão endotelial na Esclerose Sistémica:
contributo para o conhecimento da biologia e correlação com a
gravidade da doença**

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.” (Arthur Schopenhauer)

À minha família.

À Sofia e ao Ricardo, meus filhos, amores, amigos e companheiros.

Ao meu pai Mário, minha eterna paixão.

À doce Maria Emília, meu refúgio, minha mãe.

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PREFÁCIO

A Unidade de Imunologia Clínica (UIC), dirigida pelo Doutor Carlos Vasconcelos, da qual faço parte, nasceu nos anos 80 com o então chamado NIC (Núcleo de Imunologia Clínica), mas só se tornou uma unidade autónoma do Serviço de Medicina Interna em 2004. Nesta Unidade, de referência nacional para várias doenças como o LES (Lúpus Eritematoso Sistémico) e a Esclerose Sistémica, são acompanhados cerca de 3.000 doentes com patologia de foro auto-imune.

Foi em meados dos anos 90, durante o meu internato complementar, que comecei a dedicar-me à observação de doentes com este tipo de patologias e, desde cedo, me apaixonei por uma, pela qual pouco ou nada se podia fazer: a Esclerose Sistémica!

E foi no âmbito desta paixão que em 2002, juntamente com uma colega da Cirurgia Vascular, a Doutora Ivone Silva, formei a “Consulta de Raynaud e de Capilaroscopia”, após um estágio no Royal Free Hospital em Londres, e me tornei responsável pela *coorte* de doentes com Esclerose Sistémica da UIC.

A necessidade de ajudar doentes com esta patologia esteve sempre presente e, nesse contexto, fui uma das fundadoras da “Associação Portuguesa de Doentes com Esclerodermia”, da qual sou Presidente Honorária, e colaborei na organização de várias reuniões anuais com e para os doentes.

A minha tese surge da paixão sentida ao longo de todos estes anos, da certeza de que há muito a fazer neste campo e da esperança de poder contribuir para construir a diferença na vida destes doentes.

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Quero começar por agradecer aos meus orientadores, nomeadamente ao Professor Carlos Vasconcelos, meu mentor desde o início do meu internato complementar, pela sua amizade, confiança e apoio nos momentos bons e menos bons. Sem ele não teria iniciado esta caminhada!

À Professora Margarida Lima por todos os momentos partilhados, pelo incentivo contínuo, pela dedicação, apoio e amizade. Sem ela este percurso não teria acabado!

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Ao meu sobrinho Rui pela cumplicidade, partilha das angústias sentidas, incentivo contínuo e apoio incondicional. Obrigada por teres acreditado no meu trabalho.

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RESUMO

Introdução

A Esclerose Sistêmica, ou Esclerodermia (ES), é uma doença complexa, com elevada morbidade e mortalidade, e cuja patogênese permanece pouco conhecida. As características clínicas são heterogêneas e parecem refletir o contributo de diferentes fatores patogénicos, de natureza genética e/ou ambiental, imunológicos, vasculares ou fibróticos.

A vasculopatia, caracterizada por ativação endotelial e alteração do tônus vascular, parece ser o evento mais precoce e é acompanhada pela presença de citocinas pró-inflamatórias, aumento da expressão das moléculas de adesão, fatores que regulam a angiogénese, perda do controle da atividade *redox*, *stress* oxidativo e hipoxia. Apesar da hipoxia crónica, a angiogénese é insuficiente, havendo uma perda progressiva de capilares e alteração da arquitetura vascular. Envolve interações moleculares complexas, vários tipos celulares e culmina em doença vascular oclusiva grave, com isquemia tecidular e lesão orgânica. A vasculopatia pode ser observada em todos os órgãos atingidos, podendo preceder o aparecimento de fibrose.

As alterações morfológicas encontradas na capilaroscopia do leito ungueal, em mais de 95% dos doentes com ES, traduzem a lesão microvascular e a tentativa, ineficaz, de angiogénese. Os padrões capilaroscópicos descritos por Cutolo *et al.* (precoce, ativo e tardio), parecem relacionar-se com o tempo de evolução da doença e, consequentemente, com a evolução da microangiopatia.

A determinação sérica de biomarcadores tem sido utilizada para avaliar a vasculopatia, mas o seu valor na ES permanece desconhecido e não existem biomarcadores disponíveis que permitam efetuar um diagnóstico precoce e avaliar a atividade ou o prognóstico da doença.

Objetivo

Relacionar biomarcadores vasculares e imunológicos com o subtipo da doença, fases de evolução da mesma, padrões capilaroscópicos, atingimento orgânico e sua gravidade, identificando, desta forma, possíveis biomarcadores úteis para a prática clínica.

Metodologia

Da *coorte* de 190 doentes com perfil esclerodérmico (Pré-ES + ES) seguidos na Unidade de Imunologia Clínica do Centro Hospitalar do Porto, foi selecionada uma amostra de conveniência, constituída por 57 doentes (47 com critérios da ACR/EULAR para ES e 10 com critérios de Pré-ES de acordo com LeRoy e Medsger). A duração do tempo de seguimento (*follow-up*) clínico para os doentes incluídos neste estudo, observacional e prospetivo, foi de 4 anos. Vinte e cinco indivíduos adultos saudáveis (dadores benévolos de sangue) foram usados como grupo controlo. Foram critérios de exclusão a existência de fatores de risco cardiovascular e a coexistência de outras doenças.

Os doentes foram classificados nos vários subtipos (Pré-ES, ES limitada, ES difusa) e aqueles com ES foram subdivididos nas fases precoce, intermédia e tardia, de acordo com o tempo de evolução e subtipo da doença. O subtipo limitado foi ainda subdividido em CREST (calcinose, fenómeno de Raynaud, dismotilidade esofágica, esclerodactilia, telangiectasia) e não CREST.

Foram colhidos dados demográficos e os doentes foram avaliados do ponto de vista clínico com uma periodicidade mínima de 6 meses, tendo sido realizados os exames subsidiários necessários, de acordo com as boas práticas para esta patologia. A classificação do envolvimento orgânico e sua gravidade foi efetuada utilizando a escala de gravidade de Medsger. A microangiopatia foi avaliada por videocapilaroscopia e utilizado o *score* de Cutolo *et al.* para a classificar em precoce, ativa ou tardia.

Folham colhidas amostras de sangue e determinadas as concentrações séricas de VEGF, endostatina, angiostatina, ET-1, VCAM-1, ICAM-1, E-seletina e P-seletina por método de ELISA. Foram calculadas as razões VEGF/endostatina e VEGF/angiostatina. Os ANA séricos foram determinados por imunofluorescência indireta tendo como substrato células Hep-2 e foram avaliados vários anticorpos específicos e não específicos por *immunoblotting* (ACA, ATA, anti-RNAPIII, AFA, anti-Th/To, anti-PM-Scl, anti-Ro52 e anti-NOR90) ou por fluoroimunoensaio (anti-U1RNP). As citocinas séricas (IL-2, IL-4, IL-6, IL-10, TNF- α , INF- γ e IL-17A) foram avaliadas por citometria de fluxo, usando um método baseado em microesferas fluorescentes revestidas por anticorpos específicos para cada uma das citocinas. Foi também efetuada imunofenotipagem de linfócitos por citometria de fluxo de 4 cores usando o método de imunofluorescência direta, com imunomarcagem em sangue total, seguida de lise dos eritrócitos e fixação dos leucócitos, para quantificar e caracterizar as células T e as células NK no sangue periférico.

Resultados e conclusões

A lesão/ ativação endotelial (avaliada pelos níveis séricos de ET-1 e das moléculas de adesão) e as alterações da angiogénese (avaliadas pelos níveis séricos de VEGF, angiostatina, endostatina e razões VEGF/endostatina e VEGF/angiostatina) estavam presentes em todos os subtipos da doença, fases evolutivas e padrões capilaroscópicos.

Algumas moléculas, ou a razão entre elas, aumentaram ou diminuíram precocemente e acompanharam a evolução da doença (endostatina, VEGF/endostatina, P-seletina, VCAM-1); outras moléculas (VEGF) predominaram em fases mais precoces, em doentes ainda sem fibrose cutânea, e outras ainda em fases mais tardias da ES (angiostatina, ET-1, E-seletina), parecendo ter impacto na progressão da doença e na patogénese do envolvimento de vários órgãos (ET-1, E-seletina, ICAM-1, angiostatina, endostatina, VEGF/angiostatina, VEGF/endostatina). A angiostatina e a ET-1 encontraram-se aumentadas em doentes com a variante CREST, pelo que poderão estar envolvidas em mecanismos patogénicos responsáveis pelo seu desenvolvimento.

Os auto-anticorpos específicos da doença, ACA e ATA, relacionaram-se com os subtipos limitado e difuso, respetivamente, e associaram-se ao envolvimento orgânico. A diminuição dos linfócitos no sangue periférico, nomeadamente dos linfócitos T CD8+, da fração de linfócitos T CD8+ ativados, de linfócitos T CD56+, e das células NK e seus subtipos (CD56+fraco e CD56+forte), assumiram importância fundamentalmente nas fases precoce e tardia da ES, em doentes com padrões capilaroscópicos mais avançados (ativo e tardio), podendo ter relação com a patogenia da doença e contribuir para o envolvimento de órgãos.

Em suma, biomarcadores vasculares e imunológicos avaliados nesta investigação poderão ter interesse no seguimento de doentes com perfil esclerodérmico, nomeadamente no que diz respeito à monitorização e prognóstico dos diferentes atingimentos orgânicos, fundamental nas decisões terapêuticas nestes doentes.

ABSTRACT

Background

Systemic Sclerosis or Scleroderma (SSc) is a complex disease with high morbidity and mortality, whose pathogenesis remains incompletely understood. Its clinical features are heterogeneous and may reflect variable contributions from different pathogenic factors, such as genetic, environmental, immunologic, vascular or fibrotic ones.

The vasculopathy, characterized by endothelial cell activation and altered vascular tone, seems to be the earliest pathological event and is accompanied by the presence of pro-inflammatory cytokines, higher adhesion molecules expression, angiogenic regulatory factors, loss of redox control, oxidative stress and hypoxia. Despite chronic hypoxia, angiogenesis is insufficient, with progressive loss of capillaries and vascular architecture changes. It involves complex molecular interactions, several cell types, and leads to severe vessel occlusive disease, with tissue ischemia and organ injury. Vasculopathy can be observed in all affected organs and may precede the onset of fibrosis.

The abnormal capillary morphology observed in nailfold capillaroscopy in more than 95% of the patients with SSc reflect the ineffective attempt of angiogenesis. The capillaroscopic patterns described by Cutolo *et al.*, (early, active, and late) seem to relate with the time of the disease's evolution and, consequently, with the evolution of microangiopathy.

The measurement of serum biomarkers has been used to assess vasculopathy, but the specificity of most those studied in SSc remains unknown, and there are no available biomarkers which allow an early diagnosis and the assessment of the disease's activity or prognosis.

Objective

To relate vascular and immunological biomarkers with the disease subsets, evolution phase, nailfold capillaroscopic changes, organ involvement and its severity, therefore identifying potential biomarkers for clinical practice.

Methodology

From the cohort of 190 patients with Scleroderma spectrum disease (Pre-SSc + SSc) followed in the Clinical Immunology Unit of Centro Hospitalar do Porto, a convenience sample of 57 patients was selected (47 patients fulfilled the ACR/EULAR criteria for SSc and 10 fulfilled

the LeRoy and Medsger criteria for Pre-SSc). This prospective observational study had a duration of 4 years of clinical follow-up. Twenty-five healthy individuals (blood donors) were used as control group. The presence of cardiovascular risk factors or concomitant diseases were exclusion criteria.

Patients were classified into the different subtypes (Pre-SSc, limited SSc - *lcSSc*, diffuse SSc - *dcSSc*) and SSc patients were subdivided into early, intermediate and late phases, according to the time of disease evolution and SSc subtype. Additionally, *lcSSc* was subdivided in CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) and non-CREST.

Demographic data was registered and all patients were clinically assessed with a minimum periodicity of 6 months; additional tests were ordered in accordance to the best practices for this disease. Disease involvement and severity were classified using the Medsger severity scale. Microangiopathy was evaluated through nailfold videocapillaroscopy and Cutolo's score was used for its classification into early, active and late.

Peripheral blood samples were collected and VEGF, endostatin, angiostatin, ET-1, VCAM-1, ICAM-1, E-selectin and P-selectin serum concentrations were determined using ELISA assays. VEGF/angiostatin and VEGF/endostatin ratios were calculated. Serum ANA were determined by indirect immunofluorescence with Hep-2 cells as substrate and several specific and non-specific auto-antibodies were assessed by immunoblotting (ACA, ATA, anti-RNAPIII, anti-Th/To, AFA, anti-PM-Scl, anti-Ro52 and anti-NOR90) or by enzymatic fluoroimmunoassay (anti-U1RNP). Serum cytokines (IL-2, IL-4, IL-6, IL-10, TNF- α , INF- γ e IL-17A) were assessed by flow cytometry, using a cytometric bead array. Lymphocyte immunophenotyping was performed by four color flow cytometry, using a whole blood stain-wash-and-then-lyse method to analyze the changes of T lymphocytes and NK cells in the peripheral blood.

Results and conclusions

The endothelium lesion/ activation (evaluated by ET-1 and adhesion molecules serum levels) and angiogenesis changes (assessed through VEGF, angiostatin and endostatin serum levels, and by VEGF/endostatin and VEGF/angiostatin ratios) were present in all of the disease subtypes, evolution phases and capillaroscopic patterns.

Some molecules, or the ratios between them, were precociously increased or decreased and followed the disease's evolution (e.g., endostatin, VEGF/endostatin, P-selectin, VCAM-1); other molecules (e.g., VEGF) were predominant in early phases, among patients still without cutaneous fibrosis, and some others in latter SSc phases (e.g., angiostatin, ET-1,

E-selectin), seeming to have an impact on the progression of the disease and on the pathogenesis of the involvement of several organs (e.g., ET-1, E-selectin, ICAM-1, angiostatin, endostatin, VEGF/angiostatin, VEGF/endostatin). Angiostatin and ET-1 were increased among patients with the CREST variant, and so they may be involved in pathogenic mechanisms responsible for its development.

The disease specific auto-antibodies, ACA and ATA, were related to the limited and diffuse subtypes, respectively, and were associated to organic involvement. The decrease of lymphocytes in peripheral blood, namely T CD8+, activated CD8+, CD56+ lymphocytes and NK cells and their subtypes (CD56+ high and CD56+ low NK cells), was important mainly in early and late SSc phases, among patients with more advanced capillaroscopic patterns (active and late), and they may have a role in the pathogenesis of organ involvement.

In summary, vascular and immunological biomarkers assessed in this research may have interest for the follow-up of SS spectrum disease patients, namely regarding monitoring and prognosis of various organs involvement, fundamental for therapeutic decisions in these patients.

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4.2 - MICROANGIOPATIA, LESÃO ENDOTELIAL E ALTERAÇÕES DA ANGIOGÊNESE

4.2.1 IMPAIRED ANGIOGENESIS IN SYSTEMIC SCLEROSIS: WHAT´S MORE RELEVANT: THE RATIO BETWEEN ANGIOGENIC AND ANGIOSTATIC FACTORS OR THESE FACTORS INDIVIDUALLY? A COHORT STUDY

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Systemic sclerosis refractory disease: From the skin to the heart

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ABREVIATURAS

ACA – *Anti-centromere antibodies* (anticorpos anti-centrómero)
ACR – *American College of Rheumatology*
ACR/EULAR – *American College of Rheumatology / European League Against Rheumatism*
AECA – *Anticorpos anti-células endoteliais*
AFA – *Anti-fibrillar antibodies* (anticorpos anti-fibrilarina)
ANA – *Antinuclear antibodies* (anticorpos anti-nucleares)
APC – *Allophycocyanin* (aloficocianina, fluorocromo)
ATA – *Anti-topoisomerase antibodies* (anticorpos anti-topoisomerase)
BALF – *Bronchoalveolar lavage fluid* (lavado bronco-alveolar)
BCL-2 – *B cell lymphoma 2* (oncoproteína)
BDB – *Becton-Dickinson Biosciences*
BDI – *Mahler's Baseline Dyspnea Index*
CAMPHOR – *Cambridge Pulmonary Hypertension Outcome Review*
CBA – *Cytometric Bead Array*
CCL – *-C-C-chemokine ligands* (quimiocinas tipo –C-C)
CCR – *-C-C- chemokine receptors* (receptores de quimiocinas tipo –C-C)
CE – *Células endoteliais*
CESD-10 – *Center for Epidemiologic Studies Short Depression Scale*
CHF – *Congestive Heart Failure* (insuficiência cardíaca congestiva)
CHFS – *Cochin Hand Function Scale*
CREST – *Calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia* (calcinose, fenómeno de Raynaud, dismotilidade esofágica, esclerodactilia, telangiectasias)
CRST – *Calcinose, Raynaud, esclerodactilia e telangiectasias* (calcinoses, fenómeno de Raynaud, esclerodactilia, telangiectasias)
CSURI – *Capillaroscopic Skin Ulcers Risk Index*
CT – *High resolution computed tomography* (tomografia computadorizada de alta resolução)
CTL – *Cytotoxic T Lymphocytes* (linfócitos T citotóxicos)
DAS 28 – *Disease Activity Score for Rheumatoid Arthritis*
dcSSc – *Diffuse cutaneous systemic sclerosis* (esclerose sistêmica / esclerodermia cutânea difusa)
DDT – *Distância dedo – eminência tenar*
DLCO – *Diffusing capacity of the lungs for carbon monoxide* (capacidade de difusão dos pulmões para o monóxido de carbono)
DLQI – *Dermatology Life Quality Index questionnaire*
DU – *Digital ulcers* (úlceras digitais)
ECG – *Electrocardiogram* (eletrocardiograma)
ECM – *Extracellular matrix* (matriz extracelular)
EDTA-K3 – *Tri-potassium ethylenediaminetetraacetic acid* (sal tripotássico de ácido etileno-diamino-tetraacético)

ELISA – *Enzyme-Linked Immunosorbent Assay*

ePAP – *estimated Pulmonar Arterial Pressure*

ES – Esclerodermia ou Esclerose Sistêmica

ET-1 – *Endothelin-1* (endotelina-1)

ET-A – *Endothelin receptor A* (recetor A da endotelina)

ET-B – *Endothelin receptor B* (recetor B da endotelina)

EULAR – *European League Against Rheumatism*

EUSTAR – *EULAR Scleroderma Trial and Research Group*

FACIT – *Fatigue, Functional Assessment of Chronic Illness Therapy*

FcYRIIIa – *Low affinity receptor for the Fc region of IgG* (recetor de baixa afinidade para o fragment Fc da IgG)

FEVE – Fração de ejeção do ventrículo esquerdo

FGF – *Fibroblast growth factor* (fator de crescimento para os fibroblastos)

FITC – *Fluorescein isothiocyanate* (isotiocianato de fluoresceína)

FS – *Scleroderma Functional Score*

FSDS – *Female Sexual Distress Scale*

FSFI – *Female Sexual Function Index*

FTD – *Finger-tenar distance* (distância dedo-eminência tenar)

FTP – *Finger-to-Palm distance in flexion* (distância dedo-palma da mão)

FVC – *Forced capacity vital, % predicted*

FVC – *Forced vital capacity* (capacidade vital forçada)

GI – *Gastrointestinal tract* (trato gastrointestinal)

HAQ-DI – *Health Assessment Questionnaire Disability Index*

HGF – *Hepatocyte growth factor* (fator de crescimento para os hepatócitos)

HR-QoL – *Health-related quality of life* (qualidade de vida relacionada com a saúde)

HTAP – Hipertensão arterial pulmonar

ICAM – *Intracellular adhesion molecule* (molécula de adesão vascular)

IFI – *Imunofluorescência indireta*

IFN- γ – *Interferon gamma* (interferão gamma)

IL – *Interleukins* (interleucinas)

KIR - *Killer cell Immunoglobulin-like Receptors* (recetores das células citotóxicas, semelhantes às imunoglobulinas)

lcSSc – *Limited cutaneous Scleroderma* (esclerose sistêmica / esclerodermia cutânea limitada)

LES – Lúpus Eritematoso Sistêmico

LFT – *Lung function tests* (testes de função pulmonary)

LPS – *Lipopolysaccharide* (lipopolissacarídeo)

LVEF – *Left Ventricular Ejection Fraction* (fração de ejeção do ventrículo esquerdo)

MAbs – *Monoclonal antibodies* (anticorpos monoclonais)

MACTAR – *McMaster Toronto Arthritis Patient Preference Disability Questionnaire*

MANSA – *Manchester Short Assessment of Quality of Life*

MCP-1 – *Monocyte chemotactic protein type 1* (proteína quimiotóxica para os monócitos tipo 1)
 MHISS – *Mouth Handicap in Systemic Sclerosis Scale*
 MMP – *Matrix metalloproteinases* (metaloproteinases da matriz extracelular)
 MOS-SLEEP – *Medical Outcomes Study Sleep Scale*
 mRRS – *Modified Rodnan Score*;
 NCAM – *Neural Cell Adhesion Molecule* (molécula de adesão das células neuronais) (CD56)
 NF-Kb – *Nuclear Factor kappa B* (fator nuclear kappa B)
 NIC – Núcleo de Imunologia Clínica
 NK – *Natural Killer* (células citotóxicas naturais)
 NKT – *Natural Killer T cells* (células T citotóxicas naturais)
 OR – *Odds ratio* (razão de Odds)
 PAH – *Pulmonary arterial hypertension* (tensão arterial pulmonar)
 PASP – *Pulmonary artery systolic pressure* (tensão arterial sistólica pulmonar)
 PB – *Peripheral blood* (sangue periférico)
 PC5 – *Phycoerythrin Cyanin 5* (ficoeritrina cianina 5)
 PDGF – *Platelet derived growth factor* (fator de crescimento derivado das plaquetas)
 PDGF – *Fator de crescimento derivado das plaquetas*
 PE – *Phycoerythrin* (ficoeritrina)
 PFT – *Pulmonary function tests* (testes de função pulmonar)
 PGI – *Patient-generated Index*
 PHQ – *Patient Health Questionnaire*
 PHQ-8 or -9 – *Patient Health Questionnaire –8 or –9*
 PIGF – *Placental growth factor* (fator de crescimento placentário)
 Pré-ES – Pré-Esclerodermia
 Pre-SSc – *Pre-Scleroderma* (pré-esclerodermia)
 PROMIS – *Patient-Reported Outcomes Measurement Information System*
 PSAP – *Pulmonary systolic arterial pressure* (tensão arterial sistólica pulmonar)
 QoL – *Quality of life* (qualidade de vida)
 RCS – *Raynaud's Condition Score*
 RNAP – *Anti-RNA polymerase antibodies* (anticorpos anti-polimerase do ARN)
 RP – *Raynaud's phenomenon* (fenómeno de Raynaud)
 SBI – *Symptom Burden Index*
 SDF-1 – *Stromal cell-derived factor (CXCL12)* (fator derivado das células do estroma)
 SDO – *Satisfaction with Daily Occupations*
 SF – Short form
 SHAQ – *Scleroderma Health Assessment Questionnaire*
 SLE – *Systemic Lupus Erythematosus* (lupus eritematoso sistémico)
 SSc - *Systemic Sclerosis or Scleroderma* (esclerose sistémica ou esclerodermia)
 SWAP – *Satisfaction With Appearance Scale*
 TCR – *T cell receptor* (recetor da célula T)

TDI – *Transition Dyspnea Index*

TGF – *Transforming growth factor* (fator de transformação do crescimento)

TGF- β – *Transforming Growth Factor beta* (fator de transformação do crescimento, tipo beta)

TIMP – *Tissue inhibitors of metalloproteinases* (fatores tecidulares inibidores das metaloproteinases)

TNF- α – *Tumour Necrosis Factor alpha* (fator de necrose tumoral alfa)

UCLA SCTC GIT – *University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Scale*

UCLA SCTC GIT 2.0 – *The University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Scale 2.0*

UIC – Unidade de Imunologia Clínica

VCAM – *Vascular Cell Adhesion Molecule* (molécula de adesão das células vasculares)

VEGF – *Vascular Endothelial Growth Factor* (fator de crescimento das células endoteliais vasculares)

VEGFR – *Vascular Endothelial Growth Factor Receptor* (recetor do fator de crescimento das células endoteliais vasculares)

WAI – *Work Ability Index*

WPS-RA – *The modified Work Productivity Survey – Rheumatoid Arthritis*

RESUMO DA ATIVIDADE CIENTÍFICA RELACIONADA COM A TESE

PUBLICAÇÕES E COMUNICAÇÕES

DE ACORDO COM O ARTIGO 34º DO DECRETO-LEI N.º 115/2013, FORAM UTILIZADOS PARA ESTA TESE RESULTADOS CONTIDOS NOS SEGUINTE TRABALHOS PUBLICADOS:

- “SYSTEMIC SCLEROSIS REFRACTORY DISEASE: FROM THE SKIN TO THE HEART”
- “QUALITY OF LIFE IN SYSTEMIC SCLEROSIS”
- “T AND NK CELL PHENOTYPIC ABNORMALITIES IN SYSTEMIC SCLEROSIS: A COHORT STUDY AND A COMPREHENSIVE LITERATURE REVIEW”
- “DIFFERENT CONTRIBUTIONS OF ANGIOSTATIN AND ENDOSTATIN IN ANGIOGENESIS IMPAIRMENT IN SYSTEMIC SCLEROSIS: A COHORT STUDY”

TRABALHOS PUBLICADOS

Em revistas nacionais, com revisão por pares

2014

“AUTOLOGOUS STEM CELL TRANSPLANTATION IN A PATIENT WITH SEVERE SYSTEMIC SCLEROSIS”

Vaz CP, Almeida I, Guedes M, Rosário C, Branca R, Campilho P, Roncon S, Vasconcelos C, Campos A.

Acta Reumatol Port. 2014; 39:262-264.

Doi: Não disponível.

PMID: 25326407.

Fator de Impacto da Revista: 2014 (0.40); 2015 (0.41)

Tipo: Caso clínico.

Em revistas internacionais, com revisão por pares

2011

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Almeida I, Faria R, Vita P, Vasconcelos C.

Autoimmun Rev. 2011; 10: 693-701.

Doi: 10.1016/j.autrev.2011.04.025.

PMID: 21575745.

Fator de Impacto da Revista: 2011 (8.72); 2012 (7.62)

Tipo: Artigo de revisão.

2012

“ARE ANTI RO52 ANTIBODIES ASSOCIATES WITH PULMONARY INVOLVEMENT IN SCLERODERMA”

Ferreira JP, Almeida I, Marinho A, Cerveira C, Vasconcelos C.

J Pulmonar Respirat Med 2:116.

Doi:10.4172/2161-105X.1000116.

PMID: Não aplicável.

Fator de Impacto da Revista: Não aplicável.

Tipo: Artigo original.

2013

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Guedes M, Zilhão C, Palhau L, Almeida I, Silva I, Vasconcelos C, Marinho A, Vaz C, Campos A.

Pediatr Rheumatol Online J. 2013; 11(Suppl 2): 134.

Doi: 10.1186/1546-0096-11-S2-P134

PMID: Não aplicável.

PMCID: PMC4044174

Fator de Impacto da Revista: 2014 (0.26); 2013 (0.39)

Tipo: Caso clínico.

2015

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Almeida C, Almeida I, Vasconcelos C.

Autoimmun Rev. 2015; 14:1087-1096.

Doi: 10.1016/j.autrev.2015.07.012.

PMID: 26212726.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2015 (7.49)

Tipo: Artigo de revisão.

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

Silva I, Teixeira A, Oliveira J, Almeida I, Almeida R, Águas A, Vasconcelos C.

Clin Rev Allergy Immunol. 2015; 49:240-252.

Doi: 10.1007/s12016-015-8500-0.

PMID: 26142066.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2015 (5.463)

Tipo: Artigo original (com revisão da literatura)

“PREDICTIVE VALUE OF VASCULAR DISEASE BIOMARKERS FOR DIGITAL ULCERS IN SISTEMIC SCLEROSIS PATIENTS”

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

Clin Exp Rheumatol. 2015; 33(4 Suppl 91):S127-30.

Doi: Não disponível.

PMID: 26242908.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2015 (1.16)

Tipo: Artigo original.

“T AND NK CELL PHENOTYPIC ABNORMALITIES IN SYSTEMIC SCLEROSIS: A COHORT STUDY AND A COMPREHENSIVE LITERATURE REVIEW”

Almeida I, Silva SV, Fonseca AR, Silva I, Vasconcelos C, Lima M.

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Doi: 10.1007/s12016-015-8505-8.

PMID: 26445774.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2015 (5.463)

Tipo: Artigo original (com revisão da literatura)

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Silva I, Loureiro T, Teixeira A, Almeida I, Mansilha A, Vasconcelos C, Almeida R.

Eur J Dermatol. 2015; Sep-Oct 25; 25(5): 444-51.

Doi: 10.1684/ejd.2015.2605.

PMID: 26693634.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2014-2016 (2.069)

Tipo: Artigo original.

2016

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Almeida I, Oliveira Gomes A, Lima M, Silva I, Vasconcelos C.

Clin Exp Rheumatol. 2016; Feb 16.

Doi: Não disponível.

PMID: 26885625.

Fator de Impacto da Revista: 2016 (disponível em 2017); 2015 (2.724)

Tipo: Artigo original.

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Clinical Rheumatology – Manuscript ID CR-05-2016-0444

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Almeida I, Freitas AR, Lima M, Oliveira JC, Vasconcelos C.

“CELL ADHESION MOLECULES IN SYSTEMIC SCLEROSIS – WHAT SHOULD WE EXPECTED? RESULTS FROM A PORTUGUESE COHORT”

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Almeida I, Potlog N, Neves E, Lima M, Vasconcelos C.

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Melbourne, Austrália, Março de 2010.

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III Congresso Nacional de Autoimunidade | XIX Reunião Anual do NEDAI

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CAPÍTULO 1 |

APRESENTAÇÃO DA INVESTIGAÇÃO

A Esclerodermia ou Esclerose Sistêmica (ES) é uma doença complexa cuja patogénese permanece pouco esclarecida. As características clínicas são heterogêneas, o que poderá ser o reflexo do contributo de fatores patogénicos distintos, uns de natureza genética e/ou ambiental, outros imunológicos, vasculares ou fibróticos [1].

A vasculopatia, caracterizada por ativação endotelial e alteração do tónus vascular, parece ser o evento mais precoce e associa-se à presença de citocinas pró-inflamatórias, aumento da expressão das moléculas de adesão, fatores que regulam a angiogénese, perda do controle da atividade *redox*, *stress* oxidativo e hipoxia [2]. Apesar da hipoxia crónica, a angiogénese é insuficiente, havendo uma perda progressiva de capilares e alteração da arquitetura vascular [3]. Na fisiopatologia da doença estão envolvidas interações moleculares complexas e vários tipos celulares, culminando em doença vascular oclusiva grave, com isquemia tecidular e lesão orgânica [2]. A vasculopatia pode ser observada em todos os órgãos atingidos e pode preceder em meses ou anos o início de fibrose [3].

A esclerose da pele é um sinal cardinal da ES [4] e os subtipos limitado e difuso distinguem-se com base na extensão do pregueamento cutâneo [5], efetuado de acordo com os critérios de Rodman [6].

A Pré-Esclerodermia (Pré-ES) caracteriza-se pela presença de Fenómeno de Raynaud associado a auto-anticorpos específicos da doença esclerodérmica e/ou a alterações típicas na capilaroscopia periungueal, em doentes com pregueamento cutâneo normal [7]. Os doentes com alterações típicas na capilaroscopia e com anticorpos específicos da doença têm uma probabilidade entre 65.9% e 72.7% de evoluírem para ES ao fim de 5 e 10 anos, respetivamente [8].

As alterações morfológicas encontradas na capilaroscopia do leito ungueal traduzem a lesão microvascular e os padrões capilaroscópicos descritos por Cutolo *et al.* (precoce, ativo e tardio) parecem relacionar-se com o tempo de evolução da doença e, consequentemente, com a evolução da microangiopatia [9,10].

A determinação sérica de biomarcadores tem sido utilizada para avaliar a vasculopatia, mas a especificidade da maioria dos biomarcadores estudados na ES permanece desconhecida [11]. Não existem biomarcadores disponíveis que permitam efetuar um diagnóstico precoce e avaliar a atividade ou o prognóstico da doença [12] e só alguns estudos publicados na literatura os relacionaram com as características clínicas [11].

1. Objetivos

1.1 Gerais

1. Caracterizar e quantificar a microangiopatia e a lesão endotelial nos doentes com perfil esclerodérmico (Pré-ES + ES);
2. Relacionar essas alterações com os subtipos, estadios da doença, sua gravidade e outros parâmetros biológicos e imunológicos;
3. Identificar possíveis biomarcadores da doença, potencialmente úteis para a prática clínica.

1.2 Específicos

1. Utilizar a capilaroscopia para identificar e classificar a microangiopatia em precoce, ativa e tardia através da aplicação de um score proposto por Cutolo *et al.*;
2. Avaliar a lesão endotelial, de forma indireta, usando o doseamento de moléculas de adesão e da endotelina-1 (ET-1) no soro;
3. Avaliar o potencial angiogénico através do doseamento sérico de fatores pro-angiogénicos e angiostáticos;
4. Relacionar a microangiopatia com o número de linfócitos T citotóxicos e ativados, células *Natural Killer* (NK), níveis séricos de citocinas e auto-anticorpos;
5. Relacionar os parâmetros avaliados com os subtipos e tempo de evolução da doença, assim como com a sua gravidade classificada pela escala de gravidade de Medsger.

2. Questões de investigação

1. A lesão endotelial é precoce e precede a desorganização microvascular?
2. Existe relação da lesão endotelial e alterações da angiogénese (avaliadas pelos marcadores serológicos) com as alterações na microvasculatura, (avaliadas por capilaroscopia)?
3. Existe relação das lesões vasculares (avaliadas por capilaroscopia) com a ativação das células T, o desequilíbrio Th1/Th2, a diferenciação T citotóxica e as células NK?
4. Existe relação entre as alterações vasculares (avaliadas pelos marcadores serológicos) e imunológicas e o subtipo de doença, tempo de evolução, atingimento orgânico e sua severidade?
5. Os biomarcadores avaliados têm interesse na prática clínica?

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CAPÍTULO 2 |

ENQUADRAMENTO TEÓRICO

2.1 – ESCLEROSE SISTÉMICA: DA CLASSIFICAÇÃO ÀS MANIFESTAÇÕES CLÍNICAS

Embora a patogénese da ES permaneça pouco esclarecida, as hipóteses recentes defendem a existência de uma interrelação precoce entre o endotélio vascular e as células do sistema imune [1].

Apesar de descrita em 1753 por Cuzzio, só em 1847 Gintrac utilizou o nome “sclérodermie” para se referir a esta doença como uma entidade clínica, e Goetz, em 1945, confirmou o carácter multissistémico, dando-lhe o nome de “progressive systemic sclerosis” [2].

Em 1964, Winterbauer descreveu um subtipo de esclerodermia que denominou de CRST (calcinose, Raynaud, esclerodactilia e telangiectasias), posteriormente chamado de CREST por Veloy, ao associar a dismotilidade esofágica a esta síndrome [2].

Barnett, em 1978, publicou uma das primeiras classificações da doença baseada no pregueamento cutâneo, em que identificou três subgrupos, relacionando-os com diferentes prognósticos [3].

Posteriormente, outros grupos propuseram outros critérios de classificação ou modificaram os iniciais [4] mas só em 1980 surgiram os primeiros critérios de classificação validados externamente, os Critérios de Classificação da ACR (*American College of Rheumatology*) [5]. Estes critérios foram amplamente utilizados até 2013, apesar das limitações reconhecidas, altura em que surgiram os critérios da ACR/EULAR (*American College of Rheumatology / European League Against Rheumatism*) [6].

Le Roy *et al.* [7] subclassificaram a ES nos subtipos limitado e difuso com base na extensão do pregueamento cutâneo (limitada: alteração do pregueamento distal aos cotovelos e joelhos e proximal às clavículas; difusa: alteração do pregueamento proximal aos cotovelos e joelhos e distal às clavículas).

Ao longo do tempo, vários autores e, mais recentemente, a EUSTAR (*EULAR Scleroderma Trial and Research Group*) reconheceram que existe um estadio precoce da doença sem envolvimento cutâneo, que pode evoluir para a forma difusa ou limitada da ES, dando-lhe denominações diferentes: “Pré-ES”, “ES Limitada”, “Early ES” e “Very early ES” [8-11]. Estas diferentes designações representam o mesmo conceito e correspondem a doentes com Fenómeno de Raynaud associado a alterações capilaroscópicas típicas e/ou anticorpos específicos.

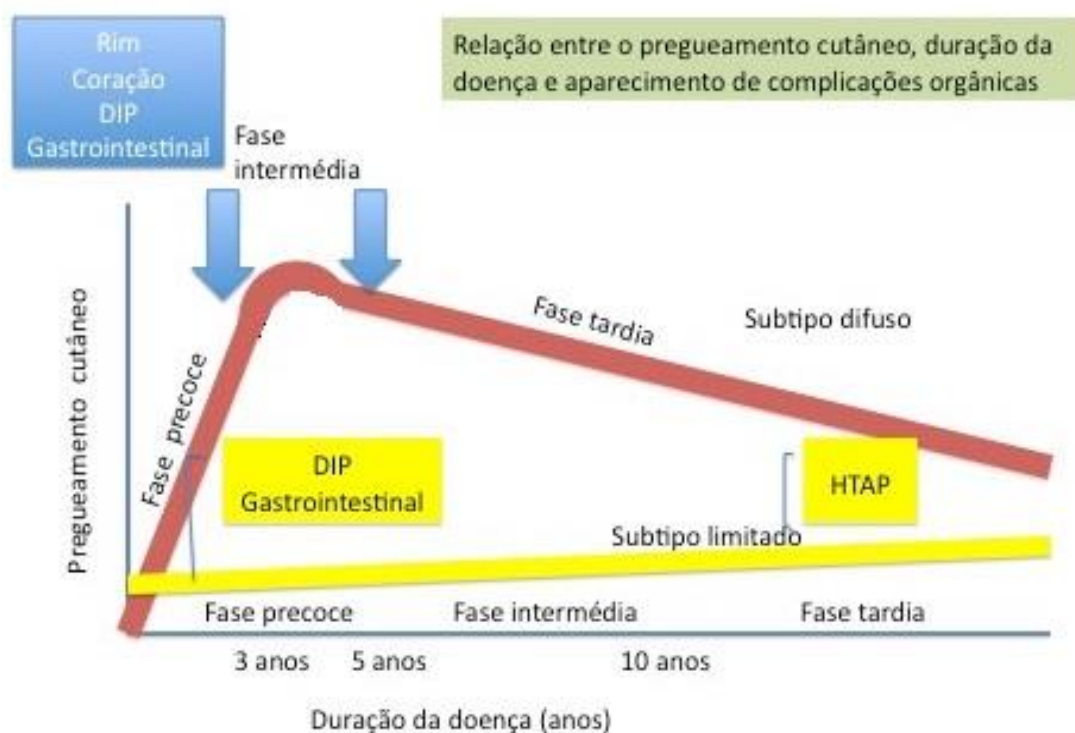
Os doentes com alterações típicas na capilaroscopia e anticorpos específicos da doença têm 65.9% e 72.7% de probabilidade de evoluírem para Esclerodermia ao fim de 5 e 10 anos, respetivamente. Se tiverem apenas padrão capilaroscópico típico associado ao Fenómeno de Raynaud, a probabilidade diminui para 22.6% aos 5 e 10 anos e se tiverem

apenas auto-anticorpos específicos da doença associados ao Fenómeno de Raynaud, passa para 21.5% e 32.3% aos 5 e 10 anos, respetivamente [9].

O termo “sine ES” foi introduzido por Poormoghim *et al.* para identificar um subtipo de doentes com Fenómeno de Raynaud ou um equivalente vascular periférico (cicatrizes ou úlceras digitais, gangrena e alterações da capilaroscopia) associado a anticorpos antinucleares e alterações orgânicas (fibrose pulmonar intersticial, hipertensão arterial pulmonar, dismotilidade esofágica ou intestinal, envolvimento cardíaco típico da ES ou falência renal compatível com crise renal esclerodérmica) não atribuíveis a outra doença. O autor considerou que este subtipo deve ser encarado como fazendo parte do espectro da ES limitada com atingimento orgânico, serologia e prognósticos semelhantes [12].

O envolvimento orgânico correlaciona-se com o subtipo da doença (limitada ou difusa) assim como com o tempo de evolução da mesma [13,14].

Figura 1 - Relação entre o pregueamento cutâneo, duração da doença e aparecimento de complicações orgânicas (adaptado de [14])



De acordo com Medsger e Steen, a doença divide-se em 3 fases (precoce, intermédia e tardia), considerando o tempo de evolução a partir do primeiro sintoma atribuído à mesma, que é diferente consoante o subtipo. Assim, a ES limitada é considerada precoce se tiver menos de 5 anos de evolução e tardia se tiver mais de dez; na ES difusa, a fase precoce

corresponde a uma evolução inferior a 3 anos e a tardia superior a seis. Os valores intermédios correspondem à fase intermédia [14].

É durante os primeiros três anos da doença difusa, altura em que a pele aumenta o seu espessamento, que surgem cerca de 90% das complicações orgânicas de foro gastrointestinal, renal, pulmonar intersticial e cardíaco mas também sintomas gerais e alterações músculo-esqueléticas. O subtipo limitado costuma ter atingimento orgânico mais discreto. A hipertensão arterial pulmonar que surge em cerca de 10% dos doentes e normalmente na fase tardia, é a complicação mais temível do subtipo limitado [15].

A gravidade da doença pode ser quantificada através da utilização da escala de gravidade de Medsger [16] que quantifica, de 0 a 4, o estado geral, a pele, o atingimento vascular periférico, o tubo digestivo, o atingimento das articulações e tendões, os músculos, os pulmões, o coração e os rins.

Quadro 1 - Escala de gravidade de Medsger (adaptado de [16])

Órgãos	0 (normal)	1 (ligeiro)	2 (moderado)	3 (severo)	4 (terminal)
Estado geral	Perda peso < 5% Hemoglobina > 12.2g/dl	Perda peso 5-10% Hemoglobina 11.0-12.2g/dl	Perda peso 10-15% Hemoglobina 9.7-10.9g/dl	Perda peso 15-19% Hemoglobina 8.3-9.6g/dl	Perda peso > 20% Hemoglobina < 8.3g/dl
Vascular periférico	Sem Raynaud (FR) ou FR que não necessita de tratamento	FR que necessita de vasodilatadores	Cicatrizes na polpa dos dedos (<i>pitting scars</i>)	Úlceras digitais	Gangrena digital
Pele	mRSS 0	mRSS 1-14	mRSS 15-29	mRSS 30-39	mRSS > 40
Articulações / tendões	Distância dedo – eminência tenar (DDT) <0.9cm	DDT-1.9cm	DDT-2-3.9cm	DDT4-4.9cm	DDT> 5cm
Músculos	Força muscular proximal normal	Fraqueza muscular proximal ligeira	Fraqueza muscular proximal moderada	Fraqueza muscular proximal severa	Necessária ajuda para deambular
Tubo digestivo	Exames radiográficos do esófago e intestino delgado normais	Hipoperistaltismo esófago distal ou anomalias do intestino delgado	Necessidade de antibióticos para o supercrescimento bacteriano	Má absorção Pseudoclusão	Necessidade de hiperalimentação artificial
Pulmão	DLCO > 80% FVC > 80% Ausência de fibrose	DLCO 70-79% FVC 70-79% Crepitações lobares inferiores Fibrose no Rx tórax	DLCO 50-69% FVC 50-69%	DLCO < 50% FVC < 50%	Oxigenoterapia
	PSAP < 35mmHg	PSAP 35-49mmHg	PSAP 50-64mmHg	PSAP > 65mmHg	
Coração	ECG: normal	ECG: defeitos na condução	ECG: arritmias	ECG arritmia com necessidade de terapêutica	Insuficiência cardíaca congestiva
	FEVE > 50%	FEVE 45-49%	FEVE 40-44%	FEVE 30-40%	FEVE < 30%
Rim	Sem crise renal	Hx crise renal	Hx crise renal	Hx crise renal	Hx crise renal
	Creatinina < 1.3mg/dL	Creatinina < 1.5mg/dL	Creatinina 1.5-2.4mg/dL	Creatinina 2.5-5.0mg/dL	Necessidade de diálise

FR: fenómeno de Raynaud; mRRS: score de Rodnan modificado; DDT, Distância dedo –eminência tenar; DLCO: capacidade de difusão do monóxido carbono; FVC: capacidade vital forçada; PSAP: pressão na artéria pulmonar estimada por ecocardiograma; ECG: electrocardiograma; FEVE: fração de ejeção do ventriculo esquerdo; Hx: história

Esta escala é uma escala composta, medindo atividade e dano em cada um dos nove órgãos avaliados, e não tem um *score* global; tem algumas limitações, nomeadamente quando usada como medida de atividade (dificuldade em distinguir a atividade do dano), mas tem utilidade para medir a gravidade e a função e para estabelecer um prognóstico da doença [16, 17].

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2.2 – ESCLEROSE SISTÉMICA: DO ENVOLVIMENTO ORGÂNICO AO TRATAMENTO – O PROTÓTIPO DA DOENÇA REFRACTÁRIA

Artigo publicado

“SYSTEMIC SCLEROSIS REFRACTORY DISEASE: FROM THE SKIN TO THE HEART”

Almeida I, Faria R, Vita P, Vasconcelos C.

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Abstract

Systemic sclerosis or scleroderma (SSc) is an heterogeneous disease involving the connective tissue and the microvasculature with fibrosis and vascular occlusion. It is difficult to define refractory SSc once it is itself a paradigm of a refractory condition: there is no evidence of when to act to stop the progression to fibrosis and irreversible microvascular damage. There is no definition of refractory disease in SSc and to propose a definition we used mainly the Medsger severity index and the EULAR 2009 treatment recommendations from the skin to the heart through peripheral vascular, musculoskeletal, gastrointestinal, renal, pulmonary hypertension and interstitial lung disease. We used some clinical setting reflecting the different reasoning when there is probable refractory disease and finally we briefly pointed out some available treatment options to refractory disease. With this reflection, we would like to open paths to a broader discussion.

KEYWORDS: Systemic sclerosis, Refractory autoimmune disease, Refractory scleroderma.

Introduction

Systemic sclerosis or scleroderma (SSc) is a rare and heterogeneous disease that involves the connective tissue and the microvasculature with fibrosis and vascular occlusion [1]. The skin is almost universally affected and the organs are more or less involved depending on the disease subgroups, mainly peripheral vascular, lung and gastrointestinal tract.

Although the mortality has been changed with the ACE inhibitors on scleroderma renal crisis [2] and new vasodilators on pulmonary hypertension [3,4], there still are few trials showing evidence of treatment for other devastating manifestations. Stopping the progression and changing the natural history of the disease is the aim of treatment on any autoimmune disease but we haven't found yet when and how to treat it: we should stop scleroderma before the fibrotic and irreversible microvascular damage stage!... we should stop scleroderma before it is scleroderma!... [5]. This could be seen as a definition of refractory disease on itself and highlighting the idea that "no drug has been proven ineffective until it has been tried in scleroderma!" (E. Bywaters). It is not coincidence that SSc is the second autoimmune condition in which autologous stem cell transplant has been used [6].

Since there are none or few clinical trials showing evidence of treatment once the diagnosis is certain every patient should be considered for entering clinical trials, especially for diffuse cutaneous SSc (dcSSc).

Virtually any cytokine and fibroblast, endothelial or autonomic vascular mediator are potential targets for intervention [7], several cases or case series reports have shown some kind of benefit and there are 140 clinical trials going on different features of SSc. They are out of the scope of this review and we focused on the main clinical relevant data and expert-based approach to discuss refractory systemic sclerosis.

Refractory Systemic Sclerosis

Traditionally, we can define refractory SSc as a non-response to standard treatment in a specific moment in time. We cannot use a static definition since medicine is an ever evolving subject and over time new therapeutic options become available. What today is refractory, may not be tomorrow, according to the state of art. There is no definition of refractory disease in systemic sclerosis but extrapolating from other diseases and SSc disease activity and damage scores, we could consider refractory disease:

- a) Disease progression or inadequate response (for some organs it can mean absence of response while for others a partial response is acceptable) despite optimized therapy according to EULAR 2009 recommendations [8], whatever the organs or systems involved
- b) Intolerance to therapy despite clinical response
- c) When the disease manifestation has no effective medical therapy, and
- d) After the exclusion of occult conditions or comorbidities that may mimic disease progression or non-response to therapy.

There are few tools to evaluate treatment response and we chose to use Medsger severity score (Table 1) [9] because it is a composite index measuring both activity and damage (showing the difficulty distinguishing each other on SSc) on each of 9 organ system separately; there is no overall score. It has limitations as a measure of activity and it is not easy to use it daily but is quite useful measuring severity and function and establishing prognosis [10].

Thus, whatever organ is involved, we'll consider lack of response if the score rises and response if the score is decreased. When the score remains the same there are three possible scenarios: either we have a response to treatment but the score doesn't have sensitivity to change (for example, Raynaud's can get better with vasodilators still scores 1 in Medsger index [9]); or we could be just measuring irreversible damage and not disease activity; or we have a partial or non-response and these might represent probable refractory disease.

Since systemic sclerosis is heterogeneous we are going through every single organ or system and discuss what could be refractory disease, using some clinical examples, and briefly pointing out which available and upcoming treatments and options are more tailored to each case.

Table 1 – Medsger Scleroderma severity scale [9]

Organ system	0 (normal)	1 (mild)	2 (moderate)	3 (severe)	4 (end stage)
General	Weight loss <5%	Weight loss 5-10%	Weight loss 10-15%	Weight loss 15-19%	Weight loss > 20%
	Hemoglobin > 12.2g/dL	Hemoglobin 11.0-12.2g/dL	Hemoglobin 9.7-10.9g/dL	Hemoglobin 8.3-9-6g/dL	Hemoglobin < 8.3g/dL
Peripheral vascular	No Raynaud phenomena (RP) or RP not requiring vasodilators	RP requiring vasodilators	Digital pitting scars	Digital tip ulcerations	Digital gangrene
Skin	mRSS 0	mRSS 1-14	mRSS 15-29	mRSS 30-39	mRSS > 40
Joint/tendon	Finger-tenar distance <0.9cm	FTD 1-1.9cm	FTD 2-3.9cm	FTD 4-4.9cm	FTD > 5cm
Muscle	Normal proximal muscle strength	Proximal muscle mild weakness	Proximal muscle moderate weakness	Proximal muscle severe weakness	Ambulation aids required
GI tract	Normal esophagram	Distal esophageal hypoperistalsis and small bowel abnormalities	Need for antibiotics for microbial proliferation	Malabsorption, pseudocclusion	Hyperalimentation required
Lung	DLCO > 80%	DLCO 70-79%	DLCO 50-69%	DLCO < 50%	Oxygen required
	FVC > 80%	FVC 70-79%	FVC 50-69%	FVC < 50%	
	No fibrosis	Lower lobe rales			
		Fibrosis seen on XR			
	ePAP < 35mmHg	ePAP 35-49mmHg	ePAP 50-64mmHg	ePAP > 65mmHg	
Heart	EKG: normal	EKG: conduction defect	EKG: arrhythmia	EKG arrhythmia requiring treatment	Congestive heart failure
	LVEF > 50%	LVEF 45-49%	LVEF 40-44%	LVEF 30-40%	LVEF < 30%
Kidney	No renal crisis	Hx renal crisis	Hx renal crisis	Hx renal crisis	Hx renal crisis
	Creatinine < 1.3mg/dL	Creatinine < 1.5mg/dL	Creatinine 1.5-2.4mg/dL	Creatinine 2.5-5.0mg/dL	Dialysis required

GI, gastrointestinal; RP, Raynaud's phenomena; mRSS, modified Rodnan score; FTD, finger-tenar distance (FTP, finger-to-palm distance in flexion); DLCO, diffusing capacity for carbon monoxide, % predicted; FVC, forced capacity vital, % predicted; ePAP, estimated pulmonar arterial pressure; EKG, electrocardiogram; LVEF, left ventricular ejection fraction; CHF, congestive heart failure.

From the Skin to the Heart

Skin

Skin involvement in SSc defines the two major clinical subsets: limited cutaneous SSc (lcSSc) which is more frequent and diffuse SSc (dcSSc) scleroderma. The skin involvement in lcSSc is not the most troublesome feature and in most cases it is primarily managed by local measures (like paraffin baths [11,12]), although it can respond to systemic treatment when it is used for other organ involvement as we have seen in some of our patients. In dcSSc the skin disease usually plateaus or improves over 3-5 years [11] but it can be quite debilitating and deforming [1,11] and it should be treated, although it is a challenge since no treatment has shown unequivocal safety and efficiency.

The skin involvement is most frequently assessed by the modified Rodnan skin score (mRSS) which is a semi-quantitative score using 17 different skin sites that score from normal thickness (0) to complete thickness preventing pinching (3) [13]. It was validated by weighing skin biopsies and measuring collagen content [14], it assesses skin thickness rather than tethering and has prognostic implications on visceral complications and global outcome [15].

It is not easy to define skin involvement as refractory since it usually improves spontaneously with time after peaking at about 18 months [11]. Calcinosis rather than thickening have no effective treatment available at all and it's considered refractory *per se* [8]

According to the EULAR 2009 recommendations methotrexate or cyclophosphamide has proven efficacy for dcSSc skin involvement [8] and should be used one or another depending on the severity of visceral involvement.

Mycophenolate mofetil (MMF) had benefit on skin involvement after antithymocyte globulin [16] and later on open-label studies [17,18]. Some randomized clinical trials have finished recruiting patients and results on MMF are awaited. Although MMF is not recommended on the EULAR 2009 consensus, we point out that it was used only data published till 2006 and, specifically on MMF, expert-opinion recommends it when no evidence of internal organ involvement or overlap features or connective disease are present [11].

Proposal of definition of Refractory Skin involvement, when:

a) the score worsen on full dose treatment (at least for 3 months), or

b) the score doesn't improve on full dose treatment (at least between 6 months and a year).

Other immunosuppressive drugs were tested in dcSSc: cyclosporine seemed to be beneficial on skin involvement but precipitated renal crisis on some patients and it should not be used [11]; azathioprine was also beneficial after cyclophosphamide [19].

Small open-label reports showed benefit of intravenous immunoglobulin on skin involvement in SSc and some of them with longer courses of disease (> 3 years) [11]. Rituximab also seemed to be beneficial in small trials [20,21].

As an antifibrotic therapy, imatinib has showing benefits on reducing skin fibrosis in vitro [22], in a number of case reports [23] and in a recent open-label clinical trial [24]. D-penicillamine was classically used as antifibrotic and it has proven benefits on some reports and retrospective studies but not on RCT's. It has several severe lateral effects and it should be considered only for selected patients [11].

More recent drugs have shown beneficial results on case reports on skin involvement but larger trials are need or ongoing (tocilizumab [25,26], abatacept [27] and others).

Raynaud phenomena and digital ulcers

Vascular involvement in SSc extends from Raynaud phenomena (RP) with no need for vasodilators to digital gangrene. Digital ulcers are a major morbidity in some patients and traditionally thought to be more common in lcSSc, but more recently, risk factors have been assessed and male sex, presence of pulmonary arterial hypertension (PAH), involvement of the oesophagus, diffuse skin sclerosis (only when PAH was present), anti-Scl70 antibodies, young age at onset of RP and elevated erythrocyte sedimentation rate (ESR) significantly impacted on the appearance of digital ulcers [28] and in a large SSc center cohort digital ulcers were significantly more frequent in dsSSc than in lcSSc (27.5% vs 13.1%) [29].

Assessment for RP and digital ulcers is mainly clinical. RP severity is based on patient's perception and more accurately by the Raynaud's Condition Score (RCS) that combines the number and duration of attacks with VAS assessment of severity [30]. Pitting scars, digital ulcers and, eventually, ischemia or gangrene are assessed clinically by the doctor. There are some functional vascular tests to assess RP but few are validated or widely available (color laser Doppler ultrasound measurements, cold challenge, and assessment of vasodilatory capacity) [30]. The nailfold capillaroscopy has great diagnostic value in the early stages [31], but its prognostic rule is not well established, although some studies suggest that worsening capillaroscopy pattern significantly predicts digital ulcers appearance [31,32].

To classify RP or digital ulcers as refractory to standard therapy it should be excluded concomitant disease: concomitant proximal artery disease (outlet syndrome and ulnar artery commitment which is commonly associated with SSc [33]), vasculitis, thrombotic disease

(antiphospholipid antibodies, for example) or calcinosis that prevent ulcer healing [33]. Telangiectasia are frequent especially in lcSSc and are considered refractory once there is no available treatment.

For treatment, EULAR 2009 recommendations [8] indicate nifedipine for RP and iloprost for severe RP, intravenous iloprost for active digital ulcers and bosentan to prevent digital ulcers, especially those with multiple ulcers, and after failure of calcium antagonist and prostanoid therapy. It also recommends losartan to lower frequency and severity of RP secondary to SSc. Even though not on EULAR recommendations it is usual practice to advise modification on life style: avoid cold (gloves, glove warmers, clothes), microtrauma, stress, caffeine, vasoconstrictor drugs (beta blockers), cease smoking [33] and take poliunsaturated fatty acids supplements (evening primrose oil) [33-35].

Proposal of definition of Refractory Digital Vasculopathy, when:

- a) RF worsens despite non pharmacological and drug therapy, or***
 - b) pitting scars worsen in number, or***
 - c) any evolution to digital ulcer and ischemia on treatment, or***
 - d) no response to treatment of new digital ulcers or ischemia.***
-

Other drugs have proven efficacy in a small trial but they lack larger studies or their results are awaited: sildenafil, a phosphodiesterase inhibitor, reduced the number of RP flares [36]; fluoxetine [33]; atorvastatin [33]. The topical micro emulsion gel of nitroglycerin MQX-503 proved to be effective on a large multicenter RCT with less lateral effects than classical topical nitrates [37] but it's not widely available yet.

Surgery is sometimes the choice for refractory cases mainly if there is infected and necrotic tissue or even if calcinosis is preventing ulcer healing [33,38]. Cervical sympathectomy is only rarely recommended. Digital sympathectomy is proving to be effective [38,39] for elective cases but it is a highly specialized procedures and performed only on very few centers.

Clinical Case: refractory disease is not a static definition

A female patient born in 1962, had RP since 9 years old. In 1999 she was diagnosed with lcSSc with digital ulcers and acro-osteolysis of the 5th finger that were refractory to nifedipine, pentoxifiline, topical hand nitroglicerine, aspirin and iv pulsed iloprost since the previous year. In 2000, her 2nd and 3rd fingers were amputated and until 2008 she was still

having recurrent digital ulcers despite treatment. She started on bosentan once it became available and since then she had less digital ulcer.

- This case illustrates that refractory disease can be different depending on available drugs, highlighting the dynamic state of refractory disease definition.

Musculoskeletal disease

Musculoskeletal involvement is frequent in SSc especially arthralgia in early phases and tendon sheath involvement in dcSSc. Together with skin involvement, musculoskeletal impairment results on contractures and deformities in the hand and consist of decreased flexion of the metacarpophalangeal joint, decreased extension of the proximal interphalangeal joint and decreased abduction of the thumb. Frank synovitis is quite rare and when effusion is present it is generally mechanical, but if it resembles rheumatoid arthritis even without a true overlap, it should be assessed with modified Ritchie index or DAS. One of the features of dcSSc is tendon sheath inflammation with friction rub mainly on wrists, knees and ankles and olecranian bursitis. Every dcSSc patient should be screened with passive and active palpation on the main sites involved, once it has predictive value for more severe skin, heart and kidney disease and these patients should be candidates for immunosuppression [40]. There aren't many tools to assess it and the finger-tenar distance (FTD) although recommended as secondary outcome in clinical trials was only validated in one. It doesn't measure only the tendon involvement but also the skin tightness and joint contractures but it's easier to use than other combined measures later proposed [41].

There are two kinds of muscle involvement in SSc: SSc related myositis, which is extremely rare and generally associated with dcSSc; and dermatomyositis / polymyositis – SSc overlap which occurs at least in 16% [1]. SSc related myositis mainly courses with proximal myalgia, moderate muscle enzymes elevation (two- to three-fold) and electromyography inflammation pattern. If proximal weakness appears, only biopsy rules out dermatomyositis / polymyositis overlap.

There are no EULAR 2009 recommendations for joint, tendons or muscle SSc disease [8], but not surprising expert opinion recommends classical DMARD's as MTX, MMF and AZA [11]. Although no specific treatment is recognized for tendon friction rub, most of our patients responded to classical DMARD's therapy (not published data).

Proposal of definition of Refractory Musculoskeletal, when:

- a) worsening or not responsive arthritis despite conventional DMARD's treatment, or***
 - b) no decrease or increase in FTD, or***
 - c) same or worsened number of friction rub tendons, or***
 - d) persistent myositis despite treatment.***
-

Each of the different musculoskeletal features can be refractory and, in the absence of other visceral involvement that would indicate other approach, patients should be addressed as other similar rheumatologic diseases: if arthritis is the main feature and is refractory to conventional DMARD's it could be eligible to anti-TNF [1], rituximab, abatacept, tocilizumab; as if myositis is refractory, with or without overlap, other options as cyclophosphamide, IvIg and rituximab could be considered. As in other rheumatologic disease, steroids may be beneficial in some dcSSc features (lung [1], joints and myositis [1,8]) but prednisolone dosage above 10mg/day is associated with increased scleroderma renal crisis [42], thus patients on steroids should be carefully monitored for blood pressure and renal function [8].

The role of rehabilitation on SSc's musculoskeletal disease is an orphan issue in literature: Poole J. reviewed the published literature and there are few and small studies [12]. Paraffin wax bath with or without exercise might improves finger extension, perceived stiffness and skin elasticity on early and long-standing disease. Dynamic splints were tried but deceptive compared to other conditions and it worsened RP in some patients. Range of motions self-administrated exercises and connective tissue massages with joint manipulations appears to improve joint motion and quality of life. Positive results were also recently attained with aerobic exercise and strengthening or resistive exercise even in patients with mild to moderate myositis. Small mouth opening was also reviewed and range of motion and stretching exercises were effective in increasing mouth opening with improvement in speaking, eating and oral hygiene ability [12].

Clinical Case: time must be given before refractory disease classification

A 57 years old female was diagnosed on the emergency room with lcSSc with 2 years of onset RP, sclerodactyly, hand contracture (FTD 6cm), restricted mouth aperture and ANA + 1/640 nucleolar pattern (negative anti-Scl70, anticentromere, anti-PMScI). She also had 9 months progressive severe SSc-related myositis with proximal tetraparesia, cervical and pharyngeal weakness (she couldn't lift her arms, walk without help or stand her neck and she was choking with her own saliva with nasal regurgitation). She was started on IvIg cycles, prednisolone 10mg/day and got slightly better (walking without help) and she started on MTX

10mg sc/week but only did one dose because of a choking episode that led to aspiration and severe respiratory failure needing non-invasive ventilation. In the doubt of probable parenchyma lung involvement, she was started on cyclophosphamide. In the next 2 months she got progressively better being able to walk without aid or cervical necklace, pinching enough to cook, needing non-invasive ventilation only at sleep time but just able to swallow her own saliva. She continued on cyclophosphamide monthly and rehabilitation therapy.

- Could we consider this case refractory? This is a lcSSc patient and severe SSc-related myositis with pharyngeal weakness which is rarely so severe and often overlooked when milder. Despite apparently responding to treatment after 6 months of cyclophosphamide, the pharyngeal weakness was the same: was it refractory or damage?

One year after, she continued on rehabilitation therapy for deglutition and general exercise and 1 year after she could swallow any food without symptoms.

- This rare case highlights that some features are quite severe and may need more time before being considered refractory, and rehabilitation was crucial.

Gastrointestinal (GI) Disease

Gastrointestinal features in SSc involve impaired gastrointestinal motility and vascular malformation (mainly watermelon stomach and bowel angiodysplasia) [1]. Dysmotility is second in frequency only to the skin: esophageal involvement occurs in 75-90%, stomach involvement in at least 50%, small bowel involvement in 40-70%, colon involvement in 20-50%, and anorectal involvement in 50-70% [43]. Dysmotility promotes reflux and bacterial overgrowth which can later lead to malabsorption and vitamin deficiencies [1,43]. Although most of the patients have some kind of GI involvement, in very few it is the main debilitating feature.

Symptoms should be actively asked on each clinical visit and if there is any evidence of dysphagia, heartburn, early satiety, bloating, vomiting, diarrhea, constipation, intestinal distention or fecal incontinence, complementary studies should be performed [44]. For esophageal involvement, once it is frequently asymptomatic, all patients should have a baseline motility examination by cine/video barium esophagram or manometry to exclude or establish esophageal dysfunction [44]. If esophageal involvement is suspected every patient should be screened not only to assess functional impairment but to exclude other concomitant disease or complication (esophageal reflux disease or carcinoma) with endoscopy. This is also true for stomach (emptying scintigraphy and endoscopy), small bowel (duodenal diameter on x-ray, hydrogen breath and D-xylose test, abdominal plain x-ray, small bowel x-ray follow-through and if appropriate jejunal culture), colon (barium enema and colonoscopy or sigmoidoscopy) and anorectal disease (motility and anoscopy) [44].

No immunosuppression, antifibrotic or other background therapy has proven to be effective on GI tract SSc involvement and only symptomatic therapy is the basis for EULAR 2009 recommendations (proton pump inhibitors, prokinetic drugs and, when malabsorption is caused by bacterial overgrowth, rotating antibiotics) [8]. Cisapride has shown to be beneficial on gastric emptying and esophageal motility in short-term courses but arrhythmias are a severe side effect [8].

Proposal of definition of Refractory Gastrointestinal Disease, when:

a) maintaining or worsening symptoms despite therapy, even when there is functional tests improvement.

In some small open-label trials octreotide has proven to be effective on small intestine and bacterial overgrowth disease, especially on intramuscular preparation [45,46]. More recently reports considered intrapyloric botulinum toxin injections, placement of a jejunostomy tube, or implantation of a gastric stimulator, as more invasive options [47].

Clinical Case: One system, different responses to treatment

A 14 years old girl presented with dcSSc with skin, peripheral vascular, joint and muscular involvement and was started on MTX 15mg sc/week. Two years later she developed dysphagia and manometry showed esophageal hypomotility. Dysphagia responded to prokinetics for almost 2 years when heartburn with regurgitation, anorexia and early satiety developed and stomach scintigram showed 19% emptiness. She continued on prokinetics and was started on cyclophosphamide. Five months later despite maintaining dysphagia, the early satiety got better and the stomach scintigram improved to 50% emptiness.

- This catastrophic case illustrates the refractory esophageal involvement but apparently without gastric refractory disease yet, highlighting how GI involvement must be seen as separate segments with different approaches and the potential role of immunosuppression therapy on GI SSc disease. No therapy is available now for the progression of this 18 years old young woman and it sadly seems that she is sometime going to need parenteral nutrition.

Renal

Mild renal SSc involvement is frequent in SSc, manifesting as mild renal dysfunction, proteinuria, and hypertension [48]. More severe is scleroderma renal crisis (SRC), defined as the new onset of accelerated arterial hypertension and/or rapidly progressive oliguric renal failure during the course of systemic sclerosis. About 80% [1,49] occurs in patients with diffuse scleroderma, and patients with greater risk are: early diffuse scleroderma; rapidly progressive skin thickening and mRRS ≥ 20 points; new anemia; presence of anti-RNA polymerase III autoantibodies [48]; friction rub tendons; large joint contractures at *baseline*; new heart events (pericardial effusion, congestive, heart failure) [50]; and history of taking moderate to high dose corticosteroid (e.g. prednisolone or equivalent at > 15 mg/day) in the previous 3 to 6 months [8,42,48]. In up to a quarter of patients with SRC, the diagnosis of systemic sclerosis is made at the time of the renal presentation.

Patients at risk should monitor arterial pressure once or twice a week, ideally screen abnormalities with an urine strip at home and measure creatinine regularly [51,52].

According to the EULAR recommendations the use of ACE inhibitors is mandatory, despite no controlled trials have been done, once it changed mortality in SSc compared to 40 years ago [1,48]. ACE inhibitors are probably useful not only as antihypertensors but also by changing the hyperreninemic status in SRC [53]. The goal for treatment is to bring the blood pressure to normal range while avoiding hypotension [48]. Captopril is the easiest to titrate blood pressure initially, before the switch to a longer acting ACE inhibitor. Creatinine may still rise after blood pressure control and even if suspected to be due to the ACE inhibitor stopping it during a renal crisis never improves renal function [48]. The benefit of ACE inhibitors extends even to those who have to started dialysis [48,49].

Not all patients respond promptly to an ACE inhibitor and other antihypertensive drugs should be used [51]. Angiotensin II receptor blocking agents and calcium channel blockers can also be safely added if required once ACE inhibitors are at full dose, but may not be sufficient to control disease in the absence of ACE inhibitor therapy. Other antihypertensive classical drugs (clonidine, alpha/beta-blockers and minoxidil) [51] are also helpful for refractory hypertension but beta-blockers should be avoided. Some of the classical antihypertensive drugs were used to control hypertension on SRC before ACE inhibitors were available and these were the first to change survival.

Around 10% of SRC are normotensive but also seems to respond to ACE inhibitors, in lower doses to prevent hypotension. In any dcSSc patients with new renal insufficiency, microangiopathic hemolytic anemia, thrombocytopenia, or other suggestions of renal crisis,

even in the absence of malignant hypertension, normotensive SRC should be considered [48,51].

For every other organ we used Medsger severity score [9] for assessing possible refractory disease and although it is useful to assess possible damage and evolution to chronic renal failure, it is not applicable to SRC. Several factors have been already mentioned as predictors of developing or poor prognosis of SRC, but when should it be thought to be refractory? Like in other microangiopathic conditions as malignant hypertension we used “maintained microangiopathy dysfunction even when blood pressure is already controlled” as refractory disease criteria.

Proposal of definition of refractory renal disease, when:

a) blood pressure is not controlled in the first 72 hours on maximal ACE inhibitor (or it's not near baseline levels)

(this time cut-off is based on published poor outcome on renal function when there is no blood pressure response)

b) worsening or failure to improve renal function despite treatment

(isolated increase of creatinine level when starting ACE inhibitor should not be seen as progression in Medsger score, once it can normalize or decrease spontaneously)

c) need for dialysis

c) maintained microangiopathy dysfunction even when blood pressure is already controlled

Risk factors for poor outcome are serum creatinine greater than 3 mg/dL before starting an ACE inhibitor [48,53], male gender, older age and presence of congestive heart failure. However, in presence of potential refractory disease, other renal comorbidities should be excluded. One word to ANCA vasculitis SSc-associated: mimics normotensive SRC but develops after several years of SSc [54], and should not be seen as coincidence if some SSc features are not typical (high fever and inflammation markers and normotensive glomerulonephritic renal failure with no explainable cause) and there is ANCA detection [55].

Intravenous prostacyclin has been reported in small trials to increase renal perfusion [51,52] and may also help to normalize blood pressure. Therefore, some centers recommend inclusion of continuous intravenous iloprost during the hypertensive phase of the renal crisis, with discontinuation when renal function improves or at initiation of dialysis. Plasma exchange can be used in profound microangiopathic haemolytic anaemia [48,51].

Temporary haemodialysis is required in some patients and renal transplantation may be an option for patients who still require dialysis at 2 years [51]. Renal transplant survival rates are similar to transplant survival rates for other connective tissue diseases, and patient

survival is better in those transplanted than in those on the waiting list for transplantation for end-stage renal failure due to scleroderma renal crisis. Recurrence of scleroderma renal crisis is rare in patients with native kidneys compliant to medication and uncommon (5%) in transplant patients [49].

Lung

The two main sets of pulmonary involvement in systemic sclerosis are pulmonary arterial hypertension and interstitial lung disease and together replaced SRC as the main causes of systemic sclerosis related deaths and, so pulmonary arterial hypertension, forced volume vital capacity < 80% of normal and decreased DLCO are independent risk factors for reduced survival in SSc patients.

Pulmonary arterial hypertension in SSc

Pulmonary arterial hypertension (PAH) related to SSc is progressive and can occur isolated or associated with interstitial lung disease. When isolated is mainly in females, related to lcSS, telangiectasia and anti-centromere antibodies [56]. Other risk factors are advanced age, longer course of disease, digital ulcers, low capillaries density on nail fold capillaroscopy and low DLCO [56,57].

Once symptoms occur late, screen is crucial. Instead of waiting till effort breathless, precordial pain, pre-syncope or frank right heart failure appears [58], frequent echocardiogram should be performed. Although EKG, chest X ray and pulmonary function test alterations can predict right ventricular overload [56, 59], the echocardiogram shows earlier signs and is the standard screening test but the cutoff of estimated systolic arterial pressure (sPAP) that should lead to right heart catheterization is not consensual [59]. Although invasive, right heart catheterization is the gold standard for PAH diagnosis and should be always completed with vasoreactivity challenge [59] which is invasive. It should be kept in mind that right ventricular dysfunction determines the outcome associated with pulmonary hypertension and occurs at lower pulmonary pressures than in patients with primary pulmonary hypertension, suggesting that the contribution of diastolic dysfunction is hypothetically related to myocardial SSc involvement.

Since there is recent effective treatment, other non-invasive tools became needed to monitor disease progression and treatment response. The assessment of clinical functional class is one of them and the exercise capacity is easily and reproducibly assessed by the 6-min walk test which relates to disease progression and survival. Haemodynamic parameters

can be monitored by echocardiogram. Although the brain natriuretic peptide (BNP) and pro-BNP blood levels can correlate with right heart overload and hyperdynamic status, there is no evidence yet that it should be serially measured [56].

To classify as refractory PAH to standard therapy it should be excluded concomitant disease, for example, pulmonary venoocclusive disease which is a cause for pulmonary hypertension.

The PAH management involves general measures (oxygen supplementation, diuretics for edema and anticoagulants) and specific treatment based on two criteria: WHO functional classification (Table 2) [60] and responsiveness to acute vasodilator testing.

Table 2 – Functional classification of pulmonary hypertension modified after the New York Heart Association functional classification according to the WHO 1998 [60]

Class I	Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnoea or fatigue, chest pain, or near syncope.
Class II	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain, or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnoea or fatigue, chest pain, or near syncope.
Class IV	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnoea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

No immunosuppression has proven to be effective on SSc-related PAH. Since EULAR 2009 recommendations, there have been some changes on the ERS/ESC Pulmonary Hypertension guidelines [60] that should be considered.

Although long-term beneficial response to calcium channel blockers treatment in vasoreactive patients is seen less often in SSc-related PAH than in idiopathic PAH, it should be the first option to be used in patients on WHO class I to III.

Bosentan improved survival compared with historic controls and was associated with a delay in the time to clinical worsening.

The experts advise sildenafil in whom bosentan was ineffective or could not be used for safety reasons. Other endothelin-receptors antagonists (ambrisentan) and phosphodiesterase

type-5 inhibitor (tadalafil) can be considered. Sitaxentan was recently discontinued for new patients after fatal hepatic failure reports [61].

Intravenous prostacyclin analogs are reserved for severe (WHO class IV and may be considered in class III) or refractory cases due to the administration by continuous intravenous infusion, the high number of possible complications (namely, pulmonary edema in patients with left heart disease or venocclusive disease) and the high costs.

Inhaled iloprost can be another option in WHO class III as it showed an increase in exercise capacity and improvement in symptoms.

Proposal of definition of refractory SSc-related PAH (based on PAH inadequate response criteria), when:

a) patients that still progress in the Medsger disease severity scale (measured by the estimated pulmonary arterial pressure) or

b) worsening in 6-min walk test or

c) WHO functional class II or III stable and not satisfactory or instable and deteriorating, or

c) WHO functional class IV not rapidly improving to class III or better.

In the case of inadequate clinical response sequential combination therapy should be considered, on a case to case basis in referral centers.

There are reports of imatinib used in association with endothelin antagonists and phosphodiesterase type-5 inhibitors that improves refractory PAH [62-64].

Autologous hematopoietic stem cell transplantation, after high-dose cyclophosphamide or total body irradiation and antithymocyte globulin therapy, is also an option in refractory cases with nearly 50% response rate [65]. While the results of ongoing trials are pending, the high rates of toxicity and mortality compared with other therapeutic options, restricts its use. Balloon atrial septostomy as a bridge to lung or heart-lung transplantation can be considered in selected patients as end-stage option.

There are several clinical trials ongoing on pulmonary hypertension in SSc that may help in the future to define a new set of recommendations.

Clinical setting: Refractory Disease needs exclusion of mimetics

In 2004, a 56 years-old Caucasian woman with anti-centromere positive SSc diagnosed two years before, developed progressively worsened dyspnea on minimal effort. Cardiac sounds, EKG and chest X-ray led to echocardiogram that confirmed right ventricular hypertrophy and dilation and showed sPAP of 68mmHg. Angio high-resolution thoracic CT

demonstrated patchy reticulonodular interstitial opacities, thickened interlobular and intralobular septae, and excluded pulmonary embolism.

Right-heart catheterization confirmed pulmonary hypertension (60mmHg) and no significant vasodilation was found with inhaled NO. Inhaled iloprost was started and the patient's clinical status rapidly worsened with acute pulmonary edema.

- This patient had venocclusive pulmonary disease, which is a rare condition, but may mimic refractory disease and is of clinical significance since iloprost and other prostacyclin analogs are considered to be contra-indicated.

Interstitial lung disease in SSc

Non-specific interstitial pneumonitis is the most common interstitial lung disease in SSc and is the main indication for immunosuppression in this disease [66,67]. The clinical manifestation are fatigue, dry cough and effort breathless, which are unspecific and not discriminative and can be absent in its early phases. The most common findings are dry bilateral lower lobe rales. They appears mostly in dcSSc and associated with Scl70 antibodies [1].

An accurate evaluation selects the higher risk patients who would benefit from treatment [68]. It should be instituted earlier in the disease course in hope of preventing progression to more than 20% extent on high resolution thorax CT and less than 70% forced vital capacity [68]. The best result that can be achieved is stabilization rather than improvement, which tends to be slow and small changes in symptoms, lung function, and/or radiographic appearance can be masked by the effect of existing fibrosis [69].

Despite its known toxicity, and modest therapeutic benefit, cyclophosphamide is still the only standard therapy recommended by EULAR 2009 [8]. It can be followed or not by maintenance therapy with azathioprine [70,71] or MMF [72-76].

In patients not eligible for cyclophosphamide, azathioprine has shown beneficial results, although not as good as cyclophosphamide [77,79].

Proposal of definition of Refractory SSc-related interstitial lung disease, when:

a) progression of lung fibrosis on high resolution thoracic CT on treatment, or

b) worsening of diffusing capacity for carbon monoxide and forced vital capacity, despite therapy.

There are very few trials of any treatment after cyclophosphamide failure, even though other drugs have been reported to be promising on induction therapy and could also be tried on refractory disease.

Mycophenolate mofetil is currently being tested as a possible alternative to cyclophosphamide with encouraging yet preliminary results [76].

Rituximab has a possible disease-modifying role by improving lung function even after failure of standard therapy [21].

There are several clinical trials ongoing on interstitial lung disease for new drugs and even autologous stem cell transplantation. Patients should be considered to enter them if refractory disease on setting.

Heart disease

Heart involvement in SSc is frequent but many times overlooked because: it can run asymptotically, its symptoms can be related to other non-cardiac features (lung, musculoskeletal or esophageal) [79] and also some screening tests are not sensitive [79,80]. When sensitive diagnostic tools are used, heart SSc disease is seen in almost all patients [81].

Although heart involvement can be secondary to other organ disease, namely renal or lung, it can also be primary reflecting myocardial fibrosis, autonomic neuropathy, small coronary vessel or pericardial sheet involvement [80]. Thus, depending on which structure is affected, several disorders can be expressed: rhythm abnormalities, pericardial effusion, ischemia, hypertrophy or fibrosis with or without heart failure [81] which is most of the time due to diastolic dysfunction. There is some evidence that myocardial involvement is a final result of microcirculation blood vessel reactivity (coronary Raynaud's [82]) with or without structural damage [81].

Symptomatic heart involvement carries poor prognosis [81] and is related to 2-year and 5-year survival rate of less than 40% and 24%, respectively [1]. Left axis deviation, large volume pericardial effusion [1] and cardiac autonomic dysfunction are independent variables that predict mortality [80] and should be screened.

If the patient is asymptomatic, 24 hours EKG monitoring (Holter) and echocardiogram should be performed to screen and prevent sudden death events [80]. Silent ischemic disease can be screened accurately with thallium scintigram [80].

There are no specific EULAR treatment recommendations [8] regarding cardiac involvement in systemic sclerosis and patients are treated according to general guidelines for each condition. Actually, vasodilators as calcium channel blockers and angiotensin converting

enzyme inhibitors proved to improve both perfusion and function abnormalities emphasizing the role of microcirculation impairment [81].

Medsger score to stage heart involvement severity use EKG alterations, left ventricular ejection fraction and the presence of congestive heart failure [9].

Proposal of definition of Refractory Heart involvement, when:

- a) EKG alterations progress in severity score despite adequate treatment and exclusion of concurrent morbidities, or***
 - b) left ventricular dysfunction progresses despite state of the art heart failure optimized treatment or intolerance, or***
 - c) heart failure symptoms and signs remain or worsen despite state of the art heart failure optimized treatment or intolerance.***
-

Once fibrosis is installed one would expect there would be nothing to do, but as in skin involvement why not to discuss if there is any role to immunosuppressives or antifibrotic therapy?

Conclusion

Systemic sclerosis is a severe disease on itself but the recent broad advances on SSc physiopathology are opening new perspectives and hopes in SSc future treatments which lead to the need of defining refractory disease, and that was our main goal.

Despite so, in the era of evidence-based medicine and with several clinical trials ongoing directed to many aspects of SSc management, when there is a refractory condition for which there is no available data and we're in the bedside of the patient, we recommend that the best approach would be discussing the patient with your local or international centers of reference in SSc.

Take-home messages

- Systemic sclerosis (SSc) is itself a paradigm of a refractory condition, thus it is difficult to define refractory SSc.
- We proposed definitions of refractory SSc disease based on the 2009 EULAR recommendations, Medsger severity score and published expert opinion, adapted to each organ or system.
- Some drugs have been proved to be effective in specific organ involvement as scleroderma renal crisis (SRC), pulmonary arterial hypertension (PAH) and digital ulcers; but other

organ involvement as the gastrointestinal tract and heart involvement are orphans and only symptomatic therapy has been recommended.

- Every SSc patient should be considered to enter clinical trials which are on going on several antifibrotic, immunomodulators and even autologous stem cell transplantation.
- While awaiting a background systemic therapy for SSc, refractory and difficult SSc patients' management should be discussed with your local or international centers of reference.

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2.3 – QUALIDADE DE VIDA NOS DOENTES COM ESCLEROSE SISTÊMICA

Artigo publicado

“QUALITY OF LIFE IN SYSTEMIC SCLEROSIS”

Almeida C, Almeida I, Vasconcelos C.

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Abstract

Systemic sclerosis (SSc) is a chronic multi-system autoimmune disease associated with disability and reduced quality of life. There is no effective treatment or cure to SSc, so it is important improve global health of these patients and reduce morbidity and mortality associated with SSc. It was made a literature review about quality of life in patients with SSc, regarding the several factors that should be considered and evaluated when attending to SSc patients. It was also considered the validated scales and questionnaires used to measure outcomes in patients with SSc. We concluded that it is important to have an interdisciplinary approach to SSc patients considering the patient's cognitive representations of the disease and what they value most like mobility and hand function, pain, fatigue, sleep, depression and body image.

Keywords: Systemic sclerosis, Scleroderma, Quality of life, QoL, Health-related quality of life measurement instruments.

Introduction

Systemic sclerosis (SSc) is a chronic multi-system autoimmune disease with extremely heterogeneous manifestations, characterized by thickening and fibrosis of the skin, vasculopathy and involvement of various internal organs in different degrees. Patients with SSc report a number of problems associated with disability and reduced quality of life (QoL) [1-3], which in turn is an important factor for disease outcomes [4-6]. Also the presence of polyautoimmunity in SSc, a common condition affecting one quarter of the patients, could influence both disease phenotype and severity. These patients seem to have a milder disease [7].

There is no effective treatment or cure to SSc [8-10], so a primary goal of care is to reduce symptoms and disability and to improve health-related quality of life (HR-QoL), with both pharmacological and non-pharmacological interventions [8,11].

Patients with rare diseases and especially with SSc face unique challenges that are not covered by generic interventions or interventions developed for other chronic diseases [8, 12], so to improve global health of these patients and reduce morbidity and mortality associated with SSc, it is important to measure outcomes with validated scales and questionnaires.

The authors decided to make a literature review about quality of life in patients with SSc.

Material and methods

A literature search was performed on October 1, 2014 using PubMed database with the keywords quality of life in conjunction with scleroderma or with systemic sclerosis. The results of the search included a total of 599 articles. After duplicates removal remained 337 articles selected. The abstracts of all original and review articles in English were read. Those thought to be relevant with full text available and pertinent secondary references were fully examined, and 117 articles were included.

Features related to quality of life in SSc patients

Knowledge about the frequency and perceived impact of the range of problems faced by individuals living with SSc, is limited [1,11]. It is important to know that there is a great difference between the assessment of the disease severity and HR-QoL [4,13]. The patient's cognitive representations of the disease are the most important determinants of physical and mental health. A recent study suggested that the fear of clinical consequences and the tendency to attribute each physical complaint to SSc are major contributors to the physical health, while the emotional responses to personal representation of the disease contribute to mental health [13,14]. The "Canadian Scleroderma Patient Survey of Health Concerns and Research Priorities" suggests that patients with SSc are more unsatisfied with healthcare than other chronically ill patients [2], because SSc involves more visible physical disfigurement that tends to worsen over time, contributing to high psychological morbidity (more depressive symptoms and anxiety) [15], regular use of healthcare and related increased costs. Physicians may ignore or use unreliable measurements to evaluate psychological distress [16]. While physicians may prioritize objective indicators of disease status, patients may perceive other aspects of their disease experience as more debilitating or distressing [1, 17, 18], like limitations in mobility and hand function, pain, fatigue, sleep disturbance, depression, sexual dysfunction and body image distress from disfiguring changes in appearance (pigment changes, hand contractures and facial telangiectasias for example) [8].

Van Lankveld *et al.* [15] reported that fatigue, functional limitations, skin deformities, pain and disfigurement were the most annoying symptoms, whereas Suarez Almazor *et al.* [18] identified physical pain, coping skills, social aspects of living with the disease, physical appearance and patient-physician relationships as particularly important to patients.

On 2011, a large Canadian National Survey with persons with SSc identified that the symptoms with the highest frequency and the most likely to have at least moderate impact on daily activities were fatigue, Raynaud's phenomenon, stiff hands, joint pain and sleeping disorders [1]. On the other hand, several symptoms with high frequency but low rated in terms of impact were itching, dry mouth and skin color change [1]. Other studies [1, 19-24] report that gastrointestinal problems, difficulty breathing, pain from various sources, depression, fatigue and pruritis were associated with disability and reduced QoL.

So to improve the HR-QoL of patients with SSc, several factors must be considered and evaluated, which will be focused in this review.

When attending to the two main SSc subsets – limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) – studies show that HR-QoL is impaired in both, but more in

dcSSc patients [13, 16, 25]. Also in both early SSc [26] and undifferentiated connective tissue disease patients, HR-QoL is impaired in physical and mental domains [13].

Patients with SSc have higher levels of fatigue and pain over the general population [19]. Raynaud's phenomenon, active ulcers, worsened synovitis and gastrointestinal symptoms were independently associated with pain by multivariate analysis [21].

Gender

Gender preferences, clinical differences and intrinsic psychological aspects also have a considerable influence on HR-QoL [16]. Generally male gender is considered a factor of poor prognosis in SSc [27-29]. While men's concern is work or study, women have more concern about daily and leisure activities [16]. Men were found to have more often renal failure, increased blood pressure, arrhythmia, inflammatory myopathy, myositis, dcSSc, positive nucleolar antinuclear antibodies, echocardiography pulmonary artery pressure > 35 mmHg, interstitial lung disease (the last one at the time of diagnosis and during follow-up) [27, 30] and higher risk to develop lung cancer [31], and less often sicca syndrome and anti-centromere antibodies [27]. Women more often exhibit arthralgias, calcinosis and lcSSc [27].

In a study, Nguyen *et al.* [27] found no association with gender concerning perceived health status, HR-QoL and reported global and location-specific disability were not found. In the same study, women also have more self-reported symptoms of anxiety, while men were free of self-reported symptoms of both anxiety and depression [27]. The authors [27] concluded that gender is not a major determinant of perceived disability and impaired HR-QoL in SSc, but functional and social issues should be considered as severe in both.

Work Productivity

Adult life is markedly influenced by employment. The ability to work is a multifactorial phenomenon influenced by factors such as physical and psychological capacity, and by specific work demands and factors outside the working life [32,33]. Long periods of absenteeism are often accompanied by loss of social status and life roles, with serious economic consequences for the individual, the employer and the society [32-35].

There are no studies using validated instruments to determine the predictors of decreased productivity in SSc, but studies that exist show that SSc has a major impact on productivity at home and at work [32-34]. Employment status was strongly associated with perceived disability and health status but not with gender [27]. In a published study more than one-third of patients were unemployed and more than 40% perceived their work ability as unsatisfactory [32]. Nguyen *et al.* [33] also suggested that employment status and socio-economic burden in SSc could be related more to perceived health status and disability than

to specific organ involvement. On the other hand, patients with better Work Ability Index (WAI) perceived a better life satisfaction but not HR-QoL [32]. There is a lack of association of functional disability and productivity at work [34]. According to Singh *et al.* [34], a lower educational level and greater physician assessment of overall health are predictors of decreased productivity at work. Limited SSc and higher score in Health Assessment Questionnaire Disability Index (HAQ-DI) are associated with decreased home productivity, and lower education level and higher HAQ-DI score are associated with SSc related disability [34]. On the other hand, Hudson *et al.* [32, 36] found a significant correlation between work disability and diffuse skin involvement, but Sandqvist *et al.* [32] found no evidence that degree of skin and lung involvement or disease duration influenced work capacity.

Perceived symptoms such as general pain, fatigue and impaired hand function (related to skin sclerosis, Raynaud phenomenon, ulcers and pain) are common in SSc and have an important impact on both work ability and employment status [32, 37-39]. Impaired grip force and dexterity also seem to influence employment status [40] that in turn is influenced by work ability, access to a job, social insurance systems and perhaps economic situation of the patient [32]. Also mouth disability, reduced mouth opening, altered dentition, speaking difficulties and sicca syndrome could influence employment status in SSc [33], especially in those jobs requiring public contact or talking. Higher costs in SSc patients were strongly associated with greater disease severity, poor health status, younger age, lower education [34, 41] and patient's perception of health status [42]. According to Minier *et al.* [42], disease duration has no significant impact on costs.

Interventions should include support in job adaptation and self-management strategies to help patients deal with pain and fatigue and to enhance the confidence to perform their work [32]. Adaptations at work attending to work intensity, ergonomic modifications at the working place and flexibility of working hours are important [32]. Patients must disclose information about SSc to employers and colleagues.

It is also important to consider that occupational exposures to pollutants and chemicals (like crystalline silica, white spirit, aromatic solvents, chlorinated solvents, trichloroethylene, ketones and welding fumes) as well as the use of some drugs have been suggested to play a role in SSc pathogenesis [43]. So, it seems advisable that these patients avoid such occupational exposures.

Sleeping disorders

Effects of sleep and sleep deprivation on cytokines and immune dysfunction are known [44, 45]. According to polysomnographic findings, SSc patients may have increased risk for sleep disturbances [44]. Prado *et al.* [46] showed that 70% of patients had reduced sleep

efficiency compared with age adjusted norms. Frech *et al.* [44] also found that patients with SSc have detrimental effects on their sleep compared with the US general population.

Although sleep duration of SSc patients is comparable to that of the general population on average, the sleep quality is poor [44]. Presence and severity of reflux symptoms, worsening dyspnea and depressed mood were independent predictors of sleep disturbances, but physician assessment of disease and demographics did not predict it [44]. Abad *et al.* [47] showed that pain is an independent predictor of sleep disturbance, but this was not confirmed in a multivariate analysis [44]. Also tender and swollen joint counts were not associated with it [44].

Depression

Half of the SSc patients have mild to severe psychological distress [16, 48, 49] and this is sometimes underestimated by physicians [16].

Depression was associated with the variables of age, symptom frequency and impact in terms of mental health, anxiety and social phobia [2]. Anxiety is also common in SSc patients [2, 49, 50] and correlates with changes in body image that in turn is a predictor of social phobia [2].

It was also shown that in general population an association between reflux symptoms and depressed mood exists [51]. Patients who were depressed rated their GI disease as significantly more severe compared with those without depressed mood [52]. Reflux symptoms and constipation are independently associated with depressed mood in SSc patients [22, 52, 53].

Therapeutic interventions in SSc may be suboptimal unless important psychological or behavioral factors are adequately addressed [54-57]. The cultural environment may offer benefic influences on QoL [4].

The use of various strategies to cope better with the disease is related to physical and mental health [13]. To improve the quality of life in patients with SSc is important to develop some strategies toward general SSc self-management, better handling with emotional distress, managing body image distress, physical and occupational therapy for hands, fatigue and energy management, as well as managing sleep and sexual function problems [8]. Dietary interventions, like iron and vitamin D supplementation, didn't interfere with psychological assessment or quality of life [58]. Although, a systematic review highlights that experimental studies in humans indicate beneficial effects of vitamin D supplementation in reducing the severity of disease activity, variability in administration may reduce its positive effects and the dose of hormone supplied in some trials may be insufficient to control the autoimmune aggression or to prevent the onset of disease [59].

Patients with SSc typically have difficulty accessing specialized services compared to people with other chronic diseases. Most of them understand access to online information on physical, psychological and social consequences of the disease as important [8], and that the rheumatologist is not often the preferred provider of information [60]. It was identified a considerable proportion of patients with need for information, in particular on medical issues like test results, medical treatment and knowing when to see a doctor [60]. This was associated with worse physical functioning and having a partner.

Sexual Function

Sexual activity is an important component of QoL and so when complications from SSc have a negative impact on sexual function, it can affect the QoL and interpersonal relationships [61-64]. Little is known about this theme in SSc patients, but sexual dysfunction is common between men and women with SSc [61]. Male erectile dysfunction is defined as the consistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance [65] that can be attributed to SSc vasculopathy and cavernosal fibrosis [66]. Female sexual dysfunction is defined as persistent or recurring decrease in sexual arousal, dyspareunia and a difficulty or inability to achieve orgasm [63] and in SSc patients is associated to skin tightening around the vaginal introitus, joint contractures, muscle weakness, changes in skin around the breasts and breast muscle, and joint pain [61,67]. Studies involving women with SSc report more sexual dysfunction than studies with women of general population or with other chronic conditions [68-70]. Sexual dysfunction in the female patients, compared to male patients, is no less prevalent but is considerably more complex [61].

Daily and long-acting PDE-5 inhibitors are safe and effective for male erectile dysfunction in SSc patients [66], but one study performed in women showed only confounding and small effects [61]. In women we should consider agents for vaginal lubrication, advice about positioning and avoiding meals prior to sexual activity, and considerations about sexual adverse effects of other treatments [61,70].

Raynaud's phenomenon and stiff hands

Contractures and deformities of the hand, consisting of decreased flexion, limited extension, reduced thumb abduction, microvascular lesions, paroxysmal vasospasm or permanent ischemia and subsequently digital ulcers (DU), skin sclerosis, tendons retractions, bone and articular involvement, and subcutaneous calcinosis contribute substantially to a large burden on global disability and social relationship in SSc [71, 72]. The presence of Raynaud's phenomenon (RP) and/or puffy hands also contributes to disability and if there is also an increase erythrocyte sedimentation rate levels, they are negatively associated with physical

health status [13]. It was showed that the presence of an autoimmune RP might be sufficient to affect HR-QoL [13].

Patients indicate that hand disability interfering with daily life and work is more important than the treatment of other internal organs [73] and so healthcare professionals should take into account improving disability, patients' satisfaction and social comfort as clinically relevant objectives of therapy [72]. RP in SSc is very frequent, severe, long lasting and frequently it is the first clinical manifestation of SSc preceding other clinical manifestations [74]. Reducing the severity and complications of RP may improve the functional outcomes and QoL [74, 75]. For that it is important a wider patient recruitment at specialist referral centers in the early stages of disease, systematic use of diagnostic tools such as capillaroscopic examination and serological markers and early use of the recently available treatments [12]. In a randomized trial, patients treated with tadalafil reported significant improvement in physical function and body pain and mental health compared with baseline [75]. Recently, the injection of autologous stromal vascular fraction from adipose tissue into the fingers of patients with SSc can be performed safely and is well tolerated, decrease of ~50% in Cochin Hand Function Scale, Raynaud's severity and hand pain at 2 months with persisting benefits at 6 months and had a favorable impact on QoL [72]. Other study [76] showed that after two weeks of a daily home exercise program, hand mobility improved among patients with SSc which leads to improved QoL and is especially effective when combined with wearing gloves to prevent cold exposure leading to RP [76]. It is also important to know the possible predictors of the development of digital ulcers (especially autoantibodies) [77] which could help in identifying patients with indication for target therapy (presence of anti-topoisomerase I autoantibodies, early first non-RP, great extent of skin fibrosis, late nailfold videocapillaroscopy scleroderma pattern and its worsening and reduced VEGF levels) [74].

Skin deformities and disfigurement

Data regarding to dermatology-specific HR-QoL in SSc patients are still limited [78-80].

SSc patients have high disease burden as compared to the general population and to patients with more common skin diseases and their QoL is much lower than that of patients with more frequent chronic conditions such as heart disease or diabetes [81]. SSc involves many physical changes on visible parts of the body [2, 82] which can cause both physical and psychological morbidity [83]. Salt and pepper appearance was the cutaneous finding that had association with high dermatology life quality index scores [16]. In SSc patients skin remission cannot be achieved like in others dermatoses (psoriasis, atopic dermatitis, pemphigus, or SLE) [10] and so the social factors and disease coping may contribute to a better mental health [84-86].

There are also several oral and maxillofacial manifestations that may interfere with the quality of life of SSc patients, like the presence of trismus, muscular atrophy, thin atrophied lips, secondary microstomia, xerostomia, rigidity of tongue and lips, widening of the periodontal ligament space, trigeminal neuralgia and resorption of the mandible [87]. Microstomia can have a profound negative impact on the relationships of patients with others and on their quality of life as it causes psychological distress secondary to facial disfigurement, functional debilitating sequels such as drooling and difficult on chewing and speech, and may also interfere with normal oral hygiene and dental treatment as it has been associated with dental caries [88].

Surgical correction of bilateral mandibular condylitis and surgical correction of malocclusion give functional occlusion, improve facial balance and improved quality of life [87].

One study with 48 participants with microstomia showed that the orofacial exercise program intervention for adults did not show significant improvement at 6 months perhaps because of the low adherence rate and insufficient frequencies, repetitions and durations of the orofacial exercises [88].

Gastrointestinal problems

Gastrointestinal involvement (GI) occurs in 90% of patients with SSc [89-91] and is associated with increased morbidity and mortality as well as a negative impact on HR-QoL [89,92], although the correlation between symptoms and GI motility disorders is difficult to establish [90].

Gastrointestinal reflux and dysphagia are the most common gastrointestinal symptoms, and like gastroparesis, intestinal pseudo-obstruction, bacterial overgrowth, intestinal malabsorption, diarrhea and / or fecal incontinence affect food intake and intestinal absorption and lead to gradual appearance of nutritional deficiency in 30% of patients [92, 93]. Reflux as also been linked to SSc-associated interstitial lung disease [52].

Gut involvement is the leading cause of morbidity and the third most common cause of mortality in patients with SSc [94]. Anorectal involvement occurs in 50-70% of SSc patients, but they are often reticent to report these symptoms to their physicians [90] and therefore are undertreated, resulting in a significant negative effect on HR-QoL [95]. Patients with anorectal dysmotility experience considerably reduced QoL in physical and social dimensions [90, 96], with increased mortality of up to 85% compared to patients with minor GI complaints.

Patients with early severe GI involvement (with malabsorption, pseudo-obstruction and need for hyper-alimentation) have a higher ratio of dcSSc to lcSSc and are less likely to suffer from interstitial lung disease [97]. Diarrhea is probably the best symptom category indicative of severe GI involvement as it is often secondary to small gut involvement and bacterial overgrowth [91]. In these patients the presence of ACA or scl-70 antibodies was less frequent

and there was a higher frequency of anti-U3RNP and anti-U1RNP and other ANAs [91, 94]. It was found an inverse relationship between symptoms of diarrhea and pulmonary fibrosis [91].

Pruritis

Pruritis is common in about a half of SSc patients and had significant association with mental and physical function [78]. SSc patients with pruritis have more significant skin involvement, more severe finger ulcers, and worse respiratory symptoms. Pruritis is independently associated with GI symptoms, with significant disability and with QoL in SSc [98]. There aren't management guidelines. Naltrexone, through opioid-mediated actions or a reduction of inflammatory mediators, modulates pruritis and have been suggested that may be an effective, highly tolerable treatment for pruritis in SSc and has been found to improve QoL in inflammatory bowel disease [98].

Treatment of pain, pruritis and prominent cutaneous findings should be taken into account in order to improve QoL of SSc patients [16].

Quality of life in SSc – measurement instruments

The assessment of HR-QoL is an essential element of healthcare evaluation and because of that several generic and specific HR-QoL instruments have been developed. The first ones were designed to be applicable across a wide range of populations and interventions, while specific HR-QoL measures were designed to be relevant to particular interventions or in certain subpopulations as in the case of the ones designed to SSc patients (Table I). HR-QoL is usually assessed using patient questionnaires that may cover physical, social, psychological, emotional, cognitive, spiritual, work- or role-related and financial impact domains.

In this paper we will summarize the different instruments that could be used to evaluate HR-QoL in SSc patients.

Table 1 – QoL measurement instruments: application, specificity and validity in SSc patients

QoL measurement instruments	Specific for SSc	Validated in SSc
Patient-Reported Outcomes Measurement Information System-29 Version 1	No	Yes
Short form 36 questionnaire Version 2	No	Yes
The patient overall health assessment	No	No
The physician assessment of overall health	No	No
Manchester Short Assessment of Quality of Life	No	Yes
Health Assessment Questionnaire Disability Index	No	Yes
Scleroderma Health Assessment Questionnaire	Yes	Yes
McMaster Toronto Arthritis Patient Preference Disability Questionnaire	No	Yes
Pain assessment/ Visual analogue scale	No	Yes
SSc-associated symptoms assessment	Yes	Yes
Symptom Burden Index	Yes	Yes
Patient-generated Index	Yes	Yes
The modified Work Productivity Survey – Rheumatoid Arthritis	No	No
Work Ability Index	No	No
Scleroderma Functional Score	Yes	Yes
Satisfaction with Daily Occupations instrument	No	Yes
Functional Assessment of Chronic Illness Therapy –Fatigue	No	Yes
Medical Outcomes Study Sleep Scale	No	Yes
Center for Epidemiologic Studies Short Depression Scale –10	No	Yes
Patient Health Questionnaire –8 or –9	No	Yes
Female Sexual Function Index	No	No
Female Sexual Distress Scale	No	No
The Cochin Hand Function Scale	No	Yes
Mouth Handicap in Systemic Sclerosis Scale	Yes	Yes
Satisfaction With Appearance Scale	No	Yes
Dermatology Life Quality Index questionnaire	No	No
Raynaud's Condition Score	Yes	Yes
The University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Scale 2.0	Yes	Yes
Mahler's <i>Baseline</i> Dyspnea Index (BDI) and Transition Dyspnea Index (TDI)	No	No
Cambridge Pulmonary Hypertension Outcome Review	No	No

Assessment of global health-related quality of life and life satisfaction

Patient-Reported Outcomes Measurement Information System –29 (PROMIS-29) Version 1

The Patient-Reported Outcomes Measurement Information System (PROMIS) framework [99], created by the US National Institutes of Health, evaluate patient-reported symptoms and outcomes using an item bank during a 7 days recall period. PROMIS-29 is a PROMIS-derived instrument that measures physical, mental, and social health [99, 100] with 7 subcomponents (symptoms, function, affect, behavior, cognition, relationships, and function) and various domains and subdomains within each subcomponent. It has demonstrated construct validity in assessing important areas of a person's life affected by chronic diseases and is easily comparable among disease states. However, it has no clinical endpoints yet defined and it may not capture commonly cited problems among patients with SSc, such as symptom burden on their social lives or emotional distress related to physical appearance [18, 101,102].

Short form 36 questionnaire (SF-36) Version 2

The short form 36 questionnaire version 2 (SF-36 v.2.) is a generic health status measure consisting of 36 items assessing 8 scales [9,103] which are summarized into Physical and Mental Component Summary scores. It has a 4-week recall period. The SF-36 instrument is not specific to any organ system and has a significant correlation to systemic involvement in SSc patients [90] and between patients and the physician. This score has been validated in SSc.

The patient overall health assessment

The patient overall health assessment [34,44,104,105] is a generic health status measure consisting in one question. The choices ranged from 1 (excellent health) to 5 (poor health).

The physician assessment of overall health

The physician assessment of overall health [34,44,105,106] consists in one question to assess the overall health of a patient in the last week. Physicians were asked to assess the

overall health of their patient in the last week. Responses are rated on a scale ranged from 0 (excellent health) to 10 (extremely poor health).

Manchester Short Assessment of Quality of Life (MANSA)

Manchester Short Assessment of Quality of Life (MANSA) [32,107] is an instrument that assesses the subjective life satisfaction as a whole and with different life domains. It has been shown to have good internal consistency and satisfactory construct validity for women with SSc [108].

Assessment of global disability and pain

Health Assessment Questionnaire Disability Index (HAQ-DI) and Scleroderma Health Assessment Questionnaire (SHAQ)

The Health Assessment Questionnaire-Disability Index (HAQ-DI) [9,105] is a self-administered 20 question instrument in 8 domains (dressing, arising, eating, walking, hygiene, reach, grip and common daily activities) designed for arthritis that assesses a patient's level of functional ability/disability and includes questions that involve both upper and lower extremities. It ranges from 0 (no disability) to 3 (severe disability) and has a 7 day recall period.

The Scleroderma Health Assessment Questionnaire (SHAQ) [105] consists of the HAQ-DI and includes other items: pain, patient global assessment, vascular, digital ulcers, lung involvement, and gastrointestinal involvement. It is specific for SSc, is a reliable outcome measure for disease severity [60] and has also a 7 day recall period.

McMaster Toronto Arthritis Patient Preference Disability Questionnaire (MACTAR)

McMaster Toronto Arthritis Patient Preference Disability Questionnaire (MACTAR) [109] is an instrument that assesses the patients' perceived disability. Patients were asked to select the 3 situations among activities of daily living that caused them maximal trouble. Each item is scored on an 11-point quantitative scale (range 0 – 10). This score has been validated in SSc [110, 111].

Pain assessment

Pain is assessed using visual analogue scale or a single-item scale [44, 105]: How much pain did you have in the past 7 days? Very mild, mild, moderate, severe and very severe?

SSc-associated symptoms assessment

Worsening of SSc-associated symptoms [44] is assessed using single-item questions from the scleroderma disease activity index, asking about any new symptoms or worsening of symptoms over the past 1 month of the skin, RP or digital ulcers, GI symptoms (upper and lower) and shortness of breath or chest pain in patients.

Symptom Burden Index (SBI)

The Symptom Burden Index (SBI) [99,105,112] is a questionnaire that measures symptom burden and has 8 domains: bowel function, calcinosis, eating, problems with hands, pain, shortness of breath, appearance, and sleep. Patients are asked 5 questions about each symptom they may experience: (i) how much of a problem was _ (the symptom)? (ii) how often was _ (the symptom) a problem? (iii) how much did _ (the symptom) interfere with daily activities? (iv) how often did _ (the symptom) interfere with daily activities? and (v) how important a problem was _ (the symptom)? Each response is scored with a 0– to 10–point scale, 0 indicating no burden and 10 severe burden. The SBI has validated in SSc [112].

Patient-generated Index (PGI)

The Patient-generated Index (PGI) [99,113] is a questionnaire completed in 4 stages, which evaluates HR-QoL on the basis of domains that individual patients identify as important to them. In the first, patients select the 5 most important areas of their lives affected by SSc. A sixth area is added for all patients: “all other areas of your life affected by your scleroderma.” In the second stage, patients score each of the 6 areas named using a scale ranging from 0 (as bad as could possibly be) to 6 (as good as could possibly be) to rate how each specific area of their lives was affected by SSc over the last month. In the third stage, patients are then asked to “spend 10 points to show which areas of your life you feel are most important to your overall quality of life.” The fourth stage is generation of an index value, done by multiplying the 6 scores for each area by the number of points spent, and then summing these products. The result is an index score that is transformed to a percentage ranging from 0 to 100, with lower scores indicating worse HRQOL. PGI is a valid instrument to assess HR-QoL in patients with SSc [99].

Assessment of work-related factors and daily activities

The modified Work Productivity Survey – Rheumatoid Arthritis (WPS-RA)

The Work Productivity Survey – Rheumatoid Arthritis (WPS-RA) [34] is a 9 question instrument, with a 30 day recall period, developed to assess prevalence of productivity outside and within the home in patients, to determine the predictors of decreased productivity outside and within the home and assess prevalence and predictors of self-reported associated disability. It was initially developed for RA and has been validated as an instrument to assess productivity. As there were not previous validated instruments to assess this in SSc patients, WPS-RA was modified by replacing the word “arthritis” with “scleroderma” throughout the survey [34], and used to assess the impact of SSc on productivity.

Work Ability Index (WAI)

Work Ability Index (WAI) [32] is a self-administered questionnaire considering the demands of work, the worker’s health status and resources. It comprises 7 items grouped according to: (i) estimation of current work ability compared with lifetime best; (ii) work ability in relation to physical and mental demands of the work; (iii) number of diagnosed diseases; (iv) estimation of work impairment due to diseases; (v) sick leave during the past year; (vi) own prognosis of work ability after 2 years; and (vii) psychological resources. Higher scores on the WAI indicate better work ability.

Scleroderma Functional Score (FS)

The Scleroderma Functional Score (FS) [32,114] is a self-administered 11-item questionnaire that assesses 11 daily activities within self-care and household chores: 9 items are related to upper extremity function and 2 items to muscle weakness and lower extremity function.

Satisfaction with Daily Occupations (SDO) instrument

The Satisfaction with Daily Occupations (SDO) [27,115] is a self-administered instrument, comprising 9 questions regarding the patients’ satisfaction with the activity areas

of work, leisure, household chores and self-care. The SDO has shown good internal consistency and construct validity for individuals with SSc [108].

Assessment of fatigue

Functional Assessment of Chronic Illness Therapy – Fatigue (FACIT-Fatigue)

The Functional Assessment of Chronic Illness Therapy – Fatigue (FACIT-Fatigue) [9, 34, 44] is a 13-item questionnaire that assesses self-reported fatigue and its impact upon daily activities and function, with a range of possible scores of 0 – 52, with lower scores reflecting more fatigue. It uses a 5-point categorical response scale of 0 (not at all) to 4 (very much). It has a 7 day recall period.

Assessment of sleep

Medical Outcomes Study Sleep Scale (MOS-SLEEP)

The Medical Outcomes Study Sleep (MOS-SLEEP) scale [9, 44, 116] yields a sleep problems index and 6 scale scores with 12 items, where quantity of sleep is scored as the average hours slept per night. It has a 4 week recall period.

Assessment of depression

Center for Epidemiologic Studies Short Depression Scale (CESD-10)

The Center for Epidemiologic Studies Depression (CESD-10) Scale [9, 44, 106] is a 10-item instrument that measures depressive symptoms using a 4-point categorical response scale (range 0 to 30) with higher scores representing greater depressive symptoms (score ≥ 10). The items include depressed mood, feelings of guilt, worthlessness and helplessness, psychomotor retardation, loss of appetite and sleep difficulties. It has a 7 day recall period.

Patient Health Questionnaire –8 or –9 (PHQ-8 or -9)

The Patient Health Questionnaire –8 or –9 (PHQ-8 or -9) [8] is a 8 or 9-item measure of depressive symptoms that is commonly used in medical populations. It has a 2 weeks recall period. Items are scored from 0–3, with the response options “not at all” (score 0), “several

days” (score 1), “more than half the days” (score 2), and “nearly every day” (score 3). Higher scores represent increased severity of depressive symptoms. Item 9 regards suicidal ideation. It has been validated in SSc.

Assessment of sexual function

Female Sexual Function Index (FSFI)

The Female Sexual Function Index (FSFI) [61, 70, 117] is a 19-item self-report questionnaire that assesses six domains of sexual functioning in women: desire, subjective arousal, lubrication, orgasm, pain, and satisfaction. Higher subscale or total scores indicate better sexual function.

Female Sexual Distress Scale (FSDS)

The Female Sexual Distress Scale (FSDS) [61, 70, 112] is a questionnaire that quantifies the personal distress that any sexual complaints cause, which is an essential component of the definition of female sexual dysfunction, higher scores representing more sexuality-related personal distress.

Assessment of specific physical disability, esthetic and skin impairment

The Cochin Hand Function Scale (CHFS)

The Cochin Hand Function Scale (CHFS) [27,118] is an 18 items questionnaire administered by physician that assesses hand disability. Each question is scored on a scale of 0 (performed without difficulty) to 5 (impossible to do). This questionnaire was validated in SSc [119].

Mouth Handicap in Systemic Sclerosis Scale (MHSS)

The Mouth Handicap in Systemic Sclerosis Scale (MHSS) [27,120] is a questionnaire administered by the physician that assesses mouth disability and contains 12 items concerning difficulties in performing activities of daily living. Each question is scored on a scale of 0 (never) to 4 (always).

Satisfaction With Appearance Scale (SWAP)

The Satisfaction with Appearance Scale (SWAP) [8,121] is a measure developed to assess body image in disfigured medical populations, like SSc, focusing on both subjective body image distress as well as the social impact (e.g., avoidance). It has four subscales measuring Social Distress, Facial Features, Non-facial Features, and Perceived Social Impact. It was validated in SSc.

Dermatology Life Quality Index questionnaire (DLQI)

The Dermatology Life Quality Index questionnaire (DLQI) [16,122] is a self-administered questionnaire which contains 10 questions and 6 domains that include symptoms and feelings, daily activities, leisure, work and school, personal relationships, and treatment. The interpretation of DLQI scores were as follows: 0–1 (no effect at all), 2–5 (small effect), 6–10 (moderate effect), 11–20 (very large effect), and 21–30 (extremely large effect). It has a 7 day recall period.

Raynaud's Condition Score (RCS)

The Raynaud's Condition Score (RCS) [105] is a daily self-assessment of Raynaud's phenomenon (RP) activity using a 0–10 ordinal scale, during the last 14 days. It incorporates the cumulative daily frequency, duration, severity, and impact of RP attacks and assesses the overall cumulative effect of RP. It has validity in SSc.

Assessment of gastrointestinal and nutrition problems

The University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Scale 2.0 (UCLA SCTC GIT 2.0)

The University of California Los Angeles Scleroderma Clinical Trial Consortium Gastrointestinal Scale 2.0 (UCLA SCTC GIT 2.0) [44, 89, 105] is a 7-multi-item scale with a total of 34 items that include reflux, distention/bloating, diarrhea, fecal soilage, constipation, emotional well-being and social functioning. It was designed to assess the range of problems that can occur in SSc, their severity and their impact in HR-QoL. The total GIT score is the average of six of seven scales (excludes constipation) and total gastrointestinal score is scored

from 0 (better HR-QoL) to 3 (worse HR-QoL) except for diarrhea scale with a maximum score of 2 (worse HR-QoL) and constipation scale with a score of 2.5 (worse HR-QoL). It was found to have acceptable feasibility, reliability and validity.

Assessment of dyspnea and lung involvement

Mahler's Baseline Dyspnea Index (BDI) and Transition Dyspnea Index (TDI)

The Mahler's Baseline Dyspnea Index (BDI) and Transition Dyspnea Index (TDI) [105,123] measure dyspnea at one point in time and then how it has changed at another time, respectively. It can be self or interviewer administered. Each index rates 3 different categories: magnitude of task, magnitude of effort, and functional impairment, that ranges from 0 (severe) to 4 (unimpaired) for the BDI and from -3 (major deterioration) to +3 (major improvement) for the TDI. It has not fully tested in SSc but has used successfully in a scleroderma lung study [124].

Cambridge Pulmonary Hypertension Outcome Review (CAMPHOR)

The Cambridge Pulmonary Hypertension Outcome Review (CAMPHOR) [105] is a pulmonary arterial hypertension (PAH) quality of life Instrument, not specifically validated in SSc-associated PAH. It assesses patient-reported symptoms, functioning and QoL, with 3 scales including overall symptoms (made up of energy, breathlessness and mood subscales), functioning and QoL with 65 items.

Conclusion

SSc patients show a number of problems associated with reduced QoL. As there is no effective treatment or cure to SSc, it is important to know the different features of disease and have an interdisciplinary approach to SSc patients, using both pharmacological and non-pharmacological interventions, and involving physicians, psychologists, physical and occupational therapists, social workers and spiritual advisers if it is an option for the patient. More emphasis should be placed on techniques for coping with the disease and biopsychosocial support [84-86], measures of overall management of SSc, acceptance of body image, management of energy and fatigue, sleep problems, sexual dysfunction, and occupational therapy for hands. It is also important to assess the impairment of disease in terms of HR-QoL and for that there are several generic and specific HR-QoL instruments that could be used, covering physical, social, psychological, emotional, cognitive, spiritual, work- or role-related and financial impact domains. This review listed the majority of them and the more reliable and valid ones, but some may have been missed by the search strategy.

Take-home messages

- SSc patients show a number of problems associated with reduced QoL.
- In SSc patients it is important reduce symptoms and disability and improve health-related quality of life, with both pharmacological and non-pharmacological interventions.
- The assessment of HR-QoL is an essential element of healthcare evaluation in SSc patients.
- There are several generic and specific HR-QoL instruments that could be used in SSc patients, covering physical, social, psychological, emotional, cognitive, spiritual, work-or role-related and financial impact domains.

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2.4 – ALTERAÇÕES IMUNOLÓGICAS NA ESCLEROSE SISTÉMICA

Embora a patogénese da ES permaneça desconhecida, as alterações vasculares, a acumulação de colagénio e a ativação do sistema imune são consideradas as principais características da doença [1,2,3]. A ativação imune é caracterizada pela produção de anticorpos, ativação linfocitária e libertação de variadas citocinas [3].

Os anticorpos antinucleares (ANA), são principalmente dirigidos para antígenos nucleolares, podendo estar presentes em mais de 95% dos doentes [4]. Utilizando o método de Imunofluorescência indireta em células Hep2 (IFI), podem ser identificados vários padrões de fluorescência, incluindo o centromérico, nucleolar, mosqueado, homogéneo e membrana nuclear, entre outros [5]. Dos autoanticorpos associados à ES, alguns são específicos para a doença (80% dos ANA), nomeadamente os anticorpos anti-centrómero (ACA), anti-topoisomerase I (ATA, anti-Topo I ou anti-Scl70), anti-polimerase do ARN (anti-RNAPIII e I), anti-fibrilharina (AFA ou anti-U3RNP) e anti-Th/To, podendo relacionar-se com a apresentação clínica e gravidade da doença [6].

Este facto sugere que os autoanticorpos estão associados à patogénese da ES, embora não pareçam exercer um papel patogénico direto uma vez que a maioria dos autoantígenos relacionados com a ES são componentes intracelulares [3]. Contudo, um estudo recente demonstrou que os anticorpos anti-Topo I se podem ligar diretamente à superfície dos fibroblastos [7] e, outro, que os ratos imunizados com Topo I desenvolveram esclerose da pele e fibrose pulmonar [8].

Apesar da relevância patológica destes autoanticorpos permanecer por esclarecer, a sua presença demonstra que existe, pelo menos, uma ativação anormal das células B [3].

De uma forma geral, estes autoanticorpos excluem-se mutuamente, não se alterando no decurso da doença [9,10,11].

Nos últimos anos têm sido descritos autoanticorpos com patogenicidade direta, associados com moléculas da superfície celular, componentes da matriz extracelular e aos recetores de sinalização [12].

Relativamente aos anticorpos contra moléculas da superfície celular, os AECA (anticorpos anti-células endoteliais), têm sido identificados em 44 a 84% dos doentes [13]. Pensa-se que os AECA induzem a apoptose das células endoteliais (CE) e a expressão das moléculas de adesão nessas células, aumentando a adesão leucocitária e participando, desta forma, na patogénese da doença [14,15].

Nos doentes com ES, também tem sido detetados anticorpos contra os fibroblastos. Estes parecem aumentar a expressão das moléculas de adesão e a ligação de leucócitos aos

fibroblastos da derme [16] e há estudos que sugerem que estes anticorpos reconhecem especificamente a Topo I na membrana celular dos fibroblastos [17].

Relativamente aos anticorpos associados a componentes da matriz extracelular (EMC), os anti-MMP-1 (anticorpos contra as metaloproteinases 1 da matriz) têm sido descritos em cerca de 75% dos doentes com ES difusa e em 30% dos doentes com ES limitada [18]. Os anticorpos de tipo IgG inibem a atividade da collagenase em cerca de 77% e contribuem para agravar a fibrose existente [13].

Embora Baroni *et al.* [19] tenham detetado, em todos os doentes com ES que estudaram, anticorpos contra os recetores do PDGF (fator de crescimento derivado das plaquetas), capazes de induzir a sua ativação, estes resultados não são consistentes na literatura [20,21].

Desde longa data que alterações da função das células B têm sido associadas à ES e estudos prévios mostraram que estes doentes apresentam expansão das células B *naïve* e ativadas, mas diminuição das células B de memória no sangue periférico, sugerindo que o aumento da expressão CD19 nestas últimas se relacione com a sua hiperatividade [22]. Outros estudos mostraram que as células B infiltram a pele afetada e o pulmão de doentes com ES [23] estando envolvidas na patofisiologia da fibrose [18,19,24]. Neste sentido, as células B produzem autoanticorpos com potencial patogénico direto, como acima referido, e citocinas pró-fibróticas como a interleucina 6 e o fator de crescimento transformador beta (TGF- β) que estimulam os fibroblastos, podendo aumentar também a produção de colagénio por mecanismos dependentes de um contato direto [19,24].

Em fases precoces da doença, mesmo antes de existir fibrose, os tecidos afetados apresentam infiltrados inflamatórios compostos por macrófagos, mastócitos e linfócitos, sendo que os linfócitos T são as células dominantes [25,26]. Os fibroblastos perivasculares estão em estreito contato com estas células [27, 28] e são estimulados por fatores solúveis para produzirem colagénio [29].

Estudos mais antigos demonstraram que as células T CD4⁺ predominavam nos infiltrados inflamatórios da pele afetada de doentes com ES [25,30,31], expressavam moléculas relacionadas com a sua ativação [32] e eram capazes de secretar citocinas fibrogénicas [33]. Mais recentemente, Fuschiotti *et al.* demonstraram que células T CD8⁺ predominavam nos infiltrados inflamatórios cutâneos em fases iniciais da doença enquanto os linfócitos T CD4⁺ eram predominantes em fases mais tardias [34].

Também foram descritas alterações de outros subtipos de células T, como os linfócitos T gamma delta, as células T reguladoras (Treg) e as células T *natural killer* (T/NK) [35-38].

Um aumento dos linfócitos T ativados (CD45RO⁺) tem sido descrito em biópsias pulmonares de doentes com ES e foram relacionados com o espessamento das paredes

alveolares [39]. Por outro lado, outros estudos têm demonstrado um aumento do número absoluto e relativo de células T CD45RO+, particularmente do subtipo CD8+, no lavado broncoalveolar de doentes com ES [31,40], sendo que um deles também revelou aumento dos linfócitos T citotóxicos CD16+ e/ou CD56+ [40].

A evidência relativamente às alterações das células T no sangue periférico dos doentes com ES é contraditória. Alguns estudos demonstraram diminuição dos níveis de células T CD8+ [41], outros de ambos os subtipos de células T (CD8+ e CD4+) [42,43] e outros não encontraram qualquer diferença relativamente aos controlos saudáveis [44,45].

Igualmente relevante é a evidência que sugere que as células T CD8+ efectoras estão aumentadas no sangue periférico de doentes com ES [46] ao contrário do subtipo de memória que se encontra diminuído [43,44,46]. A depleção de células circulantes T CD8+ de memória parece ser um achado relativamente constante nos doentes com ES, independentemente do critério fenotípico usado para definir essas células, i.e, células T CD29+ [43,44] ou CD45RA-CD27+ [46].

Alterações no número absoluto ou relativo de células T ativadas no sangue periférico também têm sido descritas, tais como o aumento percentual e numérico das células T CD4 CD25+ [44] e aumento das percentagens de células T CD4+ HLA-DR+ e CD8+ HLA-DR+ [45].

Por outro lado, Giovannetti *et al.* referiram existir uma maior proliferação de células T CD4+, um aumento de apoptose linfocitária e de células Treg em doentes com ES relativamente aos controlos [37].

Até à data, poucos estudos avaliaram as células NK no sangue periférico de doentes com ES e os resultados são contraditórios. Uns descreveram contagens normais de células NK [42] e outros relataram diminuição das percentagens e/ou números absolutos das mesmas [36,45]. Evidência fenotípica de ativação das células NK [47,48] e alterações da sua função também foram descritas no sangue periférico de doentes com ES [47,49].

Relativamente à polarização funcional das células T, células provenientes de lesões cutâneas de doentes com ES assim como células T CD8+ provenientes do lavado broncoalveolar exibem, predominantemente, um padrão T *helper* 2 (Th2) de produção de citocinas [50-51]. Adicionalmente, células T CD8+ do sangue periférico, expressam níveis elevados de GATA-3, um fator de transcrição que induz a resposta Th2 e suprime a diferenciação Th1 [52]. Para além disso, há evidência de aumento da produção de IL-13 pelas células T CD8+ do sangue periférico, que é uma citocina profibrótica com efeitos diretos e indiretos (através da estimulação do TGF- β) na ativação fibroblástica [53]. Mais recentemente, estudos têm avaliado o papel das células Th17 e suas citocinas nos doentes com ES [54] e tem sido demonstrado existir um maior número destas células no sangue periférico de doentes do que em controlos saudáveis [55-57]. Para além disso, tem sido descrito um aumento dos

linfócitos Th17 na pele de doentes com ES em estadios precoces [58] e o seu número parece ser inversamente relacionado com a extensão da fibrose cutânea [59]. Relativamente aos níveis de IL-17 A no sangue periférico, os dados existentes na literatura são contraditórios [60-62].

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2.5 – MICROANGIOPATIA, LESÃO ENDOTELIAL E ANGIOGÊNESE NA ESCLEROSE SISTÊMICA

CONTEÚDOS

Microangiopatia e Lesão Endotelial

Angiogénese e Vasculogénese

Referências

Avaliação da Microangiopatia, da Lesão Endotelial e da Angiogénese

Capilaroscopia

Referências

Biomarcadores de lesão endotelial e de angiogénese

Biomarcadores de lesão endotelial

Endotelina-1(ET-1)

Moléculas de adesão

ICAM-1 (CD54)

VCAM-1(CD106)

Seletina-E (CD62E)

Seletina-P (CD62P)

Biomarcadores de angiogénese

Fator de crescimento do endotélio vascular (VEGF)

Endostatina

Angiostatina

Referências

Microangiopatia e Lesão Endotelial

A vasculopatia, caracterizada por ativação endotelial e alteração do tônus vascular, parece ser o evento patogénico mais precoce na ES. Associa-se à presença de citocinas pró-inflamatórias, fatores que regulam a angiogénese, perda do controle *redox*, *stress* oxidativo e hipoxia. Envolve interações moleculares complexas, vários tipos celulares, nomeadamente células endoteliais e as suas células de suporte e células inflamatórias, e culmina em doença vascular severa e oclusiva [1].

O endotélio é um órgão metabolicamente ativo, complexo e altamente especializado, exercendo funções biológicas essenciais, sendo o seu funcionamento crítico para a remodelação dos vasos sanguíneos, através da angiogénese e vasculogénese, durante o crescimento e a reparação vascular [1]. Facilita a circulação sanguínea, assegura um estado anti-trombótico inibindo a agregação plaquetária excessiva, controla a adesão leucocitária e a migração celular e, por conseguinte, a inflamação, coordena o tônus vascular mantendo um equilíbrio entre as moléculas vasodilatadoras (ex.: óxido nítrico e prostaglandinas) e vasoconstritoras (ex.: ET-1 e PDGF - fator de crescimento derivado das plaquetas), previne a proliferação das células musculares lisas dos vasos, inibe a deposição de matriz extracelular e regula o transporte de nutrientes [1].

O endotélio exerce as suas funções reguladoras através da síntese de mediadores e a sua disfunção é um componente importante em doenças que se caracterizam pela existência de inflamação e fibrose como é o caso da Esclerodermia [1].

Na ES, as lesões mais precoces parecem residir nas células endoteliais, caracterizando-se por um aumento do espaço intercelular, vacuolização citoplasmática, proliferação da membrana basal e infiltração da parede vascular por linfócitos e vesículas celulares [2], sendo o fator(es) desencadeante(s) desconhecido(s). No entanto, as infeções víricas ou bacterianas, o *stress* oxidativo, a hipoxia, o fluxo sanguíneo turbulento, a hiperlipidemia, fatores irritantes do meio ambiente como o tabaco, entre outros, parecem ser despoletadores de processos inflamatórios e conduzem à lesão, ativação e disfunção endotelial [1]. Segundo Nguyen *et al.*, estas alterações parecem ser precedidas por apoptose das células endoteliais [3].

Perante a lesão, o endotélio responde rapidamente através de alterações nos níveis de óxido nítrico, prostaglandinas, ET1, fator de *Von Willebrand* e fator ativador do plasminogénio, entre outros. Posteriormente, há a libertação de fatores de crescimento potentes que estão implicados na deposição da matriz extracelular, na ativação e proliferação das células musculares lisas dos vasos sanguíneos, pericitos e outras células mesenquimatosas das paredes dos vasos. As alterações fenotípicas nas células endoteliais

e estruturas adjacentes tornam a resposta do endotélio mais lenta e conduzem a uma remodelação vascular com alterações profundas na sua arquitetura [1].

As alterações vasculares afetam predominantemente os capilares e os pequenos vasos [4]. Os capilares caracterizam-se por apresentarem ansas distorcidas e irregulares em todos os órgãos envolvidos e estas alterações podem preceder o início de fibrose em meses ou anos [4]. A vasculopatia atinge todas as camadas e, vasos de maiores dimensões, apresentam hiperplasia fibrótica da íntima e das células musculares lisas da parede, com perda de elasticidade. O espessamento da íntima conduz à oclusão do vaso, despoletando a formação de trombose *in situ* [5]. A perda progressiva de capilares e a remodelação anormal dos vasos dão origem a um fluxo sanguíneo insuficiente, causando hipoxia crónica e severa. A hipoxia é o maior estímulo para a angiogénese e leva à produção de fatores que a despoletam [5]. Contudo, apesar da existência de hipoxia grave e aumento de fatores angiogénicos, a resposta angiogénica na ES é insuficiente, perpetuando o ciclo vicioso e levando à isquemia dos tecidos [6].

Angiogénese e Vasculogénese

A angiogénese é um processo complexo que consiste na formação de novos vasos a partir de vasos pré-existentes [7,8]. É controlada por fatores estimuladores (pró-angiogénicos) e inibidores (angiostáticos) cujo nível se encontra equilibrado em tecidos saudáveis [9] e depende da funcionalidade de uma série de moléculas, incluindo fatores de crescimento e seus recetores, proteínas da matriz extracelular, moléculas de adesão e enzimas proteolíticas [10,11].

Desempenha um papel primordial durante o desenvolvimento embrionário e, posteriormente, na vida adulta, em várias situações fisiológicas e patológicas como por exemplo, a inflamação crónica [7,8].

Durante este processo, há aumento da permeabilidade vascular, extravasão de proteínas plasmáticas que vão servir de suporte temporário à migração de células endoteliais, libertação de metaloproteinases pelo endotélio que vão romper a membrana basal vascular e conduzir à invasão do estroma circundante por células endoteliais. Esta invasão celular vai dar origem à formação de novos vasos, através da proliferação das células endoteliais e da organização das novas células em estruturas tubulares tridimensionais. A formação do lúmen dos vasos e a estabilização da parede dos mesmos por pericitos são os processos finais da angiogénese, levando à formação de uma rede funcional de novos capilares [4,10].

As células do sistema imune inato e adaptativo estão envolvidas nos mecanismos de ativação, proliferação e migração das células endoteliais através da libertação de mediadores pró-angiogénicos [4].

A vasculogénese é um processo que consiste na formação de novos vasos a partir de células progenitoras, derivadas da medula [4]. Estas adquirem características de células endoteliais maduras, expressam os seus marcadores e são incorporadas em novos vasos, em locais de isquemia [12].

A vasculogénese, após o nascimento, contribui para a reparação vascular em resposta à sua lesão, através da re-endotelização rápida dos vasos e formação de colaterais em tecidos isquémicos. As células endoteliais são mobilizadas da medula óssea para a circulação em resposta ao *stress* e/ou sinais relacionados com a existência de lesão vascular, migrando para os locais lesados através do endotélio, e contribuem para a formação de novos vasos/ reparação dos existentes, trabalhando em conjunto com as células endoteliais maduras pré-existent [4,13].

A resposta angiogénica encontra-se enfraquecida na ES [4] e a evidência aponta para a existência de um desequilíbrio entre fatores angiogénicos e angiostáticos [4] assim como para uma diminuição da atividade angiogénica dos linfócitos e monócitos do sangue periférico [4]. Apesar disso, o nível de vários mediadores pró-angiogénicos, tais como o VEGF que é um dos mais potentes, tem-se revelado aumentado na pele e no sangue periférico [4, 14-17] o que contrasta com a existência de uma vascularização insuficiente [4].

A exposição prolongada ao VEGF dá origem à formação de uma rede capilar caótica, com formas irregulares e capilares ectasiados, alterações que são encontradas na capilaroscopia de doentes com ES [4].

Para além disso, os recetores do VEGF (VEGFR-1, VEGFR-2 e VEGFR-3) estão sobre expressos nas células endoteliais dos microvasos da pele [6,18]. No entanto, há estudos que apontam para a existência de uma discrepância entre a expressão do VEGF e dos seus recetores e outros para uma sinalização deficitária dos recetores ao nível das células endoteliais dos microvasos [4,18,19], podendo ambos os mecanismos contribuir para uma angiogénese insuficiente.

O aumento da expressão da isoforma do VEGF com propriedades antiangiogénicas, o VEGF 165b, formada através de um *splicing* alternativo de uma região não codificadora do exão 8, e que atua inibindo a angiogénese estimulada pela hipoxia e através de uma inibição competitiva do VEGF 165, parece ser outra explicação para a existência de uma angiogénese ineficaz [20, 21].

Outra razão apontada para a ineficácia do VEGF na ES poderá ser o tempo de exposição tecidular ou demasiado curto, resultando em vasos instáveis, ou demasiado

prolongado, resultando na fusão de microvasos imaturos e formação de uma rede caótica de capilares gigantes [6, 9, 19].

Uma explicação alternativa para a angiogénese insuficiente na ES, poderá residir no aumento de fatores angiostáticos como a endostatina [22, 24] e a angiostatina [23, 24].

Para além da angiogénese ineficaz, há evidência de que uma vasculogénese insuficiente possa contribuir para a patofisiologia da ES [4]. No entanto, os estudos publicados na literatura são contraditórios relativamente ao papel das células progenitoras das células endoteliais circulantes na ES [4].

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2.5.1 – AVALIAÇÃO DA MICROANGIOPATIA, DA LESÃO ENDOTELIAL E DA ANGIOGÊNESE

2.5.1.1 - Capilaroscopia

A videocapilaroscopia é o método mais sofisticado, não invasivo, utilizado para avaliar a microcirculação *in vivo* [1].

Esta técnica deverá ser efetuada pelo menos em 8 dedos das mãos e vários parâmetros devem ser avaliados para que seja possível identificar alterações precoces associadas às doenças autoimunes: presença de capilares alargados ($> 20\mu\text{m}$) ou megacapilares ($> 50\mu\text{m}$), microhemorragias, perda de capilares, edema, capilares ramificados (angiogénese) e desorganização da arquitetura vascular [1].

Na ES, a lesão microvascular é caracterizada por alterações progressivas dos capilares com diminuição da sua densidade e redução do fluxo sanguíneo [1].

Vários estudos propuseram graduar as alterações morfológicas da microvasculatura em doentes com ES. Maricq *et al.* [2,3] reconheceram 2 padrões esclerodérmicos *major*, o ativo e o lento. Lee *et al.* [3,4] tentaram quantificar a perda de capilares descrevendo 4 escalas de gravidade (0=sem perda de capilares; 1= uma ou duas áreas de deleção vascular discretas; 2= mais de duas áreas de deleção discretas; 3= áreas avasculares extensas e confluentes). Andrade *et al.* [3,5] propuseram o uso de 4 parâmetros para descrever a perda de capilares: número médio de ansas por mm na última fileira de capilares do 4º e 5º dedo das ambas as mãos, utilização do *score* de Lee *et al.* [4] para cada dedo das mãos, classificar de forma subjetiva a densidade capilar da última fileira como alta, moderada ou baixa e contar o número de papilas vazias de cada dedo (devem ter 1 ou mais capilares).

Mais recentemente, Cutolo *et al.* [6,7] classificaram as alterações da microvasculatura detetadas por videocapilaroscopia em 3 padrões: precoce, ativo e tardio. O padrão precoce caracteriza-se pela existência de capilares dilatados/ megacapilares (mas menos de 4 por campo), microhemorragias escassas, distribuição capilar relativamente bem preservada e ausência de perda de capilares; o padrão ativo caracteriza-se pela existência de hemorragias frequentes, mais de 6 megacapilares por mm, perda moderada de capilares (20-30%), ligeira alteração da arquitetura vascular (entre 4 a 6 capilares alterados por mm) e poucos ou nenhuns capilares ramificados. O padrão tardio caracteriza-se pela presença de poucos ou nenhuns megacapilares, alargamento irregular dos capilares, perda severa de capilares (entre 50-70%) com áreas avasculares abundantes, desorganização da arquitetura vascular e capilares ramificados. Durante a progressão da ES é observado um aumento e agravamento significativo e gradual das lesões microvasculares [1].

Com o objetivo de quantificar estas alterações da microvasculatura, Sulli *et al.* [8] propuseram uma pontuação semi-quantitativa, adotada para cada alteração dos capilares:

0=sem alterações; 1= menos de 33% de alterações/ diminuição, 2= entre 33-66% de alterações/ diminuição, 3= mais de 66% de alterações/ diminuição capilares/mm. A pontuação média para cada uma das alterações é calculada através da análise de 4 campos consecutivos de 1mm cada, em cada um dos 8 dedos estudados. As médias das pontuações dos 8 dedos são somadas e divididas por 8. O valor final representa a pontuação de cada parâmetro avaliado. Os autores encontraram uma relação entre a variação das pontuações relacionadas com a presença de perda de capilares, desorganização da arquitetura vascular e capilares ramificados e a progressão da doença esclerodérmica. Já para as pontuações médias referentes à existência de hemorragias e de capilares gigantes ou megacapilares, essa relação não foi encontrada [1].

Smith *et al.* [9] propuseram uma escala simples para calcular a pontuação média de perda de capilares (0=sem alterações; 1= <33% de redução capilar *versus* o normal (9/mm); 2=33 a 66% de redução capilar e 3= mais de 66% de redução capilar), com base nas pontuações obtidas em 8 dedos das mãos e em 4 campos de 1mm por dedo. A média das pontuações é somada e dividida por 8. Foi escolhido o valor de 1.67 (sensibilidade e especificidade de 70%) como *cutoff* prognóstico para o desenvolvimento de úlceras/ lesões tróficas no período de 6 a 12 meses.

Sebastiani *et al.* [10] propuseram uma pontuação, denominada CSURI (*capillaroscopic skin ulcers risk index*), que representa a primeira proposta de utilização de uma pontuação quantitativa preditiva do aparecimento de úlceras digitais de novo ou persistência de úlceras pré-existentes. Baseia-se na avaliação do número de capilares na fileira distal (N), número de megacapilares (M) e no diâmetro máximo dos megacapilares (D). Das imagens recolhidas, pelo menos uma imagem por dedo (do 2º ao 5º de cada mão), é escolhida uma que tiver menor N e, secundariamente, que tiver maior M. É obrigatório a existência de pelo menos 1 megacapilar para calcular a pontuação final. O índice CSURI é calculado com base na fórmula: $D \times M : N^2$. Segundo os autores, um *cutoff* de 2.94 tem um valor preditivo positivo para o aparecimento de úlceras digitais de 73.3%.

Nos últimos anos, alguns autores descreveram uma associação entre a redução da densidade capilar na capilaroscopia e a severidade da Hipertensão Arterial Pulmonar (HTAP) [11,12]. Mais recentemente, Riccieri *et al.* [13] descreveram a existência de uma relação entre a pressão na artéria pulmonar avaliada por cateterismo cardíaco direito e as alterações encontradas na capilaroscopia. Um padrão capilaroscópico mais severo (ativo/ tardio) foi descrito em 92% dos doentes com HTAP e em 42% daqueles sem HTAP.

Kayser *et al.* [14] relacionaram as alterações encontradas na capilaroscopia, em particular a existência de áreas avasculares, com a sobrevida dos doentes.

“A capilaroscopia, de forma isolada, não é suficiente para explorar a patofisiologia complexa da microangiopatia e parâmetros clínicos, demográficos e serológicos são essenciais para caracterizar o prognóstico das lesões microvasculares. Assim, são necessários estudos com maior número de doentes e com modelos preditivos mais complexos, incluindo a capilaroscopia e outras variáveis clínicas/ serológicas que possam identificar novas associações com as manifestações da ES e seu prognóstico” [3].

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2.5.1.2 - Biomarcadores de lesão endotelial e de angiogénese

Um biomarcador, de acordo com o *Biomarkers Definitions Working Group*, consiste numa “característica que é objetivamente medida e avaliada como um indicador de um processo biológico normal, processo patológico ou resposta farmacológica a uma terapêutica de intervenção” [1].

Os biomarcadores podem ser utilizados como ferramentas de diagnóstico, de prognóstico, de estadiamento e classificação da extensão de uma doença, para prever o seu curso clínico e monitorizar a resposta a uma determinada intervenção terapêutica [1].

Idealmente, os biomarcadores devem estar presentes em fases pré-clínicas, estar diretamente envolvidos no processo patogénico e correlacionarem-se com a evolução avaliada por um teste considerado *gold-standard* [2].

De acordo com Hummers LK [3], os biomarcadores vasculares podem ser utilizados na ES para prever o desenvolvimento de complicações vasculares numa população suscetível, avaliar a resposta à terapêutica quando são utilizados agentes que atuam sobre os vasos e definir mecanismos biológicos específicos e/ou atividade da doença ao nível dos vasos.

Entre os potenciais biomarcadores vasculares estudados na ES (quadro 1), neste capítulo serão abordados os utilizados na nossa investigação. Apesar de poder existir uma partilha de mecanismos de ação das várias moléculas, a ET-1 e as moléculas de adesão foram considerados essencialmente biomarcadores de lesão endotelial e os restantes marcadores de angiogénese.

Quadro 1 - Potenciais biomarcadores da doença vascular esclerodérmica (adaptado de [3])

Proteínas angiostáticas	Marcadores pró- angiogénicos
Angiostatina	MMP (<i>matrix metalloproteinases</i>)
Endostatina	HGF (<i>hepatocyte growth factor</i>)
Trombospodina	VEGF (<i>vascular endothelial growth factor</i>)
Recetores solúveis do VEGF	PIGF (<i>placental growth factor</i>)
Endoglina solúvel	PDGF (<i>platelet derived growth factor</i>)
TIMP (<i>tissue inhibitors of metalloproteinase</i>)	FGF (<i>fibroblast growth factor</i>)
	Precusores das células endoteliais circulantes
	MCP-1 (<i>monocyte chemotactic protein</i>)
	SDF-1 (<i>stromal cell-derived factor/CXCL12</i>)
	Fractalcina (CXCL1)
Marcadores endoteliais	Moléculas de adesão vascular
Factor de von Willebrand	E-seletina
Endotelina-1	P-seletina
	VCAM-1 (<i>vascular cell adhesion molecule</i>)
	ICAM-1 (<i>intracellular adhesion molecule</i>)

Biomarcadores de lesão endotelial

Endotelina-1

O sistema da endotelina é constituído por três péptidos vasoativos (ET-1, ET-2 e ET-3), os seus recetores ligados à proteína G (recetor de endotelina A e B: ET_A, ET_B) e as duas enzimas conversoras da endotelina (1 e 2) [4]. Os recetores ET_A e ET_B possuem características farmacológicas diferentes [5] e a afinidade das três isoformas é idêntica para o recetor ET_B mas gradativa para o recetor ET_A. Desta forma, a ET1 é o peptídeo que tem maior afinidade para este último recetor, seguido da ET-2 e da ET-3 [6]. Os recetores ET_A localizam-se nas células musculares lisas dos vasos e a sua estimulação provoca vasoconstrição. Os recetores ET_B encontram-se quer nas células endoteliais (cuja estimulação dá origem a vasodilatação via produção do óxido nítrico e prostaglandinas) quer nas células musculares lisas dos vasos (cuja estimulação provoca vasoconstrição) [5]. O número de recetores é regulado por uma variedade de fatores, sendo que a isquemia os aumenta [7,5] e a angiotensina II os diminui [5,8].

A ET-1 é a isoforma predominante na espécie humana e é processada sequencialmente a partir de um pré-pro-peptídeo composto por 212 aminoácidos, através de uma endopeptidase e das enzimas conversoras da endotelina, até ao peptídeo biologicamente ativo de 21 aminoácidos [9]. É produzida pelas células endoteliais e mesenquimatosas, como fibroblastos e células musculares lisas, atua de forma parácrina e autócrina e a sua produção é controlada por uma série de fatores solúveis [10].

A ET-1 exerce os seus efeitos através dos recetores ET_A e ET_B estando documentada uma elevação do seu nível plasmático em doentes com ES [10,11]. Tem efeitos vasoconstritores potentes e funciona como mediador da proliferação das células da parede vascular, fibrose e inflamação [10,12]. A nível vascular e endotelial, promove a vasoconstrição e adesão leucocitária, facilita a interação entre os leucócitos e os fibroblastos e aumenta a proliferação celular e a produção de fatores pró-fibróticos [13]. O efeito fibrogénico da ET-1 foi observado *in vitro* e a sua presença parece ser necessária para a atividade fibrogénica do TGF (*transforming growth factor*) tanto *in vivo* como *in vitro* [14].

O aumento da expressão dos recetores ET_A e a diminuição da expressão dos ET_B no endotélio de doentes com ES e o aumento dos últimos no tecido muscular liso dos vasos, parecem contribuir para a proliferação celular, hipertrofia, inflamação, fibrose e vasoconstrição [15,16,17]. Está demonstrado que inibidores não seletivos dos recetores da ET-1 [18] reduzem os níveis de ET-1, aumentam mediadores vasoativos (como o óxido nítrico) e parecem também diminuir a atividade pro-fibrótica dos fibroblastos cutâneos dos doentes com ES [19].

Moléculas de adesão

As moléculas de adesão são proteínas da superfície celular, multifuncionais, que medeiam as interações célula/ célula e célula/ matriz extracelular [20]. Participam em vários processos celulares como a motilidade, sinalização e ativação. São críticas para o desenvolvimento de respostas inflamatórias e imunes, incluindo a migração dos leucócitos dos vasos para os tecidos, fagocitose, vigilância imunológica e ativação leucócito/ célula endotelial [20].

A maioria encontra-se agrupada em famílias, com base em semelhanças estruturais e de função, incluindo as Seletinas, as Integrinas, as Caderinas e a Superfamília das Imunoglobulinas [20].

As Seletinas capturam e facilitam o rolamento dos leucócitos sobre o endotélio, que é a etapa inicial no recrutamento dos leucócitos para os tecidos extravasculares, e as CAMs permitem a adesão firme dos leucócitos às células endoteliais e a sua migração através do endotélio vascular [21].

O processo de transformação destas moléculas de adesão após a sua ativação não é completamente conhecido, mas uma percentagem é exteriorizada para fora das células [22], pensa-se que por clivagem enzimática [23].

As isoformas solúveis das moléculas de adesão mantêm a capacidade de se ligarem aos respetivos ligandos e, embora o papel funcional permaneça desconhecido [23], o seu doseamento no soro e outros fluídos orgânicos tem sido proposto como útil para avaliar a perturbação endotelial [22].

Vários estudos têm demonstrado aumento de várias moléculas de adesão no soro de doentes com ES relativamente a controlos saudáveis, nomeadamente da ICAM-1, VCAM-1, P-seletina e E-seletina [21,24-26].

ICAM-1 (CD54)

É uma proteína da Superfamília das Imunoglobulinas [27]. Encontra-se expressa em vários tipos celulares, incluindo as células endoteliais, onde tem um papel crítico numa adesão firme e na migração trans-endotelial de vários subtipos de células leucocitárias e pode ativar cascatas de sinalização celular [27].

Células endoteliais ativadas por citocinas pró-inflamatórias [23], óxido nítrico e outras moléculas [28] aumentam significativamente a expressão da ICAM-1 (normalmente expressa em baixos níveis) [23]. A expressão desta molécula de adesão também se encontra aumentada nos fibroblastos de doentes com ES [29].

O aumento da isoforma solúvel tem sido descrito em doentes com ES [21,23] e pode refletir o aumento da sua expressão nas células endoteliais mas também nos fibroblastos ou células do sistema imune [23].

VCAM-1 (CD106)

É uma glicoproteína da superfamília das Imunoglobulinas α [30]. É, também, um ligando das integrinas $\alpha 4, \alpha 4 \beta 1$ e $\alpha 4 \beta 7$ e medeia a adesão de monócitos, eosinófilos, linfócitos e basófilos [31,32]. Tem uma expressão celular mais restrita que a ICAM-1 [23] e a sua expressão é induzida por citocinas (IL1, IL4, TNF) e lipopolissacarídeos (LPS) [33-34].

Seletina-E (CD62E)

Membro da família das Seletinas e encontrada apenas nas células endoteliais ativadas [23], possui um terminal capaz de reconhecer o tetra-sacarídeo *sialil-Lewis* dos monócitos e granulócitos o que parece ser importante para a adesão de granulócitos, monócitos e células T CD4+ de memória ao endotélio [35-37]. A sua expressão é regulada por citocinas (como a IL-1, TNF alfa, interferão gama) e LPS [38,39].

Seletina-P (CD62P)

Membro da família das Seletinas, encontra-se expressa nas plaquetas e nas células endoteliais. Facilita o rolamento dos leucócitos sobre as células endoteliais e a agregação e adesão plaquetária a outras células [20]. Encontra-se armazenada em grânulos α das plaquetas e em corpos de *Weibel-Palade* nas células endoteliais, sendo rapidamente mobilizada para a superfície das células após estas serem ativadas por mediadores inflamatórios como a trombina e a histamina [20].

Biomarcadores de angiogénese

VEGF

O VEGF (também conhecido por VEGF-A) é uma glicoproteína hemodimérica de ligação à heparina [40], produzida por uma grande variedade de células, nomeadamente endoteliais, fibroblastos, macrófagos e células musculares lisas [41-46].

É um dos fatores angiogénicos conhecidos mais potentes [47] e está envolvido em várias etapas da angiogénese fisiológica e patológica [47] atuando seletivamente nas células vasculares endoteliais, com efeitos biológicos dose-dependentes [47].

O VEGF emite sinais angiogénicos para o endotélio vascular através de recetores de tirosina-cinase de alta afinidade, designados VEGFR-1, VEGFR-2, cuja expressão está

aumentada nas células endoteliais dos microvasos de doentes com ES [48,49] e, em menor extensão, através do VEGFR-3.

O VEGF165b é uma isoforma do VEGF com propriedades anti-angiogénicas e expressa-se difusamente nos tecidos humanos, estando elevado em condições patológicas não angiogénicas como a ES. Atua como inibidor competitivo do VEGF165 através da sua ligação ao VEGFR-2 [50].

Endostatina

A endostatina é um peptídeo derivado do colagénio XVIII que é produzido pelos fibroblastos e hepatócitos e é encontrado nas membranas basais dos vasos sanguíneos da pele e dos pulmões [51-52]. Estudos apontam para um aumento da síntese e proteólise do colagénio XVIII na ES [52].

O VEGF estimula a libertação de collagenases que dão origem à libertação da endostatina a partir do colagénio XVIII existente nas células endoteliais [53-54]. A endostatina é encontrada em grânulos plaquetários alfa, é libertada durante a ativação e agregação plaquetária [53-54] e é o inibidor da angiogénese induzido pelo VEGF mais potente [51], competindo com o VEGF ao nível dos seus recetores nas células endoteliais [53].

Angiostatina

A angiostatina é um fragmento terminal de aminoácidos do plasminogénio, libertado através da ação de várias proteases, incluindo o ativador tecidual do plasminogénio, a uroquinase ativadora do plasminogénio e várias metaloproteinases (MMP) da matriz como a MMP-2, MMP-3, MMP-7, MMP-9 e MMP-12 e possui atividade antiangiogénica [55].

Estudos apontam para que proteases libertadas de grânulos de linfócitos T possam ser responsáveis pelo aumento da produção de angiostatina e diminuição dos níveis séricos de plasmina em doentes com ES [55].

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CAPÍTULO 3 |

METODOLOGIA

CONTEÚDOS

Seleção da amostra

Classificação dos doentes

Avaliação clínica, exames subsidiários e classificação do envolvimento orgânico e sua gravidade

Exames de investigação

Análise estatística

Revisão da literatura

Referências

Neste capítulo apresenta-se, de forma resumida, a metodologia utilizada na investigação, uma vez a mesma ser detalhada nos capítulos dos resultados.

Este estudo, observacional e prospetivo, teve uma duração de 4 anos de *follow-up* clínico (de Março de 2011 a Março de 2015).

O estudo foi aprovado pela Comissão de Ética e pelo Conselho de Administração do hospital e todos os participantes assinaram um termo de consentimento livre e informado.

Seleção da amostra

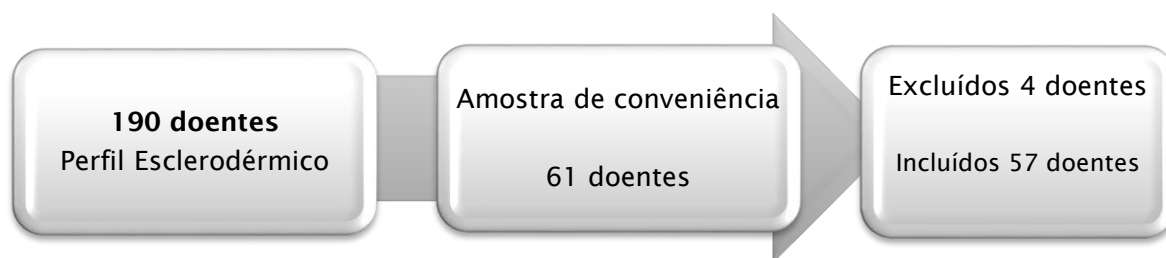
A *coorte* de doentes com perfil Esclerodérmico (Pré-ES + ES) da Unidade de Imunologia Clínica (UIC) da qual sou responsável incluía, à data do início do estudo, 190 doentes.

Foi selecionada uma amostra de conveniência, constituída por 61 doentes, entre Setembro de 2010 e Março de 2011.

Foram, posteriormente, excluídos 4 doentes, 3 por apresentarem patologias associadas que podiam interferir com os resultados da investigação (uma Infecção pelo Vírus da Imunodeficiência Humana Adquirida, uma Doença Mista do Tecido Conjuntivo e um linfoma não Hodgkin de células B) e o outro por falta de amostra de sangue.

Dos 57 doentes incluídos no estudo, 47 reuniam critérios da ACR e posteriormente da ACR/EULAR para ES e 10 critérios de LeRoy e Medsger para Pré-ES [1,2,3].

Figura 1 – Seleção da amostra



Foi selecionado um grupo controlo, dentro de uma população de dadores benévolos de sangue, constituído por 25 indivíduos adultos saudáveis.

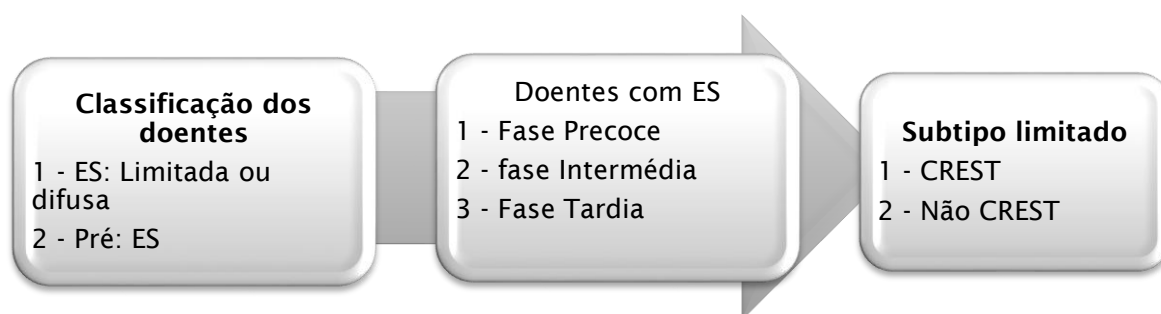
Foram considerados critérios de exclusão a existência de fatores de risco cardiovascular (hábitos tabágicos, diabetes mellitus, dislipidemia e hipertensão arterial) e a

coexistência de outras doenças associadas que pudessem interferir com os resultados da investigação.

Classificação dos doentes

Os doentes incluídos no estudo foram classificados nos vários subtipos de acordo LeRoy *et al.* e LeRoy e Medsger [2,4]. De seguida, os doentes com ES foram subdivididos, de acordo com o tempo de evolução da doença (tempo decorrido desde o início do primeiro sintoma atribuído à doença) e em consonância com o subtipo, em fases (precoce, intermédia e tardia) [5] e o subtipo limitado em CREST e não CREST [6].

Figura 2 – Classificação dos doentes



Avaliação clínica, exames subsidiários e classificação do envolvimento orgânico e sua gravidade

Os doentes foram avaliados do ponto de vista clínico com uma periodicidade mínima de 6 meses e pedidos exames subsidiários de acordo com as boas práticas para esta patologia. A classificação do envolvimento orgânico foi efetuada de acordo com o ponto de corte da escala de gravidade de Medsger que foi também utilizada para classificar a gravidade de envolvimento de cada órgão [7]. Foi realizada, sempre pelo mesmo operador, técnica de videocapilaroscopia periungueal, utilizando um videocapilaroscópio KK com lentes de ampliação de 200 vezes. A técnica foi aplicada do 2º ao 5º dedo de ambas as mãos, no início e no final do estudo, sendo as imagens guardadas e validadas por perito em videocapilaroscopia. A variação inter-observadores foi de 2.5%. Foi utilizada a classificação

de Cutolo *et al.* [8], que é uma classificação semi-quantitativa da microangiopatia esclerodérmica, e as alterações encontradas foram divididas em precoces, ativas e tardias.

Exames de investigação

As amostras de sangue destinadas à quantificação de moléculas solúveis foram colhidas em tubos Vacuette sem aditivo (*Greiner Bio-One, Austria*). Os soros, obtidos por centrifugação, foram aliquotados e congelados a -40 °C até ao momento da sua análise para as seguintes determinações analíticas: ET-1, angiostatina, endostatina, VEGF, VCAM-1, ICAM-1, P-seletina e E-seletina.

As concentrações séricas de VEGF, endostatina e angiostatina foram avaliadas através do método ELISA (VEGF-A: ELISA assay BE55101 da *IBL International*; endostatina e angiostatina: ELISA assays da *USCN Life Sciences Inc. Wuhan;China*), de acordo com as instruções do fabricante. Foram também calculadas as razões entre os níveis séricos de VEGF e os níveis séricos de angiostatina, assim como as razões entre os níveis séricos de VEGF e os níveis séricos de endostatina.

As concentrações séricas de ET-1 foram medidas usando um método de ELISA em *sandwich: Endothelin 1.21 (Biomedica Medizinprodukte GmbH, Austria)*, de acordo com as instruções do fabricante.

A determinação da concentração sérica das moléculas de adesão VCAM-1 solúvel (*IBL International Ref BE59051*), ICAM-1 solúvel (*IBL International Ref BE59011*), P-seletina solúvel (*IBL International Ref BE59081*) e E-seletina solúvel (*IBL International Ref BE59061*), foi efetuada utilizando imunoensaios ELISA da *IBL International, Hamburgo, Alemanha*, de acordo com as instruções do fabricante.

As amostras de soro foram também usadas para a determinação dos ANA séricos por imunofluorescência indireta, utilizando um *kit* comercializado (*INOVA Diagnostics Inc. San Diego, CA, USA*) tendo como substrato células HEp-2, com uma diluição inicial de 1:80, de acordo com as instruções do fabricante. Foi avaliada a intensidade e o padrão de imunofluorescência. Para além disso, foram avaliados vários anticorpos, específicos e não específicos da doença esclerodérmica usando o método de fluoroimunoensaio enzimático ou immunoblotting.

Os estudos de imunofenotipagem dos linfócitos foram efetuados por citometria de fluxo em amostras de sangue total colhidas em tubo com EDTA-K3, utilizando anticorpos monoclonais conjugados com diferentes fluorocromos - isotiocianato de fluoresceína (FITC), ficoeritrina (PE), ficoeritrina cianina 5 (PC5) e alofocianina (APC) -, e com diferentes especificidades: anti-CD3, anti-CD4, anti-CD8, anti-CD45RO, anti-HLA-DR (*Becton-Dickinson Biosciences, San José, Califórnia, EUA – BD*) e anti-CD56 (*Beckman Coulter, Hialeah, FL,*

EUA e Immunotech, Marselha, França). As marcações celulares foram efetuadas em 100 µl de sangue total contendo 0.5 a 1.5 x 10⁶ células, usando quantidades saturantes dos anticorpos monoclonais, tal como recomendado pelos fabricantes, e fazendo a incubação durante 15 minutos à temperatura ambiente. Após lise dos eritrócitos e fixação dos leucócitos com o reagente *FACS Lysing Solution*® (BD), nas condições recomendadas pelo fabricante, as amostras foram lidas no citómetro de fluxo FACSCalibur™ (BD), tendo sido adquiridas 200,000 células por tubo. A análise dos dados foi feita no programa *Paint-a-Gate* (BD).

O doseamento das citocinas IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ e IL-17A no soro foi realizado por citometria de fluxo, com um ensaio multiplex com esferas de látex, *BD™ CBA Human Th1/Th2/Th17 Cytokine Kit* (BDB), de acordo com as indicações do fabricante.

Na altura da colheita, foi assegurado que os doentes tinham efetuado o *washout* de drogas que pudessem interferir com as medições das moléculas em causa, tendo sempre em conta o estado clínico do doente.

Análise estatística

Todas as análises estatísticas foram efetuadas no *software IBM Statistical Package for Social Sciences* (SPSS 22.0). A descrição dos resultados foi realizada com base nas estatísticas descritivas adequadas e serão referidas em cada um dos capítulos respetivos.

Revisão da literatura

A pesquisa dos artigos científicos utilizados nesta tese foi efetuada na base *medline*, via *pubmed*.

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CAPÍTULO 4 |

RESULTADOS

4.1 – ALTERAÇÕES IMUNOLÓGICAS

Nesta secção são avaliados os níveis séricos de várias citocinas Th1/Th2/Th17 por citometria de fluxo, anticorpos específicos e não específicos de doença esclerodérmica por *immunoblotting* ou fluoroimunoensaio e quantificados os linfócitos T e células NK no sangue periférico por citometria de fluxo. Os resultados são relacionados com os subtipos de doença esclerodérmica, fases da ES, padrões capilaroscópicos e envolvimento orgânico e sua gravidade.

4.1.1 – SUPPORTING THE NEED FOR ONGOING DISCUSSION IN SYSTEMIC SCLEROSIS FIELD: FINDINGS OF A COHORT STUDY

Artigo para submeter a publicação

Almeida I, Potlog N, Neves E, Lima M, Vasconcelos C.

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Abstract

Objective: To characterize a Scleroderma population regarding auto-antibodies, clinical findings and to evaluate its correlation.

Methods: Non-random convenience sample of 57 Scleroderma patients. The presence of specific and non-specific auto-antibodies was evaluated and correlated with disease subtypes, organ involvement and capillaroscopic pattern.

Results: Fifty seven patients were evaluated: 34 patients with limited cutaneous SSc (lcSSc), 13 patients with diffuse cutaneous SSc (dcSSc) and 10 patients with Pre-SSc. Anti-centromere antibodies (ACA) were the most prevalent (59.6%), followed by anti-topoisomerase (ATA) (26.3%) and anti-RNA polymerase (RNAP) (3.5%). Antibodies identified in the lcSSc group were predominantly ACA, while in the dcSSc we observed a higher prevalence of ATA. The presence of ATA was significantly related to gastrointestinal tract involvement, while ACA were associated with less skin and pulmonary interstitial involvement. Anti-Ro 52 antibodies, although not performed in all patients, were positive in almost all, primarily in those with limited SSc. Cardiac involvement was similar in both lcSSc and dcSSc subtypes, although it was greater in the first one. Subclinical organ involvement in patients with Pre-SSc was reported, compatible with the diagnosis of sine-Scleroderma, although not fulfilling 2013 ACR/EULAR criteria for SSc.

Conclusions: The value of ACA and ATA in the evaluation of organ involvement in patients with SSc was proven, and the high prevalence of antibodies anti-Ro 52 in SSc patients, namely with the limited subtype, was confirmed. Apparently, cardiac involvement may be as or more frequent in the lcSSc subtype, when compared with the dcSSc, supporting the need for systematic screening of cardiac involvement in all patients with SSc. ACR/EULAR 2013 criteria are not yet sensitive enough for the early diagnosis of some forms of SS, namely sine-SSc.

Introduction

Scleroderma or Systemic Sclerosis (SSc) is a connective tissue disease, of unknown etiology, with heterogeneous clinical manifestations and variable clinical evolution [1]. Microvascular damage, fibroblast dysfunction with fibrosis, and the production of autoantibodies are essential processes involved in its pathogenesis [2]. Antinuclear antibodies (ANA) are primarily directed against nucleolar antigens, and they may be present in more than 95% of patients [3]. Using the indirect immunofluorescence method on Hep-2 cells, several fluorescent patterns may be identified, including centromeric, nucleolar, speckled, homogeneous and nuclear membrane, amongst others [4]. In regards to autoantibodies associated with SSc, some are specific to the disease (80% of ANA), namely anti-centromere antibodies (ACA), anti-topoisomerase I (ATA, anti-TOPOI or anti-Scl 70), anti-RNA polymerase (anti-RNAPIII and I), anti-fibrillarin (AFA or anti-U3-RNP) and anti-Th/To, and they may correlate with the disease's severity and clinical presentation [5]. In general, these autoantibodies are mutually exclusive, and do not change during the course of the disease [6-8].

Anti-centromere antibodies (centromeric fluorescent pattern) frequency in SSc patients varies in accordance with the different studies and ethnic groups involved, ranging between 20 and 40% [5]. These are mainly associated with limited cutaneous SSc (lcSSc), namely with the CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) variant [9] and, in about 20%, with pulmonary arterial hypertension [3]. On the other hand, they seem to play a protective role against pulmonary interstitial involvement and scleroderma renal crisis [3]. In ACA-positive patients, digital ulcers, gangrene and acro-osteolysis are frequent, and musculoskeletal involvement is very rare [3].

Anti-topoisomerase I antibodies are usually associated with a nucleolar fluorescent pattern, and their frequency may vary in SSc, in accordance with the method used [10]. According to Tamby *et al.*, ATA have been detected in 36.9% of patients using indirect immunofluorescence, enzyme-linked immunosorbent assays (ELISA) and immunoblotting methods, but only in 24.3% when ELISA method is used on its own [11,12].

Anti-RNA polymerase I and III antibodies present, for the most part, a nucleolar pattern and are usually coexistent [13,14]. Their prevalence varies according to the studied population, and their frequency is between 4 and 31.5% in the European population [15-18].

Anti-topoisomerase and anti-RNAPIII antibodies are, usually, associated with the diffuse form of SSc (dcSSc) [13,19], and both antibodies have been related with a worse prognosis and an elevation in mortality [20-22]. In addition, ATA have been associated with higher pulmonary interstitial, heart and musculoskeletal involvement [3,9]. According to Walker

et al., the presence of ATA is associated with a higher prevalence of intestinal symptoms, cardiac arrest, diastolic dysfunction and renal hypertension [19]. Beyond that, these antibodies, like ACA, have been associated with digital ulcers, gangrene and acro-osteolysis [3]. Anti-RNAPIII seem to be correlated with higher renal involvement (Scleroderma Renal Crisis) [3], severe diffuse cutaneous involvement [3] and, according to some authors, with gastric antral vascular ectasia [23,24]. Four recent studies described a possible association between the presence of these antibodies and malignancy [18,25,26]. On the other hand, the presence of anti-RNAPIII antibodies seems to play a protective role concerning the severity of pulmonary interstitial involvement [3].

Anti-fibrillarin (anti-U3-RNP), anti-Th/To and anti-PM/Scl antibodies are usually associated with the nucleolar pattern [5].

Anti-fibrillarin antibodies, which are present in 4-10% of patients with SSc [3,27-29], are detected more frequently among male and African American patients [3,29-32], they are frequently associated with musculoskeletal manifestations and pulmonary arterial hypertension [28,33]. Steen *et al.* demonstrated an association between this autoantibody, the diffuse subtype and severe multiorgan involvement, especially pulmonary arterial hypertension and pulmonary fibrosis, gastrointestinal involvement and peripheral neuropathy [3]. Sharif *et al.* have described the association of these antibodies with digital ulcers, pericarditis and gastrointestinal involvement and, in contrast to Steen *et al.*, with less pulmonary involvement [31].

Anti-Th/To antibodies, whose prevalence in SSc is 2-5%, are mostly detected in patients with the limited subtype [34]. They are associated with severe gastrointestinal involvement [3], and, in contrast to ACA, not only are associated with pulmonary arterial hypertension but also with pulmonary interstitial disease, and therefore have a worse prognosis [3]. On the other hand, patients with these antibodies have less skin involvement and less severe digital vasculopathy [35].

Other antibodies may be identified in patients with SSc, such as anti-Ro52, anti-Nor90, anti-U1RNP and anti-PM/Scl [5]. According to Hudson *et al.* [36], anti-Ro52 antibodies, even though detectable in other autoimmune diseases, are one of the most frequently encountered in SSc (20%). They may serve as a marker of pulmonary interstitial disease and overlap syndrome, Sjögren [37] and/or Myositis [36], and it has been suggested that they have diagnostic and prognostic value [36]. Anti-Nor90 antibodies are less frequent and, when present, are associated with the limited subtype, with mild organ involvement and a more favourable prognosis [38]. Anti-U1RNP antibodies, whose prevalence in SSc varies between 2 and 14% [3,32,39], are more frequently detected in the limited subtype [40] and in overlap syndromes with Mixed Connective Tissue Disease [3], and renal involvement is rare in its

presence [5]. Graf *et al.* have reported an association between these antibodies and a higher risk of pulmonary arterial hypertension and lower survival [22]. Observed in approximately 3% of patients with SSc, anti PM-Scl antibodies are considered a serologic marker of overlap syndrome SSc/Myositis [41-44], and are associated with digital ulcers [45] and pulmonary fibrosis [46]. On the other hand, they seem to play a protective role against pulmonary arterial and gastrointestinal involvement [46].

Study objectives included the prevalence of several auto-antibodies (specific and non-specific) in SSc, and their correlation with disease subtypes, organ involvement and capillaroscopic pattern.

Material and Methods

Patients

Of the 190 SSc patients followed in the Clinical Immunology Unit of the Centro Hospitalar do Porto, a non-random convenience sample of 61 patients observed during consultations between September 2010 and March 2011 was selected. Subsequently, 4 patients were excluded: 2 because of having an overlap syndrome, 1 because of having non-Hodgkin lymphoma, and another because of being infected with human immunodeficiency virus type 1. The study was approved by the Ethics Committee and the Board of Directors of the Hospital and all patients signed an informed consent form.

Forty seven patients fulfilled the ACR (American College of Rheumatology) [47] criteria for SSc diagnosis and the other 10 did not present skin involvement, therefore being classified as Pre-Scleroderma (Pre-SSc), according to what will be subsequently described.

The ACR/ EULAR (The European League Against Rheumatism) criteria of 2013 [48] were met by those 47 patients that had met the ACR criteria, and by 1 of the 10 without skin involvement due to having digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement.

Clinical evaluation

Patients were divided, depending on the kind of skin involvement, into dcSSc and lcSSc, according to LeRoy *et al.* [49] and Pre-SSc according to LeRoy and Medsger [50]. Patients with lcSSc were, afterwards, subdivided in two groups, CREST and non-CREST, according to Lonzetti *et al.* [51].

Over the course of time, several authors, and more recently the EUSTAR (EULAR Scleroderma Trial and Research Group), recognized the existence of an early stage of scleroderma without skin involvement but with the possibility of evolving towards limited or diffuse SSc, having designated it as: “pre-SSc” [52], “early SSc” [53], “limited SSc” [50], “very early SSc” [54]. We chose to use “pre-SSc” to define these patients without skin involvement but with Raynaud phenomenon associated with typical capillaroscopic findings of Scleroderma and/ or specific antibodies to the disease.

The diffuse and limited SSc types were, consequently, divided into early, intermediate and late stages, according to the first symptom related with the disease and in agreement with the SSc subtype [55]. In the limited subtype, an early stage is defined as a progression time less than 5 years, and a late stage greater than 10 years. The intermediate stage is situated

between 5 and 10 years. In the diffuse subtype, the early stage is defined as a time of progression less than 3 years, late stage as greater than 6, and intermediate between 3 and 6 years.

Demographic, clinical, serologic (presence and type of auto-antibodies) and capillaroscopic data were evaluated. The degree of organ involvement was determined by clinical history, physical exam and diagnostic tests.

In the absence of a standardized method to assess the disease's severity, the Medsger's severity scale, a composite scale that simultaneously evaluates activity and damage, was used [55]. In spite of presenting limitations, this scale proved to be useful in evaluating the severity and function and has prognostic value [56]. Medsger's severity scale assesses the involvement of 9 systems - general, peripheral vascular, lungs, heart, kidneys, muscles, joints/tendons, skin and gastrointestinal (GI) - scored on a scale of 0 (without documented involvement or without need of treatment, e.g. Raynaud) to 4 (terminal involvement).

We chose to evaluate organ involvement, using the Medsger scale cut-off point, except in osteoarticular evaluation (it does not value joint involvement, but only the distance from the thumb to the thenar eminence, which was not evaluated systematically in our patients).

Modified Rodnan Skin Score was the tool of choice for evaluating skin involvement [57]. Muscular involvement was evaluated by physical examination and complemented by biochemical study (creatine phosphokinase, aldolase and glutamate oxaloacetate transaminase).

Chest teloradiography, high resolution computed tomography (CT), spirometry and diffusing capacity for carbon-monoxide and two-dimensional echocardiogram (evaluation of pulmonary arterial pressure: PSAP) were used to evaluate respiratory involvement. PSAP was considered altered when greater than 35 mmHg. Right heart catheterization was performed in patients with PSAP greater than 40 mmHg to confirm Pulmonary Hypertension.

Intraventricular conduction disturbances and/or arrhythmias were detected by electrocardiogram (ECG) and/or Holter, and systolic dysfunction was evaluated by echocardiogram, namely through calculation of the left ventricular ejection fraction.

Gastrointestinal tract involvement was documented by high digestive endoscopy, esophageal manometry, and, in symptomatic patients, by gastric scintigraphy. Involvement of the lower GI tract, whenever justified, was documented by barium tests, glucose hydrogen breath test, ultrasound, colonoscopy and ano-rectal manometry.

Joint evaluation was made through physical exam, and complemented by imaging exams (radiography and/ or joint echography) whenever justified. DAS 28 (Disease Activity

Score Calculator for Rheumatoid Arthritis) was used for documenting the activity of this involvement.

Lastly, a score introduced by Cutolo *et al.* was used to classify microvascular abnormalities observed in nailfold capillaroscopy [58]. Three patterns of microvascular damage were described: early, active and late.

Serologic analysis

Serum ANA was determined by indirect immunofluorescence using a commercialized ANA kit (INOVA Diagnostics Inc. San Diego, CA, USA) with HEp-2 cell lines as substrate at an initial serum dilution of 1:80 according to the manufacturer's instructions. Fluorescence intensity and pattern were evaluated. The following auto-antibodies were analyzed, using different methodologies: ACA, ATA, anti-RNAPIII, anti-Th/To, AFA, and anti-PM/Scl, anti-Ro52 and anti-NOR90 by immunoblotting (EUROLINE, Euroimmun, Germany) and anti-U1RNP by enzymatic fluoroimmunoassay (EliA, ThermoFisher Scientific Uppsala, Sweden). It should be noted that these antibodies were not studied in all patients.

Statistical analysis

Statistical analysis was performed using the IBM SPSS program version 20.0 for Windows (SPSS Inc, IL, USA). The descriptive statistics consisted of the calculation of the median values and the absolute (n) and relative (%) frequencies. We decided not to use inferential statistics for the comparison of different types of the disease, as the reduced size of some of the groups invalidates the applicability of the suitable tests in most of the cases. The correlation between the presence of antibodies and the fact of being or not being a CREST patient, and with organ involvement, was only assessed for ACA and ATA, as the reduced number of people that had or had not each one of the remaining antibodies would invalidate a correct interpretation of the results. In order to assess the correlation between the presence of each antibody and organ involvement, odds ratio (OR) were calculated, and the level of critical significance for rejecting the null hypothesis was presented. The null hypothesis was rejected when $p < 0.05$.

Results

Socio-demographic and clinical characterization

The socio-demographic and clinical characteristics of the study population are summarized in Table 1. In this study, 55 patients were female and 2 male, with a median age of 54 years old, ranging between 18 and 82 years old.

Table 1 – Clinical characteristics of patients (n = 57)

Age (years) (<i>median; minimum, maximum</i>)	54 (18 – 82)
Gender (n, %)	
Female	55 (96.5%)
Male	2 (3.5%)
Type of SSc (n, %)	
Pre-SSc	10 (17.5%)
Limited cutaneous SSc	34 (59.6%)
CREST	16 (28.1%)
Non-CREST	18 (31.6%)
Diffuse cutaneous SSc	13 (22.8%)
Phase of SSc	
Limited cutaneous SSc (n = 34) (n, %)	
Early	5 (14.7%)
Intermediate	8 (23.5%)
Late	21 (61.8%)
Diffuse cutaneous SSc (n = 13) (n, %)	
Early	2 (15.4%)
Intermediate	2 (15.4%)
Late	9 (69.2%)
Capillaroscopic pattern (n = 55 *) (n, %)	
Normal/Minimal change	4 (7.3%)
Early	12 (21.8%)
Active	21 (38.2%)
Late	18 (32.7%)

* Two patients did not undergo capillaroscopy. Abbreviations: SSc, Scleroderma; CREST, Calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.

Concerning the SSc subtype, 34 patients (59.6%) presented the limited cutaneous form and 13 (22.8%) the diffuse cutaneous form. Sixteen of the patients with lcSSc met the criteria for CREST. Ten patients did not present skin involvement and were classified as Pre-SSc. In regards to capillaroscopic findings made in 55 of the 57 patients, it was demonstrated that 91.7% presented a scleroderma pattern, most being in the active phase (38.2%).

Organ involvement

Through the analysis of organ involvement in the 47 patients with the limited and diffuse forms, we noticed the existence of skin and vascular involvement in 100% of the cases.

In regards to the involvement of other organs, the difference found in pulmonary and gastrointestinal involvement in patients with the diffuse subtype stands out.

Of the 44 patients that underwent evaluation of the GI tract, half presented hypomotility of the esophageal body and 6 presented a diminished lower esophageal sphincter pressure. The presence of esophagitis in 6 patients, erythematous gastropathy in 5 and gastritis in another 5 should be noted. Other alterations found: gastric dysmotility (2 patients), intestinal dysmotility, gastric (3 patients) and intestinal (1 patient) angiodysplasia, and anorectal myopathia (3 patients).

All 47 patients underwent pulmonary function tests (PFT), high resolution CT scan and echocardiogram to evaluate pulmonary involvement. Thirty five patients (74.5%) presented alterations in one or more tests and 27 (57.4%) had CT scan abnormalities, most of which (20) were considered major (fibrosis and/ or alveolitis affecting at least the lung bases). Seven patients presented alterations classified as minor; these include septal hypertrophy, limited zones of fibrosis fibrosis/ground glass opacities. Fifteen patients (31.9%) had PSAP greater than 35 mmHg in transthoracic echocardiogram, and 7 presented values greater than 40 mmHg, therefore having undergone right heart catheterization. In two of these patients, the existence of pulmonary arterial hypertension was confirmed.

In regards to cardiac involvement, there were no echocardiographic changes in myocardial function or in the pericardium. Forty two patients underwent ECG/Holter to assess the existence of rhythm and/ or conduction disturbances, and 34% (16 patients) had alterations, including 12 patients with lcSSc (40.0%) and 4 patients with dcSSc (33.3%).

Ten patients presented mild inflammatory joint complaints, however only 1 developed arthritis.

None of the patients in the study presented either renal or general health involvement, and only 1 with the diffuse subtype presented alterations of muscle strength (Table 2).

Table 2 – Organ involvement by Scleroderma subtype

Organ involvement	Pre-SSc (n = 10)	lcSSc (n = 34)	dcSSc (n = 13)
General	0 (0%)	0 (0%)	0 (0%)
Skin	0 (0%)	34 (100%)	13 (100%)
Vascular	10 (100%)	34 (100%)	13 (100%)
Gastrointestinal	3 (37.5%) [n = 8]	15 (46.9%) [n = 32]	7 (58.3%) [n = 12]
Pulmonary	3 (33.3%) [n = 9]	24 (70.6%)	11 (84.6%)
Cardiac	1 (14.3%) [n = 7]	12 (40.0%) [n = 30]	4 (33.3%) [n = 12]
Muscular	0 (0%)	0 (0%)	1 (7.7%)
Osteoarticular	2 (20.0%)	9 (27.3%) [n = 33]	1 (7.7%)
Renal	0 (0%)	0 (0%)	0 (0%)

Results are presented as the number and (%) of cases in which the autoantibodies were found to be positive. The size of sub-samples in which the antibodies were tested is indicated between square parentheses whenever different from total.

Abbreviations: SSc, Scleroderma; Pre-SSc, Pre-Scleroderma, lcSSc, limited cutaneous Scleroderma; dcSSc, diffuse cutaneous Scleroderma.

Through the analysis of organ involvement in the 10 patients with Pre-SSc (without skin involvement), we observed that 100% had vascular involvement, although only half required vasodilator treatment.

Of the 8 patients that underwent esophageal manometry/upper gastrointestinal endoscopy, 3 presented hypomotility of the esophageal body, 1 presented a diminished lower esophageal sphincter pressure, 1 had esophagitis and 1 had congestive gastropathy. All patients underwent PFT and echocardiogram and only 2 did not undergo pulmonary high resolution CT. Two patients presented altered PFT, 1 associated with minor alterations in chest CT, and the other one presented a PSAP of 42mmHg (normal catheterism).

In regards to cardiac involvement, only one patient presented alterations (complete right bundle branch block).

Two of the 10 patients presented mild arthralgias with inflammatory characteristics.

The results of the analysis of organ involvement, by SSc subtype, are presented in Table 2.

Serological features

Serological characteristics are presented in Table 3.

Table 3 – Frequency of patients with each type of antibodies

	Total (n = 57^{**})	Pre-SSc (n = 10^{**})	lcSSc (n = 34^{**})	dcSSc (n = 13^{**})
ANA (n = 57 [*])	57 (100%) [n = 57]	10 (100%) [n = 10]	34 (100%) [n = 34]	13 (100%) [n = 13]
ACA (n = 57 [*])	34 (59.6%) [n = 57]	9 (90%) [n = 10]	21 (61.8%) [n = 34]	4 (30.8%) [n = 13]
ATA (Scl70) (n = 57 [*])	15 (26.3%) [n = 57]	1 (10%) [n = 10]	7 (20.6%) [n = 34]	7 (53.8%) [n = 13]
RNAP (n = 56 [*])	2 (3.5%) [n = 56]	0 (0%) [n = 10]	1 (3.0%) [n = 33]	1 (7.7 %) [n = 13]
AFA (n = 56 [*])	1 (1.8%) [n = 56]	0 (0%) [n = 10]	1 (3.0%) [n = 33]	0 (0.0%) [n = 13]
Anti-Th/To (n = 56 [*])	1 (1.8%) [n = 56]	0 (0%) [n = 10]	1 (3.0%) [n = 33]	0 (0.0%) [n = 13]
Anti-PMScl (n = 56 [*])	2 (3.6%) [n = 56]	0 (0%) [n = 10]	2 (6.1%) [n = 33]	0 (0.0%) [n = 13]
Anti-U1-RNP (n = 54 [*])	1 (1.9%) [n = 54]	0 (0%) [n = 10]	1 (3.2%) [n = 31]	0 (0.0%) [n = 13]
Anti-Ro52 (n = 23 [*])	19 (82.6%) [n = 23]	3 (60.0%) [n = 5]	12 (100%) [n = 12]	4 (66.7%) [n = 6]
Anti-NOR 90 (n = 12 [*])	8 (66.7%) [n = 12]	3 (100%) [n = 3]	4 (66.7%) [n = 6]	1 (33.3%) [n = 3]

Abbreviations: SSc, Scleroderma, lcSSc, limited cutaneous Scleroderma; dcSSc, diffuse cutaneous Scleroderma; ACA, anti-centromere antibodies, AFA, anti-fibrillar antibodies; ANA, anti-nuclear antibodies; ATA, anti-topoisomerase antibodies, RNAP, anti-RNA polymerase antibodies

* Number of patients in whom each antibody was assessed.

** Total size of sample and sub-samples.

The number of patients analyzed in each case is presented in square parentheses.

We found that 100% of patients had ANA. The most frequent immunofluorescence patterns were centromeric (n = 30; 52.6%) and speckled (n = 20; 35.1%), and two patients had both patterns simultaneously (3.5%). The prevalence of a nucleolar pattern was lower, and was detected in 5 patients (8.8%).

In regards to antibodies specific to the disease, ACA were the most prevalent (59.6%), followed by ATA (26.3%) and anti-RNAP (3.5%). AFA or anti-U3-RNP and anti-Th/To antibodies were the least prevalent, having been found in only 1 patient (1.8%).

Most of the patients with ACA belonged to the limited subtype [21] while, of the 15 patients with ATA, 7 belonged to the diffuse subtype and 7 to the limited subtype.

Relative to anti-Pm/Scl and anti-U1-RNP antibodies, the first were detected in 2 patients and the later in 1 patient.

Of the 23 patients in whom the presence of antibodies anti-Ro52 was investigated, and of the 12 in whom the presence of antibodies anti-NOR 90 was investigated, 19 (82.6%) and 8 (66.7%), respectively, presented these auto-antibodies.

Correlation between serological and other characteristics

Regarding the association between disease subtype and antibodies encountered, we noticed that the most prevalent antibodies in patients with lcSSc were ACA (61.8%), followed by ATA (20.6%) (Table 3). On the other hand, in dcSSc, the most prevalent antibodies were ATA (53.8%), followed by ACA (30.8%).

Additionally, we compared the prevalence of ACA and ATA in patients with lcSSc, with and without CREST. We found that patients with CREST presented a larger proportion of ACA ($13/16 = 81.3\%$ vs. $8/18 = 44.4\%$) and a smaller proportion of ATA ($1/16 = 6.3\%$ vs. $6/18 = 33.3\%$) comparatively to patients without CREST.

Let it be noted that in all patients with lcSSc in whom the presence of antibodies anti-Ro52 was evaluated, the results were positive.

The OR related to the prediction of organ involvement based in the presence of antibodies are shown in Table 4.

Table 4 – Antibodies and organ involvement

	ACA (n = 57 *)	ATA (n = 57*)
Skin	0.126 (0.059)	3.818 (0.224)
Vascular	0.341 (0.351)	1.474 (0.738)
Gastrointestinal	0.542 (0.284) [n = 52]	6.286 (0.012) [n = 52]
Pulmonary	0.286 (0.055) [n = 56]	2.308 (0.247) [n = 56]
Cardiac	0.857 (0.802) [n = 49]	2.500 (0.160) [n = 49]
Osteoarticular	1.520 (0.540) [n = 56]	0.477 (0.379) [n = 56]

Abbreviations: ACA, anti-centromere antibodies; ATA, anti-topoisomerase antibodies.

Results presented as Odds Ratio (OR) (p).

* Number of patients in whom each antibody was assessed. Number of patients analyzed indicated between square parentheses whenever different from total

General health and renal involvement were not analyzed, as there were no patients with this kind of involvement. Likewise, muscular involvement was also not evaluated as there was only one patient with alteration of muscle strength.

A trend towards an association between ACA and less skin and pulmonary interstitial involvement was observed, moreover, the presence of ATA was significantly related to a higher GI involvement.

Limited and diffuse SSc patients were studied separately depending on the presence of ACA and ATA antibodies and their association with CT scan alterations. Nine patients were not analyzed due to being seronegative for both antibodies (6 had lcSSc and 3 had dcSSc) and one patient due to having both antibodies (dcSSc).

In patients with lcSSc with ATA there is a higher proportion of patients with CT scan alterations ($5/7 = 71.4\%$), compared to that observed in patients with ACA ($8/21 = 38.1\%$). All patients with dcSSc analyzed (3 with ACA and 6 with ATA) presented CT scan alterations.

A similar analysis was performed regarding the presence or absence of cardiac involvement. We found that, in the lcSSc group, the percentage of patients with cardiac involvement was slightly greater in those with ATA ($4/7 = 57.1\%$) than in those with ACA ($8/19 = 42.1\%$). In patients with dcSSc, the proportion of cardiac involvement tends to be greater in patients with ATA than in patients with ACA ($3/6 = 50.0\%$ vs. $1/3 = 33.3\%$, respectively).

The relationship between capillaroscopic pattern and antibodies encountered was also studied (results not displayed), and no significant association was found.

Discussion

Antinuclear antibodies are detected in more than 95% of SSc patients [3], and it seems that those which are disease-specific, namely ACA and ATA, are related to the disease subtype (lcSSc vs. dcSSc) and are associated with, or able to predict, clinical evolution and organ involvement [19].

Although ACA have a high specificity for the limited subtype and ATA are primarily associated with the diffuse subtype, ACA have been described in approximately 5-7% patients with the diffuse subtype [3,19], and ATA are associated in about 31-36% of the cases with the limited subtype [19].

In our study, all patients were ANA-positive, with most (52 patients) presenting antibodies which are specific or related to the disease. Of the five patients positive only for ANA, 3 presented a nucleolar pattern frequently observed in SSc which, in this case, may be associated with specific auto-antibodies not yet identified.

Auto-antibodies detected in our patients with dcSSc were mostly ATA (anti-TOPOI, anti-Scl70) while in the lcSSc group were mostly ACA, which is consistent with the literature [3]. However, 30.8% of the cases with dcSSc had ACA, which is clearly greater than the published value.

On the other hand, of the 14 patients with limited or diffuse SSc ATA positive, half belonged to the limited subtype, which is also higher than what is described in the literature. However, the low number of patients with this positive antibody results may contribute to an overestimation of this value.

Anti-Ro52 antibodies were found in most patients in whom it was searched for, in accordance with the fact that they are considered to have a high prevalence in this population, as supported by Hudson *et al.* [36].

On the other side, a strong association seems to exist between these antibodies and the limited subtype; something that, according to what is known, had never been reported before.

Regarding organ involvement, our study, similarly to what has been described in the literature [3,9,16,19,59], demonstrated a tendency of ACA to associate with less skin and pulmonary interstitial involvement. This evidence may be justified, either by the higher prevalence of these antibodies in the limited subtype or by its protective role against pulmonary involvement.

Although elsewhere a positive relationship between ATA and intestinal involvement has been described [19], our study highlighted an association between the presence of these antibodies and esophageal involvement.

Our results showed a similar proportion of cardiac involvement in patients with limited and diffuse forms of SSc, being even higher in lcSSc, which was not found in other studies [60-62].

Regardless of only 10 patients diagnosed as Pre-SSc having been evaluated, we have observed, as did Valentini *et al.* [63], that many of these patients already had some degree of organ involvement, namely of the GI tract and isolated changes in the PFT.

Such data supports the need to research the existence of pre-clinical organ involvement, as defended by the same author.

Even though none of the patients with gastrointestinal involvement meet the 2013 ACR/EUSTAR criteria, the 3 patients with hypomotility of the esophageal body, according to Poormoghim *et al.* [64], could be classified as being sine-SSc, although it is not clear whether or not both of these denominations (Pre-SSc and sine-SSc) represent different subtypes of the disease [65].

In summary

High prevalence of specific and non-specific autoantibodies in SSc.

ATA preferentially associate with dcSSc and ACA preferentially associate with lcSSc.

ACA are associated with lower pulmonary and skin involvement, whereas ATA are associated with higher esophageal affection ($p < 0.05$).

High prevalence of Ro52 antibodies, in patients with Pre-SSc as in patients with SSc, especially with in those with lcSSc.

Evidence of subclinical organ involvement in Pre-SSc would support the need for a systematic evaluation of all organs potentially affected.

Cardiac rhythm and conduction disturbances, which are prevalent in both lcSSc and dcSSc, would support the need to perform ECG and Holter in all patients.

Patients with Pre-SSc and esophageal involvement fulfill the criteria for the diagnosis of sine SSc, but not the ACR/EUSTAR criteria for the diagnosis of SSc.

Study limitations: the size of the sample, namely the reduced number of patients with dcSSc, may have conditioned the interpretation of some results.

Conclusions

Although the ACR/EUSTAR criteria for the diagnosis of SSc comprises a great advance, they are still not sensitive enough for the diagnosis of some patients in early stages, namely with sine-SSc.

Given that the group of patients with lcSSc was the most prevalent in our study, the data found in relation to cardiac involvement in this disease indicate the need for systematic screening of this organ involvement in these patients, namely with ECG and Holter.

Beyond the value of specific antibodies in the diagnosis of the disease which justified their inclusion in the new classification criteria, their prognostic value in this study was once again highlighted due to their relationship with the type of organ involvement.

Attending to the high prevalence of antibodies anti-Ro52, mainly observed in the limited subtype, and to their prognostic value previously reported in other studies, particularly in what concerns pulmonary interstitial involvement, we consider that their determination should always be performed in patients with SSc. However, more studies should be conducted to prove these data.

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4.1.2 – T AND NK CELL PHENOTYPIC ABNORMALITIES IN SYSTEMIC SCLEROSIS: A COHORT STUDY AND A COMPREHENSIVE LITERATURE REVIEW

Artigo publicado

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Abstract

Scleroderma (SSc) is a rare and heterogeneous immune-mediated disease involving the connective tissue and microvasculature whose pathogenesis remains unclear. Data concerning T- and NK-cell abnormalities and cytokine levels in the peripheral blood (PB) from patients with SSc are scarce and the results are contradictory. The present study aimed to analyze the changes of T lymphocytes, NK cells and T helper (Th) related cytokines in the PB of patients with SSc in comparison to healthy individuals, and its relation to disease subtype and stage, organ involvement and nailfold capillaroscopic changes. A non-random convenience sample of 57 Scleroderma patients was utilized. Fifty-five out of the 57 patients studied were women (97%); 10 patients presented Pre-SSc and 47 SSc: 34 limited cutaneous SSc (lcSSc) and 13 diffuse cutaneous SSc (dcSSc). Patients with SSc were classified in early (n=7), intermediate (n=10) and late (n=30) disease. Blood samples were analyzed by flow cytometry for total T-cells, CD4+ and CD8+ T cell subsets, total NK cells and CD56+low and CD56+high NK cell subsets. T cells were further analyzed for the expression of the CD56 adhesion molecule and activation-related markers (HLA-DR, CD45RO). In addition, the serum levels of Th1, Th2 and Th17 related cytokines were measured by flow cytometry.

Twenty five healthy individuals recruited from the blood bank were used as controls. Patients had lower numbers of total lymphocytes and T cells comparing to healthy controls. Both CD4+ and CD8+ T cells were decreased, but differences were statistically significant only for CD8+ and CD8+CD45RO+ T cells. These alterations were seen in patients with SSc, but not in patients with Pre-SSc, and, in general, they were more pronounced in patients with dcSSc than in patients with lcSSc, in patients with vascular involvement than in those without as well as in patients having active and late nailfold capillaroscopic patterns. CD56+ T cells were also decreased in SSc patients, especially in those with active/late capillaroscopic patterns or with severe lung disease. Diminished numbers of circulating NK cells were also observed in patients with lcSSc and in those with early disease. No statistically significant changes were found in serum cytokine levels, as compared with controls. Patients with SSc had major alterations in circulating CD8+ and CD56+ T cells, as well as in NK cells, suggesting that these cells may play a relevant role in SSc pathogenesis, probably operating at different phases and/or at different organs. In addition the serum levels of Th1, Th2 and Th17 cytokines did not provide useful information for evaluating T cell polarization in SSc.

Keywords: Systemic Sclerosis, T cells, CD8, CD4, CD56, NK cells, cytokines.

Introduction

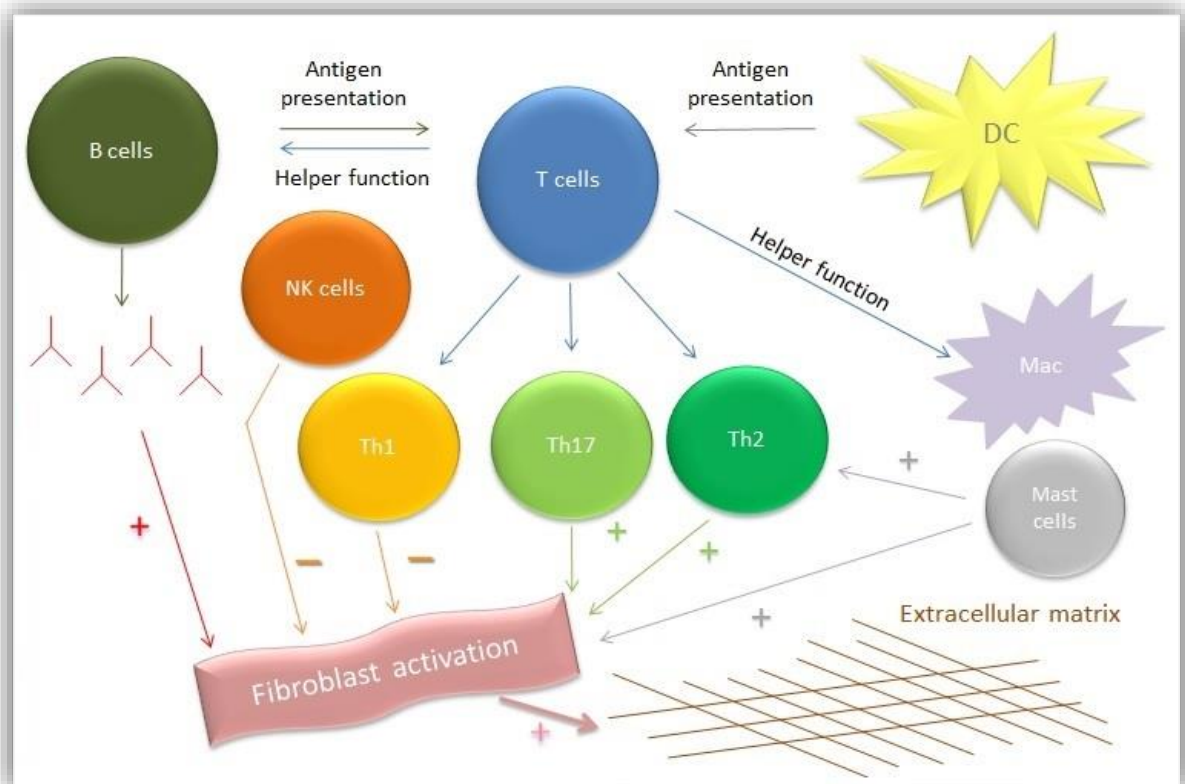
Scleroderma or Systemic Sclerosis (SSc) is a rare and heterogeneous immune-mediated disease that typically presents with vascular abnormalities and visceral and cutaneous fibrosis whose pathogenesis remains unclear [1-8]. Its pathological hallmark is tissue fibrosis resulting from excessive fibroblast activation, with overproduction of collagen and other extracellular matrix (ECM) components [9-11], and the mechanisms involved seem to be, at least partly, immune-mediated [12-14]. The most recent hypotheses favours an interrelationship between the vascular endothelium [15], tissue fibroblasts [9, 10], and the cells of the immune system [11, 16, 17] (Figure 1). Genetic and environmental factors might also contribute to disease susceptibility.

Abnormal B cell function has been associated with SSc for a long time, and many autoantibodies have been identified in the sera of SSc patients; some of them are specific to the disease, such as anti-centromere, anti-topoisomerase 1 and anti-RNA polymerase I/III antibodies, but their role remains poorly understood [18,19]. Previous studies have showed that SSc patients have expanded naive B cells and diminished activated memory B cells in the peripheral blood [20], and that B cells infiltrate the affected skin and lung from patients with SSc [21], where they are involved in the pathophysiology of fibrosis [22-24]. In accordance, B cells are able to produce autoantibodies - e.g. stimulatory anti-platelet-derived-growth factor (PDGF) receptor autoantibodies - and profibrotic cytokines - e.g. interleukin (IL)-6 and transforming growth factor beta (TGF- β) that can stimulate fibroblasts, and they can also enhance collagen production, by a contact-dependent mechanisms [22,23]. In addition, B cells may produce autoantibodies that inhibit the function of metalloproteinases, therefore decreasing collagen degradation [24].

In early phases of the disease, even before fibrosis occurs, the affected tissues exhibit an inflammatory infiltrate composed of macrophages, mast cells and lymphocytes, in which T cells represent the dominant cell type [25, 26]. Perivascular fibroblasts are in close contact with these cells [11, 27], and they are stimulated by soluble factors to produce collagen [18]. Earliest studies revealed that CD4⁺ T cells were the predominant cell type in the inflammatory infiltrate of the lesional skin [25, 29, 30], did express activation-related molecules [31], and were able to secrete fibrogenic cytokines [32]. More recently, Fuschiotti *et al.* described that in early disease the cutaneous lymphocyte infiltrate was composed mainly of CD8⁺ T cells, whereas CD4⁺ T cells predominated in advanced disease [33]. Changes in other T cell subsets, such as gamma delta T cells, natural killer T (NKT) cells, and regulatory T cells (Tregs) have also been described [34-37].

Pulmonary involvement in SSc is characterized by alveolitis and interstitial fibrosis, and increased numbers of activated CD45RO+ T cells were observed in lung biopsies from SSc patients, with possible relationship to the alveolar wall thickness [38]. In addition, other studies have shown an increase in relative and absolute numbers of CD45RO+ T cells, particularly of the CD8+ subtype, in the bronchoalveolar lavage fluid (BALF) from patients with SSc [30,39]. These CD8+ T cells have skewed repertoires of the T cell receptor (TCR) alpha and beta chain variable regions (TCRV-alpha and -beta), as a result of antigen-driven oligoclonal expansions [40]. One of these studies also revealed an increase of CD16+ and/or CD56+ cytotoxic T lymphocytes (CTL) in the BALF of patients with SSc, especially in those with severe lung impairment [39].

Figure 1 – Mechanisms involved in tissue fibrosis



The mechanisms involved in tissue fibrosis seem to be, at least partly, immune-mediated. The affected tissues exhibit an inflammatory infiltrate composed of lymphocytes, macrophages, and mast cells. Fibroblasts are stimulated to produce collagen. DC, dendritic cells; Mac, macrophages; Th, T helper.

Evidence regarding T cell abnormalities in the peripheral blood (PB) from patients with SSc is contradictory. While some studies have showed decreased levels of CD8+ T cells [41], others studies revealed decreased levels of both T cell subsets [42,43], and even others found no significant difference from healthy controls [44,45]. Also relevant is the evidence suggesting

that effector CD8⁺ T cells are increased in the PB from patients with SSc [46], contrary to the memory subtype that is reduced [43,44, 46]. Depletion of circulating memory CD8⁺ T cells appears to be a relatively constant finding in SSc, irrespectively to the phenotypic criteria used to define these cells, i.e., CD29⁺ [43,44] or CD45RA-CD27⁺ [46] T cells. Changes in relative and/or absolute counts of activated T cells have also been reported in the PB from SSc patients, such as increased percentages and numbers of CD4⁺CD25⁺ T cells [44] and increased percentages of CD4⁺HLADR⁺ and CD8⁺HLADR⁺ T cells [45]. In addition, Giovannetti *et al.* found that CD4⁺ T cell proliferation, lymphocyte apoptosis and Treg cell frequencies were significantly higher in SSc patients than those observed in controls [46].

Human natural killer (NK) cells can be divided into two subsets based on their cell-surface expression of CD56 – CD56⁺low and CD56⁺high – each with distinct phenotypic and functional properties [47]. CD56⁺low NK-cells predominate in the PB, are more naturally cytotoxic and they express FcγRIIIa (CD16), the low affinity receptor for the Fc region of IgG, as well as killer cell immunoglobulin-like receptors (KIR); by contrast, CD56⁺high NK cells are CD16⁻/+low and KIR⁻, constitutively express the high affinity receptor for IL-2 (CD25), thereby exhibiting a high proliferative response after the binding of low amounts of this cytokine, and they have the capacity to produce abundant cytokines, but have low natural cytotoxicity [47]. Thus, NK cells, the innate immune cytotoxic effectors, are also able to produce immunoregulatory cytokines, and there is increasing evidence for their involvement autoimmune diseases [48, 49].

To date, only a few studies have assessed the NK cells in the PB from patients with SSc, with discrepant results. Among them, some studies have reported normal NK cell counts [42] whereas other studies have described decreased percentages and/or absolute numbers of NK cells [35, 45], which were found to be more pronounced in dcSSc and in late-stage disease [45]; other studies revealed that the frequency and absolute number of NK cells are increased in dcSSc, being normal in lcSSc [50].

Phenotypic evidence of NK cell activation [50,51], and abnormalities of the NK cell function, as evaluated by cytokine production and NK cell activity, have also been described in the PB from patients with SSc, and they seem to depend on the experimental conditions (non-stimulated vs. stimulated NK cells) and to vary according to the disease subtype (lcSSc vs. dcSSc) [50,52].

The contribution of genes codifying for killer cell receptors, such as KIR, has also been investigated [53]. Momot *et al.* observed that KIR genotypes characterized by the presence of the activating KIR2DS2 and the absence of the corresponding inactivating receptor, KIR2DL2, are more frequent in patients with SSc than in healthy individuals, suggesting that the genetic combination KIR2DS2+/KIR2DL2- is associated with scleroderma [53].

Cytokines are important mediators of the immune response. Type 1 cytokines – interferon gamma (IFN- γ), IL-2, and tumour necrosis factor alpha (TNF- α) promote pro-inflammatory cell-mediated immune responses, whereas type 2 cytokines (e.g. IL-4, IL-5, IL-6, IL-10, IL-13) induce antibody synthesis [54]. In addition, some Th2 cytokines, such as IL-4, IL-13 and TGF- β , participate in ECM remodelling by activating collagen deposition, whereas Th1 cytokines have an opposite effect [55]. Interleukin-17 is a pro-inflammatory cytokine with effects on epithelial, endothelial and fibroblast cells [56]. Deregulated cytokine production and skewed development Th1 and Th2 subsets, which secrete type 1 and type 2 cytokines, respectively, and Th17 cells, which produce IL-17A and F, but also IL-21, IL-22, IL-23 and IL-26, have been implicated in the pathogenesis of inflammatory, autoimmune and fibrogenic diseases [55-58].

In that concerning functional T cell polarization in SSc, T cells from the lesional skin of SSc patients exhibit predominantly a Th2 pattern of cytokine production [59]. CD8⁺ T cells present in the BALF from patients with SSc also produce mainly Th2 cytokines, a feature that associates with greater decline in pulmonary function [60]. In addition, peripheral blood CD8⁺ T cells from patients with SSc express high levels of GATA-3, a transcription factor that has been shown to induce Th2 responses, while suppressing Th1 differentiation [61]. Moreover, there is evidence of an increased production of IL-13 by CD8⁺ T cells from SSc patients, a well-known profibrotic Th2 cytokine with a direct effect on fibroblast activation, which may also act indirectly via stimulation of TGF- β [55]. In accordance, it was shown that peripheral blood CD8⁺ T cells from patients with SSc produce high levels of IL-13 and that this abnormality is more pronounced in diffuse than in limited cutaneous SSc [46]. Also in line, high numbers of CD8⁺ T cells and IL-13⁺ cells were found in the skin lesions of SSc patients, particularly during the early phase of the disease, and IL-13-producing circulating CD8⁺ T cells from patients with SSc were found to express skin-homing receptors and to induce a IL-13 dependent profibrotic phenotype in dermal fibroblasts [33].

More recently, other studies have reported on the role of Th17 cells and their hallmark cytokines in SSc patients [62]. Studies in rodents indicated that IL-17 acts in a pro-fibrotic manner, while data in humans suggest that Th17 cells and IL-17A may play a role in controlling rather than promoting fibrosis [62], by reducing the synthesis of type I collagen and enhancing the production of ECM degrading enzymes by dermal fibroblasts [63,64]. For instance, it has been shown that patients with SSc have a higher frequency of Th17 cells in the PB than healthy controls [65-68] and the Th17 profile was mostly attributable to CD4⁺ T cells expressing the -C-C- chemokine receptor CCR6, a chemokine receptor that has been shown to allow homing into the skin and other tissues including the lung, particularly under inflammatory conditions, by interacting with the -C-C-chemokine ligand CCL20 [65]. In addition, increased IL-17A mRNA

levels were identified in involved skin and lung [69, 70], and IL-17+ lymphocytes were detected in the skin in early SSc [68], where their numbers were inversely correlated with the extent of skin involvement [71]. In that concerning the levels of IL-17A in the serum of SSc patients, some inconsistencies exist in the literature, as previous studies have reported them to be increased [69,72], or decreased [73].

Our study aimed to analyze the changes of T lymphocytes and NK cells in the PB, and the serum levels of Th1, Th2 and Th17 cytokines, in patients with SSc and its relation to the disease subtype, disease stage, organ involvement and nailfold capillaroscopic changes.

Material and Methods

Study approval and ethics statement

The study was approved by the Administration Board and the Ethical Committee of the Centro Hospitalar do Porto, and all participants gave written informed consent.

Study population

Sixty one consecutive patients were selected from a cohort of 190 patients with SSc, at the Clinical Immunology Unit of Centro Hospitalar do Porto, between September 2010 and March 2011. Four patients were later excluded; three because of overlapping pathologies (mixed connectivitis, infection with human immunodeficiency virus, non-Hodgkin lymphoma) and one that missed blood sampling. Forty-seven patients fulfilled the American College of Rheumatology criteria for SSc [64], while the remaining ten patients did not present skin involvement and were diagnosed as Pre-Scleroderma (Pre-SSc), as explained below.

The 2013 EULAR (The European League Against Rheumatism) / ACR criteria [75], were met by those 47 patients that had met the ACR criteria, and by 1 of the 10 patients without skin involvement due to having digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement.

In the study population, fifty-five were women and two were men, with a median age of 54 years, ranging from 18 to 82 years (Table 1). Twenty-five healthy individuals (blood donors) were used as controls.

Table 1 – Clinical characteristics of the study population

Age (median; range)	
Years old	54 (18-82)
Gender (n; %)	
Males	2 (3.5%)
Female	55 (96.5%)
Disease type (n; %)	57 (100%)
Pre-SSc	10 (17.5%)
SSc	47 (82.5%)
SSc clinical subset (n; %)	
lcSSc	34 (59.7%)
CREST	16 (47.1%)
Non-CREST	18 (52.9%)
dcSSc	13 (22.8%)
SSc evolution phase (n; %)	
Early SSc	7 (14.9%)
Intermediate SSc	10 (21.3%)
Late SSc	30 (63.8%)
Capillaroscopic pattern (n; %) ^{a)}	
Normal/minor alterations	4 (7.3%)
Early	12 (21.8%)
Active	21 (38.2%)
Late	18 (32.7%)

Abbreviations: CREST, calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; SSc, Systemic Sclerosis; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; Pre-SSc, Pre-Scleroderma.

No patient was on immunosuppressive treatment at the time of this study

a) Two patients were not able to be classified once nailfold capillaroscopy was not available by the time data was collected.

Clinical assessment

Patients were divided, depending on the kind of skin involvement, into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), according to LeRoy *et al.* [76] and Pre-SSc according to LeRoy and Medsger [77].

Over time, several authors and, more recently, the EULAR Scleroderma Trial and Research group (EUSTAR) [78], have recognized that there is an early stage of disease in which there is no skin affection but that may evolve to limited or diffuse SSc, and named it differently - “pre-scleroderma” [79], “limited scleroderma” [77], “early SSc” [80], and “very early SSc” [81]. Even though, they represent nearly the same concept. We defined Pre-SSc according to LeRoy and Medsger criteria, where patients must present Raynaud’s phenomenon plus scleroderma-type nailfold capillary changes and/or scleroderma-type autoantibodies [77].

Limited and diffuse cutaneous SSc were then subdivided into early, intermediate and late SSc, according to time since first disease related symptom and in consonance with subtype of disease: early disease was defining as having less than five years of evolution for lcSSc and less than three years for dcSSc; late disease was defined as having at least ten years and six years for lcSSc and dcSSc, respectively; intermediate values correspond to intermediate phases of limited and diffuse SSc [81]. Additionally, lcSSc was further subdivided in CREST (calcinosis, Raynaud phenomenon, oesophageal dysmotility, sclerodactyly, and telangiectasia) and non-CREST groups [82].

To assess disease severity we used the Medsger scale, which classifies involvement of each of the nine organ systems from 0 (no documented involvement or without need for treatment) to 4 (end stage disease) [83]. Although it’s utility as a prognostic measure, as a composite index measuring both activity and damage, this scale has some limitations [84].

The degree of organ involvement was determined by medical history, physical examination, and diagnostic tests. Pulmonary involvement (interstitial and arterial) was assessed by chest ray, high resolution computerized tomography (CT) scan of the chest, in addition to lung function tests (LFT) and diffusing capacity for carbon-monoxide (DLCO), and two-dimensional echocardiography, with evaluation of the pulmonary arterial systolic pressure (PASP). Patients with lung fibrosis and/ or alveolitis affecting at least the lung bases were considered as having major pulmonary alterations; criteria for minor alterations included septal hypertrophy and limited zones of fibrosis and/or ground glass opacities. Forced vital capacity

(FVC) and DLCO were considered abnormal when lower than 80%. Pulmonary arterial systolic pressure was considered altered when greater than 35 mmHg. Heart disease was evaluated using electrocardiography and / or Holter and echocardiography. Right side heart catheterization was made in patients with PASP greater than 40 mmHg to confirm pulmonary hypertension.

The involvement of the upper digestive tract was documented by endoscopy, oesophageal manometry and, in symptomatic patients, complemented by gastric emptying scintigraphy. The low digestive tract involvement, when applicable, was evaluated by barium studies, respiratory hydrogen and glucose tests, ultrasound, colonoscopy, and anorectal manometry.

The patients included in the study showed no renal manifestations or deterioration in general health, only one patient showed changes in muscle strength, so these scores are not presented. The distance from the thumb to thenar eminence was not evaluated systematically, so the corresponding scores (joint / tendon) are also not shown.

Finally, a score introduced by Cutolo et al. was used to classify nailfold microvascular changes as early, active and late capillaroscopic patterns of microvascular damage [85].

Patients' characteristics are summarized in Tables 1 and 2.

At the time of the study, none of the patients was receiving corticosteroids or immunosuppressive drugs.

Table 2 – Organ involvement according to Medsger Scale in patients with lcSSc and dcSSc (n=47)

Organ specific disease severity scale ^{a)}	Number of cases (%)
Skin score	
0	0 (0.0%)
1	36 (76.6%)
≥ 2	11 (23.4%)
NA / NC	0 (0.0%)
Peripheral vascular score	
0	0 (0.0%)
1	19 (40.4%)
≥ 2	28 (59.6%)
NA / NC	0 (0.0%)
Lung score	
0	12 (25.5%)
1	17 (36.2%)
≥ 2	18 (38.3%)
NA / NC	0 (0.0%)
Gastrointestinal tract score	
0	22 (46.8%)
1	22 (46.8%)
≥ 2	0 (0.0%)
NA / NC	3 (6.4%)
Heart score	
0	26 (55.3%)
1	6 (16.8%)
≥ 2	8 (17.0%)
NA / NC	7 (14.9%)

Abbreviations: SSc, Systemic Sclerosis; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; NA / NC, not available or non-classified (SSc patients were not able to be classified once exams were not available by the time data was collected)

a) Organ specific Medsger disease severity scale, ranging from 0 (no documented involvement) to 4 (end stage disease).

Biological samples

Two PB samples (4.5 ml each) were collected in tubes containing tri-potassium ethylenediaminetetraacetic acid (EDTA-K3) (for lymphocyte immunophenotyping) or without anticoagulant (for measurement of serum cytokines).

Laboratory studies

Lymphocyte immunophenotyping was performed by four colour flow cytometry, using a whole blood stain-wash-and-the-lyse method, as previously described in detail [86]. Briefly, after adjusting cell concentrations to 5 - 15 x 10⁶ cells/μl, 100μl of cell suspension were incubated for 15 minutes at room temperature with the appropriate volume of fluorochrome conjugated monoclonal antibodies (mAbs), followed by erythrocyte lysis and cell fixation, using FACS lysing solution[®], from Becton-Dickinson Biosciences, San José, California, USA (BDB), according to the manufactory's instructions. For immunostaining, we used fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin cyanin 5 (PC5) and allophycocyanin (APC) conjugated mouse mAbs against human CD3, CD4, CD8, CD45RO, HLA-DR (BDB) and CD56 molecules (Beckman Coulter, Hialeah, FL, USA and Immunotech, Marseille, France). Briefly, T-cells (CD3+) and T-cell subsets (CD3+CD4+ or CD3+CD8+) were first quantified and subsequently characterized for the expression of the CD56 cytotoxic cell-associated molecule (NCAM, neural cell adhesion molecule) [87], and the well known activation-related markers, HLA-DR and CD45RO [88, 89], the later being recognized for a long time to be also useful for identification of memory T cells [90]. Natural killer cells (CD3-CD56+) were also quantified and the sub-classified as CD56+dim (predominantly cytotoxic) and CD56+bright (mainly immunoregulatory), according to the levels of CD56 expression [86].

Serum cytokines were assayed by flow cytometry using the BDTM CBA Human Th1/Th2/Th17 Cytokine Kit (BDB) that uses multiplex cytometric bead array (CBA) technology, whose theoretical limit of detection is comparable to conventional Enzyme-Linked Immunosorbent Assays (ELISA). Cytokines evaluated included IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A. Sample processing was performed accordingly to the manufacturer's instructions.

In both cases, data acquisition was done on a FACSCalibur™ flow cytometer (BDB) with a two laser-line configuration, an air-cooled argon blue laser (488 nm) and a red diode laser (635 nm), using the CellQUEST™ software program (BDB). Data analysis was performed using the BD Paint-A-Gate Pro™ Software and the FCAP Array™ software (BDB), for cell immunophenotyping and serum cytokines, respectively. The specific concentration of each cytokine analyzed was determined through the comparison of the mean fluorescence intensity of samples to the standard curve.

Statistical analysis

Statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS 20.0) software (SPSS Inc, IL, USA). Data are presented as median, minimum and maximum values. *Kolmogorov–Smirnov* statistics was used to evaluate sample normality distribution. Once some of the studied variables did not have a normal distribution and given the small size of the statistical sample, non-parametric tests were used. The Mann Whitney U and the Kruskal-Wallis test were used for single comparisons of continuous variables between two or more groups, respectively. A probability $P < 0.05$ was considered statistically significant. “Trends” toward statistical significance, which need to be confirmed in other studies, were considered whenever $0.05 > P > 0.10$.

Results

T cells in Scleroderma

A statistically significant reduction in the number of total lymphocytes and total T cells was observed in the PB from patients under study, compared to healthy controls ($p=0.01$ and $p=0.02$, respectively) (Table 3).

Considering separately CD4+ and CD8+ T cells, a statistically significant decrease in CD8+ T cell counts was found in patients compared to controls ($p=0.007$); a trends to a decrease of CD4+ T cells was also noticed, although without statistical significance.

With reference to the immunophenotypic characteristics of peripheral blood T cells, the numbers of activated T cells, either CD45RO+ or HLA-DR+, were lower in patients than in normal individuals, although differences were not statistically significant, except for CD8+CD45RO+ T cells ($p=0.030$) (Table 3). The numbers of CD56+ CTL were also significantly decreased in patients, comparing to healthy individuals ($p=0.008$).

Curiously, none of these parameters were significantly altered in the PB from patients with Pre-SSc, as compared to controls, whereas patients with SSc had lymphopenia ($p=0.006$), decreased numbers of T cells ($p=0.001$), CD45RO+ ($p=0.039$) and CD56+ ($p=0.003$) T cells, as well as decreased numbers of CD8+ ($p=0.001$) and CD8+CD45RO+ T cells ($p=0.004$) (Table 3).

Table 3 – Immunophenotypic features of peripheral blood T cells and NK cells from patients under study, Pre-SSc and SSc, as compared to healthy controls

	Healthy controls (n=25)	Patients			p values ^{a)}		
		Pre-SSc + SSc (n=57)	Pre-SSc (n=10)	SSc (n=47)	Patients vs. Controls	Pre-SSc vs. Controls	SSc vs. Controls
Lymphocytes	1971 (947-3209)	1618 (764-4893)	1858 (1164-4893)	1477 (764-4772)	0.01	0.401	0.006
T cells							
CD3+	1486 (551-2187)	1176 (420-4082)	1378 (933-3289)	1120 (420-4082)	0.02	0.740	0.001
CD45RO+	1004 (175-1499)	761 (240-2485)	1088 (761-2442)	712 (240-2485)	0.125	0.339	0.039
HLADR+	251 (80-529)	197 (47-1214)	304 (145-810)	187 (47-1214)	0.193	0.425	0.087
CD56+	63 (5-394)	36 (2-478)	57 (4-257)	34 (2-478)	0.008	0.174	0.003
T cell subsets							
CD3+CD4+	942 (442-1492)	802 (337-3903)	889 (646-1903)	771 (337-3903)	0.075	0.598	0.058
CD45RO+	741 (356-1278)	679 (237-3407)	774 (620-1782)	596 (238-3407)	0.814	0.219	0.519
HLADR+	100 (51-212)	118 (31-472)	147 (60-278)	113 (31-372)	0.752	0.424	0.539
CD3+CD8+	468 (110-845)	326 (18-1386)	405 (255-1386)	316 (18-1368)	0.007	0.867	0.001
CD45RO+	269 (87-451)	174 (9-825)	287 (174-825)	171 (9-820)	0.030	0.219	0.004
CD8+HLADR+	116 (29-334)	105 (5-818)	197 (74-551)	96 (5-818)	0.588	0.082	0.249
NK cells							
CD3-CD56+	194 (90-806)	154 (48-595)	239 (85-595)	151 (48-586)	0.168	0.447	0.068
NK cell subsets							
CD56+low	177 (76-796)	143 (41-582)	228 (75-568)	136 (41-582)	0.191	0.495	0.085
CD56+high	12 (4-25)	10 (1-74)	17 (5-28)	9 (1-74)	0.297	0.133	0.083

Results are presented as numbers of the indicated cell populations /mm³.

Abbreviations: SSc, Systemic Sclerosis; Pre-SSc, Pre-Scleroderma.

a) Mann-Whitney U test. P<0.05 was considered to be statistically significant.

The colouring is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies.

Clinical subtypes

Regarding the SSc clinical subtypes (lcSSc and dcSSc), we observed that total lymphocytes, total T cells, activated CD45RO⁺ and HLA-DR⁺ T cells, total CD8⁺ T cells, activated CD8⁺CD45RO⁺ T cells and total CD4⁺ T cells were significantly decreased in dcSSc (p values varying from 0.047 to 0.004) as compared to normal individuals (Table 4). A less pronounced decrease in the numbers of activated CD4⁺HLA-DR⁺, CD4⁺CD45RO⁺ and CD8⁺HLA-DR⁺ T cells was also observed, although differences did not reach statistical significance (Table 4). In addition, patients with dcSSc have diminished numbers of CD56⁺ CTL (p=0.008), as compared to controls. Most of these changes were also found in patients with lcSSc, although they were less evident and differences were statistically significant only for lymphocytes (p=0.035), total T cells (p=0.041), total CD8⁺ T cells (p=0.005), CD8⁺CD45RO⁺ T cells (p=0.017), and CD56⁺ CTL (p=0.011). No statistically significant differences were observed when patients with lcSSc and dcSSc were compared to each other (Table 4). Moreover, no differences were observed between lcSSc patients with CREST as compared to those without CREST, except for a marginal decrease in the number of CD4⁺CD45RO⁺ T cells in the later group (p=0.035) (Table 5).

Table 4 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with SSc, according to lcSSc and dcSSc clinical subtypes

	Healthy controls (n=25)	SSc clinical subtypes		p values ^{a)}		
		lcSSc (n=34)	dcSSc (n=13)	lcSSc vs. Controls	dcSSc vs. Controls	lcSSc vs. dcSSc
Lymphocytes	1971 (947-3209)	1658 (764-4772)	1366 (877-2535)	0.035	0.004	0.147
T cells						
CD3+	1486 (451-4082)	1177 (505-4082)	932 (419-1839)	0.041	0.009	0.161
CD45RO+	1004 (175-1499)	713 (239-2484)	694 (353-1354)	0.107	0.032	0.419
HLADR+	251 (80-529)	196 (47-1214)	167 (50-367)	0.232	0.041	0.264
CD56+	63 (5-394)	37 (2-477)	28 (2-214)	0.011	0.008	0.202
T cell subsets						
CD3+CD4+	942 (442-1492)	842 (364-3903)	618 (337-1434)	0.141	0.047	0.128
CD45RO+	741 (356-1278)	668 (237-3407)	567 (318-1254)	0.771	0.275	0.419
HLA-DR+	100 (51-212)	118 (3-478)	88 (33,167)	0.844	0.236	0.428
CD3+CD8+	468 (110-845)	319 (18 to 1368)	247 (8-507)	0.005	0.006	0.651
CD45RO+	269 (87-451)	171 (9-820)	146 (54-206)	0.017	0.007	0.405
HLA-DR+	116 (29-334)	105 (5-818)	57 (14-234)	0.591	0.054	0.154
NK cells						
CD3-CD56+	194 (90-806)	149 (48-586)	156 (80-288)	0.095	0.162	0.812
NK cell subsets						
CD56+low	177 (76-796)	136 (41-582)	149 (68-280)	0.101	0.236	0.831
CD56+high	12 (4-25)	9 (1-74)	10 (4-26)	0.041	0.678	0.254

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

Abbreviations: SSc, Systemic Sclerosis; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc.

a) Mann-Whitney U test. P<0.05 was considered to be statistically significant and is indicated in bold.

The colouring is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies.

Table 5 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with lcSSc, according to absence or presence of CREST

	lcSSc		p values ^{a)}
	With CREST (n=16)	Without CREST (n=18)	lcSSc with CREST vs. lcSSc without CREST
Lymphocytes	1576 (1091-2860)	1719 (764-4772)	0.918
T cells			
CD3+	1189 (845-2468)	1173 (505-4082)	0.605
CD45RO+	737 (403-1762)	666 (240-2485)	0.317
HLADR+	223 (53-1214)	159 (47-453)	0.121
CD56+	39 (10-478)	34 (2-98)	0.836
T cell subsets			
CD3+CD4+	854 (440-1449)	802 (364-3903)	0.972
CD45RO+	682 (416-1236)	628 (237-3407)	0.035
HLA-DR+	122 (31-472)	91 (39-178)	0.121
CD3+CD8+	327 (180-1368)	253 (18-501)	0.138
CD45RO+	192 (45-820)	142 (9-287)	0.836
HLA-DR+	125 (27-818)	90 (5-317)	0.262
NK cells			
CD3- CD56+	149 (80-586)	144 (48-474)	0.972
NK cell subsets			
CD56+low	137 (74-582)	136 (41-461)	0.890
CD56+high	10 (4-35)	8 (1-74)	0.535

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

The coloring is using is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies.

CREST calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia, SSc systemic sclerosis, LcSSc limited cutaneous Ssc.

Mann-Whitney U test; p<0.05 was considered to be statistically significant.

Evolution phases

Patients with early SSc had significantly lower numbers of lymphocytes ($p=0.015$), total T cells ($p=0.030$), CD45RO+ ($p=0.011$) and HLA-DR+ ($p=0.002$) T cells, CD56+ T cells ($p=0.043$), total CD8+ T cells ($p=0.005$), CD8+CD45RO+ ($p=0.020$) and CD8+HLA-DR+ ($p=0.007$) T cells, as well as CD4+HLA-DR+ T cells ($p=0.015$), as compared to controls (with a partially recover during the intermediate phase) (Table 6).

Table 6 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with Scleroderma, lcSSc and dcSSc subtypes, according to evolution phase

	Healthy controls (n=25)	SSc evolution phase			p values ^{a)}				
		E-SSc (n=7)	I-SSc (n=10)	L-SSc (n=30)	E-SSc vs. Controls	I-SSc vs. Controls	L-SSc vs. Controls	E-SSc vs. I-SSc	I-SSc vs. L-SSc
Lymphocytes	1971 (947-3209)	1118 (764-2151)	1700 (1136-4772)	1476 (877-2860)	0.015	0.208	0.015	0.305	0.349
T cells									
CD3+	1486 (451-4082)	868 (505-1600)	1175 (796-4082)	1094 (419-2468)	0.030	0.189	0.022	0.329	0.453
CD45RO+	1004 (175-1499)	562 (240-1029)	744 (462-2485)	704 (354-1762)	0.011	0.535	0.085	0.051	0.382
HLADR+	251 (80-529)	75 (53-284)	263 (62-399)	196 (47-1214)	0.002	0.971	0.199	0.011	0.512
CD56+	63 (5-394)	36 (2-98)	38 (10-89)	28 (0-478)	0.043	0.028	0.013	0.770	0.949
T cell subsets									
CD3+CD4+	942 (442-1492)	660 (364-1430)	813 (440-3903)	760 (337-1449)	0.116	0.290	0.101	0.380	0.755
CD45RO+	741 (356-1278)	556 (237-948)	636 (416-3407)	624 (318-1236)	0.374	0.535	0.761	0.495	0.925
HLA-DR+	100 (51-212)	67 (31-149)	128 (39-186)	113 (33-472)	0.015	0.715	0.896	0.097	0.629
CD3+CD8+	468 (110-845)	186 (142-388)	385 (167-147)	300 (18-1368)	0.005	0.080	0.006	0.051	0.595
CD45RO+	269 (87-451)	81 (34-210)	191 (86-320)	161 (9-820)	0.020	0.243	0.016	0.025	0.288
HLA-DR+	116 (29-334)	27 (10-174)	142 (21-243)	97 (5-818)	0.007	0.744	0.427	0.025	0.553
NK cells									
CD3-CD56+	194 (90-806)	116 (67-215)	135 (48-338)	158 (80-586)	0.030	0.125	0.272	0.435	0.333
NK cell subsets									
CD56+low	177 (76-796)	110 (58-155)	119 (41-329)	149 (68-582)	0.019	0.134	0.370	0.435	0.318
CD56+high	12 (4-25)	8 (5-74)	8 (3-26)	10 (1-35)	0.146	0.241	0.166	0.770	0.876

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

Abbreviations: SSc, Systemic Sclerosis; E-SSc, Early Systemic Sclerosis; I-SSc, Intermediate Systemic Sclerosis; L-SSc, Late Systemic Sclerosis.

a) Mann-Whitney U test. $P<0.05$ was considered to be statistically significant and is indicated in bold. The colouring is used to highlight the cases with $p<0.05$ (green cells), and those having a “trend” toward statistical significance ($0.05>p<0.10$) (yellow cells), which needs to be confirmed in other studies.

Nailfold capillaroscopy changes

Fifty-one from the 55 patients with available nailfold capillaroscopy presented relevant microvascular changes. Among them, patients with “active” and “late” capillaroscopic patterns showed statistically significantly lower lymphocyte counts than normal individuals ($p=0.039$ and $p=0.031$, respectively) (Table 7). T cell analysis revealed lower counts of both total CD8+ and CD8+CD45RO+ T cells in both groups, as compared to controls ($p=0.024$ and $p=0.069$ for patients with “active” pattern; $p=0.005$ and $p=0.025$ for patients with “late” pattern), as well as lower CD56+ T cell counts ($p=0.028$ and $p=0.009$, for “active” and “late” patterns, respectively). In contrast, no differences were found in patients with “early” capillaroscopic changes ($p>0.05$). Moreover, differences for CD4+ T cells did not achieve statistical significance in none of the above mentioned groups.

Table 7 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with Scleroderma, Pre-SSc and SSc, according to the capillaroscopic pattern

	Healthy controls (n=25)	Capillaroscopic pattern (CP) (Pre-SSc and SSc patients, n=51) ^{a)}			p values ^{b)}				
		Early CP (n=12)	Active CP (n=21)	Late CP (n=18)	Early CP vs. Controls	Active CP vs. Controls	Late CP vs. Controls	Early vs. Active CP	Active vs. Late CP
Lymphocytes	1971 (947-3209)	1734 (1091-4893)	1533 (764-4772)	1742 (1019-2373)	0.299	0.039	0.031	0.454	0.822
T cells									
CD3+	1486 (451-4082)	1218 (845-3289)	1120 (419-4082)	1324 (744-1825)	0.548	0.046	0.080	0.171	0.632
CD45RO+	1004 (175-1499)	911 (403-2442)	761 (240-2485)	938 (508-1374)	0.850	0.305	0.072	0.258	0.693
HLADR+	251 (80-529)	287 (53-810)	214 (50-1214)	234 (167-365)	0.742	0.182	0.115	0.291	0.866
CD56+	63 (5-394)	45 (74-257)	33 (7-478)	47 (14-182)	0.363	0.028	0.009	0.364	0.761
T cell subsets									
CD3+CD4+	942 (442-1492)	889 (660-1903)	746 (337-3903)	1099 (412-1371)	0.571	0.062	0.506	0.159	0.535
CD45RO+	741 (356-1278)	774 (463-1782)	677 (237-3407)	1019 (360-1227)	0.328	0.574	0.883	0.258	0.866
HLA-DR+	100 (51-212)	153 (31-278)	107 (33-472)	124 (69-178)	0.264	0.372	0.848	0.071	0.454
CD3+CD8+ T	468 (110-845)	405 (185-1386)	287 (82-1368)	340 (202-507)	0.525	0.024	0.005	0.159	0.955
CD45RO+	269 (87-451)	200 (45-825)	171 (54-820)	169 (83-287)	0.770	0.069	0.025	0.351	0.778
HLA-DR+	116 (29-334)	176 (27-551)	96 (19-818)	137 (40-223)	0.440	0.515	0.313	0.312	0.673
NK cells									
CD3-CD56+	194 (90-806)	173 (85-595)	161 (48-435)	143 (80-474)	0.481	0.396	0.110	0.984	0.592
NK cell subsets									
CD56+low	177 (76-796)	161 (74-568)	141 (41-425)	130 (70-461)	0.481	0.396	0.133	0.827	0.612
CD56+high	12 (4-25)	11 (5-28)	11 (3-74)	9 (1-26)	0.643	0.635	0.257	0.620	0.414

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

Abbreviations: SSc, Systemic Sclerosis; CP, capillaroscopic pattern;

a) Two patients did not perform capillaroscopy and another 4 patients had normal or nearly normal capillaroscopic patterns.

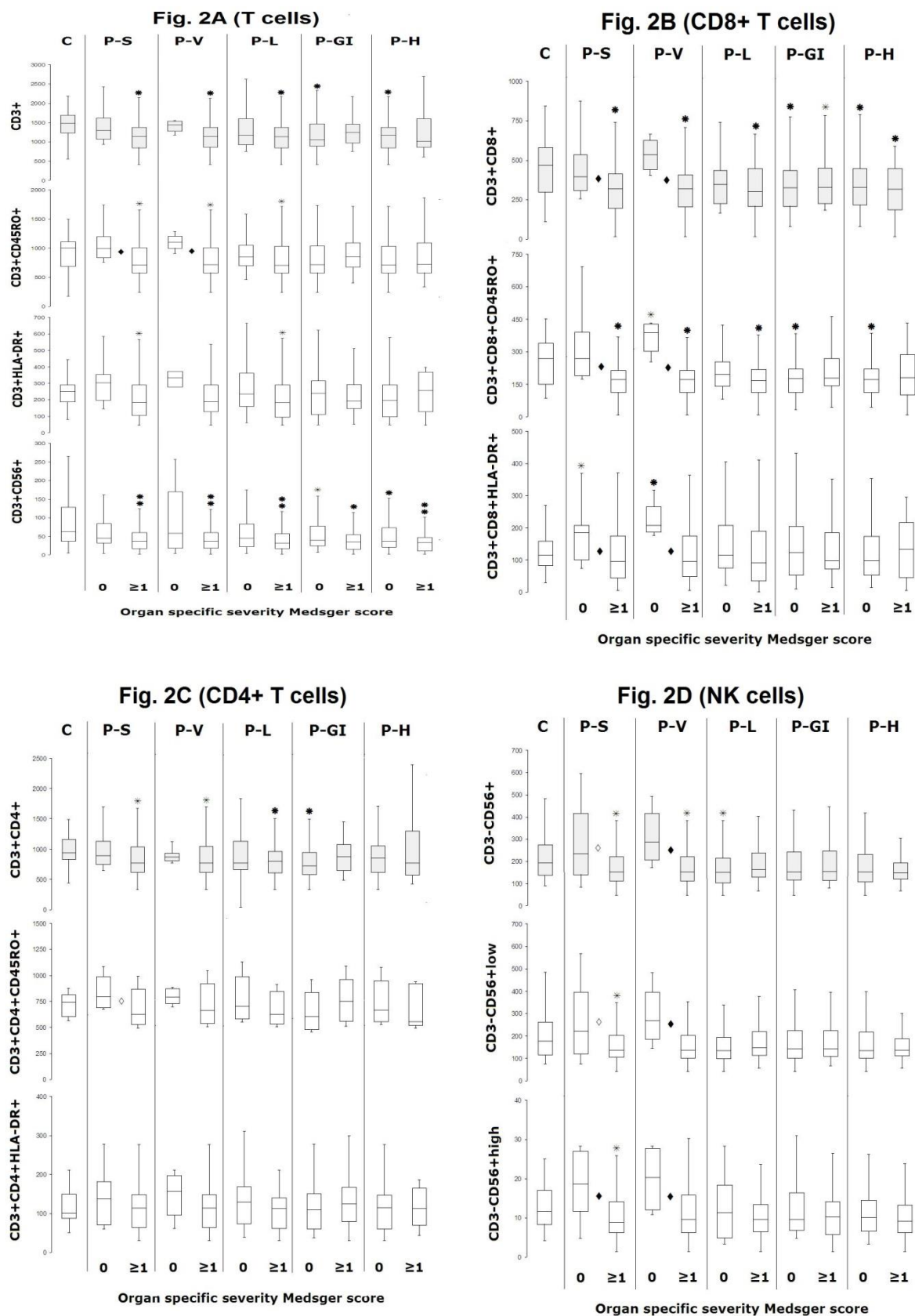
b) Mann-Whitney U test. P<0.05 was considered to be statistically significant. The colouring is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies.

Organ involvement

T cell analysis according to organ involvement, as evaluated by the organ specific Medsger disease severity scale is depicted in Figure 2, panels A to C, and detailed results are available as supplementary material (Appendix A).

In general, the numbers of total T cells were significantly reduced in patients with skin, vascular and lung involvement when compared to controls ($p=0.010$, $p=0.015$ and $p=0.011$, respectively). CD56⁺ T cells were also markedly reduced in patients with skin, vascular and lung involvement, but also in those with gastrointestinal and heart involvement, as compared to controls ($p=0.003$, $p=0.005$, $p=0.004$, $p=0.026$ and $p=0.003$, respectively) (Figure 2A). Similar results were obtained for CD8⁺ T cells ($p=0.001$, $p=0.002$, $p=0.003$, $p=0.076$ and $p=0.020$, respectively) (Figure 2B). In contrast, the decrease of CD4⁺ T cells observed in these patients was much less pronounced and did not reach statistical significance, except for the lung ($p=0.043$) (Figure 2C).

Figure 2 – Box and whisker plots summarizing the absolute counts (cells/ μ l) of T cell (a), CD8+ T cells (b), CD4+ T cells (c), and NK cell (d) in patients with SSc



Legend Figure 2: Box and whisker plots summarizing the absolute counts (cells / μl) of T cells (panel A), CD8+ T cells (panel B), CD4+ T cells (panel C), and NK cells (panel D) in patients with SSc, classified according the Medsger organ-specific disease severity scale (score = 0 or score ≥ 1).

The body of the boxes goes from the first quartile (Q1) to the third quartile (Q3). Within the boxes, a line is drawn at the median of the data set. Two horizontal lines (whiskers) extend from the boxes to the minimum and maximum values, excluding the outliers. Black stars (*) above boxes indicate the cases in which differences reached statistical significance ($p < 0.05$) when the correspondent patient group was tested against controls; empty stars (*) above the boxes indicate a "trend" toward statistical significance ($0.05 > p > 0.10$), which needs to be confirmed in other studies. Black diamonds (◆) between boxes indicate the cases in which differences reached statistical significance ($p < 0.05$) when the correspondent patient groups were tested against each other; empty diamonds (◇) between the boxes indicates a "trend" toward statistical significance ($0.05 > p > 0.10$), which needs to be confirmed in other studies.

Abbreviations: C, Controls (healthy individuals); P-S, Patients-Skin score; P-V, Patients-Vascular score; P-L, patients-Lung score; P-GI, Patients-Gastrointestinal score; P-H, Patients-Heart score.

A trend for decrease of CD45RO+ T cells was also observed in patients with skin, vascular and lung affection, although differences between patients and controls reached statistical significance only for the skin ($p = 0.039$). The same applies to HLA-DR+ T cells, even though differences were not statistically significant in any case (Figure 2A). Statistical significance was however achieved for the expression of the CD45RO activation/memory related marker if only the CD8+ T cell subset was considered ($p = 0.004$, $p = 0.008$ and $p = 0.002$, respectively). In addition, CD8+CD45RO+ and CD8+HLA-DR+ T cells were more reduced in the PB from patients with skin score ≥ 1 (SSc) than in those with skin score = 0 (Pre-SSc) ($p = 0.002$ and $p = 0.025$, respectively) and similar results were obtained for the vascular involvement ($p = 0.006$ and $p = 0.026$, respectively) (Figure 2B).

Using a higher cut-off for the disease severity Medsger scores, i.e., organ specific scores ≥ 2 vs. ≤ 1 , there were no statistically significant differences for the mentioned T cell subsets and activation related markers, except for the lung, concerning CD56+ T cells ($p = 0.013$).

When comparing patients without specific organ involvement with controls, statistically significant differences were not observed for the skin, vascular and lung groups. However, patients without gastrointestinal and heart involvement had lymphopenia ($p = 0.013$ and $p = 0.024$, respectively), decreased T cells ($p = 0.027$ and $p = 0.055$, respectively) and decreased CD56+ T cells ($p = 0.060$ and $p = 0.039$, respectively) (Figure 2A), as well as diminished numbers of CD8+ T cells ($p = 0.011$ and $p = 0.015$, respectively) and CD8+CD45RO+ T cells ($p = 0.027$ and $p = 0.032$, respectively) (Figure 2B).

Concerning lung involvement (Tables 8 and 9), we observed that both patients with normal and abnormal LFT had lower lymphocyte counts ($p = 0.014$ and $p = 0.036$) and lower T cell counts ($p = 0.029$ and $p = 0.042$), as well as lower CD8+ ($p = 0.011$ and $p = 0.027$), and CD8+CD45RO+ ($p = 0.043$ and $p = 0.061$) counts, as compared with controls (Table 8).

However, CD56+ T cells were selectively decreased in patients with abnormal LFT ($p < 0.001$), but not in those having normal LFT. Similarly, patients with major alterations in the chest CT had lower T cell counts ($p = 0.018$), as well as lower counts of CD8+ ($p = 0.005$), CD56+ ($p < 0.006$) and CD8+CD45RO+ ($p = 0.019$) T cells, as compared with controls (Table 9). CD56+ T cells were also lower in patients with minor alterations ($p=0.014$; Table 9).

Table 8 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with SSc, according to the respiratory function tests

	Healthy controls (HC) (n=25)	SSc groups according to the results of RFT ^{a)}		p values ^{b)}		
		Normal RFT (SSc0) (n=25)	Abnormal RFT (SSc1) (n=28)	SSc0 vs. HC	SSc1 vs. HC	SSc0 vs. SSc1
Lymphocytes	1971 (947-3209)	1476 (877-4893)	1734 (764-4772)	0.014	0.036	0.461
T cells						
CD3+	1486 (451-4082)	1120 (419-3289)	1178 (505-4082)	0.029	0.042	0.590
CD45RO+	1004 (175-1499)	835 (354-2442)	717 (240-2485)	0.178	0.139	0.853
HLADR+	251 (80-529)	214 (47-1213)	183 (57-574)	0.245	0.203	0.952
CD56+	63 (5-394)	45 (2-478)	24 (2-257)	0.216	<0.001	0.027
T cell subsets						
CD3+CD4+	942 (442-1492)	774 (337-1903)	808 (364-3903)	0.087	0.113	0.736
CD45RO+	741 (356-1278)	681 (318-1782)	639 (237-3407)	0.805	0.708	0.880
HLA-DR+	100 (51-212)	123 (31-472)	113 (33-183)	0.949	0.458	0.505
CD3+CD8+	468 (110-845)	316 (18-1386)	327 (142-664)	0.011	0.027	0.533
CD45RO+	269 (87-451)	175 (9-825)	169 (34-433)	0.043	0.061	0.952
HLA-DR+	116 (29-334)	96 (5-818)	105 (10-416)	0.447	0.722	0.814
NK cells						
CD3-CD56+	194 (90-806)	158 (48-595)	157 (67-586)	0.230	0.277	1.000
NK cell subsets						
CD56+low	177 (76-796)	141 (41-568)	145 (58-582)	0.260	0.310	0.973
CD56+high	12 (4-25)	8 (1-74)	11 (4-37)	0.101	0.618	0.304

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

Abbreviations: HC, healthy controls; SSc, Systemic Sclerosis; RFT, respiratory function tests.

a) Patients with decreased diffusing capacity for carbon-monoxide (DLCO <80%) and/or with decreased forced vital capacity (FVC <80%) were considered to have abnormal RFT, whereas those with normal DLCO (≥80%) and normal FVC (≥80%) were considered to have normal RFT.

b) Mann-Whitney U test. P<0.05 was considered to be statistically significant.

The colouring is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies.

Table 9 – Immunophenotypic features of peripheral blood T cells and NK cells from patients with SSc, according to the pulmonary abnormalities in the high resolution chest computerized tomography

	Healthy controls (HC) (n=25)	SSc groups according to the pulmonary alterations in the chest CT ^{a)}			p values ^{b)}					
		Normal chest CT	Minor pulmonary alterations	Major pulmonary alterations	SSc0	SSc1	SSc2	SSc0	SSc0	SSc1
		(SSc0) (n=25)	(SSc1) (n=10)	(SSc2) (n=18)	vs. HC	vs. HC	vs. HC	vs. SSc1	vs. SSc2	vs. SSc2
Lymphocytes	1971 (947-3209)	1570 (1081-4893)	1781 (1366-2535)	1388 (764-4772)	0.049	0.093	0.030	0.458	0.328	0.314
T cells										
CD3+	1486 (451-4082)	1179 (750-3289)	1217 (798-1839)	1041 (419-4082)	0.114	0.154	0.018	0.942	0.268	0.270
CD45RO+	1004 (175-1499)	857 (401-2442)	744 (615-1354)	672 (240-2485)	0.248	0.307	0.146	0.855	0.326	0.231
HLADR+	251 (80-529)	231 (47-1214)	187 (81-368)	162 (50-574)	0.491	0.133	0.247	0.626	0.491	0.837
CD56+	63 (5-394)	41 (2-478)	32 (10-257)	35 (2-156)	0.168	0.014	0.006	0.427	0.431	0.598
T cell subsets										
CD3+CD4+	942 (442-1492)	831 (440-1903)	882 (486-1434)	660 (337-3903)	0.197	0.465	0.055	0.855	0.209	0.292
CD45RO+	741 (356-1278)	754 (397-1782)	717 (425-1254)	561 (237-3407)	0.691	0.971	0.268	0.798	0.209	0.314
HLA-DR+	100 (51-212)	115 (33-472)	137 (55-183)	100 (31-178)	0.920	0.584	0.301	0.450	0.309	0.179
CD3+CD8+	468 (110-845)	381 (18-1386)	302 (190-664)	294 (82-511)	0.118	0.058	0.005	0.609	0.247	0.443
CD45RO+	269 (87-451)	186 (9-825)	160 (83-424)	173 (45-413)	0.184	0.125	0.019	0.584	0.350	0.774
HLA-DR+	116 (29-334)	130 (5-819)	91 (34-317)	80 (14-416)	0.977	0.381	0.460	0.584	0.571	0.737
NK cells										
CD3-CD56+	194 (90-806)	153 (48-595)	186 (108-474)	142 (67-586)	0.143	0.913	0.176	0.324	0.883	0.213
NK cell subsets										
CD56+low	177 (76-796)	141 (41-568)	178 (105-461)	137 (58-582)	0.101	0.971	0.290	0.243	0.863	0.314
CD56+high	12 (4-25)	13 (5-74)	11 (3-26)	7 (1-14)	0.662	0.942	0.001	0.488	0.002	0.084

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³.

Abbreviations: HC, healthy controls; SSc, Systemic Sclerosis; CT, computerized tomography.

a) Major pulmonary alterations included fibrosis and/ or alveolitis affecting at least the lung bases; minor pulmonary alterations comprised septal hypertrophy, limited zones of fibrosis and/or ground glass opacities.

b) Mann-Whitney U test. $P < 0.05$ was considered to be statistically significant.

The colouring is used to highlight the cases with $p < 0.05$ (green cells), and those having a “trend” toward statistical significance ($0.05 > p > 0.10$) (yellow cells), which needs to be confirmed in other studies.

NK cells in Scleroderma

Regarding NK cell counts, including the CD56+low (cytotoxic) and CD56+high (immunoregulatory) NK cell subsets, no statistically significant changes were observed in our study population, although these cells showed a trend to be decreased in SSc, but not in Pre-SSc, as compared to normal individuals (Table 3).

Clinical subtypes

Regarding the SSc clinical subtypes, we observed that both NK cell counts were lower in patients with lcSSc and dcSSc relative to controls, although differences reached statistical significance only for CD56+high NK cells in patients with lcSSc (Table 4). In addition, no statistically significant differences were observed in patients with CREST comparing to those without CREST (Table 5).

Evolution phases

Patients with “early” disease had statistically significantly lower numbers of NK cells in the PB, as compared to normal individuals ($p = 0.030$), due to a decrease in the CD56+low NK cell compartment ($p = 0.019$) (Table 6).

Nailfold capillaroscopy changes

In that concerning the capillaroscopic pattern, there were no statistically significant differences between the NK cell (both CD56+low and CD56+high) count from patients with early, active and late capillaroscopic patterns and normal individuals (Table 7).

Organ involvement

Patients with skin score=0 (Pre-SSc) had higher NK cell counts (both CD56+low and CD56+high) and patients with skin score $\geq$ 1 (SSc) had a lower NK cell counts (both CD56+low and CD56+high) than normal individuals, although the differences did not reach statistical significance; similar results were observed for patients with vascular involvement, but not for the lung, gastrointestinal, and heart organ systems (Figure 2D) (Appendix A, Supplementary material).

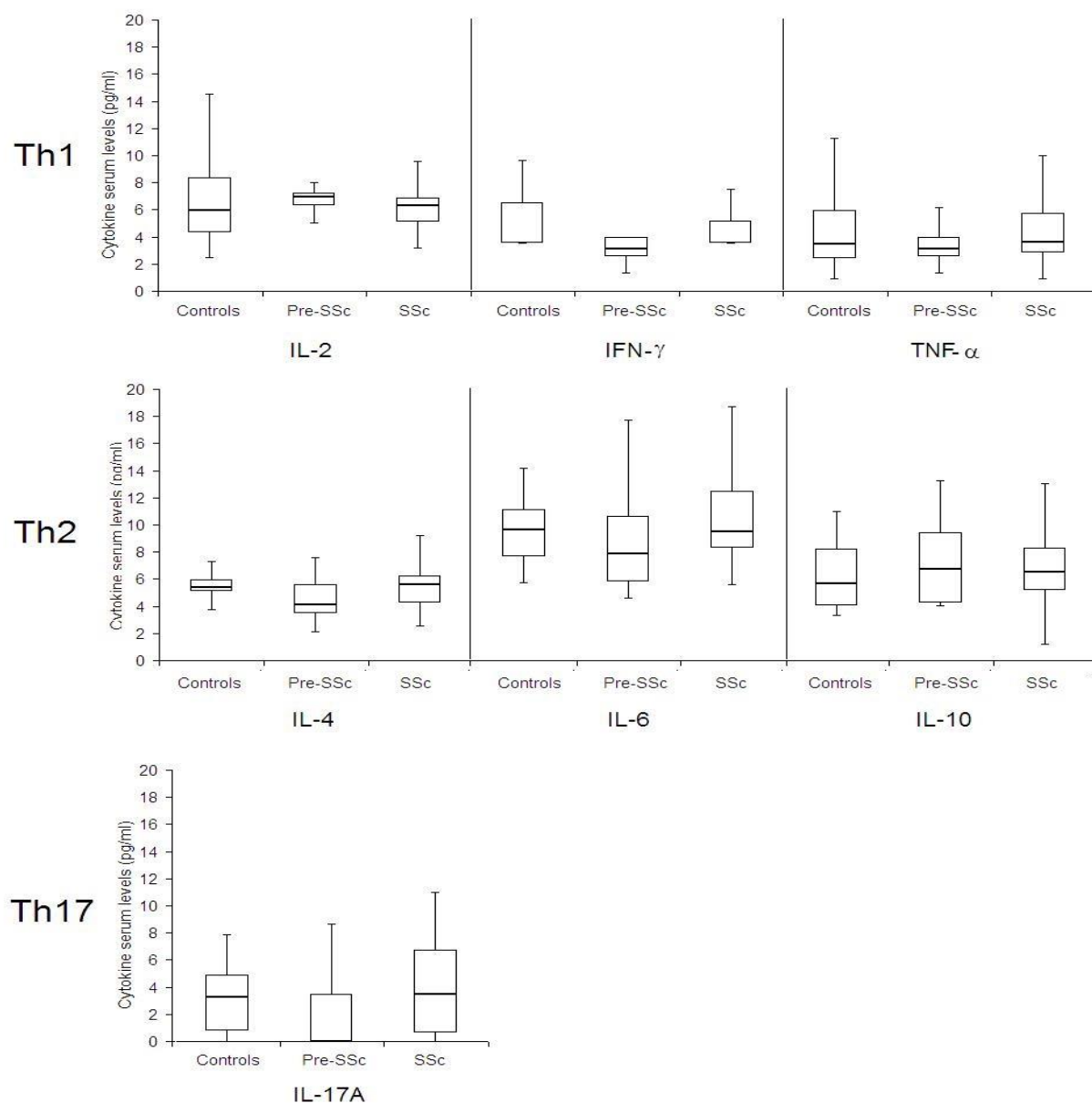
In addition, patients with vascular involvement (vascular score $\geq$ 1) had lower numbers of CD56+low and CD56+high NK cells when compared with patients without vascular involvement or without need for treatment (vascular score = 0) ($p=0.049$ in both cases), and patients with skin involvement (skin score $\geq$ 1, SSc) had lower counts of CD56+high NK cells when compared with patients without skin involvement (skin score =0, Pre-SSc) (Figure 2D) (Appendix A, Supplementary material).

We found no differences on the total NK cell and CD56+low NK cell counts in patients with normal or abnormal LFT, as compared to controls (Table 8); however, patients with major pulmonary alterations in the chest CT had significantly lower numbers of CD56+high NK cells as compared to controls ($p=0.001$), as well as compared to patients with no alterations ($p=0.002$) (Table 9).

Serum levels of Th1, Th2 and Th17 cytokines in Scleroderma

Regarding the serum levels of Th1 (IL-2, IFN- γ , TNF- α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL-17A) cytokines, we found no statistically significant differences between patients with Pre-SSc, patients with SSc, and healthy controls or according to subtypes, stage, organ involvement and capillaroscopy ($p>0.05$ in all cases) (Figure 3).

Figure 3 – Box and whisker plots summarizing the levels (pg/μl) of T helper (Th) 1 (IL-2, IFN-γ, and TNF-α), Th2 (IL-4, IL-6, and IL-10), and Th17 (IL-17 A) cytokines in the sera from patients (Pre-SSc and SSc) and controls (healthy individuals).



Box and whisker plots summarizing the levels (pg/μl) of T helper (Th) 1 (IL-2, IFN-γ and TNF-α), Th2 (IL-4, IL-6 and IL-10) and Th17 (IL-17A) cytokines in the sera from patients (Pre-SSc and SSc) and controls (healthy individuals).

Cytokine measurement was performed by flow cytometry, using the BD TM CBA Human Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences). The body of the boxes goes from the first quartile (Q1) to the third quartile (Q3). Within the boxes, a line is drawn at the median of the data set. Two horizontal lines (whiskers) extend from the boxes to the minimum and maximum values, excluding the outliers.

Abbreviations: IL, interleukin, IFN-γ, interferon gamma, TNF-α, tumour necrosis factor alpha, Pre-SSc, pre scleroderma; SSc, scleroderma.

Discussion

The interrelationship between the microvascular endothelium, fibroblasts and cells of the immune system is important for the pathogenesis of SSc, and T lymphocytes seem to be major players in this process [1–5; 12, 16, 17]. Accordingly, we found relevant alterations of peripheral blood T cells from patients with SSc, as compared to healthy individuals.

Significant changes observed in our study population, composed by Pre-SSc and SSc patients, were restricted to patients with SSc and consisted of T cell lymphopenia, mainly affecting the CD8⁺ and CD56⁺ T cell subsets, with evidence for decreased absolute numbers of activated T cells, as evaluated by the expression of CD45RO and to a less extent HLA-DR, which was more pronounced in the CD8⁺ T cell compartment. At this point, our study indicates that depletion of CD8⁺ and CD56⁺ T cells in the PB is an immunological mark of SSc, suggesting an important role of these T cell subsets in disease pathogenesis.

These results are in accordance with previous studies indicating that CD8⁺ T cells are major players SSc [16], and extend these findings to CD56⁺ T cells.

Considering CD8⁺ T cells, both lcSSc and dcSSc patients, but not those with Pre-SSc, showed a significant decreased of CD8⁺ and CD8⁺CD45RO⁺ T cells compared to healthy controls [45]. In addition, activated CD8⁺CD45RO⁺ and CD8⁺HLA-DR⁺ T cells were more pronounced reduced in the PB from patients with SSc than in patients with Pre-SSc and in patients with vascular involvement than in patients without, which might be explicated at least in part by CD8⁺ T cell recruitment to the tissues. As regards to nailfold capillaroscopy, patients with “active” and “late” capillaroscopic patterns showed statistically significantly lower CD8⁺ and CD8⁺CD45RO⁺ T cell counts, sustaining that these cells might be involved in microvascular changes observed in these patients. To our knowledge, no previous published studies referred to this issue. Finally, CD8⁺ T cell depletion was more pronounced in early disease, confirming a relevant role of these cells early during the disease course. Decreased numbers of circulating CD8⁺ T cells in early SSc could be explained by migration of this T cell subset to the lesional skin [33], although other mechanisms, such as increased T cell apoptosis may also operate [91-93]. In accordance, serum levels of the soluble Fas/APO-1 receptor have been shown to be increased in SSc [91]. Also in line, reports on the expression of pro-apoptotic (Fas) and anti-apoptotic (BCL-2) molecules and transcription factors (NF- κ B) in T cells from patients with SSc have suggested that the increased CD4/CD8 ratio in PB from patients with SSc could be due to an increased spontaneous apoptosis of SSc CD8 lymphocytes [93], as well as to a greater resistance of CD4⁺ lymphocytes to apoptotic stimuli [94].

In contrast, we found no statistically significant differences in the CD4⁺ T cell compartment, except in patients with dcSSc, who had low CD4⁺ T cell counts. This observation

is in agreement with the fact CD4⁺ T cells predominate in the skin of SSc patients in advanced disease stages [25, 29,30,33].

Discrepant results found in the literature concerning the CD4⁺ and CD8⁺ T cell subsets in the PB from patients with SSc are probably due to different characteristics of the studied populations (T cell patterns may diverge in early and late stages of disease and/or in different clinical SSc subsets), as well as from different ways of expressing the results, i.e., relative or both relative and absolute numbers of circulating T cells. In accordance, considering 10 studies published from 1991 to 2014 [35,41–45,51,95-97], with a total of 331 patients with SSc, the percentage of CD8⁺ T cells was found to be significantly decreased in 5 studies [35,41–43,95] and normal in another five [44,45,51,96,97], whereas the percentage of CD4⁺ T cells was found to be normal in 9 studies [35,41–45,51,95,97], and significantly decreased in only one [96]. However, taking into consideration only the 5 studies reporting the absolute cell counts [35,42 – 44,96], CD8⁺ T cell and CD4⁺ T cell numbers were found to be significantly decreased in 3 studies [35,42,43] and 2 studies [42,43], respectively, whereas normal CD8⁺ and CD4⁺ T cell counts were found in the remaining two [44,96] and three [35,44,96] studies, respectively. Thus, in general, T cell depletion seems to be more pronounced in the CD8⁺ than in the CD4⁺ T cell compartment, as documented in our study.

In our work, a reduction of the circulating CD56⁺ T cells, which are known to have cytotoxic functions [87], was also observed in patients with SSc. In accordance, CD56⁺ CTL were significantly decreased in patients with lcSSc and dcSSc, and in early, intermediate and late disease stages, as well as in patients with skin, vascular, lung, gastrointestinal and heart involvement, as compared to controls. Similar results were found in patients with active and late capillaroscopic patterns, but not in those with early capillaroscopic changes. However, we found no statistically significant differences between lcSSc and dcSSc, between lcSSc with and without CREST, and between early, intermediate and late SSc, neither between patients with or without organ involvement, concerning the absolute number of CD56⁺ T cells. Curiously, patients with Medsger lung scores (≥ 2) had statistically significant lower numbers of CD56⁺ T cells than patients with lung scores ≤ 1 and similar results were observed in patients with alterations in the chest CT, suggesting that CD56⁺ T cell depletion in the PB might be useful as a biomarker of lung disease severity. In line with this finding, previous studies have shown increased numbers of CD56⁺ CTL in the BALF of patients with SSc, especially in those with severe lung impairment [39].

As previously observed in other immune-mediated diseases, such as psoriasis [98], we provide the first clear evidence showing that decreased CD56⁺ T cell counts in the PB are a characteristic of SSc. Cytotoxic T cell mediated endothelial cell injury and apoptosis, either by Fas or granzymes/perforin-related mechanisms, have been proposed as one of the

mechanisms involved in the pathogenesis of SSc, ultimately leading to microcirculatory dysfunction and eventual organ failure [99]. In addition peripheral blood T cells from patients with early systemic sclerosis have been shown to kill autologous fibroblasts in co-culture [100]. Together with our results, these observations would suggest that the activation of CTL-mediated pathways may be involved in vascular and fibroblast damage in scleroderma and in initiating and propagating the autoimmune response.

Our study also provides evidence for the existence of NK cell alterations in the PB from SSc patients, although in general they were less pronounced than those observed in the T cell compartment. In accordance, CD56+low and CD56+high NK cells showed a trend to be decreased in SSc, but not in Pre-SSc, as compared to normal individuals. In addition, CD56+high NK cells were significantly decreased in the PB from patients with lcSSc, but not from patients with dcSSc, and patients with early disease had statistically significantly lower numbers of CD56+low NK cells, relative to the healthy controls. In contrast, no statistically significant differences were observed in patients with CREST compared to patients without CREST, neither in patients with early, active and late capillaroscopic patterns, as compared to healthy individuals. Curiously, patients with skin and lung involvement had lower CD56+low and CD56+high NK cell counts in comparison to controls, whereas higher counts of both NK cell populations were observed in patients without skin and vascular involvement. Natural killer cells were also diminished when patients with evidence of skin or lung involvement were compared with patients without. Moreover, we found that SSc patients with abnormal LFT and major alterations in the chest CT had lower counts of CD56+high NK cells. These changes appear to be organ specific, as they were not found in the heart and gastrointestinal system. It must however be emphasized that these observations need to be confirmed in further studies with a large number of patients, as statistical significance was not achieved for some of the above mentioned observations.

In general, the results presented herein would suggest that altered NK cell numbers and function contributes to immunological abnormalities in SSc. Data available in the literature is sometimes discrepant. For instance, Gambichler *et al.* found that the absolute count of NK cells in the PB from patients with SSc did not significantly differ from controls [42], Riccieri *et al.* reported that NK cells were marginally but not significantly decreased [35], Ercole *et al.* found that patients with dcSSc and late-stage disease had smaller percentages of NK-cells [45] and Horikawa *et al.* described that the frequency and absolute number of NK cells were increased in dcSSc, whereas they were normal in lcSSc [50]. Again, as previously stated for T cells, these apparently conflicting observations may be related to the way of reporting the results and to the characteristics of the studied population.

As also mentioned for CTL, NK cell-mediated cytotoxic pathways may be involved the pathogenesis of SSc. In accordance, NK cells from patients with SSc (both lcSSc and dcSSc) were found to exhibit activated phenotypes [50], and similar results were reported in patients with late stage disease SSc [51]. Evidence for NK cell dysfunction has also been found. For instance, Horikawa *et al.* observed that IFN- γ , IL-5 and IL-10 production by non-stimulated NK cells was increased in SSc patients compared to normal controls, whereas on stimulation, a reduced amount of IFN- γ was produced [50]. They also found that despite the augmented cytokine production by non-stimulated NK cells, natural cytotoxic activity and granzyme B secretion were reduced in NK cells from SSc patients [50]. Using the ^{51}Cr release assay, Wanchu *et al.* found that patients with lcSSc had significantly higher *baseline* NK cell mediated cytotoxicity than controls and the augmentation following in vitro stimulation with IFN- γ was negligible, whereas no statistically significant differences were observed in patients with dcSSc [52]. Finally, apoptosis of dermal microvascular endothelial cells, a key event in the pathogenesis of SSc, was attributed to anti-endothelial cell antibody-dependent cell-mediated cytotoxicity via the Fas pathway [101].

In that concerning the serum levels of the analyzed Th1, Th2, and Th17 cytokines, we found no evidence for relevant changes in patients with SSc, compared to controls. Concentrations of almost all of the cytokines studied in the control's and patient's serum were nearby the low theoretical limit of detection of the CBA cytokine kit used, which, accordingly to the manufactory' information, is in general comparable to conventional ELISA but depends on the cytokine analyzed, ranging from 2.4 pg/ml (for IL-6) to 18.9 pg/ml (for IL-17A).

Measurement of cytokine levels in the PB or "in situ" have provided evidence for an unbalance between "Th1/Th2" or "Th17/Treg" cytokines in the development of SSc [102]. Indeed, the Th2 cytokine response, with the production of IL-4, IL-10 and TGF- β , leads to tissue fibrosis, whereas Th1 and Th17 cytokines promote inflammation in SSc patients [102]. It should however be emphasized that there have been conflicting results, probably due to the heterogeneity of the disease and differences in study methods. For instance, some of the studies reporting increased IL-17 serum levels in patients with SSc used ELISA techniques to detect total IL-17, and not specifically the IL-17A, and the minimum significant detection theoretical level of the assay was considerably lower than that of the CBA assay we have used [69,7]. Thus, as previously stated in other studies performed in different clinical settings, we conclude that measuring serum/plasma concentration of cytokines using CBA or ELISA techniques depends on the cytokine and the method used and may be not sensitive enough to detect subtle differences between patients and healthy individuals [103]. It should also be emphasized that cytokines regulate cellular immune interactions by operating mainly at microenvironmental level, being produced by lymphocytes, monocytes, macrophages, and

also by locally resident cells, such as fibroblasts and endothelial cells [104]. Thus, assessing cytokines by measuring their level in serum and plasma, although often done by practical reasons, may not reflect the local cytokine microenvironment in normal and pathological tissues [105].

Conclusions

In conclusion, our study confirms that T cells may have a relevant role in the pathogenesis of SSc and suggests that CD8⁺ T cells are an important part in this process. In accordance, CD8⁺ and activated/memory CD8⁺ T cell counts are decreased in the PB from patients with SSc, being related to SSc clinical subtypes, evolution stages, organ involvement, as well as to the microvascular abnormalities as evaluated by capillaroscopy. CD56⁺ T cells and NK cells also appear to be involved: NK cell depletion occur early in disease evolution, whereas depletion of circulating CD56⁺ T cells seems to be a promising biomarker of the severity of lung disease and active and late capillaroscopic changes, even though more studies are required. Decreased numbers of CD8⁺, activated/memory CD8⁺ and CD56⁺ T cells and NK cells in the PB may result at least partially from the recruitment of these cells to the affected tissues, although other mechanisms may also operate. Finally, assessing cytokine production by measuring their levels in the serum may not reflect the abnormal cytokine microenvironment that has been reported in patients with SSc. SSc remains amongst the most enigmatic of human autoimmune diseases with multiple difficulties in understanding of the genetics, the epigenetics, the primary versus secondary immune response, the treatment and indeed even the classification [106-114].

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Appendix A – Supplementary material: Immunophenotypic features of peripheral blood T cells from patients, Pre-SSc and SSc, according to the Medsger scale.

	Skin		p Values ^{a)}	Vascular		p Values ^a	Lung		p Values ^a	Gastrointestinal		p Values ^a	Heart		p Values ^a
	(n=57)			(n=57)			(n=56) ^{b)}			(n=52) ^{c)}			(n=47) ^{d)}		
	Score 0 (n=10)	Score ≥1 (n=47)		Score 0 (n=5)	Score ≥1 (n=52)		Score 0 (n=18)	Score ≥1 (n=38)		Score 0 (n=28)	Score ≥1 (n=24)		Score 0 (n=32)	Score ≥1 (n=15)	
Lymphocytes	1858	1477	0.401	1990	1570	0.522	1613	1587	0.063	1443	1739	0.013	1542	1533	0.024
	(1164-4893)	(764-4772)	0.006	(1164-2076)	(764-4893)	0.008	(1096-4893)	(764-4772)	0.011	(764-4893)	(1091-2860)	0.138	(764-4893)	(860-2536)	0.050
			0.173			0.310			0.752			0.131			0.737
T cells															
CD3+	1378	1120	0.740	1442	1145	0.850	1179	1133	0.179	987	1258	0.027	1175	1014	0.055
	(933-3289)	(419-4082)	0.010	(1178-1557)	(419-4082)	0.015	(750-3289)	(419-4082)	0.011	(419-4082)	(762-2468)	0.143	(419-4082)	(612-1839)	0.088
			0.084			0.152			0.423			0.210			0.804
CD45RO+	1088	712	0.339	1107	721	0.206	848	704	0.420	714	857	0.074	720	690	0.185
	(761- 2442)	(240-2485)	0.039	(911-1284)	(240-2485)	0.064	(462-2442)	(240-2485)	0.079	(240-2485)	(403-1762)	0.621	(240-2485)	(339-1374)	0.148
			0.009			0.042			0.161			0.145			0.738
HLADR+	304	187	0.425	333	191	0.128	236	183	0.672	236	196	0.336	197	234	0.174
	(145-810)	(47-1214)	0.087	(277-373)	(47-1214)	0.112	(62-1214)	(47-574)	0.095	(50-810)	(53-1214)	0.337	(50-1214)	(47-397)	0.420
			0.115			0.111			0.216			0.777			0.838
CD56+ ^{e)}	57	34	0.867	58	36	0.850	45	32	0.250	38	36	0.060	37	33	0.039
	(4-257)	(2-478)	0.003	(4-257)	(2-478)	0.005	(4-478)	(2-257)	0.004	(7-215)	(2-478)	0.026	(2-478)	(2-142)	0.003
			0.173			0.620			0.269			0.440			0.215

	Skin		p	Vascular		p	Lung		p	Gastrointestinal		p	Heart		p
	(n=57)			(n=57)			(n=56) ^{b)}			(n=52) ^{c)}			(n=47) ^{d)}		
	Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1	
	(n=10)	(n=47)		(n=5)	(n=52)		(n=18)	(n=38)		(n=28)	(n=24)		(n=32)	(n=15)	
T cell subsets															
CD3+CD4+	889	771	0.598	869	772	0.376	773	802	0.324	716	889	0.039	855	771	0.179
	(646-1903)	(337-3903)	0.058	(773-967)	(337-3903)	0.082	(440-1903)	(337-3903)	0.043	(337-3903)	(486-1449)	0.421	(337-3903)	(427-1434)	0.214
			0.224			0.546			0.362			0.203			0.756
CD45RO+	774	596	0.219	790	663	0.343	706	627	0.949	596	754	0.374	677	559	0.928
	(620-1782)	(237-3407)	0.519	(708-918)	(237-3407)	0.679	(416-1782)	(237-3407)	0.603	(237-3407)	(425-1236)	0.491	(237-3407)	(313-1254)	0.547
			0.092			0.356			0.325			0.203			0.568
HLA-DR+	147	113	0.424	156	113	0.343	129	113	0.582	107	130	0.355	116	113	0.624
	(60-278)	(31-472)	0.539	(62-211)	(31-472)	0.615	(39-472)	(31-211)	0.401	(37-278)	(31-472)	0.497	(31-472)	(43-186)	0.691
			0.133			0.195			0.183			0.146			0.690
CD3+CD8+	405	316	0.830	535	319	0.282	348	303	0.155	322	331	0.011	324	331	0.015
	(255-1386)	(18-1368)	0.001	(405-664)	(18-1386)	0.002	(167-1386)	(18-664)	0.003	(82-1386)	(185-1368)	0.076	(82-1386)	(18-589)	0.020
			0.019			0.012			0.382			0.405			0.947
CD45RO+	287	171	0.219	388	173	0.058	195	169	0.196	175	186	0.027	171	181	0.022
	(174-825)	(9-820)	0.004	(254-433)	(9-825)	0.008	(83-825)	(9-433)	0.020	(34-825)	(45-820)	0.240	(45-825)	(9-433)	0.104
			0.002			0.006			0.291			0.327			0.659
HLA-DR+	197	96	0.082	208	96	0.016	116	96	0.949	116	98	0.833	98	112	0.453
	(74-551)	(5-818)	0.249	(177-317)	(5-818)	0.322	(21-818)	(5-416)	0.369	(10-551)	(14-818)	0.778	(14-818)	(5-295)	0.729

	Skin		p Values ^{a)}	Vascular		p Values ^a	Lung		p Values ^a	Gastrointestinal		p Values ^a	Heart		p Values ^a
	(n=57)			(n=57)			(n=56) ^{b)}			(n=52) ^{c)}			(n=47) ^{d)}		
	Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1		Score 0	Score ≥1	
	(n=10)	(n=47)		(n=5)	(n=52)		(n=18)	(n=38)		(n=28)	(n=24)		(n=32)	(n=15)	
			0.025			0.026			0.353			0.777			0.889
NK cells															
CD3-CD56+	239	151	0.447	288	151	0.164	151	162	0.093	153	161	0.131	152	148	0.136
	(85-595)	(48-586)	0.068	(173-495)	(48-595)	0.088	(48-595)	(67-586)	0.391	(48-595)	(80-586)	0.479	(48-595)	(67-474)	0.134
			0.076			0.039			0.372			0.447			0.643
NK cell subsets															
CD56+low	228	136	0.495	267	137	0.206	136	149	0.104	141	150	0.140	136	138	0.201
	(75-568)	(41-582)	0.085	(145-481)	(41-582)	0.107	(41-568)	(58-582)	0.440	(41-568)	(68-562)	0.554	(41-568)	(58-461)	0.163
			0.088			0.049			0.382			0.447			0.738
CD56+high	17	9	0.133	20	11	0.114	11	10	0.617	9	10	0.336	10	9	0.154
	(5-28)	(1-74)	0.083	(11-28)	(1-74)	0.148	(3-28)	(1-74)	0.169	(4-74)	(1-35)	0.273	(3-35)	(1-27)	0.244
			0.035			0.049			0.785			0.735			0.855

Results are presented as median (minimum – maximum) numbers of the indicated cell populations /mm³. Abbreviations: SSc, Systemic Sclerosis.

Organ specific Medsger disease severity scale: from 0 (no documented involvement) to 4 (end stage disease). a) Mann-Whitney U test. P values for Patients score 0 vs. Controls, Patients score ≥ 1 vs. Controls and Patients score ≥ 1 vs. Patients score = 0, were indicated successively. P<0.05 was considered to be statistically significant. The coloring is used to highlight the cases with p<0.05 (green cells), and those having a “trend” toward statistical significance (0.05>p<0.10) (yellow cells), which needs to be confirmed in other studies. b) Lung score was not available in 1 case; c) Gastrointestinal score was not available in 5 cases; d) Heart score was not available in 10 cases; e) CD56+ T cells: lung score ≤1 vs. lung score ≥ 2: p=0.013

4.2 MICROANGIOPATIA, LESÃO ENDOTELIAL E ALTERAÇÕES DA ANGIOGÊNESE

Nesta secção são avaliados os níveis séricos de várias moléculas de adesão (ICAM-1, VACM-1, E-seletina, P-seletina), ET-1, angiostatina, endostatina e VEGF por ELISA e calculadas as razões VEGF/angiostatina e VEGF/endostatina.

Os resultados são relacionados com os subtipos de doença esclerodérmica, fases da ES, padrões capilaroscópicos e envolvimento orgânico e sua gravidade.

4.2.1 – IMPAIRED ANGIOGENESIS IN SYSTEMIC SCLEROSIS: WHAT’S MORE RELEVANT: THE RATIO BETWEEN ANGIOGENIC AND ANGIOSTATIC FACTORS OR THESE FACTORS INDIVIDUALLY? A COHORT STUDY

Artigo para submeter a publicação

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Abstract

Objective: To determine the concentrations of circulating vascular endothelial growth factor (VEGF) and the VEGF/endostatin and VEGF/angiostatin ratios in patients with Systemic Sclerosis spectrum disease (Pre-SSc and SSc) and to assess their relationships with disease subsets, evolution phase, organ involvement and nailfold capillaroscopic changes.

Methods: VEGF, endostatin and angiostatin serum levels were measured by ELISA in 48 patients with SSc spectrum disease and 25 healthy controls. VEGF serum levels, as well as VEGF/endostatin and VEGF/angiostatin ratios, were related with disease subsets, clinical evolution phases, organ involvement and nailfold capillaroscopic changes.

Results: Compared to controls, VEGF serum levels were significantly higher in Pre-SSc patients, while the VEGF/endostatin ratio was significantly lower in all disease subtypes, in all SSc developmental stages and in all capillaroscopic patterns. Lower values of VEGF/angiostatin ratio were found among patients with skin involvement and lower levels of VEGF in patients with low forced vital capacity.

Discussion We recognize the importance of VEGF as an early marker of the disease and the VEGF/endostatin ratio as an early marker of both the disease and its evolution. VEGF/angiostatin ratio was related to the skin involvement, and VEGF appears to be related to pulmonary damage.

Keywords: Systemic Sclerosis, Scleroderma, VEGF, Endostatin, Angiostatin

Introduction

Systemic sclerosis or scleroderma (SSc) is a disease of the connective tissue characterized by widespread microangiopathy, immunological changes and fibrosis of the skin and internal organs [1].

The microvascular involvement results in vasospastic phenomena that promotes the proliferation of the intima and culminates in vascular occlusion [2]. These changes lead to chronic tissue hypoxia [3], which, in turn, is the most important stimulus of angiogenesis [4].

Raynaud's phenomenon and capillaroscopy changes, such as the reduction of the density of skin capillaries and the appearance of giant capillaries, are found early in the disease and these changes suggest poor vasodilatory capacity and a dysfunction of angiogenesis and of vascular repair mechanisms [5].

In fact, effective angiogenesis does not occur in patients with SSc [6,7], and an inadequate vascularization contrasts with the over-expression of pro-angiogenic factors such as the vascular endothelial growth factor (VEGF, also known as VEGF-A) [3, 8, 9].

The VEGF is one of the most potent angiogenic factors known so far [1] and it is involved in various stages of physiological and pathological angiogenesis [1], acting selectively on vascular endothelial cells with dose dependent biological effects [1]. It sends angiogenic signals to the vascular endothelium through the receptors of high affinity tyrosine kinase, known as VEGFR-1 and VEGFR-2, whose expression is increased in endothelial cells of micro-vessels of patients with SSc [3, 10] and, to a lesser extent, also through VEGFR-3.

The encoding gene of VEGF is composed of eight exons separated by 7 introns, six of which result in distinct isoforms [11, 12], the main one being VEGF165. There is evidence, however, that two families of VEGF proteins are formed from an alternative splicing of an untranslated region of exon 8. One of these isoforms is also a pro-angiogenic factor while the other one has anti-angiogenic properties [12]. The most studied anti-angiogenic isoform is VEGF165b, which is a competitive inhibitor of VEGF165, binding to VEGFR-2 but not activating it.

High concentrations of VEGF165b [11], an imbalance between the concentrations found in VEGF165 and its receptors [10], the time of tissue VEGF165 exposure (too short or too long) [3, 5, 7] and an inadequate response of endothelial cells to VEGF [5], appear to be possible explanations for the existence of an ineffective angiogenesis.

Increased concentrations of angiogenesis inhibitors have been described, namely endostatin [13, 14, 15], and angiostatin [14], as well as a lower VEGF/endostatin ratio in patients with SSc compared to healthy individuals [15]. This imbalance between angiogenic and anti-angiogenic factors may explain the loss of capillaries in SSc even in the presence of high VEGF levels.

The purpose of this study was to relate serum levels of VEGF and the VEGF/endostatin and VEGF/angiostatin ratios with the subtype and stage of SSc, organ involvement and its severity (according to the Medsger Scale), and the capillaroscopy pattern in comparison with healthy subjects.

Patients and Methods

Study approval

The study was approved by the Ethics Committee and the Board of Directors of the Hospital and all patients signed an informed consent form.

Patients

Sixty-one patients were selected consecutively from a 190 patient-population with SSc spectrum disease attending the Clinical Immunology Unit of Centro Hospitalar do Porto, Portugal, between September 2010 and March 2011. Four patients were later excluded, three because of overlapping pathologies (mixed connective tissue disease, infection with human immunodeficiency virus, B non-Hodgkin lymphoma) and a fourth one who missed the blood sampling. From the remaining 57 patients, 48 were included in this study (for 9 patients no sample was available for VEGF measurement).

Thirty nine patients met the American College of Rheumatology (ACR) criteria for SSc [16] while the remaining nine did not present skin involvement and were classified as Pre-SSc, as explained below.

The 2013 criteria of ACR/EULAR (The European League Against Rheumatism) [17] were met by those 39 patients that had met the ACR criteria, and by 1 of the 9 without skin involvement due to the presence of digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement.

In the study population, 46 were women and 2 were men, with a median age of 56 years, ranging from 19 to 80 years. Patients' sociodemographic and clinical characteristics are summarized in Tables 1 and 2. Twenty-five healthy individuals were used as controls.

Table 1 – SSc patient characteristics (n=48)

Age (years)	<i>Median (minimum; maximum)</i>	56 (19; 80)
Sex	<i>Males: Females</i>	2:46
Disease subset	<i>n (%)</i>	
Pre-SSc		9 (18.8)
dcSSc		12 (25.0)
lcSSc		27 (56.2)
CREST		12 (25.0)
Non-CREST		15 (31.2)
Disease stage	<i>n (%)</i>	
Early		6 (12.5)
Intermediate		10 (20.8)
Late		23 (47.9)
Capillaroscopic pattern	<i>n (%)</i>	
Normal/minor alterations		4 (8.3)
Early		11 (22.9)
Active		19 (39.6)
Late		14 (29.2)

Abbreviations: SSc, Systemic Sclerosis or Scleroderma; lcSSc, limited cutaneous Scleroderma; dcSSc, diffuse cutaneous Scleroderma.

Table 2 – Organ involvement according to the Medsger Severity Scale

Organ	Score*	Pre-SSc [n = 9]	lcSSc [n = 27]	dcSSc [n = 12]
Skin	0	9 (100)	-	-
	1	-	27 (100)	3 (25.0)
	2	-	-	8 (66.7)
	3	-	-	1 (8.3)
Peripheral vascular	0	4 (44.4)	-	-
	1	4 (4.4)	15 (55.6)	1 (8.3)
	2	-	2 (7.4)	4 (33.3)
	3	1 (11.1)	10 (37.0)	7 (58.3)
	4	-	-	-
Gastrointestinal tract	Non-classified	2 (22.2)	1 (3.7)	1 (8.3)
	0	5 (55.6)	16 (59.3)	5 (22.7)
	1	2 (22.2)	10 (37.0)	6 (50.0)
Lung	Non-classified	1 (11.1)	-	-
	0	5 (55.6)	10 (37.0)	2 (16.7)
	1	3 (33.3)	8 (29.6)	6 (50.0)
	2	-	8 (29.6)	1 (8.3)
	3	-	1 (3.7)	1 (8.3)
	4	-	-	2 (16.7)
Heart	Non-classified	3 (33.3)	3 (11.1)	1 (8.3)
	0	5 (55.6)	15 (55.6)	7 (58.3)
	1	1 (11.1)	1 (3.7)	2 (16.7)
	2	-	4 (14.8)	2 (16.7)
	3	-	4 (14.8)	-
Osteoarticular	Non-classified	-	-	-
	0	7 (77.8)	19 (70.4)	11 (91.7)
	1	2 (22.2)	8 (29.6)	1 (8.3)

Abbreviations: SSc, Systemic Sclerosis or Scleroderma; lcSSc, limited cutaneous Scleroderma; dcSSc, diffuse cutaneous Scleroderma.

Results presented as n (%).

* From "0" (no documented involvement or without need of treatment) to "4" (end-stage disease).

At the time of the study, none of the patients were receiving corticosteroids or immunosuppressive drugs and they underwent a one-to-two week period of washout from vasodilator drugs, in no cold weather, and according to the clinical status of the patients.

Clinical assessment

Patient disease subsets were classified as Pre-SSc, according to LeRoy and Medsger [18], and limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc) scleroderma, as previously described by LeRoy *et al.* [19]. Over time, several authors and, more recently, the EULAR Scleroderma Trial and Research group (EUSTAR), have recognized that there is an early stage of this disease in which there is no skin involvement but that may evolve to limited or diffuse SSc, and named it differently: “limited scleroderma” [18], “pre-scleroderma” [20], “early SSc” [21] and “very early SSc” [22], even though they represent nearly the same concept [23]. We used the definition of Pre-SSc according to LeRoy and Medsger's criteria, where patients must have Raynaud's phenomenon plus scleroderma-type nailfold capillary changes and/or scleroderma-type autoantibodies [18].

Limited and diffuse SSc were then subdivided into early, intermediate and late SSc, according to the time since the first symptom related to the disease and to the subset of disease [24]. Early stage lcSSc has less than five years of evolution and late phase has at least ten years. On the contrary, early stage dSSc has less than three years of evolution and late phase a duration exceeding six years. Intermediate values correspond to intermediate phases of lcSSc and dcSSc.

Additionally, lcSSc was further separated in CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) and non-CREST groups, according to Lonzetti *et al.* [25].

There is a lack of a standardized method to assess disease severity. We used Medsger's severity scale [26], which classifies involvement of each of nine organ system (general, peripheral vascular, skin, joints/tendons, muscles, gastrointestinal tract, lungs, heart and kidneys) from 0 (no documented involvement or without need of treatment, e.g. Raynaud) to 4 (end-stage disease), without an overall score. It has limitations due to its lack of sensitivity to score changes in the presence of SSc severity improvement [27]. Even though, it is a quite useful tool as a prognostic measure [28].

A complete clinical profile was established for each patient at the time of study enrolment and the degree of organ involvement was determined by medical history, physical examination, and complementary tests.

Organ involvement was evaluated using Medsger's scale cut-off point, except for osteoarticular evaluation, since it does not value joint involvement but only the distance between thumb and thenar eminence, which was not evaluated systematically in our patients.

The Modified Rodnan Skin Score was the tool chosen to evaluate skin involvement [29].

Joint evaluation was made through physical exam, and complemented by imaging exams (radiography and/or joint echography) whenever justified. DAS 28 (*Disease Activity Score Calculator for Rheumatoid Arthritis*) was used to classify the activity of this involvement [30].

Muscular involvement was evaluated by physical examination and complemented by biochemical study (creatine phosphokinase, aldolase and glutamate oxaloacetate transaminase).

Gastrointestinal tract involvement was documented by upper gastrointestinal endoscopy, esophageal manometry and, in symptomatic patients, by gastric scintigraphy. Involvement of the lower gastrointestinal tract, whenever justified, was documented using barium tests, glucose hydrogen breath test, ultrasound, colonoscopy and anorectal manometry.

Chest teleradiography, high resolution computed tomography, spirometry, diffusing capacity of the lungs for carbon monoxide (DLCO) corrected for hemoglobin and alveolar volume, and two-dimensional echocardiogram with evaluation of pulmonary artery systolic pressure (PSAP) were used to evaluate pulmonary involvement. PSAP was considered abnormal when higher than 35 mmHg. Right heart catheterization was performed in patients with PSAP above or equal to 40 mmHg in order to confirm pulmonary hypertension. Forced vital capacity (FVC) and DLCO were considered abnormal when lower than 80%. Patients with lung fibrosis and/or alveolitis affecting at least the lung bases were considered to have major pulmonary alterations; criteria for minor alterations included septal hypertrophy and limited zones of fibrosis and/or ground glass opacities.

Conduction disturbances and/or arrhythmias were detected by electrocardiogram and/or Holter, and systolic dysfunction was evaluated with two-dimensional echocardiogram, namely through calculation of the left ventricular ejection fraction.

Finally, a score introduced by Cutolo *et al.* [31] was used to classify nailfold microvascular changes as observed by capillary microscopy (early, active and late capillaroscopic patterns of microvascular damage).

Laboratory analysis

Serum concentrations of VEGF, endostatin and angiostatin were measured using ELISA assay (VEGF-A using *ELISA* assay BE55101 from IBL International; endostatin and angiostatin using ELISA assays from USCN Life Sciences Inc. Wuhan; China) according to the instructions provided by the manufacturer. The VEGF/endostatin and VEGF/angiostatin ratios were calculated. The VEGF assay that has been used is specific of VEGF-A, and the

employed antibody was raised against VEGF-A₁₆₅, but the manufacturer did not provide information about the specificity to the isoforms of VEGF-A.

Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS 22.0) software (SPSS Inc, IL, USA). Descriptive statistics included the absolute and relative frequencies, as well as median, minimum and maximum values. The Kolmogorov-Smirnov test was used to assess the normality of distributions.

As several of the studied variables did not present a Normal distribution, and given the small size of the sample, non-parametric tests were used. Mann-Whitney U test was used to compare mean ranks of two independent samples. Although corresponding to ordinal variables, the small number of disease phases led to another analysis using Kruskal-Wallis tests instead of the use of correlations to compare the 3 disease phases. The Spearman rank correlation test was used to evaluate the association between two variables.

As to the relationship of each organ's Medsger severity scale with VEGF serum levels, and VEGF/endostatin and VEGF/angiostatin ratios, although this scale had a sufficient number of points in order to be used to compute correlation coefficients, patients exhibited a very diverse number of different scores (from 2 to 4); therefore, for each organ we compared patients at the lowest score (0 or 1) with the remaining (score ≥ 2). The null hypothesis was rejected when $p < 0.05$.

Results

VEGF serum levels were significantly higher ($p=0.016$) and the VEGF/endostatin ratio was significantly lower ($p<0.001$) in patients with SSc spectrum disease, compared to controls; the differences in VEGF/angiostatin ratios did not reach statistical significance (Table 3).

Subgroup analysis showed that the VEGF serum levels were significantly higher ($p=0.009$) and the VEGF/angiostatin ratios were marginally higher ($p=0.057$), whereas the VEGF/endostatin ratios were lower ($p=0.018$) in patients with Pre-SSc when compared to controls; in addition, the VEGF/endostatin ratio was also significantly lower in lcSSc and dcSSc subtypes ($p<0.001$ for both). (Table 3)

Table 3 – VEGF serum levels and VEGF/Angiostatin and VEGF/Endostatin ratios in the study population and in healthy controls, and according to Systemic Sclerosis subsets.

	VEGF pg/ml	<i>p</i>*	VEGF/angiostatin ratio	<i>p</i>*	VEGF/endostatin ratio	<i>p</i>*
Healthy controls [n=25]	473 (148; 1135)		10.8 (5.1; 86.2)		270.1 (22.3; 14775.0)	
Study population [n=48]	695 (143; 4479)	0.016	13.5 (3.2; 92.3)	0.417	27.7 (3.8; 513.8)	<0.001
Pre-SSc [n=9]	918 (277; 4479)	0.009	27.4 (3.9; 92.3)	0.057	68.1 (8.3; 459.3)	0.018
SSc [n=39]	642 (143; 3902)	0.056	12.4 (3.2; 40.9)	0.765	25.9 (3.8; 513.8)	<0.001
lcSSc [n=27]	628 (143; 3902)	0.081	12.2 (3.4; 40.9)	0.793	25.9 (7.9; 513.8)	<0.001
dcSSc [n=12]	684 (226; 1233)	0.158	13.8 (3.2; 38.7)	0.808	26.8 (3.8; 124.0)	<0.001
lcSSc /Non-CREST [n=15]	565 (143; 3902)	0.213	10.8 (3.4; 40.9)	0.754	25.6 (7.9; 112.8)	<0.001
lcSSc /CREST [n=12]	635 (371; 1283)	0.102	12.7 (4.3; 29.5)	0.917	26.3 (10.6; 513.2)	<0.001

Abbreviations: VEGF, Vascular Endothelial Growth Factor; SSc, Systemic Sclerosis or Scleroderma; lcSSc, limited cutaneous Scleroderma; dcSSc, diffuse cutaneous Scleroderma; CREST, calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia. P value: Mann Whitney U test. * Comparison of each group of patients with the healthy controls. Results presented as median (minimum; maximum). VEGF serum levels, and VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (9 Pre-SSc, 39 SSc; 27 lcSSc, 12 dcSSc; 15 non-CREST, 12 CREST), 48 (9 Pre-SSc, 39 SSc; 27 lcSSc, 12 dcSSc; 15 non-CREST, 12 CREST), and 47 (9 Pre-SSc, 38 SSc; 26 lcSSc, 12 dcSSc; 15 non-CREST, 11 CREST) patients with SSc spectrum disease, respectively, and in 25, 23 and 23 controls, respectively.

When we compared all subgroups we verified that VEGF and VEGF/angiostatin ratios were higher in Pre-SSc patients than in those with SSc (VEGF: $p=0.068$; VEGF/angiostatin: $p=0.036$). No differences were found between lcSSc and dcSSc (VEGF: $p=0.951$; VEGF/angiostatin: $p=1.000$) or between CREST and non-CREST patients (VEGF: $p=0.903$; VEGF/angiostatin: $p=0.897$).

The analysis according to disease evolution phase showed that the values of VEGF and of the VEGF/angiostatin ratio did not differ significantly from those observed in controls ($p>0.05$ in all cases); as to the VEGF/endostatin ratio, it was significantly lower in all stages of the disease relative to controls ($p=0.004$, $p<0.001$ and $p<0.001$, for early, intermediate and late SSc, respectively; (Table 4).

Table 4 – VEGF serum levels, and VEGF/angiostatin and VEGF/endostatin ratios according to the Scleroderma evolution phase

	VEGF (pg/ml)	p^*	VEGF/ angiostatin ratio	p^*	VEGF/ endostatin ratio	p^*
Healthy controls [n=25]	473 (148 - 1135)		10.8 (5.1 - 86.5)		270.1 (22.3 – 14775.0)	
Early SSc [n=6]	816 (340; 1592)	0.147	15.6 (4.3; 40.9)	0.590	33.9 (24.0; 105.0)	0.004
Intermediate SSc [n=10]	597 (233; 971)	0.358	12.3 (3.4; 35.1)	0.938	20.1 (10.6; 34.5)	<0.001
Late SSc [n=23]	677 (143; 3902)	0.081	12.2 (3.2 ; 38.7)	0.784	27.9 (3.8; 513.8)	<0.001

Abbreviations: VEGF, Vascular Endothelial Growth Factor; SSc, Systemic Sclerosis or Scleroderma; NA, not applicable.

Results presented as median (minimum; maximum). VEGF serum levels, and VEGF/angiostatin and VEGF/endostatin ratios were available in 39 (6 early, 10 intermediate, 23 late), 39 (6 early, 10 intermediate, 23 late), and 38 SSc patients (6 early, 10 intermediate, 22 late), respectively, and in 25, 23 and 23 controls, respectively. P value: Mann Whitney U test.

* Comparison of each group of patients with the healthy controls.

When we compared the values of VEGF, VEGF/endostatin, and VEGF/angiostatin between the three SSc phases (early vs. intermediate vs. late; Kruskal-Wallis test), we verified no significant differences between them (VEGF: $p=0.707$; VEGF/angiostatin: $p=0.745$; VEGF/endostatin: $p=0.111$).

The analysis according to the capillaroscopic pattern showed that VEGF serum levels were significantly higher compared to controls only in patients with the active pattern ($p=0.011$). In contrast, the VEGF/endostatin ratio had significantly lower values in all capillaroscopic

patterns, even when changes were minor ($p=0.011$, $p=0.001$, $p<0.001$ and $p<0.001$, for patients with normal/ minor capillaroscopic changes, and with early, active and late patterns, respectively) (Table 5).

Table 5 – Relation between capillaroscopic patterns and VEGF, VEGF/angiostatin and VEGF/endostatin serum levels according to the capillaroscopic pattern.

Capillaroscopic pattern	VEGF (pg/ml)	p^*	VEGF/ angiostatin ratio	p^*	VEGF/ endostatin ratio	p^*
Healthy controls [n=25]	473 (148; 1135)		10.8 (5.1; 86.5)		270.1 (22.3; 14775.0)	
Normal/minor capillaroscopic alterations [n=4]	659 (484; 699)	0.360	12.9 (8.9; 29.5)	0.733	22.9 (17.1; 47.1)	0.011
Early capillaroscopic pattern [n=11]	832 (143; 4479)	0.126	17.6 (3.9; 92.3)	0.277	27.5 (7.9; 459.3)	0.001
Active capillaroscopic pattern [n=19]	724 (233; 1571)	0.011	13.6 (3.4; 33.0)	0.250	33.2 (3.8; 513.8)	<0.001
Late capillaroscopic pattern [n=14]	644 (226; 3902)	0.245	11.1 (3.2; 40.9)	0.661	25.3 (8.5; 112.7)	<0.001

Abbreviations: VEGF, Vascular Endothelial Growth Factor. Results presented as median (minimum; maximum).

VEGF serum levels, and VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (4 no/minor capillaroscopic alterations, 11 early; 19 active, 14 late capillaroscopic patterns), 48 (4 no/minor capillaroscopic alterations, 11 early; 19 active, 14 late capillaroscopic patterns) and 47 (3 no/minor capillaroscopic alterations, 11 early; 19 active, 14 late capillaroscopic patterns) patients with SSc spectrum disease, respectively, and in 25, 23 and 23 controls, respectively.

P value: Mann Whitney U test. * Comparison of each group of patients with the healthy controls.

When we compared the values of VEGF, VEGF/endostatin, and VEGF/angiostatin between the three capillaroscopic patterns (early vs. active vs. late; Kruskal-Wallis test) we verified no significant differences between them (VEGF: $p=0.909$; VEGF/angiostatin: $p=0.598$; VEGF/endostatin: $p=0.863$).

The analysis made to organ involvement and its severity showed that VEGF serum levels and VEGF/endostatin ratios were marginally lower ($p=0.068$ and $p=0.084$, respectively)

in patients with cutaneous involvement, as compared to patients where such involvement did not exist (who indeed correspond to patients with Pre-SSc), and a similar difference reached statistical significance for VEGF/angiostatin ratio ($p=0.036$) (Tables 6 and 7). No relationship was found between VEGF, VEGF/endostatin or VEGF/angiostatin and the severity of any type of organic involvement besides to those mentioned above.

Table 6 – VEGF serum levels in patients with SSc and Pre-SSc, according to organic involvement and Medsger severity scale.

	Organ involvement			Severity of organ involvement		
	Score = 0	Score ≥ 1	p^*	Score = 1	Score ≥ 2	p^{**}
Skin	918 (277; 4479)	642 (143; 3902)	0.068	667 (143; 3902)	541 (226; 1233)	0.505
Peripheral Vascular	854 (277; 1314)	677 (143; 4479)	0.408	699 (143; 4479)	660 (226; 3902)	0.942
Gastrointestinal	738 (143; 4479)	660 (340; 3902)	0.650	-	-	-
Pulmonary	553 (233; 4479)	728 (143; 3902)	0.544	777 (143; 1592)	699 (226; 3902)	0.630
Cardiac	677 (143; 4479)	712 (340-3902)	0.762	1092 (340; 1314)	620 (341; 3902)	0.572

Abbreviations: VEGF, Vascular Endothelial Growth Factor; SSc, Systemic Sclerosis or Scleroderma; Pre-SSc, Pre-Scleroderma. Results presented as median (minimum; maximum).

Skin: VEGF serum levels were available in 48 (9 score=0, 30 score=1, 9 score ≥ 2) patients with SSc spectrum disease and in 25 controls. **Peripheral vascular:** VEGF serum levels were available in 48 (5 score=0, 19 score=1, 24 score ≥ 2) patients with SSc spectrum disease and in 25 controls. **Gastrointestinal:** VEGF serum levels were available in 48 (26 score=0, 18 score=1, 0 score ≥ 2) patients with SSc spectrum disease and in 25 controls. **Pulmonary:** VEGF serum levels in 48 (17 score=0, 17 score=1, 13 score ≥ 2) patients with SSc spectrum disease and in 25 controls. **Cardiac:** VEGF serum levels were available in 48 (27 score=0, 4 score=1, 10 score ≥ 2) patients with SSc spectrum disease and in 25 controls. P value: Mann Whitney U test.

*Score 0 vs. score ≥ 1 ** Score 1 vs. score ≥ 2

Table 7 – VEGF/angiostatin and VEGF/endostatin ratios in patients with SSc and Pre-SSc, according to organic involvement and Medsger severity scale.

	VEGF/angiostatin ratio						VEGF/endostatin ratio					
	Organ involvement			Severity of organ involvement			Organ involvement			Severity of organ involvement		
	Score=0	Score≥1	p*	Score=1	Score≥2	p**	Score=0	Score≥1	p*	Score=1	Score≥2	p**
Skin	27.4 (3.9; 92.3)	12.4 (3.2; 40.9)	0.036	13.3 (3.4; 40.9)	11.2 (3.2; 38.7)	0.317	68.1 (8.3; 459.3)	25.9 (3.8; 513.8)	0.084 0.117	27.7 (7.9; 513.8)	15.1 (3.8; 89.3)	0.154
Peripheral Vascular	27.4 (3.9; 40.1)	13.3 (3.2; 92.3)		11.1 (3.4; 92.3)	13.5 (3.2; 40.9)		68.1 (8.3; 459.3)	27.6 (3.8; 513.8)	0.084 0.756	26.9 (7.9; 513.8)	28.1 (3.8; 139.3)	0.939
Gastrointestinal	15.0 (3.2; 92.3)	12.3 (4.3; 38.1)	0.352	;	;	;	33.2 (7.9; 513.8)	28.1 (3.8; 459.3)	0.605	;	;	-
Pulmonary	12.2 (3.4; 40.1)	15.6 (3.2; 40.9)	0.507	21.2 (4.3; 40.9)	11.4 (3.2; 38.7)	0.127	23.1 (8.3; 513.8)	33.3 (3.8; 459.3)	0.176	34.6 (3.8; 459.3)	31.6 (8.5; 112.7)	0.894
Cardiac	14.8 (3.2; 92.3)	12.3 (4.3; 40.9)	0.869	22.0 (4.3; 33.0)	11.3 (6.4; 40.9)		27.5 (3.8; 513.8)	33.3 (8.5; 112.7)	0.554	50.4 (24.0; 68.1)	23.1 (8.5; 112.7)	

Abbreviations: VEGF, Vascular Endothelial Growth Factor; SSc, Systemic Sclerosis or Scleroderma; Pre-SSc, Pre-Scleroderma.

Results presented as median (minimum; maximum).

Skin: VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (9 score=0, 30 score=1, 9 score ≥ 2) and 47 (9 score=0, 29 score=1, 9 score ≥ 2) patients with SSc spectrum disease, respectively, and in 23 controls, in both cases. **Peripheral vascular:** VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (5 score=0, 19 score=1, 24 score ≥ 2) and 47 (5 score=0, 18 score=1, 24 score ≥ 2) patients with SSc spectrum disease, respectively, and in 23 controls, in both cases. **Gastrointestinal:** VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (26 score=0, 18 score=1, 0 score ≥ 2) and 47 (25 score=0, 18 score=1, 0 score ≥ 2) patients with SSc spectrum disease, respectively, and in 23 controls, in both cases. **Pulmonary:** VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (17 score=0, 17 score=1, 13 score ≥ 2) and 47 (17 score=0, 17 score=1, 12 score ≥ 2) patients with SSc spectrum disease, respectively, and in 23 controls, in both cases. **Cardiac:** VEGF/angiostatin and VEGF/endostatin ratios were available in 48 (27 score=0, 4 score=1, 10 score ≥ 2) and 47 (27 score=0, 4 score=1, 9 score ≥ 2) patients with SSc spectrum disease, respectively, and in 23 controls, in both cases. P value: Mann Whitney U test. *Score 0 vs. score ≥ 1 ** Score 1 vs. score ≥ 2

A sub-analysis made to pulmonary involvement, in which the FVC, DLCO and PSAP values were considered, revealed that the VEGF serum levels were significantly lower in patients with low FVC as compared to patients with normal FVC (median VEGF serum levels of 366, ranging from 226 to 531, and of 724, ranging from 143 to 3902, respectively; $p=0.015$); no significant differences were found when comparing patients with normal or abnormal DLCO and PSAP values, neither when comparing patients with and without abnormalities in chest CT. Similarly, no significant results were found for VEGF/endostatin or VEGF/angiostatin values in this sub-analysis (data not shown).

Sub-analysis for vascular involvement displayed significant differences between Raynaud disease with or without digital ulcers ($p>0.050$; data not shown).

Discussion

Angiogenesis is a highly regulated cascade of events that culminates in the formation of new blood vessels and requires an appropriate balance between endogenous stimulators and inhibitors. In SSc, some of these sequential steps are disrupted and contribute to its pathophysiology [3, 7, 9].

This study showed that the circulating levels of VEGF were higher in SSc compared to healthy controls, as it had already been described in previous studies [11, 32-37], and within the range of values reported in cancer patients [38-40], where angiogenesis plays a major role [41]. The values of angiostatic factors, endostatin and angiostatin have been previously described by the authors as being significantly higher in patients with SSc disease compared to controls [14]. In accordance, endostatin was found to be higher in all subtypes and in all stages of SSc, whereas angiostatin was higher in lcSSc who met the CREST criteria and in dcSSc but not the Pre-SSc, and in intermediate and late, but not in early disease stages [14].

As to the VEGF/endostatin ratio, we noticed that, as described by other authors [15], the values were significantly lower in SSc patients as compared with the control group. However, unlike reported by Bartkowiak *et al.* [15], that VEGF values were low, at least partially, due to the immunosuppressive therapy, in our study these values appeared to depend on an increase of endostatin that surpasses the increase of VEGF. In turn, the values of the VEGF/angiostatin ratio were higher, although without reaching statistical significance. This relationship has not been previously studied in the literature, but it seems to suggest that the increase in angiostatin is overlapped by the increase in VEGF.

According to Hummers *et al.* [42], but contrarily to the description by other authors [9, 37, 43], no difference was found in VEGF values between limited and diffuse subtypes, as well as no relationship with the disease evolution. However, and in accordance to Distler *et al.* [9], we found that VEGF values were significantly higher in patients with Pre-SSc compared to the controls, unlike what has been recently described by Cossu *et al.*, who, although having found higher values of VEGF in patients compared to controls, report that this difference was not statistically significant [44].

Regarding the VEGF/endostatin ratio, we observed lower values for all disease subtypes and all clinical phases of disease when compared to the controls; this is in consonance with our prior results for endostatin [14]. On the other hand, Bartkowiak *et al.* found a lower VEGF/endostatin ratio only among patients with dcSSc, whereas patients with lcSSc showed a non significant similar trend [15].

As for the organ involvement and its severity, we found that lower VEGF values were related, albeit marginally, with skin involvement but, unlike other authors' conclusions, it was not possible to establish a relationship with its severity [39, 40]. In this study, we couldn't

establish relationships between VEGF values and other organic involvement or its severity, namely, as in previous studies, we couldn't establish a relationship between VEGF levels and gastrointestinal [43] or cardiac involvement [43, 45].

One of the associations more frequently described by other authors concerns the relationship between higher serum levels of VEGF and pulmonary involvement, namely the elevation of PSAP (≥ 35 mmHg) [35], the decrease in DLCO [35], the decrease in vital capacity [43] or pulmonary fibrosis [43]. In a recent study, De Santis *et al.* (2016) reported the existence of high serum levels of VEGF in SSc patients, directly relating those values with the changes found in chest CT and, conversely, with the values of DLCO, suggesting that VEGF can act as a biomarker of interstitial lung disease [46]. In our study, although we have not been able to demonstrate a relationship between serum levels of VEGF and changes in chest CT, PSAP or DLCO, we found a statistically significant relationship between lower levels of VEGF and low FCV.

As regards the relationship between VEGF/angiostatin and VEGF/endostatin ratios and organ involvement and severity, we found a statistically significant relationship between lower levels of the former and skin involvement. None of these ratios was related to another type of organ involvement or its severity.

When we relate these data with our prior results regarding endostatin and angiostatin [14] we justify this relation essentially with the presence of lower levels of VEGF in patients with this organ involvement. Unlike Bartkowiak *et al.* [15], we did not find any relationship between the VEGF/endostatin ratio and pulmonary involvement. Surprisingly, and taking into account our previously published results of a relationship between higher levels of angiostatin and the severity of pulmonary involvement [14], we found no relationship between the VEGF/angiostatin ratio and the severity of this organic involvement. This fact suggests that the imbalance of these relationships does not represent a major role in the pulmonary involvement or its severity.

Regarding capillaroscopy, the data from this study suggest the existence of an inverse relationship between serum levels of VEGF and the extent of capillary loss as observed by capillaroscopy, which is in accordance with previous studies [37].

As for the VEGF/angiostatin and VEGF/endostatin ratios, we notice that only for the latter the values are significantly decreased in all scleroderma patterns, but also in patients with changes considered as minor. When we analyse these data and relate them with our prior results regarding endostatin and angiostatin [14], we find that the results for the VEGF/endostatin seem to depend fundamentally on an increase in endostatin exceeding VEGF from the existence of minor changes up to the late changes in the scleroderma pattern; as to VEGF/angiostatin, the two molecules are increased, with a dominant increase of VEGF, but this relationship does not seem to be relevant to the changes found in capillaroscopy.

In summary, we demonstrated that scleroderma is associated with a VEGF increase, which was exceeded by the increase in endostatin but not in angiostatin, the effect being a significant decrease of the VEGF/endostatin ratio in patients as compared to controls. VEGF was increased in early stages, still without cutaneous involvement (Pre-SSc), and showed an inverse relationship with skin involvement, raising the possibility of functioning as protector of this organic involvement. On the other hand, the associations between lower VEGF levels and low FVC support the hypothesis of VEGF's role in lung damage.

The VEGF/endostatin ratio may have a role in disease evolution, whereas the VEGF/angiostatin ratio seems to play an important role in the development of skin lesions. However, more studies, with larger numbers of patients, are needed to confirm our results.

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4.2.2 – DIFFERENT CONTRIBUTIONS OF ANGIOSTATIN AND ENDOSTATIN IN ANGIOGENESIS IMPAIRMENT IN SYSTEMIC SCLEROSIS: A COHORT STUDY

Artigo publicado

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Abstract

Objectives: To determine the concentrations of circulating endostatin and angiostatin in patients with Systemic Sclerosis (SSc) and to assess its relationship to disease subsets, evolution phase, organ involvement and nailfold capillaroscopic changes.

Methods: Endostatin and angiostatin serum levels were measured by ELISA in a cohort of 57 patients with SSc, and correlated with disease subsets, evolution phase, organ involvement and nailfold capillaroscopic changes.

Results: Endostatin and angiostatin serum levels were significantly higher in patients with SSc than in healthy controls. Also, angiostatin was elevated in diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc), but not in Pre-SSc, while endostatin was increased in all SSc subsets. Moreover, endostatin was augmented in lcSSc, with or without CREST syndrome, whereas angiostatin was increased exclusively in patients with CREST. Analysis according to disease evolution phase found that endostatin was elevated in all phases while angiostatin was only significantly higher in intermediate and late phases of disease. Analysis regarding organ involvement revealed that angiostatin was significantly higher in patients with osteoarticular involvement and with more serious lung affection; no significant differences were found for endostatin. Finally, endostatin was significantly increased in all nailfold capillaroscopy stages, while angiostatin was only elevated in active and late phases.

Discussion: In accordance to previous studies, we found that endostatin and angiostatin concentrations are elevated in SSc patients. Additionally, we recognised the important role that endostatin might play as an early disease marker and realized that angiostatin is a marker of late disease and relates to lung disease severity.

Keywords: Systemic Sclerosis, Angiostatin, Endostatin

Introduction

Systemic Sclerosis or Scleroderma (SSc) is an autoimmune connective tissue disease characterized by vascular injury and widespread fibrosis involving the skin and various internal organs. Angiogenesis and vasculogenesis impairment plays a major role, although disease pathogenesis remains not fully elucidated [1-3].

Angiogenesis is a complex process dependent on the tight balance between pro-angiogenic and angiostatic factors, which is seen normally in healthy tissues. Pathological angiogenesis occurs when a hypoxic environment or inflammatory state induces a deregulation of this homeostasis and angiogenic growth factors outweigh the inhibitors [1].

Proteolytic fragments of several extracellular proteins have shown to have antiangiogenic activity, contributing to abnormal wound healing and vascular repair in SSc patients. Angiostatin, a cleavage product of plasminogen, is one of these anti-angiogenic factors; endostatin, a heparin sulphate proteoglycan found in almost all epithelial and endothelial basement membranes that results from the cleavage of type XVIII collagen, is another angiostatic factor [1].

Several studies have previously investigated endostatin concentrations in SSc patients, with contradictory results [4-10]. While most found that endostatin levels are elevated in SSc patients [4, 6-10], one failed to achieve this relation [5]. On the contrary, we found only one study in the MEDLINE database (PubMed) relating angiostatin with SSc, and it concluded that angiostatin is increased in SSc patients [11].

The aim of this study was to determine the concentrations of circulating endostatin and angiostatin in patients with SSc and to assess a relationship between these concentrations and disease subsets, evolution phase, type of organ involvement (skin, peripheral vascular, lung, heart, gastrointestinal, kidney, muscle, and osteoarticular) and nailfold capillaroscopic changes.

Patients and Methods

Patients

Sixty-one consecutive patients were selected from a 190-patients-population with SSc, at the Clinical Immunology Unit of Centro Hospitalar do Porto – Portugal, between September 2010 and March 2011. Four patients were later excluded, three because of overlapping pathologies (mixed connective tissue disease, infection with human immunodeficiency virus, B non-Hodgkin lymphoma) and a fourth one who missed the blood sampling. The study was approved by the Ethics Committee and the Board of Directors of the Hospital and all patients signed an informed consent form.

Forty-seven patients fulfilled the American College of Rheumatology (ACR) criteria for SSc [12] while the remaining ten did not present skin involvement and were classified as Pre-Scleroderma (Pre-SSc), as explained below.

The ACR/ EULAR (American College of Rheumatology / European League Against Rheumatism) criteria of 2013 [13] were met by those 47 patients that had met the ACR criteria, and by 1 of the 10 without skin involvement due to having digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement.

In the study population, 55 were women and 2 were men, with a median age of 54 years, ranging from 18 to 82 years. Patients' characteristics are summarized in Tables 1 and 2. Twenty-five healthy individuals were used as controls.

Table 1 – Clinical characteristics of Systemic Sclerosis patients (n = 57)

Age (years)	54 (18-82)
Gender	
Females	55 (96.5%)
Males	2 (3.5%)
Disease subset	
Pre-SSc	10 (17.5%)
dcSSc	13 (22.8%)
lcSSc	34 (59.6%)
CREST	17 (29.8%)
Non-CREST	17 (29.8%)
Disease phase	
Early	7 (14.9%)
Intermediate	10 (21.3%)
Late	30 (63.8%)
Capillaroscopic pattern	
Normal/ minor alterations	4 (7.3%)
Early	12 (21.8%)
Active	21 (38.2%)
Late	18 (32.7%)

Age is presented as median (range). Other results are presented as n (%).

Abbreviations: SSc, Scleroderma; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; CREST, calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.

Clinical assessment

Patient disease subset was classified as Pre-Scleroderma (Pre-SSc) according to LeRoy and Medsger [14], and limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc) scleroderma, as previously described by LeRoy *et al.* [15]. Over time, several authors and, more recently, the EULAR Scleroderma Trial and Research group (EUSTAR) have recognized that there is an early stage of disease in which there is no skin involvement but that may evolve to limited or diffuse SSc, and named it differently: “limited scleroderma” [14], “pre-scleroderma” [16], “early SSc” [17], and “very early SSc” [18]; even though, they represent nearly the same concept [19]. We used the definition of Pre-SSc according to LeRoy and Medsger criteria,

where patients must have Raynaud's phenomenon plus scleroderma-type nailfold capillary changes and/or scleroderma-type autoantibodies [14].

Limited and diffuse SSc were then subdivided into early, intermediate and late SSc, according to the time since first symptom related to the disease and in consonance with subset of disease [20]. Early phase of lcSSc has less than five years of evolution and late phase has at least ten years. On the contrary, early phase of dSSc has less than three years of evolution and late phase a duration superior to six years. Intermediate values correspond to intermediate phases of limited and diffuse SSc.

Additionally, limited SSc was further separated in CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) and non-CREST groups according to Lonzetti *et al.* [21].

There is a lack of a standardized method to assess disease severity. We decided to use Medsger's severity scale [22], which classifies involvement of each of nine organ system (general, peripheral vascular, skin, joints/tendons, muscles, gastrointestinal (GI) tract, lungs, heart and kidneys) from 0 (no documented involvement or without need of treatment, e.g. Raynaud) to 4 (end stage disease), without an overall score. It has limitations as a measure of activity due to its lack of sensitivity to change score in the presence of SSc severity improvement [23]. Even though, it is a quite useful tool as a prognostic measure [24].

A complete clinical profile was established for each patient at the time of study enrolment and the degree of organ involvement was determined by medical history, physical examination, and complementary tests.

Organ involvement was evaluated using the Medsger scale cut-off point, except for osteoarticular evaluation, since it does not value joint involvement but only the distance between thumb and thenar eminence, which was not evaluated systematically in our patients.

The Modified Rodnan Skin Score was the tool chosen for evaluating skin involvement [25].

Joint evaluation was made through physical exam, and complemented by image exams (radiography and/ or joint echography) whenever justified. DAS 28 (Disease Activity Score Calculator for Rheumatoid Arthritis) was used to classify the activity of this involvement [26].

Muscular involvement was evaluated by physical examination and complemented by biochemical study (creatine phosphokinase, aldolase and glutamate oxaloacetate transaminase).

Gastrointestinal tract involvement was documented by upper gastrointestinal endoscopy, esophageal manometry, and, in symptomatic patients, by gastric scintigraphy. Involvement of the lower GI tract, whenever justified, was documented using barium tests, glucose hydrogen breath test, ultrasound, colonoscopy and anorectal manometry.

Table 2 – Organ involvement according to Medsger Severity Scale*

ORGAN	SCORE	Pre-SSc	Limited SSc	Diffuse SSc
		(n = 10)	(n = 34)	(n = 13)
Skin	0	10 (100)	-	-
	1	-	33 (97.1)	3 (23.1)
	2	-	1 (2.9)	9 (69.2)
	3	-	-	1 (7.7)
Peripheral vascular	0	5 (50.0)	-	-
	1	4 (40.0)	18 (52.9)	1 (7.7)
	2	-	3 (8.8)	4 (30.8)
	3	1 (10.0)	11 (32.4)	8 (61.5)
	4	-	2 (5.9)	-
Gastrointestinal tract	Non-classified	2 (20.0)	2 (5.9)	1 (7.7)
	0	5 (50.0)	17 (50.0)	5 (38.5)
	1	3 (30.0)	15 (44.1)	7 (53.8)
Lung	Non-classified	1 (10.0)	-	-
	0	6 (60.0)	10 (29.4)	2 (15.4)
	1	3 (30.0)	11 (32.4)	6 (46.2)
	2	-	11 (32.4)	2 (15.4)
	3	-	1 (2.9)	1 (7.7)
	4	-	1 (2.9)	2 (15.4)
Heart	Non-classified	3 (30.0)	4 (11.8)	1 (7.7)
	0	6 (60.0)	18 (52.9)	8 (61.5)
	1	1 (10.0)	2 (5.9)	2 (15.4)
	2	-	5 (14.7)	2 (15.4)
	3	-	5 (14.7)	-
Osteoarticular	Non-classified	-	1 (2.9)	-
	0	8 (80.0)	24 (70.6)	12 (92.3)
	1	2 (20.0)	9 (26.5)	1 (7.7)

Results presented as n (%). Abbreviations: SSc, Scleroderma. * From "0" (no documented involvement or without need of treatment) to "4" (end stage disease)

Renal impairment was evaluated by biochemical study.

Chest teloradiography, high resolution computed tomography, spirometry, diffusing capacity of the lungs for carbon monoxide (DLCO) corrected for hemoglobin and alveolar volume, and two-dimensional echocardiogram with evaluation of pulmonary artery systolic pressure (PSAP) were used to evaluate pulmonary involvement. PSAP was considered abnormal when greater than 35 mmHg. Right heart catheterization was performed in patients with PSAP above or equal to 40 mmHg in order to confirm pulmonary hypertension. Forced vital capacity (FVC) and DLCO were considered abnormal when lower than 80%. Patients with lung fibrosis and/or alveolitis affecting at least the lung bases were considered to have major pulmonary alterations; criteria for minor alterations included septal hypertrophy and limited zones of fibrosis and/or ground glass opacities.

Conduction disturbances and/or arrhythmias were detected by electrocardiogram and/or Holter, and systolic dysfunction was evaluated with two-dimensional echocardiogram, namely through calculation of the left ventricular ejection fraction.

Finally, a score introduced by Cutolo *et al.* [27] was used to classify nailfold microvascular changes as observed by capillary microscopy (early, active and late capillaroscopic patterns of microvascular damage).

Laboratory analysis

Serum concentrations of endostatin and angiostatin were measured using ELISA assays from USCN Life Sciences Inc. (Wuhan, China), accordingly to the instructions provided by the manufacturer.

Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS 22.0) *software* (SPSS Inc, IL, U.S.A.). Descriptive statistics include the presentation of frequencies, medians, minimums and maximums. The Kolmogorov-Smirnov test was used to assess the normality of distributions.

Once studied variables didn't present a normal distribution and given the small size of the statistical sample, non-parametric tests were used. The Mann-Whitney test was used to compare mean ranks of two independent samples. Although corresponding to an ordinal variable, the small number of phases of disease led to the analysis using Kruskal-Wallis tests to compare the 3 disease phases instead of the use of correlations. Regarding the relationships of each organ's Medsger severity scale with angiostatin and endostatin serum levels, despite

this scale having a sufficient number of points in order to be used to compute correlation coefficients, patients exhibited a very diverse number of different scores (from 2 to 4); therefore, for each organ we compared patients at the lowest score (0 or 1) with the remaining. The null hypothesis was rejected when $p < 0.05$.

Results

Statistically significant differences were found in serum levels of angiostatin ($p=0.005$) and endostatin ($p < 0.001$) between the study population and healthy controls (Table 3).

Table 3 – Angiostatin and endostatin levels in study population and healthy controls, and according to Systemic Sclerosis subsets

	Angiostatin (ng/ml)	<i>p</i> value (patients vs. controls)	Endostatin (ng/ml)	<i>p</i> value (patients vs. controls)
Healthy controls (n=25)	34.6 (0.0-127.0)	0.005	1.6 (0.0-11.5)	<0.001
Study population (n=57)	48.5 (19.3-126.5)		20.2 (0.0-178.8)	
Pre-SSc (n=10)	46.9 (21.3-76.7)	0.108	17.7 (1.8-69.8)	<0.001
lcSSc (n=34)	48.5 (19.3-126.5)	0.014	20.6 (0.0-73.0)	<0.001
Non-CREST (n=17)	48.5 (19.3-126.5)	0.069	18.8 (9.8-40.3)	<0.001
CREST (n=17)	54.6 (21.3-85.4)	0.023	21.8 (0.0-73.0)	<0.001
dcSSc (n=13)	54.6 (24.4-101.1)	0.025	21.5 (6.3-178.8)	<0.001

Results presented as median (range) values. Abbreviations: SSc, Scleroderma; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; CREST, calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia

Disease subsets analysis revealed that angiostatin concentration was significantly higher in patients with lcSSc ($p=0.014$) and dcSSc ($p=0.025$), but not in patients with Pre-SSc, in comparison with controls, while elevated serum endostatin concentrations were noted in all SSc subsets ($p < 0.001$) (Table 3).

Serum levels of angiostatin were significantly higher in CREST patients when compared with healthy controls ($p=0.023$); however, no statistically significant differences were found between lcSSc patients without CREST syndrome and controls. In contrast, serum endostatin levels were higher in both groups of lcSSc, with or without CREST ($p < 0.001$) (Table 3).

Analysis according to disease evolution phase revealed that angiostatin serum levels were significantly higher in intermediate and late SSc ($p=0.037$ and $p=0.015$, respectively), but not in patients with early SSc ($p>0.05$), as compared to controls, while endostatin

concentrations were significantly higher in all disease evolution phases ($p<0.001$) (Table 4). Moreover, we realised that the endostatin serum levels were higher in intermediate and late SSc as compared to early SSc, although differences reached statistical significance only for patients with intermediate SSc ($p=0.003$).

Table 4 – Angiostatin and Endostatin serum levels according to the Scleroderma evolution phase

	Angiostatin (ng/ml)	<i>p value</i> (patients vs. controls)	Endostatin (ng/ml)	<i>p value</i> (patients vs. controls)
Healthy controls (n=25)	34.6 (0.0-127.0)	NA	1.6 (0.0-11.5)	NA
Early SSc (n=7)	40.4 (30.9-115.5)	0.138	15.0 (9.8-28.6)	<0.001
Intermediate SSc (n=10)	54.0 (21.3-85.3)	0.037	34.4 (16.4-38.3)	<0.001
Late SSc (n=30)	48.5 (19.3-126.5)	0.015	20.1 (0.0-178.7)	<0.001

Results presented as median (range) values. Abbreviations: SSc, Scleroderma.

Angiostatin: Early vs Intermediate ($p=0.922$); Early vs. Late ($p=0.954$); Intermediate vs Late ($p=0.988$); Early vs. Intermediate + Late ($p=0.489$). **Endostatin:** Early vs Intermediate ($p=0.003$); Early vs. Late ($p=0.535$); Early vs. Intermediate + Late ($p=0.169$); Intermediate vs Late ($p=0.040$).

Analysis comparing angiostatin and endostatin levels in patients presenting organ involvement (score ≥ 1) versus without organ involvement (score=0), revealed that angiostatin levels were significantly higher in patients with osteoarticular disease; no significant results were found on the other organ analysis or regarding to endostatin. A zero Medsger skin score corresponds indeed to the Pre-SSc subset (Table 5).

Respecting to the severity of organ involvement, we observed that angiostatin serum levels were higher in patients presenting more serious lung involvement (score ≥ 2) ($p<0.05$). Likewise, no significant differences were found on the other organ analysis or regarding to endostatin (Table 5). Sub-analysis for lung involvement also found no significant differences between normal and abnormal CT-scan, PSAP and pulmonary function tests.

Table 5 – Angiostatin and endostatin levels in Pre-Scleroderma and Scleroderma patients, according to organ involvement and Medsger severity scale

ORGAN	Angiostatin (ng/ml)						Endostatin (ng/ml)					
	Involvement			Severity			Involvement			Severity		
	Score = 0	Score ≥ 1	p	Score = 1	Score ≥ 2	p	Score = 0	Score ≥ 1	p	Score = 1	Score ≥ 2	p
Skin	46.8 (21.3-76.7)	50.0 (19.3-26.5)	0.495	46.3 (19.3-115.6)	70.9 (24.4-126.5)	0.092	17.7 (1.8-69.8)	20.9 (0.0-178.8)	0.413	20.6 (0.0-73.0)	24.4 (9.8-178.8)	0.642
Peripheral vascular	39.8 (21.3-71.1)	49.2 (19.3-126.5)	0.225	53.3 (19.3-81.3)	48.1 (21.3-126.5)	0.699	19.3 (1.8-69.8)	20.6 (0.0-178.8)	0.966	20.0 (0.0-73.0)	21.8 (3.8-178.8)	0.513
Gastrointestinal tract	48.1 (19.3-115.6)	54.6 (24.4-126.5)	0.264	- -	- -	-	19.4 (0.0-38.3)	20.9 (1.8-178.8)	0.714	- -	- -	-
Lung	50.7 (21.3-101.1)	48.5 (19.3-126.5)	0.965	42.7 (19.3-126.5)	70.9 (24.4-115.6)	0.037	32.4 (2.5-69.8)	18.4 (0.0-178.8)	0.138	18.2 (1.8-178.8)	19.6 (0.0-73.0)	0.579
Heart	51.6 (19.3-115.6)	54.6 (23.5-126.5)	0.395	47.0 (39.8-80.0)	60.6 (23.5-126.5)	0.673	20.4 (2.5-178.8)	19.3 (0.0-40.3)	0.585	19.3 (11.9-21.8)	24.8 (0.0-40.3)	0.752
Osteoarticular	45.8 (21.3-101.1)	58.4 (40.4-126.5)	0.024	- -	- -	-	20.8 (0.0-73.0)	18.6 (8.3-178.8)	0.632	- -	- -	-

Results presented as median (range) values. The number of participants in each group can be obtained from the results presented in Table 2.

The patients included in the study showed no renal manifestations or deterioration in general health and only one patient showed changes in muscle strength, so these scores are not presented.

Fifty-one patients from the study population presented changes on nailfold capillaroscopy. Analysis of angiostatin and endostatin concentrations in its three different stages shows that endostatin was significantly increased in all the 3 stages compared to controls, while angiostatin was only elevated in active and late phases (Table 6).

Table 6 – Relation between capillaroscopic patterns and angiostatin and endostatin serum levels in Pre-Scleroderma and Scleroderma patients (n=51)

Capillaroscopic pattern	Angiostatin (ng/ml)	<i>p value</i> (patients vs. controls)	Endostatin (ng/ml)	<i>p value</i> (patients vs. controls)
Early (n=12)	48.3 (19.3-76.7)	0.119	27.9 (1.8-69.8)	<0.001
Active (n=21)	46.1 (23.5-115.6)	0.024	19.4 (2.5- 178.8)	<0.001
Late (n=18)	70.9 (23.5-126.5)	0.010	21.0 (5.2-44.0)	<0.001

Results presented as median (range) values.

Discussion

Systemic sclerosis is associated with a disruption of vascular homeostasis and an unbalance between proangiogenic and antiangiogenic factors [1-3].

The present study shows that endostatin, a proteolytic fragment of type-XVIII collagen that acts as an angiogenesis inhibitor, is significantly higher in the sera from patients with SSc than in healthy controls, supporting previous studies which had already established this relationship [4, 6-10], including two studies that assessed larger cohorts of SSc patients [8, 10], and in opposition to the results obtained by Distler *et al.* [5]. With respect to angiostatin, and in agreement with the only study we found in the literature [11], we observed that its serum levels were significantly higher in SSc patients than in healthy controls, suggesting that this molecule, a cleavage product of plasminogen with anti-angiogenic properties, also plays an important role in SSc pathogenesis. In addition to confirm these observations, our study add relevant information to what is already present in the literature.

Concerning the disease subsets, in addition to confirm prior findings of higher endostatin serum levels in limited and diffuse SSc subsets than in healthy controls [7], we realised that endostatin levels are also increased in Pre-SSc. In contrast, we also observed, for the first time, that angiostatin was significantly higher in patients with lcSSc and dcSSc, but not in patients with Pre-SSc.

Previous studies have also tried to establish a relationship between the concentration of these antiangiogenic factors and SSc disease phases [5,9]. We observed that endostatin was elevated in all clinical phases of disease, in opposition to Distler *et al.* [5]. Furthermore, we realised that there was a significant difference between endostatin levels in early and intermediate disease phases in a similar manner of Farouk *et al.*, who found significantly increased levels in late stage of disease defined as disease duration superior to 3 years [9].

Moreover, we found, for the first time, that serum endostatin levels were increased in patients with lcSSc, with and without CREST, while angiostatin concentrations were only increased in patients with CREST, making us wonder if there could be different pathogenic mechanisms in this specific clinical picture of the SSc spectrum.

As expected and, once more, in opposition to Distler *et al.* [5], a positive correlation was achieved between serum levels of endostatin and all the three stages of nailfold capillaroscopic changes, and between angiostatin and active and late stages of capillaroscopic changes.

Some studies had already tried to establish a relationship between endostatin levels and lung, heart, kidney, skin and peripheral vascular involvement, but not with other organ involvement or with angiostatin [4,5,7,9].

In regard to endostatin and lung disease, our results are in consonance with previous studies found in literature, which did not find a significant interrelation between endostatin levels and lung involvement [5,7]. In addition we found that, high serum angiostatin levels were positively correlated with the severity of lung involvement (Mesdger score ≥ 2). Hebbar *et al.* established a positive correlation between endostatin and abnormalities on chest x-ray; however, the comparison of this population with all the other patients (including those with no alterations and with altered pulmonary function tests), may have influenced the results [4]. Other study found a positive correlation between endostatin levels and lung disease, defined as DLCO $<75\%$ and FVC $<80\%$ [9]; we conducted a similar analysis, which showed no significant results. Using different methodological approaches, an inverse correlation was achieved between endostatin concentration and FVC [8] and DLCO [10]. As for pulmonary hypertension, two studies found a positive relationship with endostatin levels [8,10]. In accordance, Hummers *et al.* recognized that patients presenting high right ventricular systolic pressure (≥ 40 mmHg) and pulmonary arterial hypertension confirmed by right heart catheterization, had higher concentrations of endostatin (our similar analysis showed no significant differences) [8]; and supporting these findings, Reiserter *et al.* observed that increased circulating endostatin was independently associated with pulmonary arterial hypertension [10].

Some studies have also analysed the relationship between skin score and peripheral vascular involvement and endostatin, with contradictory results [4,5,7,9]. Regarding to skin, two failed to achieve a significant correlation between skin involvement and endostatin [5,7], as we observed, but two others concluded that endostatin levels were higher in patients with higher skin scores [4,9], although a different classification for cutaneous sclerosis (Barnett classification) was used for the first one and a histopathological evaluation in the second one. As for cutaneous ulcers, only one found a positive correlation [4].

In contrast to our results regarding to cardiovascular system, a prior study demonstrated endostatin concentrations in SSc patients with heart involvement were significantly higher than in those without such changes [6].

Surprisingly, attending to the antiangiogenic effect of angiostatin in synovial membrane [28], we realised that angiostatin serum levels, but not endostatin serum levels, were higher in patients presenting osteoarticular involvement.

Taken altogether, these achievements allow us to conclude that endostatin might play an important role as an early marker of disease and that persists over disease evolution. In addition, the systematic observation of elevated angiostatin serum levels in more advanced stages of disease – in limited and diffuse SSc, in intermediate and late phases of disease, with active and late capillaroscopic changes, or in a global view in patients with and more severe

organ involvement, allow us to hypothesize that angiostatin has a greater impact in disease progression, is a marker of late disease and relates to lung disease severity.

In summary, we demonstrated that SSc is associated with the elevation of two anti-angiogenic factors, endostatin and angiostatin. Endostatin increases in an early stage of disease, highlighting its possible role as an early marker that might help us in the clinical assessment of SSc – may it predict evolution from Pre-Scleroderma phase to *true* Systemic Sclerosis? Prospective longitudinal studies are required to assess this hypothesis.

Furthermore, angiostatin is not only a marker of late and severer lung disease but also it is related with disease progression and it might be implicated in a distinct pathogenic mechanism responsible for the development of CREST in patients with limited SSc. Likewise further studies are needed to assess this relationship.

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4.2.3 – ROLE OF ENDOTHELIN-1 IN THE PHYSIOPATHOLOGY AND SEVERITY OF SYSTEMIC SCLEROSIS

Artigo enviado para publicação

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Abstract

Introduction/Objectives: To determine the concentrations of circulating endothelin in patients with Systemic Sclerosis (SSc) and to assess its relationship to disease subsets, evolution phase, organ involvement, nailfold capillaroscopic changes and autoantibodies.

Method: Endothelin-1 (ET-1) serum levels were measured by ELISA in a cohort of 48 patients with SSc spectrum disease, and related with disease subsets, clinical evolution phase, organ involvement, nailfold capillaroscopic changes and autoantibodies.

Results: Endothelin-1 serum levels were significantly higher in patients with SSc than in healthy controls. Also, ET-1 was elevated in diffuse SSc (dcSSc) and limited SSc (lcSSc), but not in Pre-SSc. Moreover, in lcSSc, ET-1 was increased exclusively in patients with CREST. Analysis according to disease evolution phases revealed that ET-1 serum levels were elevated in all phases, although differences reached statistical significance only in late phases of disease, being also significantly higher in patients with more serious lung and vascular involvement. Finally, regarding nailfold capillaroscopic changes and autoantibodies, ET-1 was only elevated in active and late phases, and was associated with anti-centromere antibodies.

Conclusions: In accordance to previous studies, we found that endothelin concentrations are elevated in SSc patients. Additionally, we realized that ET-1 is not only a marker of more severe lung and vascular disease, but it is also related with disease progression and it might be implicated in a distinct pathogenic mechanism, responsible for the development of CREST in patients with limited SSc, and in the pathogenesis of pulmonary disease.

Keywords: Systemic Sclerosis, Scleroderma, Endothelin

Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a heterogeneous autoimmune disease characterized by extensive tissue fibrosis, vascular changes and auto-antibodies directed against cellular antigens [1]. Its physiopathology is complex and involves fibroblast dysfunction, excessive collagen production, with the consequent accumulation of extracellular matrix deposits, and endothelial/vascular injuries [2].

Endothelial dysfunction is one of the earliest mechanisms of disease in SSc [3], and small vessel involvement significantly contributes to the illness' morbidity and mortality [4].

The endothelin system is composed by three vasoactive peptides (endothelin-1, 2 and 3), their G-protein receptors (endothelin receptors type A and B), and two converting enzymes (1 and 2) [5]. Endothelin-1 (ET-1) is the predominant isoform in humans and is sequentially processed from a 212-aminoacid pre-pro-peptide to a biologically active 21-aminoacid peptide [6]. It has potent vasoconstrictive effects and acts as a cellular proliferation, inflammation and fibrosis mediator [7].

Fibrinogenic effects of ET-1 were observed *in vitro*, and its presence seems necessary for the fibrinogenic properties of the transforming growth factor (TGF) both *in vitro* and *in vivo* [8]. In ET-1 gene knock-out mice, reduction of tumor necrosis factor (TNF) alpha, connective tissue growth factor (CTGF) and TGF-beta levels was documented, with subsequent decrease of bleomycin-induced cutaneous fibrosis [9]. At the blood vessels and the endothelium, ET-1 also promotes vasoconstriction, enables the interaction between leucocytes and fibroblasts and increases cellular proliferation and the production of pro-fibrotic factors [10]. Endothelin-1 acts by binding to type A and type B receptors: type A receptors, expressed in mesenchymatous cells, have vasoconstrictor effects and seem to be overexpressed in patients with SSc [11]; type B receptors are expressed in endothelial cells, where they have vasodilator properties (due to nitric oxide liberation), and in smooth muscle cells, where they have the same effects as type A receptors. These receptors seem to be under expressed on the endothelial cells and overexpressed on vascular smooth muscle cells of patients with SSc, contributing to cellular proliferation and hypertrophy, vasoconstriction, inflammation and fibrosis [12,13].

Reduction of serum levels of ET-1 (and improvement of other vasoactive mediators' levels) were shown when patients were treated with non-selective ET-1 receptor inhibitors [14]. There is also evidence that ET-1 antagonism may have positive effects regarding cutaneous fibroblasts' pro-fibrotic activity in patients with SSc [15]. Non-selective ET-1 receptor inhibitors are approved for SSc treatment [3], in patients with pulmonary hypertension [16] and with digital ulcers [17]. There seems to exist potential effectiveness of this therapy in cases of SSc-

related cutaneous fibrosis [18] and, although their use in scleroderma renal crisis hasn't shown clear benefits, there are some case reports that suggest this possibility [19].

The purpose of this work was to relate ET-1 serum levels with SSc subsets, evolution phases, type and severity of organ involvement (using the Medsger scale), nailfold capillaroscopic changes and anti-centromere (ACA) and anti-topoisomerase 1 antibodies (Scl-70).

Material and Methods

Patients

Fifty-two consecutive patients were selected from a 190-patients-population with SSc, at the Clinical Immunology Unit of Centro Hospitalar do Porto – Portugal, between September 2010 and March 2011. Four patients were later excluded, three because of overlapping pathologies (mixed connective tissue disease, infection with human immunodeficiency virus, B non-Hodgkin lymphoma) and a fourth one who missed the blood sampling.

Thirty nine patients fulfilled the American College of Rheumatology (ACR) criteria for SSc [20] while the remaining nine did not present skin involvement and were classified as Pre-Scleroderma (Pre-SSc). The ACR/ EULAR (European League Against Rheumatism) criteria of 2013 [21] were met by those 39 patients, and by 1 of the 9 without skin involvement due to having digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement.

In the study population, 46 were women and 2 were men, with a median age of 56 years, ranging from 19 to 80 years. Patients' characteristics are summarized in Tables 1 and 2. Twenty-five healthy individuals were used as controls.

Table 1 – SSc patient's characteristics (n=48)

Age (years)	
Median (range)	56 (19-80)
Gender	
Male: Female	02:46
Disease subset (n, %)	
Pre-SSc	9 (18.8)
Diffuse SSc	12 (25.0)
Limited SSc	27 (56.2)
CREST	12 (25.0)
Non-CREST	15 (31.2)
Disease phase (n, %)	
Early	6 (12.5)
Intermediate	10 (20.8)
Late	23 (47.9)
Capillaroscopic pattern (n. %)	
Normal/minor alterations	4 (8.3)
Early	11 (22.9)
Active	19 (39.6)
Late	14 (29.2)

Age is presented as median (and range) values. Others results are presented as n (%). Abbreviations: SSc, scleroderma; CREST, Calcinosis, Raynaud phenomenon, esophageal dysmotility, Sclerodactyly, and Telangiectasia;

Table 2 – Organ involvement according to Medsger Severity Scale*

Organ	SCORE	Pre-SSc (n = 9)	Limited SSc (n = 27)	Diffuse SSc (n = 12)
Skin	0	9 (100)	-	-
	1	-	27 (100)	3 (25)
	2	-	-	8 (66,7)
	3	-	-	1 (8.3)
Peripheral vascular	0	4 (44,4)	-	-
	1	4 (4.4)	15 (55.6)	1 (8.3)
	2	-	2 (7.4)	4 (33,3)
	3	1 (11.1)	10 (37.0)	7 (58,3%)
	4	-	-	-
Gastrointestinal tract	Non-classified	2 (22.2)	1 (3.7)	1 (8.3)
	0	5 (55.6)	16 (59.3)	5 (22,7)
	1	2 (22.2)	10 (37.0)	6 (50,0)
Lung	Non-classified	1 (11.1)	-	-
	0	5 (55.6)	10 (37.0)	2 (16.7)
	1	3 (33.3)	8 (29.6)	6 (50.0)
	2	-	8 (29.6)	1 (8.3)
	3	-	1 (3.7)	1 (8.3)
	4	-	-	2 (16.7)
Heart	Non-classified	3 (33.3)	3 (11.1)	1 (8.3)
	0	5 (55.6)	15 (55.6%)	7 (58.3)
	1	1 (11.1)	1 (3.7)	2 (16.7)
	2	-	4 (14.8)	2 (16.7)
	3	-	4 (14.8)	-
Osteoarticular	Non-classified	-	-	-
	0	7 (77.8)	19 (70.4)	11 (91.7)
	1	2 (22.2)	8 (29.6)	1 (8.3)

Results presented as n (%). Abbreviations: SSc, Scleroderma. * From “0” (no documented involvement or without need of treatment, e.g. Raynaud) to “4” (endstage disease)

At the time of the study, none of the patients were receiving corticosteroids or immunosuppressive drugs and a washout of the vasodilator drugs was done, during one or two weeks, in no cold weather, and according to the clinical *status* of the patients.

The study was approved by the Ethics Committee and the Board of Directors of the Hospital and all patients signed an informed consent form prior to their inclusion in the study.

Clinical assessment

Patient disease subset was classified as Pre-Scleroderma (Pre-SSc) according to LeRoy and Medsger [22], and limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc)

scleroderma, as previously described by LeRoy *et al.* [23]. Over time, several authors and the EULAR Scleroderma Trial and Research group (EUSTAR) have recognized that there is an early stage of disease in which there is no skin involvement but that may evolve to limited or diffuse SSc, and named it differently (“limited scleroderma”, “pre-scleroderma”, “early SSc” and “very early SSc” [22,24–26], even though they represent nearly the same concept [27]. We used the definition of Pre-SSc according to LeRoy and Medsger criteria, where patients must have Raynaud’s phenomenon plus scleroderma-type nailfold capillary changes and/or scleroderma-type autoantibodies [22].

Limited and diffuse SSc were then subdivided into early, intermediate and late SSc, according to the time since the first disease-related symptom and in consonance with the subset of disease [28]. Early phase of lcSSc has less than five years of evolution and late phase has at least ten years. On the contrary, early phase of dSSc has less than three years of evolution and late phase a duration superior to six years. Intermediate values correspond to intermediate phases of limited and diffuse SSc. Limited SSc was further separated in CREST (calcinosis, Raynaud phenomenon, gastrointestinal dysmotility, sclerodactyly, telangiectasia) and non-CREST groups according to Lonzetti *et al.* [29].

There is a lack of standardized method to assess disease severity. We decided to use Medsger’s severity scale [30], which classifies involvement of each of nine organ system (general, peripheral vascular, skin, joints/tendons, muscles, gastrointestinal (GI) tract, lungs, heart and kidneys) from 0 (no documented involvement or without need of treatment, e.g. Raynaud) to 4 (end stage disease), without an overall score. It has limitations as a measure of activity due to its lack of sensitivity to change score in the presence of SSc severity improvement [31]. Even though, it is useful as a prognostic measure [32].

A complete clinical profile was established for each patient at the time of study enrolment and the degree of organ involvement was determined by medical history, physical examination, and complementary tests.

Organ involvement was evaluated using the Medsger’s scale cut-off point, except for osteoarticular evaluation since it does not value joint involvement but only the distance between thumb and thenar eminence, which was not evaluated systematically in our patients.

The Modified Rodnan Skin Score was used to evaluate skin involvement [33].

Joint evaluation was made through physical exam and image tests (radiography and/or joint echography) whenever justified. DAS 28 (*Disease Activity Score Calculator for Rheumatoid Arthritis*) was used to classify the activity of this involvement [34].

Muscular involvement was evaluated by physical examination and biochemical studies (creatine phosphokinase, aldolase and glutamate oxaloacetate transaminase).

Gastrointestinal tract involvement was documented by upper gastrointestinal endoscopy, esophageal manometry, and, in symptomatic patients, by gastric scintigraphy. Involvement of

the lower GI tract, whenever justified, was documented using barium tests, glucose hydrogen breath test, ultrasound, and colonoscopy and anorectal manometry.

Chest telerradiography, high resolution computed tomography, spirometry, diffusing capacity of the lungs for carbon monoxide (DLCO) corrected for hemoglobin and alveolar volume, and two-dimensional echocardiogram with evaluation of pulmonary artery systolic pressure (PSAP) were used to evaluate pulmonary involvement. PSAP was considered abnormal when greater than 35 mmHg. Right heart catheterization was performed in patients with PSAP above or equal to 40 mmHg in order to confirm pulmonary hypertension. Forced vital capacity (FVC) and DLCO were considered abnormal when lower than 80%. Patients with lung fibrosis and/or alveolitis affecting at least the lung bases were considered to have major pulmonary alterations; criteria for minor alterations included septal hypertrophy and limited zones of fibrosis and/or ground glass opacities.

Conduction disturbances and/or arrhythmias were detected by electrocardiogram and/or Holter. Systolic dysfunction was evaluated with two-dimensional echocardiogram, namely through calculation of the left ventricular ejection fraction.

Finally, a score introduced by Cutolo *et al.* [35] was used to classify nailfold microvascular changes as observed by capillary microscopy (early, active and late capillaroscopic patterns of microvascular damage).

Laboratory analysis

Serum concentrations of ET-1 were measured using ELISA sandwich assay, Endothelin [1-21] from Biomedica (Biomedica Medizinprodukte GmbH, Austria) accordingly to the instructions provided by the manufacturer.

Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS 22.0) software (SPSS Inc, IL, U.S.A.). Descriptive statistics included the absolute and relative frequencies, median, minimum and maximum values. The Kolmogorov-Smirnov test was used to assess the normality of distributions.

Once studied variables didn't present a normal distribution and given the small size of the sample, non-parametric tests were used. The Mann-Whitney U test was used to compare mean ranks of two independent samples. Although corresponding to ordinal variables, the small number of phases of disease led to the analysis using Kruskal-Wallis tests to compare the 3 disease phases instead of the use of correlations. Regarding the relationships of each organ's Medsger severity scale with ET-1 serum levels, despite this scale having a sufficient number of points in order to be used to compute correlation coefficients, patients exhibited a very diverse and sometimes low number of different scores (from 2 to 4); therefore, for each

organ we compared patients at the lowest score (0 or 1) with the remaining. The null hypothesis was rejected when $p < 0.05$.

Results

Serum levels of ET-1 were significantly increased in the study population, as compared to healthy controls ($p<0.001$) (Table 3).

Table 3 – Endothelin-1 serum levels in the study population and healthy controls, and according to Systemic Sclerosis subsets

	Endothelin-1 (ng/ml)	<i>p value</i> (patients vs. controls)
Healthy controls (n=25)	0.48 (0.01-15.9)	<0.001
Study population (n=48)	1.88 (0.16-6.89)	
Pre-SSc (n=9)	1.63 (0.16-6.89)	0.064
lcSSc (n=27)	1.95 (0.31-22.35)	<0.001
Non-CREST (n=15)	1.91 (0.35-22.35)	0.775
CREST (n=12)	3.7 (0.31-16.05)	0.015
dcSSc (n=12)	1.63 (0.19-22.17)	0.05

Results are presented as median (and range) values. Abbreviations: SSc, scleroderma; lcSSc, limited cutaneous scleroderma; dcSSc, diffuse cutaneous scleroderma; CREST, Calcinosis, Raynaud phenomenon, esophageal dysmotility, Sclerodactyly, and Telangiectasia;

Pre-SSc vs lcSSc ($p=0,385$); Pre-SSc vs dcSSc ($p=0,875$); lcSSc vs dcSSc ($p=0,288$)

Disease subsets analysis revealed that ET-1 concentration was significantly higher in patients with lcSSc ($p<0.001$) and higher in patients with dcSSc ($p=0.05$), but not in patients with Pre-SSc, in comparison with controls (Table 3).

Serum ET-1 levels were also significantly increased in CREST patients as compared to controls ($p=0.015$); however, no statistically significant differences were found between lcSSc patients without CREST syndrome and controls (Table 3).

Analysis according to disease evolution phase revealed that ET-1 serum levels were significantly higher in late ($p=0.002$), but not in early ($p=0.106$) or intermediate ($p=0.076$) SSc

phases, as compared to controls (Table 4). When early and intermediate SSc were analyzed together, we observed a statistically significant difference between these patients and the control group.

Table 4 – Endothelin-1 serum levels according to the Scleroderma evolution phase

	Endothelin-1	<i>p value</i>
	(ng/ml)	(patients vs. controls)
Healthy controls (n=25)	0.48 (0.01-15.9)	NA
Early SSc (n=6)	1.13 (0.47-21.45)	0.106
Intermediate SSc (n=10)	1.10 (0.28-22.35)	0.076
Late SSc (n= 23)	2.51 (0.19-22.17)	0.002

Results are presented as median (range) values.

Abbreviations: SSc, scleroderma; NA, not applicable.

Endothelin: Early vs Intermediate ($p=1.000$); Early vs. Late ($p=0.370$); Intermediate vs Late ($p=0.361$); Early + Intermediate vs Late ($p= 0.252$); Intermediate + Late vs Early ($p= 0.361$); Early + Intermediate vs Healthy controls ($p= 0.029$).

The comparison of ET-1 serum levels in patients presenting organ involvement (score $\geq$ 1) versus without organ involvement (score=0), revealed no significant differences; the “zero” Medsger skin score corresponds indeed to the Pre-SSc subset (Table 5).

Table 5 – Endothelin-1 serum levels in Pre-Scleroderma and Scleroderma patients, according to organ involvement and Medsger severity scale

Endothelin-1 (ng/ml)						
ORGAN	Involvement			Severity		
	Score = 0	Score ≥ 1	p	Score = 1	Score ≥ 2	p
Skin	1.63 (0.16-6.89)	1.91 (0.19-22.35)	0.56	1.90 (0.30-22.40)	2.15 (0.20-22.20)	0.479
Peripheral vascular	1.02 (0.16-3.97)	1.99 (0.19-22.35)	0.148	2.02 (0.30-7.40)	1.60 (0.20-22.40)	0.595
Gastrointestinal tract	2.36 (0.28-22.35)	1.84 (0.16-22.17)	0.471	- -	- -	-
Lung	1.95 (0.28-4.4)	1.82 (0.16-22.4)	0.906	1.10 (0.20-10.10)	6.40 (0.30-22.40)	0.013
Heart	1.99 (0.19-22.35)	2.32 (0.26-21.45)	0.74	1.8 (0.30-16.10)	2.30 (0.30-21.50)	0.825
Osteoarticular	1.90 (0.2-22.4)	2.30 (0.2-21.50)	0.716	- -	- -	-

The number of participants in each group can be obtained from the results presented in Table 2.

Vascular Score 0+1+2 versus 3: 2.7 (0.2-10) versus 6.2 (0.2-22); p=0.040.

Forty four patients presented changes on nailfold capillaroscopy. Analysis of ET-1 concentrations in different capillaroscopic stages showed that it was significantly increased in active and late phases (Table 6).

Table 6 – Relation between capillaroscopic patterns and Endothelin-1 serum levels in Pre-Scleroderma and Scleroderma patients (n=48)

Capillaroscopic pattern	Endothelin-1 (ng/ml)	p value (patients vs. controls)
Early (n=11)	1.57 (0.16-6.89)	0.114
Active (n=19)	2.99 (0.19-22.35)	0.003
Late (n= 14)	2.70 (0.35-22.17)	0.003

Results presented as median (range) values.

Early vs Late (p= 0.064); Early vs Active (p=0.208); Active vs Late (p=0.660)

Respecting to the severity of organ involvement, we observed that ET-1 serum levels were higher in patients presenting more serious lung involvement (score $\geq$ 2) (p=0.013). Sub-analysis for lung involvement revealed significant differences between patients with normal ($\geq$ 80%) vs. abnormal DLCO (p=0.004), but not between patients with normal vs. abnormal CT-scan, PSAP and pulmonary function tests (DLCO + FVC).

Sub-analysis for vascular involvement displayed significant differences between Raynaud disease with or without digital ulcers (p=0.040). No significant differences were found in the analysis of the other organs.

Higher ET-1 levels were found for patients with ACA but not with ANA or anti-Scl70 antibodies (data not shown).

Discussion

In SSc, endothelial activation and dysfunction associated with ET-1 liberation are essential pathological aspects. Endothelin-1 has potent vasoconstrictive effects and is involved in the activation and proliferation of fibroblasts, leucocyte adhesion and in the proliferation of smooth muscle cells in the vascular wall [36].

The present study shows that the ET-1 serum levels are significantly higher in patients with SSc than in healthy controls, supporting previous studies which had already established this relationship [37–40] but also opposing others that either demonstrated no increase [41] or a decrease in these values [42].

Some authors had previously studied ET-1 concentrations in limited and diffuse subsets of disease and they have found higher concentrations in both cases when compared to healthy population [37,43,44], as we did. On the contrary, other studies have found differences between both subsets, with more elevated levels in patients with diffuse SSc [41]. We observed that ET-1 serum levels are not significantly increased in patients with Pre-SSc, and to the best of our knowledge there are no published studies on this subject.

Previous studies had tried to establish a relationship between the concentration of ET-1 and the duration of SSc disease. These studies found elevated serum levels of ET-1 in all the disease phases [43,44]. In our work, we observed that ET-1 was elevated in all clinical phases of disease, but difference reached statistical significance only in late SSc, which can be explained by the small number of patients included in the early and intermediary stages. In accordance, when both the early and the intermediary stages of the disease were evaluated together, we found a statistical significant difference between patients and controls. Moreover, we observed that ET-1 was increased in patients with lcSSc with CREST syndrome but not in those without, making us wonder if there could be different pathogenic mechanisms for this specific clinical picture of the SSc spectrum.

Kim *et al.* observed an association between the size of nailfold capillaries (evaluated by capillaroscopy) and ET-1 serum levels [45]. Sulli *et al.* also observed higher serum levels of ET-1 in the active and late stages of capillaroscopic changes but not in the early stages of capillaroscopy [43]. Our results also demonstrate this relationship.

Some studies have revealed a relationship between the serum endothelin levels and lung, kidney, skin and peripheral vascular involvement. Regarding lung disease, we found a statistically significant negative relation between serum ET1 levels and DLCO alterations ($p=0.004$), in opposition to the results obtained by others [41,44]. We also observed that ET-1 levels were positively related with the severity of lung involvement (Mesdger score ≥ 2). However, we didn't find a relationship between ET-1 levels and CT scan (in line with Morelli *et al.* [37] and Schmidt *et al.* [44]), PSAP (in line with Morelli *et al.* [37] but, in opposition to Schmidt

et al. [44] and others [38,41,45]) and FVC (in line with Schmidt *et al.* [44]). Maybe this can be due to the small number of patients in our study.

Some studies have also analyzed the relationship between skin score and peripheral vascular involvement and ET-1, with contradictory results. We failed to achieve a significant relationship between skin involvement and ET-1, as observed by others [41,45]. In vascular disease, our results are in consonance with previous studies [43,45], which found a significant interrelation between ET-1 levels and digital ulcers.

In agreement with other groups [44], we also found a relationship between ET-1 levels and ACA, but not with anti-Scl-70 antibodies.

The systematic observation of elevated ET-1 levels in limited and diffuse SSc, in all phases of disease, namely in the late one, with active and late capillaroscopic changes, or in a global view in patients with and more severe organ involvement, allow us to hypothesize that ET-1 has a great impact in disease progression, and relates to lung and vascular disease severity. Also, the fact that ET-1 levels have a negative association with DLCO makes us to consider that it may have a role as an early marker of SSc lung involvement, either in its vascular or in its interstitial aspects.

In summary, we demonstrated that SSc is associated with increased ET-1 serum levels. Furthermore, ET-1 is not only a marker of more severe lung and vascular disease, but it is also related with disease progression and it might be implicated in a distinct pathogenic mechanism, responsible for the development of CREST in patients with limited SSc, and in the pathogenesis of pulmonary disease.

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4.2.4 – CELL ADHESION MOLECULES IN SYSTEMIC SCLEROSIS – WHAT SHOULD WE EXPECTED? RESULTS FROM A PORTUGUESE COHORT

Artigo para submeter a publicação

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Abstract

Objectives: To determine the concentrations of adhesion molecules in the serum from patients with Systemic Sclerosis (SSc) spectrum disease and to assess their relationship with disease subsets, evolution phase, organ involvement and nailfold capillaroscopic changes.

Methods: E-selectin, P-selectin, Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1) serum levels were measured by ELISA in a cohort of 48 patients with SSc spectrum disease, and related with disease subsets, clinical evolution phase, organ involvement and nailfold capillaroscopic changes.

Results: Comparing patients and healthy controls, statistically significant differences were found for serum levels of VCAM-1, ICAM-1, E-selectin, and P-selectin. Patients have lower levels of ICAM-1 and higher levels of the other three molecules. This trend was found in all SSc subsets. Moreover, analysis according to disease evolution phase found that VCAM-1 and P-selectin serum levels were elevated in all disease phases, while E-selectin was only significantly higher in the late SSc and ICAM-1 was significantly lower in intermediate and late disease phases. Regarding nailfold capillaroscopy patterns, P-selectin and VCAM-1 were significantly increased and ICAM-1 significantly decreased in all patterns, while E-selectin was only elevated in late pattern. Analysis regarding organ involvement revealed that ICAM-1 serum levels were higher among patients with lung and heart involvement and VCAM-1 levels were significantly higher in patients with osteoarticular involvement. Analysis regarding severity of organ involvement revealed that E-selectin serum levels were higher in patients presenting more serious lung involvement and ICAM-1 in patients with more serious heart involvement.

Discussion: In accordance to previous studies, we found higher serum levels of VCAM-1, P-selectin and E-selectin in SSc spectrum disease patients but, in opposition, we found lower levels of ICAM-1. Additionally, we recognized the important role of P-selectin and VCAM-1 as early disease and persistent markers throughout the course of the disease and realized that E-selectin is not only a marker of late disease and relates to lung disease severity. Furthermore, ICAM-1 might be implicated in the pathogenesis of heart and lung involvement.

Keywords: Systemic Sclerosis, Scleroderma, Adhesion molecules, ICAM-1, VCAM-1, P-selectin, E-selectin.

Introduction

Systemic Sclerosis (SSc) is a connective tissue disease with unclear aetiology and pathogenesis, characterized by microvascular changes and immunologic abnormalities leading to fibrosis of the skin and internal organs [1]. Histopathologic hallmark of early SSc stage is endothelial damage and perivascular inflammatory infiltration with an accumulation of lymphocytes [2].

Molecules known to mediate leucocyte adhesion include 3 major families: selectins, like E-selectin and P-selectin, which promote the contact of lymphocyte with endothelial cells; Integrins, which help lymphocyte attachment to blood vessels; and immunoglobulin superfamily molecules, including vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1), which facilitate leukocyte endothelial transmigration [3].

VCAM-1 (CD106) is expressed on endothelial and epithelial cells, dendritic cells and macrophages [4] and allows for evaluation of the degree of endothelial injury and activity [5].

ICAM-1 (CD54) is constitutively expressed on endothelial and epithelial cells and fibroblasts, and is induced by pro-inflammatory cytokines [6].

Among selectins, E-selectin (CD62E) is specifically expressed by activated endothelial cells, whereas P-selectin (CD62P) is also expressed in platelets [6]. A major difference between them is the time needed for expression: P-selectin is quickly mobilized to the surface of endothelium or platelets, while E-selectin expression is induced by inflammatory cytokines, several hours after [6].

Although there are several studies suggesting that cell adhesion molecules play a major role in SSc pathogenesis [1, 2, 7, 8], only some of them have related these molecules with organ involvement [5, 7-11], and only few have related these molecules with disease subsets and its behaviour over the disease [3, 12] and capillaroscopic changes [13-14].

The aim of this study was to determine the concentrations of circulating VCAM-1, ICAM-1, and E and P selectins in patients with SSc and to correlate their values with disease subsets, evolution phase, type of organ involvement and nailfold capillaroscopic changes.

Patients and Methods

Study approval

The study was approved by the Ethics Committee and the Board of Directors of the Hospital and all patients signed an informed consent form.

Patients

Between September 2010 and March 2011 sixty-one patients were consecutively selected from a 190-patients-population with SSc, at the Clinical Immunology Unit of Hospital Geral de Santo António, Centro Hospitalar do Porto, Portugal. Four patients were later excluded, three because of overlapping pathologies (mixed connective tissue disease, infection with human immunodeficiency virus, B non-Hodgkin lymphoma) and a fourth one who missed the blood sampling. From the remaining 57 patients, 48 were included in this study (in 9 cases there were no sample available for adhesion molecules measurement).

Thirty nine patients fulfilled the American College of Rheumatology criteria for SSc, while the remaining nine did not present skin involvement and were diagnosed as Pre-Scleroderma, as explained in clinical assessment. The ACR/EULAR (The European League Against Rheumatism) criteria of 2013 [15] were met by those 39 patients that had met the ACR criteria, and by 1 of the 9 without skin involvement due to having digital ulcers. For practical reasons, the analysis of this last patient was made together with those that did not present skin involvement. Patient's characteristics are summarized in Tables 1 and 2. Regarding the characteristics of study population, 46 were women and two were men with a median age of 56 years (ranging from 19 to 80 years). Twenty five healthy individuals were used as controls.

Table 1 – SSc patient's characteristics (n=48)

Age (years)	<i>Median (range)</i>	56 (19-80)
Sex	<i>Male: Female</i>	2:46
Disease subset n (%)		
Pre-SSc		9 (18.8%)
Diffuse SSc		12 (25.0%)
Limited SSc		27 (56.2%)
CREST		12 (25.0%)
Non-CREST		15 (31.2%)
Disease phase n (%)		
Early		6 (12.5%)
Intermediate		10 (20.8%)
Late		23 (47.9%)
Capillaroscopic pattern n (%)		
Normal/minor alterations		4 (8.3%)
Early		11 (22.9%)
Active		19 (39.6%)
Late		14 (29.2%)

Abbreviations: SSc, Scleroderma; CREST, Calcinosis, Raynaud phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia.

Table 2 - Organ involvement according to Medsger Severity Scale*

ORGAN	SCORE	Pre-SSc	Limited SSc	Diffuse SSc
		(n = 9)	(n = 27)	(n = 12)
Skin	0	9 (100)	-	-
	1	-	27 (100)	3 (23.1)
	2	-	-	8 (66.7)
	3	-	-	1 (8.3)
Peripheral	0	4 (44.4)	-	-
Vascular	1	4 (44.4)	15 (55.6)	1 (8.3)
	2	-	2 (7.4)	4 (33.3)
	3	1 (11.1)	10 (37.0)	7 (58.3)
	4	-	-	-
Gastrointestinal	Non-classified	2 (22.2)	1 (3.7)	1 (8.3)
	0	5 (55.6)	16 (59.3)	5 (22.7)
	1	2 (22.2)	10 (37.0)	6 (50.0)
Lung	Non-classified	1 (11.1)	-	-
	0	5 (55.6)	10 (37.0)	2 (16.7)
	1	3 (33.3)	8 (29.6)	6 (50.0)
	2	-	8 (29.6)	1 (8.3)
	3	-	1 (3.7)	1 (8.3)
	4	-	-	2 (16.7)
Heart	Non-classified	3 (33.3)	3 (11.1)	1 (8.3)
	0	5 (55.6)	15 (55.6)	7 (58.3)
	1	1 (11.1)	1 (3.7)	2 (16.7)
	2	-	4 (14.8)	2 (16.7)
	3	-	4 (14.8)	-
Osteoarticular	Non-classified	-	-	-
	0	7 (77.8)	19 (70.4)	11 (91.7)
	1	2 (22.2)	8 (29.6)	1 (8.3)

Results presented as n (%). Abbreviations: SSc, Scleroderma. * From "0" (no documented involvement or without need of treatment, e.g. Raynaud) to "4" (end stage disease)

Clinical assessment

Taking into account the disease subsets, patients were classified as having Pre-Scleroderma (Pre-SSc), limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) [16].

Despite the existence of numerous definitions from the early stage of the disease in which there is no skin involvement, such as “early scleroderma”; “very early scleroderma”; “pre-scleroderma” or “limited scleroderma” [17-20], the authors used the Le Roy and Medsger definition of Pre-Scleroderma where Raynaud plus scleroderma-type nailfold capillary changes and/or scleroderma-type autoantibodies must be present [17]. Despite not having skin involvement, this stage may evolve to limited or diffuse SSc.

Patients with lcSSc were subdivided in CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) and non-CREST groups, according to Lonzetti *et al.* [21].

Regarding evolution phase, patients that fulfilled the criteria for SSc were divided according to the time evolution of the first symptom related to the disease, into early, intermediate and late SSc [22]. In patients with lcSSc, early phase and late phases were defined as having less than five years or 10 years or more of evolution, respectively. The correspondent *cut-off* values to define early and late disease phases for patients with dcSSc were 3 years and 6 years of evolution, respectively. Intermediate phase of both limited and diffuse SSc ranges between the two numeric values.

To assess disease severity we used Medsger’s severity scale where each 9 organ (general, peripheral vascular, skin, joints/tendons, muscles, gastrointestinal (GI) tract, lungs, heart and kidneys) can range from 0 (no documented involvement or without need of treatment, e.g. Raynaud) to 4 (end stage disease) without an overall score. It has limitations as a measure of activity due to its lack of sensitivity to change score in the presence of SSc severity improvement [23]. Even though, it is a quite useful tool as a prognostic measure [24].

At the time of study enrolment a complete clinical profile was established for each patient and the degree of organ involvement was made taking into account medical history, physical examination and complementary tests.

Organ involvement was evaluated using the Medsger’s scale cut-off point, except for osteoarticular evaluation, since it does not value joint involvement but only the distance between thumb and thenar eminence, which was not evaluated systematically in our patients.

The Modified Rodnan Skin Score was the tool chosen for evaluating skin involvement [25].

Joint evaluation was made through physical exam, and complemented by image exams (radiography and/ or joint echography) whenever justified. DAS 28 (*Disease Activity Score Calculator for Rheumatoid Arthritis*) was used to classify the activity of this involvement [26].

Muscular involvement was evaluated by physical examination and complemented by biochemical study (creatine phosphokinase, aldolase and glutamate oxaloacetate transaminase).

Gastrointestinal tract involvement was documented by upper gastrointestinal endoscopy, esophageal manometry, and, in symptomatic patients, by gastric scintigraphy. Involvement of the lower GI tract, whenever justified, was documented using barium tests, glucose hydrogen breath test, ultrasound, colonoscopy and anorectal manometry.

Chest teloradiography, high resolution computed tomography, spirometry, diffusing capacity of the lungs for carbon monoxide (DLCO) corrected for hemoglobin and alveolar volume, and two-dimensional echocardiogram with evaluation of pulmonary artery systolic pressure (PSAP) were used to evaluate pulmonary involvement. PSAP was considered abnormal when greater than 35 mmHg. Right heart catheterization was performed in patients with PSAP above or equal to 40 mmHg in order to confirm pulmonary hypertension. Forced vital capacity (FVC) and DLCO were considered abnormal when lower than 80%. Patients with lung fibrosis and/or alveolitis affecting at least the lung bases were considered to have major pulmonary alterations; criteria for minor alterations included septal hypertrophy and limited zones of fibrosis and/or ground glass opacities.

Conduction disturbances and/or arrhythmias were detected by electrocardiogram and/or Holter, and systolic dysfunction was evaluated with two-dimensional echocardiogram, namely through calculation of the left ventricular ejection fraction.

Finally, a score introduced by Cutolo *et al.* [27] was used to classify nailfold microvascular changes as observed by capillaroscopy (early, active and late capillaroscopic patterns of microvascular damage).

Laboratory analysis

Blood samples were collected in plain Vacutainer tubes, centrifuged and aliquoted as soon as possible. The serum aliquots were frozen at -40 °C until analysis. Serum concentrations of soluble VCAM-1 (IBL International; Ref BE59051), soluble ICAM-1 (IBL International; Ref BE59011), soluble P-selectin (IBL International; Ref BE59081) and soluble E-selectin (IBL International; Ref BE59061), were measured using ELISA assays from IBL International, Hamburg, Germany, accordingly to the manufactory instructions.

Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for Social Sciences (SPSS 20.0) software (SPSS Inc, IL, U.S.A.).

Data are presented as mean and standard deviation. Kolmogorov–Smirnov statistics was used to evaluate sample normality distribution. Studied variables didn't present a normal distribution.

Non-parametric tests were used for single comparisons of continuous variables between two (Mann Whitney U test) or three (Kruskal-Wallis test) groups. Spearman's correlation coefficient (r_s) was used to evaluate the association between two variables.

Regarding the relationships of each organ's Medsger severity scale with adhesion molecules serum levels, despite this scale having a sufficient number of points in order to be used to compute correlation coefficients, patients exhibited a very diverse number of different scores (from 2 to 4); therefore, for each organ we compared patients at the lowest score (0 or 1) with the remaining. The null hypothesis was rejected when $p < 0.05$.

Results

The serum levels of cell adhesion molecules were highly variable in patients as in controls (Table 3). Considering all the participants combined (controls and patients), positive associations were found between P-selectin and E-selectin ($r_s = 0.394$; $p < 0.001$) and between P-selectin and VCAM-1 ($r_s = 0.473$; $p < 0.001$), and a negative association between P-selectin and ICAM-1 ($r_s = -0.349$; $p < 0.001$). However, considering separately controls and patients, the association between P-selectin and E-selectin was significant among controls ($r_s = 0.564$; $p < 0.001$) but not among patients ($r_s = 0.028$; $p = 0.851$), and the association between P-selectin and VCAM-1 among patients ($r_s = 0.491$; $p < 0.001$) but not among controls ($r_s = 0.039$; $p = 0.849$). The association between P-selectin and ICAM-1 was non-significant in both groups (controls: $r_s = -0.141$; $p = 0.384$; patients: $r_s = -0.068$; $p = 0.649$; Figure 1). No statistically significant associations were observed between the serum levels of the three referred molecules and age ($p > 0.05$ for all correlations; data not presented).

Comparing the study population and healthy controls highly statistically significant differences were found in serum levels of P-selectin ($p < 0.001$), E-selectin ($p = 0.012$), VCAM-1 ($p < 0.001$), ICAM-1 ($p < 0.001$) (table 3). For the first three molecules, patients had higher levels comparing with controls, but ICAM-1 serum levels were inferior in study population (Table 3).

Figure 1 – Controls/Patients, Controls e Patients - the association between E-selectin, VCAM-1 and ICAM-1 for both groups

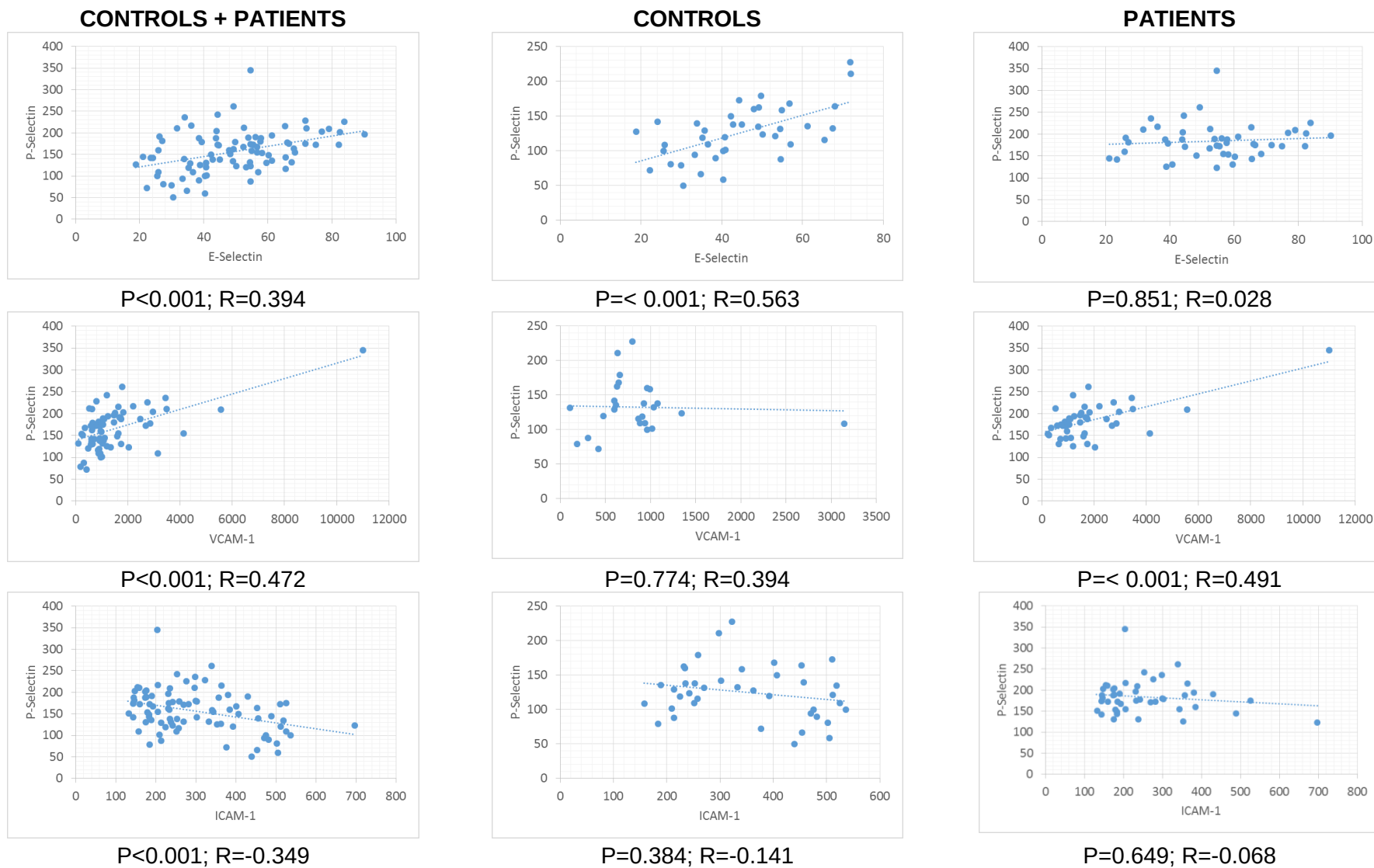


Table 3 – VCAM-1, ICAM-1, E-selectin and P-selectin levels in study population and healthy controls, and according to Systemic Sclerosis subsets.

	VCAM-1 (ng/ml)	<i>p</i> *	ICAM-1 (ng/ml)	<i>p</i> *	E-selectin (pg/ml)	<i>p</i> *	P-selectin (pg/ml)	<i>p</i> *
Healthy controls (n=40)	833 (112-3143)		337 (158-536)		43 (19-72)		100 (50-228)	
Study population (n=48)	1469 (167-11000)	<0.001	205 (130-697)	<0.001	55 (21-90)	0.012	181 (123-610)	<0.001
Pre-SSc (n=9)	962 (595-2856)	0.035	205 (144-344)	0.004	55 (36-68)	0.050	172 (143-216)	<0.001
SSc (n=39)	1472 (167-11000)	<0.001	218 (130-697)	<0.001	54 (21-90)	0.028	188 (123-610)	<0.001
lcSSc (n=27)	1684 (237-11000)	<0.001	193 (130-697)	<0.001	53 (21-90)	0.074	190 (123-610)	<0.001
dcSSc (n=12)	1123 (167-4147)	0.019	238 (143-381)	0.028	57 (23-75)	0.065	175 (126-262)	<0.001
lcSSc / non-CREST (n=15)	1823 (237-11000)	0.002	177 (130-526)	0.001	48 (27-84)	0.205	190 (130-610)	<0.001
lcSSc / CREST (n=12)	1469 (363-5565)	0.003	234 (155-697)	0.056	54 (21-90)	0.113	186 (123-242)	<0.001

Results presented as median (range) values. * *p* values: Patients vs. Controls (Mann-Whitney U test) . Abbreviations: SSc, Scleroderma; dcSSc, diffuse cutaneous Scleroderma; lcSSc, limited cutaneous Scleroderma; CREST, Calcinosis, Raynaud phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia; VCAM-1, Vascular Cell Adhesion Molecule-1; ICAM-1, Intercellular Cell Adhesion Molecule-1. One patient missing for ICAM-1.

Regarding disease subsets, we found that P-selectin and VCAM-1 serum levels were significantly higher in Pre-SSc ($p=0.001$; $p=0.035$), lcSSc ($p<0.001$; $p<0.001$) and dcSSc ($p<0.001$; $p=0.019$) patients in comparison with controls. In contrast, ICAM-1 concentrations were significantly lower in Pre-SSc ($p=0.004$), lcSSc ($p<0.001$) and dcSSc ($p=0.028$). For E-selectin, there is a tendency regarding Pre-SSc ($p=0.050$) and a statistically significant difference in SSc ($p=0.028$) with E-selectin concentrations being superior in the study population. However, when we analysed the disease subsets (lcSSc and dcSSc), there is only a central tendency again ($p=0.074$ and $p=0.065$, respectively) (Table 3).

Concerning patients with lcSSc, non-CREST and CREST, statistically significant differences were observed between study population and healthy controls for P-selectin ($p<0.001$, in both cases) and VCAM-1 ($p=0.002$ and $p=0.003$, respectively) (Table 3). These differences were not observed for E-selectin. ICAM-1 concentrations were lower in non-CREST and in CREST patients, although differences reached statistical significance only in the first case ($p=0.001$ and $p=0.056$, respectively).

When we compared all subgroups we verified that no differences were found, for all adhesion molecules, between Pre-SSc and SSc, lcSSc and dcSSc, CREST and non-CREST patients ($p>0.050$ in all cases).

Analysis according to disease evolution phases revealed that P-selectin and VCAM-1 serum levels were significantly higher in all phases as compared to controls (Table 4). Regarding E-selectin, there were no differences in early SSc phase, but as the disease evolves there is a tendency to increased concentration values in intermediate and late SSc stages ($p=0.069$ and $p=0.050$, respectively). ICAM-1 concentrations were significantly lower in intermediate and late disease stages, as compared to controls ($p=0.029$ and $p<0.001$, respectively) (Table 4).

Table 4 – VCAM-1, ICAM-1, E-selectin and P-selectin levels in study population and healthy controls and according to Systemic Sclerosis evolution phase

	VCAM-1 (ng/ml)	p^*	ICAM-1 (ng/ml)	p^*	E-selectin (pg/ml)	p^*	P-selectin (pg/ml)	p^*
Healthy controls (n=40)	833 (112-3143)		337 (158-536)		43 (19-72)		100 (50-228)	
Early SSc (n=6)	1419 (742-4147)	0.004	394 (205-526)	0.535	50 (21-67)	0.696	181 (145-235)	0.002
Intermediate SSc (n=10)	1645 (237-11000)	0.015	195 (146-697)	0.029	56 (26-82)	0.069	177 (123-345)	<0.001
Late SSc (n=23)	1472 (167-11000)	0.002	191 (130-365)	<0.001	52 (23-90)	0.050	193 (126-610)	<0.001

Results presented as median (range) values; * p values: Patients vs. Controls (Mann-Whitney U test)

Abbreviations: SSc, Scleroderma; VCAM-1, Vascular Cell Adhesion Molecule-1; ICAM-1, Intercellular Cell Adhesion Molecule-1.

Comparisons between the three disease evolution phases (Kruskal-Wallis test) showed no significant differences for VCAM-1 ($p=0.808$), E-selectin ($p=0.529$) and P-selectin. For ICAM-1 differences were found between the three phases ($p=0.013$); post-hoc analysis (Mann-Whitney test) revealed significant differences between early and late phases ($p=0.004$) but not between early and intermediate ($p=0.065$) or intermediate and late ($p=0.255$).

Due to the small size of some groups, an additional analysis was made comparing adjacent phases combined with controls. Significant differences were found between phases

early + intermediate and controls for VCAM-1 ($p=0.001$) and P-selectin ($p<0.001$), but not for ICAM-1 ($p=0.198$) or E-selectin ($p=0.115$). When phases intermediate and late were combined, significant differences (vs. controls) were found for all the molecules (VCAM-1: $p=0.001$; ICAM-1: $p<0.001$; E-selectin: $p=0.018$; P-selectin: $p<0.001$).

Regarding the pattern of nailfold capillaroscopy, P-selectin levels were significantly higher in all patterns as compared to controls (table 5) ($p<0.001$ in all cases). For E-selectin there was only statistical significance for the late pattern ($p=0.005$), with a tendency in the active pattern ($p=0.083$), both capillaroscopic patterns presenting higher values. VCAM-1 serum levels were significantly higher in all capillaroscopic patterns ($p=0.014$, $p=0.003$ and $p<0.001$ for early, active and late, respectively), whereas ICAM-1 concentrations were significantly lower in all capillaroscopic patterns ($p=0.007$, $p=0.002$ and $p=0.006$ for early, active and late, respectively) (Table 5).

Table 5 – Relation between capillaroscopic patterns and VCAM-1, ICAM-1, E-selectin and P-selectin serum levels in Pre-SSc and SSc patients (n=47)

	VCAM-1 (ng/ml)	p^*	ICAM-1 (ng/ml)	p^*	E-selectin (pg/ml)	p^*	P-selectin (pg/ml)	p^*
Healthy controls (n=40)	833 (112-3143)		337 (158-536)		43 (19-72)		100 (50-228)	
Early (n=11)	1117 (268-3496)	0.014	205 (136-489)	0.007	55 (21-75)	0.208	173 (143-216)	<0.001
Active (n=19)	1647 (237-11000)	0.003	190 (144-697)	0.002	54 (23-79)	0.083	189 (123-345)	<0.001
Late (n=14)	1488 (363-4147)	<0.001	232 (146-430)	0.006	58 (27-90)	0.005	178 (126-262)	<0.001

Results presented as median (range) values; * p values: Patients vs. Controls (Mann-Whitney U test). Abbreviations: SSc, Scleroderma; VCAM-1, Vascular Cell Adhesion Molecule-1; ICAM-1, Intercellular Cell Adhesion Molecule-1.

Comparisons between the three capillaroscopic patterns (Kruskal-Wallis test) showed no significant differences for any of the molecules (VCAM-1: $p=0.893$; ICAM-1: $p=0.717$; E-selectin: $p=0.182$; P-selectin: $p=0.288$).

Analysis comparing patients presenting organ involvement (score $\geq$ 1) with patients without organ involvement (score=0), showed that patients with articular disease had significantly lower VCAM-1 and a tendency to lower P-selectin serum levels ($p=0.007$ and

$p=0.066$, respectively); no statistically significant differences in the serum levels of VCAM-1, ICAM-1, P-selectin and E-selectin were observed for the other organs analysed ($p>0.050$ in all cases). However, patients having lung and/or heart involvement showed a tendency to have higher ICAM-1 levels, as compared with patients without involvement of these organs ($p=0.099$ and $p=0.089$, respectively). A zero Medsger skin score corresponds indeed to the Pre-SSc subset.

Respecting to the severity of organ involvement, when comparing patients with score=1 with patients with score ≥ 2 we observed that ICAM-1 levels had a tendency to be higher in patients with more serious heart involvement (Table 6) and that E-selectin serum levels had a tendency to be higher in patients presenting more serious lung involvement (score ≥ 2) ($p=0.066$ in both cases) (Table 7). No significant differences were found in the other organ analysis regarding to the remaining molecules (Tables 6 and 7).

Sub-analysis for lung involvement revealed no significant differences for TC changes, pulmonary function tests and PSAP considered separately ($p>0.050$; data not shown).

Sub-analysis for vascular involvement displayed no significant differences between Raynaud disease with or without digital ulcers ($p>0.050$; data not shown).

Table 6 – VCAM-1 and ICAM-1 levels in Pre-SSc and SSc patients, according to organ involvement and Medsger severity scale.

ORGAN	VCAM-1						ICAM-1					
	(ng/ml)						(ng/ml)					
	Involvement			Severity			Involvement			Severity		
	Score = 0	Score ≥ 1	p	Score = 1	Score ≥ 2	p	Score = 0	Score ≥ 1	p	Score = 1	Score ≥ 2	p
Skin	962 (595-2856)	1472 (167-11000)	0.468	1597 (167-11000)	1193 (643-4147)	0.386	205 (144-344)	218 (130-697)	0.808	190 (130-697)	282 (143-381)	0.164
Peripheral vascular	1592 (914-2856)	1465 (167-11000)	0.774	1641 (237-11000)	1155 (167-11000)	0.385	242 (184-302)	205 (130-697)	0.535	190 (130-697)	232 (133-526)	0.411
Gastrointestinal	1339 (167-11000)	1356 (237-5565)	0.703	- -	- -	-	204 (133-526)	218 (143-697)	0.694	- -	- -	-
Lung	1641 (237-11000)	1469 (167-11000)	0.991	1117 (167-4147)	1511 (363-11000)	0.296	185 (130-697)	238 (133-526)	0.099	218 (133-489)	277 (174-526)	0.456
Heart	1206 (167-11000)	1597 (363-4147)	0.491	1280 (914-4147)	1701 (363-3437)	0.777	185 (133-526)	273 (174-697)	0.089	191 (174-269)	290 (176-697)	0.066
Osteoarticular	1647 (167-11000)	852 (237-1721)	0.007	- -	- -	-	205 (130- 697)	268 (143-526)	0.456	- -	- -	-

The number of participants in each group can be obtained from the results presented in Table 2. Results presented as median (range) values.
Abbreviations: SSc, Scleroderma; VCAM-1, Vascular Cell Adhesion Molecule-1; ICAM-1, Intercellular Cell Adhesion Molecule-1.

Table 7 – E-selectin and P-selectin levels in Pre-SSc and SSc patients, according to organ involvement and Medsger severity scale

ORGAN	E-selectin (pg/ml)						P-selectin (pg/ml)					
	Involvement		<i>p</i>	Severity		<i>p</i>	Involvement		<i>p</i>	Severity		<i>p</i>
	Score = 0	Score ≥ 1		Score = 1	Score ≥ 2		Score = 0	Score ≥ 1		Score = 1	Score ≥ 2	
Skin	56 (36-68)	54 (21-90)	0.665	53 (21-90)	60 (23-75)	0.440	172 (143-216)	188 (123-610)	0.144	189 (123-610)	173 (126-262)	0.223
Peripheral Vascular	45 (36-66)	55 (21-90)	0.654	55 (26-90)	55 (21-84)	0.909	178 (149-216)	185 (123-610)	0.704	188 (123-610)	175 (126-345)	0.810
Gastrointestinal	56 (26-83)	54 (21-90)	0.961	- -	- -	-	182 (131-345)	177 (123-262)	0.506	- -	- -	-
Lung	58 (26-82)	52 (21-90)	0.333	44 (21-90)	58 (34-84)	0.066	178 (123-610)	187 (126-345)	0.973	183 (141-215)	194 (126-345)	0.380
Heart	56 (21-82)	55 (26-84)	0.932	55 (45-83)	55 (26-84)	0.777	176 (131-345)	185 (123-235)	0.977	180 (155-202)	185 (123-235)	0.777
Osteoarticular	55 (26-90)	53 (21-68)	0.394	- -	- -	-	188 (123-610)	164 (141-211)	0.066	- -	- -	-

The number of participants in each group can be obtained from the results presented in Table 2. Results presented as median (range) values.

Abbreviations: SSc, Scleroderma.

Discussion

Adhesion molecules play an important role in Scleroderma pathogenesis. Selectins mediate tethering and rolling of leucocytes on the endothelium [10], whereas VCAM-1 and ICAM-1 promote leucocyte transmigration over the endothelium [3].

Several studies have compared adhesion molecules levels between SSc patients and healthy controls in serum [3, 5, 10-12, 28-30] and also in tissues [28, 31-32] and, in line with those, in our study we found higher serum levels of VCAM-1, P and E selectins in SSc spectrum disease patients (Pre-SSc and SSc). In opposition, we observed lower levels of ICAM-1 in SSc spectrum disease patients, with the exception of higher levels found in the early phase of the SSc disease. Despite not having a definite answer to this last result, according to Wolf *et al.* [33], one possible explanation could be related to the presence of agonist anti-ICAM-1 antibodies in serum from patients with SSc that activate reactive oxygen species production and VCAM-1 expression. In the mentioned study it is not specified what happens to ICAM-1 serum levels in the presence of these antibodies.

Regarding disease subsets, the results reported in the literature are contradictory. Valentini *et al.* described superior levels of E-selectin and ICAM-1, but not of VCAM-1, in patients with Pre-SSc, as compared to healthy controls [14]. On the other hand, Kumanovics *et al.* found no differences in E-selectin levels between patients with Pre-SSc and healthy controls [30]. Concerning limited vs diffuse SSc, some authors have described high levels of E-selectin and VCAM-1 in both subsets [5, 34], while others have reported higher levels of VCAM-1 in diffuse [13, 34] or limited [35] SSc.

In our study, when patients were compared to controls, both selectins and VCAM-1 exhibited higher levels in all disease subsets (despite the results didn't reach statistical significance for E-selectin) whereas lower levels were observed for ICAM-1.

Several studies have tried to relate the evolution phase of SSc with adhesion molecules levels. Valim *et al.* found superior levels of E-selectin in the first four years of disease [13]. Hasegawa *et al.* evaluated ICAM-1, and E and P selectins in early SSc (< 3 years) and concluded that the serum levels of these adhesion molecules were superior in patients as compared to controls [12]. In our study, we found higher levels of the 3 molecules and also VCAM-1 in early phase, although statistical significance was achieved only for P-selectin and VCAM-1. For patients in intermediate and late phases, ICAM-1 was significantly lower (compared to controls); as for the other molecules, patients' values were also higher, being these differences statistically significant for P-selectin and VCAM-1 in both intermediate and late phases and for E-selectin in late phase.

Concerning the capillaroscopic patterns, Valim *et al.* found a relationship of E-selectin levels with capillary deletion, but not with capillary ectasia [13]. Similarly, in our study we found

that E-selectin levels were significantly higher in the late capillaroscopic pattern. On the other hand, we found, as far as we know for the first time that levels of P-selectin and VCAM-1 were significantly higher and levels of ICAM-1 were significantly lower in all capillaroscopic patterns.

Some studies had already tried to establish a relationship between adhesion molecules and organ involvement and disease severity and activity [4,36]. Regarding peripheral vascular involvement, our study, like others [5,11,34,38], revealed no significant differences between the levels of adhesion molecules and the presence of digital ulcers, in opposition to Sfikaki *et al.* who found higher ICAM-1 levels in those patients [38]. Some studies have also analyzed the relationship between skin score and adhesion molecules and, in consonance with our results, most failed to achieve a significant association [5, 11, 30, 37], with the exception of Veale *et al.* who described an opposite correlation between ICAM-1 and the skin score [39]. Concerning lung involvement, Nomura *et al.* studied the same adhesion molecules as we did in patients with and without interstitial lung disease (ILD), and they concluded that only P-selectin and ICAM-1 had statistically higher levels in ILD patients [29]. Similarly, Hasegawa *et al.* showed higher levels of ICAM-1 in ILD patients with an opposite correlation between ICAM-1 and FVC in anti-Scl70 patients [12], and Ihn *et al.* showed higher ICAM [40], VCAM and E-selectin [34] levels in patients with lung involvement evaluated by spirometry and DLCO [34]. Moreover, Pendergrass *et al.* [9] found a correlation between ICAM-1 and VCAM-1 molecules and PAH in limited SSc, and Iannone *et al.* [38] found higher VCAM-1 levels in patients without PAH versus PAH patients. By opposition, in our study we didn't find a relationship between higher VCAM-1 levels and lung involvement or severity (CT scan, PSAP, DLCO and FVC) but we found a marginal relationship between higher ICAM levels and lung involvement among patients.

In regard to E-selectin and lung disease, besides the results described above, some studies established a positive relationship between its levels and fibrosis on chest x-ray [11], diffuse fibrosis on CT scan [30], and DLCO and FVC alterations [11,12,30], considering E-selectin as a pulmonary fibrosis evolution and severity biomarker [30]. Our results are in consonance with these studies, relating positively E-selectin levels with the severity of lung involvement.

Also besides the results previously described for P-selectin, Yanaba *et al.* report that raised serum levels of P-selectin glycoprotein ligand 1 (PSGL-1) are associated with a lower frequency and severity of pulmonary involvement, which, in turn, seems to show a role of P-selectin in pulmonary fibrosis [10]. Furthermore, Iversen *et al.* found a correlation between P-selectin levels and DLCO but not with FVC [11]. In our study, we have not found a relationship between P-selectin levels and pulmonary involvement or severity.

Once more, to the best of our knowledge, our results concerning the relationship between ICAM-1 levels and heart involvement and severity are the first reported in the literature.

Inh *et al.* described positive relationships between VCAM-1 [34] and ICAM-1 [40] levels and osteoarticular involvement; by opposition we found a negative relationship between lower VCAM-1 levels and this involvement.

Ihn *et al.* [34] demonstrated that ICAM-1 and VCAM-1 serum levels were significantly correlated in patients with SSc but E-selectin levels showed no correlation with either ICAM-1 or VCAM-1 levels; in our study we found a positive correlation between P-selectin and E-selectin in controls but not in SSc spectrum disease patients, and, inversely, a positive correlation between P-selectin and VCAM-1 levels in patients but not in the control group.

The discrepancy of results between patients and healthy controls may contribute to clarify the pathogenesis of SSc.

In summary, we have demonstrated that SSc spectrum disease is associated with higher levels of selectins and VCAM-1, and lower levels of ICAM-1; in early phase, SSc is associated to higher levels of all adhesion molecules, but as the disease evolves, the levels of ICAM-1 become significantly lower. No differences were found in adhesion molecules between diffuse and limited SSc. Although ICAM-1 and E-selectin appear to have a role in pulmonary pathogenesis, E-selectin is related to pulmonary severity. ICAM-1 also appears to have a role in heart SS pathogenesis and severity and VCAM-1 and P-selectin a role in osteoarticular involvement.

P-selectin and VCAM-1 were consistently elevated in all phases, subsets and capillaroscopic patterns, making these molecules early and persistent markers throughout the course of the disease.

The systematic observation of elevated E-selectin levels in more advanced stages of disease, with late capillaroscopic changes, or in a global view in patients with more severe organ involvement, allow us to hypothesize that E-selectin has a broad impact in disease progression and relates to lung pathogenesis and severity.

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4.3 – EVOLUÇÃO DOS DOENTES AO LONGO DO FOLLOW-UP

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Introdução

As manifestações clínicas da Esclerose Sistêmica (ES) são heterogêneas e a sua evolução é variável [1]. A doença caracteriza-se pela existência de um envolvimento vascular generalizado, lesão endotelial [2] angiogénese insuficiente, desregulação imunológica e fibrose progressiva da pele e órgãos internos [3].

A esclerose da pele é um sinal cardinal da ES [4]. Geralmente inicia-se pelos dedos das mãos e as manifestações cutâneas, que ocorrem na maioria dos doentes, não só servem para o diagnóstico da doença, mas também para a subclassificar nos seus subtipos [4].

Os subtipos limitado e difuso distinguem-se com base na extensão do pregueamento cutâneo [5], efetuado de acordo com os critérios de Rodman [6]. O subtipo limitado é o mais prevalente [7] e associa-se frequentemente à presença de anticorpos anti-centrómero (ACA), enquanto o subtipo difuso se associa à presença de anticorpos anti-topoisomerase (Scl70) [8]. Diferenças entre estes dois subtipos são a velocidade com que a doença progride assim como a extensão e gravidade do envolvimento da pele e de outros órgãos. O subtipo limitado costuma ter um início mais insidioso e associa-se, fundamentalmente, a alterações vasculares; por outro lado, o subtipo difuso costuma ter um início mais rápido, atingimento cutâneo mais extenso e envolvimento orgânico mais precoce [4].

O *score* cutâneo constitui uma medida de gravidade da doença e tem significado prognóstico, principalmente no subtipo difuso [9]. Neste subtipo, o pregueamento cutâneo atinge, geralmente, o pico máximo entre os 3 e os 5 anos de evolução da doença e, posteriormente, tende a melhorar [10].

Os doentes com maior pico de *score* cutâneo são os que têm mais probabilidade de melhorar e os que não melhoram são os que têm maior risco de envolvimento orgânico [4].

A Pré-Esclerodermia (Pré-ES) caracteriza-se pela presença de Fenómeno de Raynaud associado a autoanticorpos específicos da doença esclerodérmica e/ou alterações típicas na capilaroscopia periungueal e pregueamento cutâneo normal [11]. Os doentes com alterações típicas na capilaroscopia e anticorpos específicos da doença têm uma probabilidade situada entre 65.9% e 72.7% de evoluírem para ES ao fim de 5 e 10 anos, respetivamente. Se tiverem apenas padrão capilaroscópico típico associado ao Fenómeno de Raynaud, há uma redução do risco para 22.6% aos 5 e 10 anos e se tiverem apenas autoanticorpos específicos da doença associados ao Fenómeno de Raynaud, a probabilidade de evolução altera-se para 21.5% e 32.3% aos 5 e 10 anos, respetivamente [12].

O Fenómeno de Raynaud caracteriza-se por episódios de isquemia digital, provocados por vasospasmo, despertado, fundamentalmente, pelo frio ou pelo *stress*, manifestando-se por alteração da cor da pele que passa de branca (ou isquémica) a cianótica e vermelha (ou hiperémica) [13]. É observado em mais de 90% dos doentes com ES [14] sendo,

frequentemente, a primeira manifestação da doença. Inicialmente com implicações apenas funcionais, episódios repetidos conduzem a alterações estruturais e isquemia digital que pode progredir e dar origem a úlceras ou isquemia crítica com gangrena [15].

As úlceras digitais (UD) ocorrem em mais de 50% dos doentes e caracterizam-se pela existência de lesões necróticas nas extremidades dos dedos das mãos ou pés ou sobre proeminências ósseas [16]. São muito dolorosas, incapacitantes, de cicatrização lenta e associam-se frequentemente a complicações infecciosas [17]. Podem ser múltiplas [18], crônicas (permanecem mais de 6 meses) e, em 32% dos casos, persistentes [18,19]. Por ano, em média, cerca de 30% dos doentes desenvolvem úlceras digitais [20]. São mais frequentes em doentes com o subtipo difuso [8,21], com o anticorpo anti-Scl70 [22-26] e associam-se mais frequentemente ao padrão capilaroscópico tardio [27].

Tem sido estabelecida uma associação entre a presença de úlceras digitais e um curso mais grave da doença, nomeadamente a nível da pele e do pulmão (doença vascular pulmonar e/ou intersticial) [23,28-30]. O atingimento orgânico nos doentes com úlceras parece ser mais precoce do que naqueles sem úlceras [23] e as úlceras poderão anunciar a existência de atingimento orgânico mesmo nos doentes com *very early scleroderma* ou Pré-ES [31].

O envolvimento gastrointestinal afeta mais de 90% dos doentes com ES [5,32,33], sendo a sua extensão e gravidade variáveis [34]. Apesar de todo o tubo digestivo poder estar envolvido, o esófago é o órgão mais atingido [34].

A prevalência do envolvimento digestivo parece ser sobreponível em ambos os subtipos da doença [22,34] e não ter relação com o tipo de anticorpos [34, 22], exceto quando o envolvimento esofágico é grave ou quando há atingimento gástrico [22,35]. Este envolvimento contribui de forma significativa para a morbilidade da doença esclerodérmica, influenciando a qualidade de vida dos doentes [34].

A prevalência da doença cardíaca é variável conforme a definição e os testes que são utilizados [36] e, de acordo com Ferri *et al.*, pode atingir 30-35% [16]. Uma meta-análise internacional posterior [37] refere que o atingimento cardíaco pode estar presente em cerca de 10% dos doentes ao longo da evolução da doença (variação entre 8 e 28%, de acordo com as séries) e outros estudos mostram que a prevalência pode chegar próximo dos 100%, dependendo da sensibilidade dos exames utilizados [38,39,40].

O envolvimento cardíaco, frequentemente assintomático, pode afetar as várias estruturas, nomeadamente o pericárdio, miocárdio e endocárdio e pode expressar-se por alterações da condução, arritmias, insuficiência valvular, derrame pericárdio, isquemia do miocárdio, hipertrofia miocárdica e insuficiência cardíaca [36], a maioria das vezes, diastólica. Por outro lado, pode ser secundário ao envolvimento de outros órgãos, nomeadamente do rim e do pulmão [36]. Ocorre em ambos os subtipos da doença, mas há estudos que apontam para uma maior incidência no subtipo difuso [16,41] e outros para uma maior incidência no

limitado [42]. É mais frequente em doentes com anticorpos anti-Scl70 [37] e quando sintomático, este envolvimento associa-se a mau prognóstico (sobrevivência aos 2 e 5 anos inferior a 40 e 20%, respetivamente) [43].

A doença pulmonar é, atualmente, a grande causa de morte nos doentes com ES [44], sendo a hipertensão arterial pulmonar (HTAP) e a doença intersticial (DIP) as suas duas grandes vertentes. A HTAP ocorre, de forma isolada, sem alterações evidentes do interstício pulmonar, em 12 a 16% dos doentes e está mais associada ao subtipo limitado e à presença de anticorpos ACA [45,46]. A prevalência da DIP varia conforme a população estudada, a definição utilizada e os métodos usados para a diagnosticar [47], estando mais associada ao subtipo difuso e à presença de anticorpos anti-Scl70 [8].

Embora estudos baseados em autópsias revelem a existência de pelo menos algum grau de envolvimento do interstício pulmonar em quase todos os doentes com ES [48], 55 a 65% têm alterações na tomografia computadorizada de alta resolução do tórax e mais de 96% nas PFR [49,50]. Contudo, apenas cerca de um terço tem sintomatologia significativa [47].

Neste capítulo é descrita a evolução clínica, entre 2011 e 2015, dos doentes incluídos no estudo e a sua relação com os subtipos da doença, autoanticorpos ACA e anti-Scl70, biomarcadores da angiogénese (VEGF, Endostatina, Angiostatina, VEGF/angiostatina, VEGF/endostatina) e de lesão endotelial (ICAM-1, VCAM-1, P-seletina, E-seletina, ET-1) e linfócitos T e células NK no sangue periférico.

Metodologia

A metodologia já foi previamente descrita no capítulo 3.

Os doentes sem avaliação de um determinado órgão no início do estudo e/ou último *follow-up*, foram excluídos da análise evolutiva desse órgão (Tabela 1).

Tabela1 – Doentes incluídos na análise orgânica evolutiva

Órgão	n
Pele	57
Vascular periférico	57
Tubo digestivo	51
Pulmão	55
Coração	49

Os doentes foram tratados de acordo com o envolvimento orgânico existente e segundo as recomendações da EULAR de 2009 e/ou opinião de peritos nesta área (veja-se a sub-secção 2.2).

Avaliação serológica

Os métodos utilizados para avaliar cada uma das moléculas referidas neste capítulo assim como os autoanticorpos e os linfócitos T e as células NK, já foram descritos previamente (vejam-se capítulo 3 e sub-secções 4.1 e 4.2).

Análise Estatística

A análise estatística foi efetuada no programa IBM SPSS versão 22.0 para Machintosh. A estatística descritiva consistiu no cálculo de frequências absolutas (n) e relativas (%) e de medianas. Utilizou-se o teste de Kolmogorov-Smirnov para testar a normalidade da distribuição das variáveis cardinais. Dado a maioria das variáveis não apresentarem distribuição normal, e dado o pequeno tamanho da amostra, optou-se pelo uso de testes não paramétricos em toda a análise. O teste de Mann-Whitney foi usado para comparar ordens médias de amostras independentes. Para analisar a evolução da doença, i.e., a mudança e/ou

estabilidade (grau de concordância) das manifestações orgânicas, de acordo com o *score* de Medsger, nos pontos extremos do estudo, i.e., 2011 e 2015, recorreu-se a tabelas de contingência e ao cálculo do Kappa de Cohen (κ). Com base na sugestão de Landis & Kock (1977) os valores de κ foram classificados do seguinte modo: $\kappa < 0.40$ = concordância reduzida (i.e., instabilidade no estadiamento de 2011 para 2015, ou seja uma alteração substancial nas transições do estadiamento); $0.40 < \kappa < 0.75$ = concordância moderada (estabilidade moderada, i.e., permanência moderada de casos nos respectivos estadiamentos de 2011 para 2015); e $\kappa > 0.75$ = concordância elevada (manutenção elevada de casos nos mesmos estadiamentos de 2011 para 2015, ou seja transições reduzidas). A hipótese nula foi rejeitada quando $p < 0.05$.

Resultados

As características sócio-demográficas e clínicas, bem como o envolvimento orgânico por subtipo de Esclerodermia e de acordo com o score de Medsger, já foram previamente descritas (veja-se sub-secção 4.1.1).

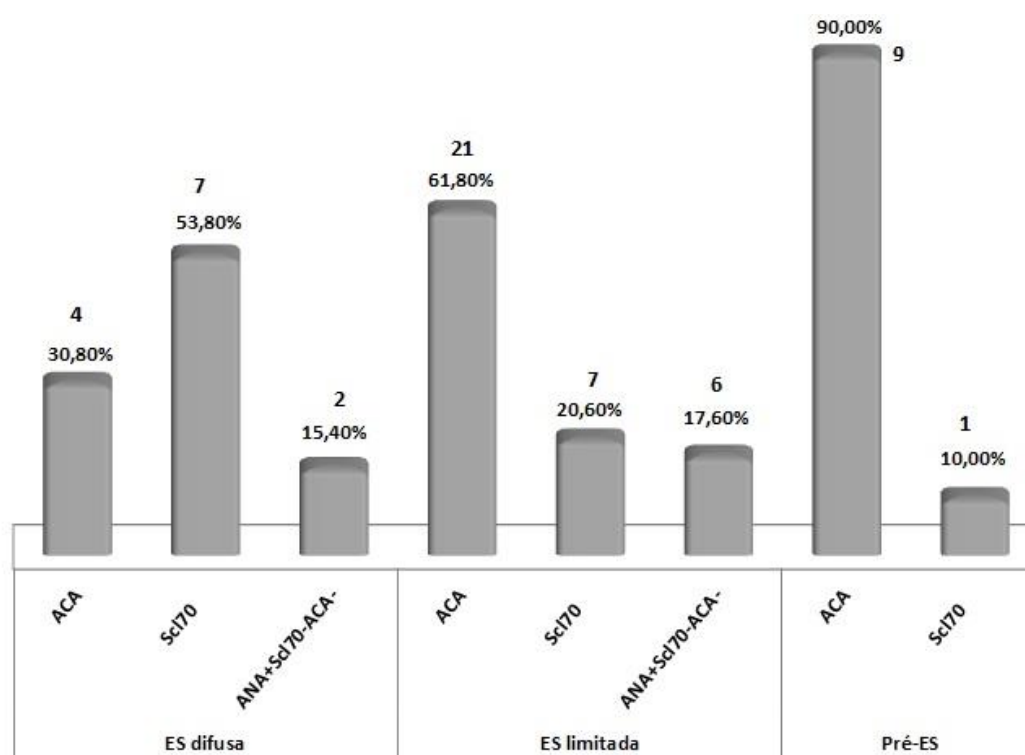
No início do estudo, a amostra era composta por 10 doentes com Pré-ES, 13 com ES difusa e 34 com ES limitada (Tabela 2).

Tabela 2 – Distribuição dos doentes por subtipo de doença no início do estudo

Subtipo da Doença	n	%
ES difusa	13	22.80%
ES limitada	34	59.60%
Pré-ES	10	17.50%
Total Geral	57	100%

O gráfico 1 apresenta a distribuição dos doentes de acordo com os anticorpos e o subtipo da doença esclerodérmica. Vinte e um doentes (61.8%) com o subtipo limitado tinham o anticorpo ACA e 7 (53.8%) com o subtipo difuso o anti-Scl70. Nove dos 10 doentes com Pré-ES tinham o anticorpo ACA.

Gráfico 1– Distribuição dos doentes de acordo com os subtipos e autoanticorpos



Relativamente à evolução clínica, de acordo com o *score* de Medsger e tendo em conta o valor de κ de Cohen, verificamos que os doentes evoluíram de forma relativamente instável, i.e., mudaram o *score* de estadiamento da doença, no atingimento vascular periférico e pele; a evolução gastrointestinal, pulmonar e cardíaca ocorreu de forma mais estável (tabela 3).

Tabela 3 – Evolução orgânica (mudança e estabilidade nos estadiamentos) de acordo com os *scores* da escala de gravidade de Medsger[#]

ÓRGÃO	Score		Score final					
	Inicial	Total	0	1	2	3	4	
Pele (n=57)	0	10	4	6	0	0	0	$\kappa:0.432$
	1	36	0	36	0	0	0	$p < 0.001$
	2	10	0	7	3	0	0	
	3	1	0	0	1	0	0	
	4	0	0	0	0	0	0	
Vascular Periférico (n=57)	0	5	0	5	0	0	0	$\kappa:0.307$
	1	23	1	16	1	5	0	$p = 0.001$
	2	7	0	2	0	5	0	
	3	20	0	4	0	16	0	
	4	2	0	0	0	2	0	
Tubo Digestivo (n=51)	0	26	17	8	1	0	0	$\kappa:0.579$
	1	25	1	24	0	0	0	$p < 0.001$
	2	0	0	0	0	0	0	
Pulmão (n=55)	0	17	16	1	0	0	0	$\kappa:0.629$
	1	20	1	13	5	1	0	$p < 0.001$
	2	13	0	1	10	2	0	
	3	2	0	0	0	0	2	
	4	3	0	0	0	2	1	
	-	-	-					
Coração (n=49)	0	32	23	2	5	1	1	$\kappa:0.647$
	1	5	0	4	0	0	1	$p < 0.001$
	2	7	0	0	7	0	0	
	3	5	0	0	0	4	1	
	4	0	0	0	0	0	0	

De “0” (sem envolvimento orgânico documentado ou ausência de necessidade de tratamento no caso do Raynaud) a “4” (estádio final); # resultados à data da última visita

No entanto, como o valor de κ de Cohen é influenciado pela maior ou menor concentração de valores nas diferentes categorias e esta é bastante variável entre órgãos, serão sobretudo valorizadas as proporções de doentes que mantiveram, pioraram ou melhoraram determinado *score* (tabela 4).

Tabela 4 – Evolução do envolvimento orgânico ao longo do estudo

Órgão	Agravamento	Melhoria	Estabilidade	Total
	n (%)	n (%)	n (%)	n
Pele	6 (10.5%)	8 (14.0%)	43 (75.4%)	57
Vascular	16 (28.1%)	9 (15.8%)	32 (56.1%)	57
Digestivo	9 (17.7%)	1 (2%)	41 (80.3%)	51
Pulmão	11 (20%)	4 (7.3%)	40 (72.7%)	55
Coração	11 (22.4%)	0 (0%)	38 (77.6%)	49

Relação entre a evolução clínica, subtipos de doença e autoanticorpos

Pele

No que diz respeito à pele, ao longo dos 4 anos de *follow-up* clínico, 6 doentes agravaram o *score*, 8 melhoraram e 43 mantiveram-se estáveis (tabelas 3, 4 e 5).

Os doentes com *score* de Medsger mais elevado (≥ 2) no início do estudo tiveram maior atingimento orgânico no *follow-up* (63.7% alterações cardíacas, 81.8% alterações digestivas, 81.8% úlceras digitais e 91.1% alterações pulmonares) do que aqueles com *score* de Medsger mais baixo (*score*=1; 51.5% alterações cardíacas, 54.3% alterações digestivas, 50% úlceras digitais e 72% alterações pulmonares).

Tabela 5 – Evolução do Score de Medsger para a pele ao longo do *follow-up* e relação com o subtipo e fase da doença.

36 doentes mantiveram-se no score 1 • 33 com subtipo limitado (5 na fase precoce, 8 na intermédia e 20 na tardia) • 3 com subtipo difuso (todos na fase tardia)
4 doentes mantiveram-se no score 0 (= Pré-ES)
3 doentes mantiveram-se no score 2 • Todos com o subtipo difuso (2 na fase precoce e 1 na intermédia)
8 doentes diminuíram de score cutâneo • 7 passaram do score 2 para 1: 6 subtipo difuso (1 na fase intermédia e 5 na fase tardia) e 1 subtipo limitado na fase tardia • 1 passou do score 3 para 2: subtipo difuso na fase tardia
6 doentes aumentaram de score cutâneo: 0 para 1 • Pré-Esclerodermia (score 0) para Esclerodermia Limitada (score 1)

Vascular periférico

No início do estudo, todos os doentes tinham envolvimento vascular periférico mas 5 (8.8%) não precisavam de tratamento para o Fenómeno de Raynaud (score 0 de Medsger); 92.3% [12] dos doentes com ES difusa, 47.1% [16] dos doentes com ES limitada e 1 doente (10%) com Pré-ES (29 doentes no total), tinham úlceras digitais ativas ou *pitting scars* (tabela 6).

Ao longo dos 4 anos de *follow-up* 16 doentes agravaram, 9 melhoraram e 32 mantiveram-se estáveis (tabelas 3 e 4).

Dezoito (81.8%) dos 22 doentes com úlceras digitais no *baseline* tiveram úlceras recorrentes e 4 melhoraram sob terapêutica. Ao longo do *follow-up*, 10 dos doentes sem história de úlceras digitais desenvolveram úlceras digitais de novo. No final do estudo, o número de doentes com ES limitada e com úlceras ou *pitting* (ativas ou passadas) aumentou para 64.7% (22 doentes; tabela 6).

Tabela 6 – Evolução do envolvimento vascular periférico durante o *follow-up* e relação com o subtipo da doença e autoanticorpos

	ACA (n= 34)	ScI70 (n=15)	ANA (n=57)	Pré-ES (n=10)	ES Limitada (n=34)	ES Difusa (n=13)
22 doentes com úlceras digitais/isquemia no <i>baseline</i>	8 [#] (23.5%)	8 [#] (53.3%)	7* (12.3%)	1(10.0%)	13 (38.2%)	8 (61.5%)
18 doentes com úlceras digitais recorrentes no <i>follow-up</i>	7 [#] (20.6%)	7 [#] (46.7%)	5* (8.8%)	1(10.0%)	11 (32.4%)	6 (46.1%)
7 doentes sem úlceras digitais mas com <i>pitting</i> no <i>baseline</i>	3 (8.8%)	2 (13.3%)	2* (3.5%)	0 (0%)	3 (8.8%)	4 (30.7%)
5 doentes com úlceras digitais de novo no <i>follow-up</i>	2 (5.9%)	1 (6.7%)	2* (3.5%)	0 (0%)	2 (5.9%)	3 (23.1%)
28 doentes sem úlceras digitais/ <i>pitting</i> no <i>baseline</i>	18 (52.9%)	4 (26.7%)	6* (10.5%)	9 (90%)	18 (52.9%)	1 (7.7%)
5 doentes com úlceras digitais de novo no <i>follow-up</i>	3U (8.8%)	1U (6.7%)	1U* (1.8%) 1P* (1.8%)	0 (0%)	5U [#] (14.7%) 1P& (2.9%)	0 (0%)

Abreviaturas: Pré-ES: Pré-Esclerodermia; ES, Esclerodermia; U, Úlcera; P, *Pitting scar*; ACA: anticorpos anticentrômero; ScI70: anticorpos anti-topoisomerase; ANA: anticorpos antinucleares.

Percentagens em função dos autoanticorpos ou do subtipo da doença.

Um doente com ACA e ScI70 positivos; * ANA positivos, ACA e ScI70 negativos.

Quanto ao padrão serológico, verificamos que 73.3% dos doentes com anti-ScI70 e 41.2% dos doentes com ACA tiveram úlceras ou *pitting scars* ao longo do *follow-up* (tabela 6).

Dos 22 doentes que tinham úlceras ativas/isquemia no início do estudo, com ou sem *pitting scars* associadas, 50% (11 doentes) apresentavam padrão tardio na capilaroscopia, 31.8% (7 doentes) padrão ativo e 3 doentes padrão precoce (13.6%) e 1 (4.5%) alterações *minor*.

Dos 35 doentes sem úlceras ativas no início do estudo (incluindo os 7 doentes que tinham *pitting scars*), 2 não realizaram capilaroscopia, 3 (8.6%) tinham alterações *minor*, 10 padrão precoce (28.6%), 10 padrão ativo (28.6%) e 7 (20%) padrão tardio.

Dos 10 doentes que desenvolveram úlceras ativas de novo durante o *follow-up*, 1 (10%) tinha padrão precoce na capilaroscopia, 6 (60%) padrão ativo e 3 (30%) padrão tardio.

Dos doentes com úlceras digitais no início ou durante o estudo, 65.6% tinham ou desenvolveram alterações digestivas, 59.4% doença pulmonar intersticial (5 com alterações *minor* na TAC do tórax), 50% alterações cardíacas, 15.6% aumento da PSAP (3 com HTAP confirmada por cateterismo) e 5 (15.6%) alterações isoladas da DLCO (tabela 7).

Tabela 7 – Doentes com úlceras digitais ativas durante o estudo e relação com envolvimento orgânico

	DLCO diminuída	DIP	PSAP aumentada	Alterações ritmo/condução	Insuficiência cardíaca	Alterações GI
Úlceras digitais no <i>baseline</i> (n=22)	5 (22.7%)	13 (59.1%)	4 [#] (18.2%)	9 (40.9%)	3 (13.6%)	15 (68.2%)
Úlceras digitais de novo no <i>follow-up</i> (n=10)	-	6 (60%)	1 (10%)	4 (40%)	0 (0%)	6 (60%)

Abreviaturas: DLCO, capacidade de difusão do monóxido de carbono; DIP, doença intersticial pulmonar; GI, gastrointestinal; PSAP, pressão na artéria pulmonar.

Percentagens em função da presença de úlceras digitais no *baseline* ou de úlceras digitais de novo no *follow-up*.

Cateterismo normal em 2 doentes.

Tubo Digestivo

Ao longo do *follow-up*, 66.7% dos doentes (34/51) tiveram alterações digestivas, caracterizadas pela existência de dismotilidade esofágica e, num caso, por dismotilidade do delgado associado a supercrescimento bacteriano. Um dos doentes tinha dismotilidade gástrica grave associada à dismotilidade esofágica.

No início do estudo, 46.9% dos doentes com subtipo limitado (15/32), 58.3% com subtipo difuso (7/12) e 42.9% com Pré-ES (3/7), apresentavam alterações digestivas, sendo que o valor aumentou, no final do estudo, para 59.4% (19/32), 83.3% (10/12) e 71.4% (5/7) no subtipo limitado, difuso e Pré-ES, respetivamente (tabela 8).

A motilidade digestiva do doente que apresentava dismotilidade esofágica e gástrica no início do estudo normalizou durante o *follow-up* com a terapêutica instituída. Dos 9 doentes que desenvolveram alterações durante o *follow-up*, 8 tinham dismotilidade esofágica e outro dismotilidade do delgado com super-crescimento bacteriano.

Quanto ao padrão serológico, verificamos que 92.3% dos doentes com anti-Scl70 e 70% dos doentes com ACA tiveram envolvimento digestivo (tabela 8).

Tabela 8 – Evolução das alterações digestivas ao longo do estudo e relação com o subtipo da doença e autoanticorpos

	ACA (n= 30)	Scl70 (n=13)	ANA (n=51)	Pré-ES (n=7)	ES Limitada (n=32)	ES Difusa (n=12)
25 doentes com alterações digestivas no <i>baseline</i>	14* (46.7%)	10* (76.9%)	2 [#] (3.9%)	3 (42.9%)	15 (46.9%) Fases:1P; 3I; 11T	7 (58.3%) Fases: 2P; 1I; 4T
1 doente melhorou no <i>follow-up</i>	0 (0%)	0 (0%)	1 [#] (2.0%)	0 (0%)	0 (0%)	1 (8.3%)
26 doentes sem alterações digestivas no <i>baseline</i>	16 (53.3%)	3 (23.1%)	7 [#] (13.7%)	4 (57.1%)	17 (53.1%) Fases: 4P; 5I; 8T	5 (41.7%) Fases:1I; 4T
9 doentes com alterações digestivas de novo no <i>follow-up</i>	7 (23.3%)	2 (15.4%)	0 (0%)	2 (28.6%)	4 (12.5%) Fases:1P; 3T	3 (25%) Fases: 3T

Abreviaturas: Pré-ES, Pré Esclerodermia; ES, Esclerodermia; ACA, anticorpos anti-centrômero; Scl70: anticorpos anti-topoisomerase; ANA, anticorpos anti-nucleares; Fases: P-precoces; I-intermédia; T-tardia.

Percentagens em função dos autoanticorpos ou dos subtipos da doença.

ANA positivos, Scl70 e ACA negativos * Um doente com ACA e Scl70 positivos.

Coração

Durante o estudo 11 doentes agravaram as suas manifestações cardíacas e 38 mantiveram-se estáveis (tabela 9).

As alterações cardíacas caracterizaram-se pela presença de arritmias/alterações da condução ou insuficiência cardíaca e manifestaram-se em 53.1% dos doentes.

Dos doentes que tinham envolvimento cardíaco no início do estudo, dois desenvolveram insuficiência cardíaca e faleceram. Nove dos 32 doentes que não tinham envolvimento cardíaco no início do estudo, desenvolveram alterações do ritmo ou da condução, e um outro insuficiência cardíaca (tabela 9).

Tabela 9 – Envolvimento cardíaco durante o estudo e relação com o subtipo da doença e autoanticorpos

	ACA (n= 30)	Scl70 (n=15)	ANA (n=49)	Pré-ES (n=7)	ES Limitada (n=30)	ES Difusa (n=12)
17 doentes com alterações cardíacas <i>baseline</i>	10 (33.3%)	7 (46.7%)	0 ^{&} (0%)	1 (14.3%)	12 (40%) Fases: 2P; 1I; 9T	4 (33.3%) Fases: 1P; 2I; 1T
2 doentes com agravamento cardíaco durante <i>follow-up</i>	0 (0%)	2 (13.3%)	0 ^{&} (0%)	0 (0%)	1 [#] (3.3%) Fases: 1T	1 [#] (8.3%) Fases: 1P
32 doentes sem alterações cardíacas <i>baseline</i>	20* (66.7%)	8* (53.3%) 4* (26.7%)	4 ^{&} (8.2%)	6 (85.7%)	18 (60%) Fases: 2P; 7I; 9T	8 (66.7%) Fases: 1P; 7T
9 doentes com alterações cardíacas de novo durante <i>follow-up</i>	5* (16.7%)	Insuficiência Cardíaca	1 ^{&} (2.0%)	1 (14.3%)	4 (13.3%) Fases: 2I; 2T Insuficiência Cardíaca	4 (33.3%) Fases: 4T

Abreviaturas: Pré-ES, Pré Esclerodermia; ES, Esclerodermia; ACA, anticorpos anticentrômero; Scl70, anticorpos anti-topoisomerase; ANA, anticorpos anti-nucleares; Fases: P-precoces; I-intermédias; T-tardias.

Percentagens em função dos autoanticorpos ou dos subtipos da doença.

* Um doente com ACA e Scl70 positivos; [&] ANA positivos, ACA e Scl70 negativos; [#] faleceu

No início do estudo, foram encontradas alterações cardíacas em 40.0 %, 33.3% e 14.3% dos doentes com subtipo limitado, difuso e Pré-ES, respetivamente, tendo aumentado para 53.3%, 66.7% e 28.6% durante o *follow-up* (tabela 9).

Quanto ao padrão serológico, verificamos que 73.3% dos doentes com anti-Scl70 e 50% daqueles com ACA tiveram envolvimento cardíaco (tabela 9).

Os dois doentes que faleceram tinham atingimento multiorgânico (DIP + HTAP + úlceras digitais e um digestivo) e anticorpos anti-Scl70 positivos.

Pulmão

No início do estudo, utilizando o ponto de corte da escala de gravidade de Medsger, 38/55 doentes (69.0%) tinham envolvimento pulmonar (tabela 10).

No entanto, se associarmos as alterações *minor* (hipertrofia dos septos, zonas limitadas de fibrose/ opacidades em vidro despolido) encontradas na TAC do tórax de alta resolução, essas alterações sobem para 74.5% e se analisarmos a ES separada da Pré-ES esse número aumenta para 80.9 %.

Durante o estudo, 11 doentes agravaram, 4 melhoraram e 40 mantiveram-se estáveis do ponto de vista pulmonar (tabelas 3,4 e 10).

Tabela 10 – Envolvimento pulmonar ao longo do estudo e relação com o subtipo da doença e autoanticorpos.

	ACA (n= 32)	Scl70 (n=15)	ANA (n=55)	Pré-ES (n=8) ^{&}	ES Limitada (n=34)	ES Difusa (n=13)
38 doentes com alterações pulmonares <i>baseline</i>	19*(59.4%)	12*(80%)	8 [#] (14.5%)	3 (37.5%)	24 (70.6%) Fases: 5P; 3I; 16T	11 (84.6%) Fases: 2P;1I;8T
4 doentes com melhoria durante o <i>follow-up</i>	3*(9.4%)	2*(13.3%)	0 (0%)	1 (12.5%)	1 (2.9%)	2 (15.4%)
10 doentes com agravamento durante o <i>follow-up</i>	2 (6.3%)	5 (33.3%)	3 [#] (5.5%)	0 (0%)	6 (17.6%) Fases: 2P; 2I; 2T	4 (30.8%) Fases: 2P; 1I;1T
17 doentes sem alterações pulmonares no <i>baseline</i>	13 (40.6%)	3 (20%)	1 [#] (1.8%)	5 (62.5%)	10 (29.4%) Fases: 5I;5T	2 (15.4%) Fases: 1I; 1T
1 doente com alterações pulmonares de novo no <i>follow-up</i>	0 (0%)	1 (6.7%)	0 (0%)	0 (0%)	0 (0%)	1 (7.7%) Fases:1I

Abreviaturas: Pré-ES, Pré Esclerodermia; ES, Esclerodermia; ACA, anticorpos anticentrômero; Scl70, anticorpos anti-topoisomerase; ANA, anticorpos antinucleares; Fases: P-precoce; I-intermédia; T-tardia;

Percentagens em função dos autoanticorpos ou dos subtipos da doença

* Um doente com ACA e Scl70 positivos; [&] Dois doentes com Pré-ES sem exames comparativos no início ou final de *follow-up*;

[#]ANA positivos, Scl70 e ACA negativos.

Dezasseis dos 55 doentes (12 ES limitadas, 3 difusas e 1 Pré-ES) tinham PSAP superior a 35 mmHg no ecocardiograma transtorácico no início do estudo, sendo que 8 efetuaram cateterismo cardíaco, tendo-se confirmado a existência de HTAP em 2 destes doentes (25%). Durante o *follow-up*, 3 doentes agravaram o valor da PSAP, tendo sido confirmada a presença de HTAP num deles. Dos 3 doentes com HTAP confirmada (37.5%), 2 pertenciam ao subtipo limitado e um ao difuso, sendo que todos tinham ACA positivos.

Dezoito doentes (12 ES limitada e 6 ES difusa; 9 Scl70, 6 ACA, 3 ANA+ACA-Scl70-) tinham alterações consideradas *major* na TAC do tórax de alta resolução (fibrose e/ou alveolite afetando pelo menos as bases pulmonares) no início do estudo, associadas ou não a alterações nas PFR e 6 agravaram ao longo do *follow-up*, sendo que 4 faleceram, dois por insuficiência cardíaca e 2 por HTAP associada, como descrito acima.

Dez dos 55 doentes (2 Pré-ES) tinham alterações isoladas da difusão nas PFR no início do estudo e nenhum desenvolveu alterações ecocardiográficas ou da TAC do tórax de alta resolução durante o *follow-up*, apesar de um ter agravado a DLCO. Dois doentes melhoraram de *score* por aumento discreto da DLCO sem qualquer tipo de intervenção.

Dos doentes sem envolvimento pulmonar prévio, apenas um agravou durante o *follow-up*, por diminuição da DLCO associada a alterações *minor* na TAC do tórax de alta resolução (tabela10).

Evolução global

À data da conclusão do estudo, 6 doentes com Pré-ES evoluíram para ES limitada, 4 doentes com ES limitada e 2 com difusa faleceram. Foi perdido o *follow-up* de 2 doentes com ES limitada.

A taxa de sobrevivência nos doentes com ES foi de 100% aos 5 anos, 97.4% (38/39) aos 10anos, 88% (22/25) aos 15 anos, 77.7% (14/18) aos 20 anos, 73.3% (11/15) aos 25 anos, 54.5% (6/11) aos 30 anos, 50% (5/10) aos 35 anos, e de 40% (4/10) aos 40 anos.

A taxa de sobrevivência no subtipo limitado foi de 100% aos 5 e 10 anos (34/34 e 27/27, respetivamente), 89% (16/18) aos 15 anos, 76.9% (10/13) aos 20.8% (9/12) aos 25 anos, 55.6% (5/9) aos 30 anos, 50% (4/8) aos 35 anos e 37.5% (3/8) aos 40 anos; no subtipo difuso a taxa de sobrevivência foi de 100% (13/13) aos 5 anos, 91.7% (11/12) aos 10 anos, 87.5% (7/8) aos 15 anos, 80% (4/5) aos 20 anos, 75% (3/4) aos 25 e 50% (1/2) dos 30 aos 45 anos.

Cinco doentes desenvolveram Síndrome de Sjogren com anticorpos anti-SSA associados e dois CBP (cirrose biliar primária) (tabela 11).

Tabela 11 - Resultado à data da conclusão do estudo

	Início do estudo	Final do estudo	N.º mortes	Causa de morte	Perda <i>follow-up</i>	Doenças associadas
Pré-ES	10	4	-	-	-	2 Sjogren 1 CBP
ES limitada	34	32	4	1 HTAP+DIP 1 HTAP 1 IC 1 Arritmia	2	2 Sjogren 1 CBP
ES difusa	13	11	2	1 HTAP 1IC	-	1 Sjogren

Abreviaturas: Pré-ES, Pré Esclerodermia; ES, Esclerodermia; HTAP, hipertensão arterial pulmonar; DIP, doença intersticial pulmonar; IC, insuficiência Cardíaca; CBP, cirrose biliar primária.

Relação entre a evolução clínica e biomarcadores de angiogénese e de lesão endotelial

Numa visão global, e no que diz respeito à relação entre as moléculas estudadas e o envolvimento orgânico de novo ou o seu agravamento no *follow-up*, encontramos uma relação entre o VEGF e a endostatina e o agravamento vascular e pulmonar, respetivamente, e entre a endostatina e a razão VEGF/endostatina e o envolvimento de novo da pele e do tubo digestivo.

Dos 32 doentes sem envolvimento cardíaco, dos 17 sem envolvimento pulmonar, dos 26 sem envolvimento do tubo digestivo e dos 10 sem envolvimento cutâneo no *baseline*, 9, 1, 6 e 10 desenvolveram alterações destes órgãos durante o *follow-up*, respetivamente. Os 5 doentes com Fenómeno de Raynaud sem necessidade de tratamento no *baseline* passaram a fazer terapêutica no *follow-up*.

Os doentes que não tinham envolvimento cutâneo no *baseline* mas que o desenvolveram durante o *follow-up*, apresentavam valores significativamente inferiores de endostatina e uma razão VEGF/Endostatina mais elevada do que aqueles que não o desenvolveram; já os doentes que não tinham envolvimento do tubo digestivo no *baseline* mas que o desenvolveram no *follow-up*, tinham valores significativamente mais baixos de endostatina e mais elevados da razão VEGF/Endostatina do que aqueles que não o desenvolveram (tabela 12).

Tabela 12- Evolução dos doentes sem envolvimento da pele ou do tubo digestivo no *baseline**

Variáveis	Sem envolvimento cutâneo no <i>follow-up</i> (n=4)	Com envolvimento cutâneo no <i>follow-up</i> (n=6)	Valor de p	Variáveis	Sem envolvimento digestivo no <i>follow-up</i> (n=17)	Envolvimento digestivo no <i>follow-up</i> (n=9)	Valor de p
VEGF	886	1205 (531; 4479)	0.624	VEGF	628	1020	0.145
(n= 9)	(277; 1773) (n=4)	(n=5)		(n= 25)	(143; 1773) (n=17)	(226; 1592) (n=8)	
Endostatina	33.95	9.5	0.011	Endostatina	33.4	14.1	0.012
(n=10)	(33,4; 69,8) (n=4)	(1,8; 19,3) (n=6)		(n=26)	(0,0; 38,3) (n=17)	(2,5; 24,4) (n=9)	
Angiostatina	53,7	45,7	0.831	Angiostatina	48.1	46.1	0.706
(n=10)	(21.3; 71.1) (n=4)	(30.4; 76.7) (n=6)		(n=26)	(19.3; 115.6) (n=17)	(26.7; 85.4) (n=9)	
ICAM-1	223.6	183,8	0.462	ICAM-1	196.9	245.8	0.951
(n=9)	(183,6; 334,2) (n=4)	(144,4; 302,3) (n=5)		(n=24)	(132.5; 526.1) (n=16)	(144.97; 429.5) (n=8)	
VCAM-1	1920,5	958,1	0.86	VCAM-1	1471,7	1358,6	0.56
(n=9)	(914.1; 2855.95) (n=4)	(594.8; 1592.3) (n=5)		(n=25)	(167,2; 11000) (n=17)	594.8; 1737.1) (n=8)	
P seletina	165,9	172,4	1	P seletina	181,1	1887,8	0.713
(n=9)	(143.1; 216.2) (n=4)	(148.8; 179.3) (n=5)		(n=24)	(143.1; 344.9) (n=16)	(130.5; 241.9) (n=8)	
E seletina	65.8	54.6	0.221	E seletina	56,2	55,8	0.759
(n=9)	(36.1; 68.4) (n=4)	(39.3; 60.3) (n=5)		(n=24)	(25.9; 82.1) (n=16)	(38.5; 82.5) (n=8)	
Endotelina-1	1,45	3,1	0.624	Endotelina-1	1,6	2,95	0.268
(n=9)	(1.0; 2.0) (n=4)	(0.2; 6.9) (n=5)		(n=25)	(0.3; 22.4) (n=17)	(0.6; 16.1) (n=8)	
VE	19,9	139,3	0.014	VE	24,9	82,1	0.008
(n=9)	(8.3; 51.4) (n=4)	(68.1; 459.3) (n=5)		(n=24)	(7.9; 105,1) (n=16)	(9,3; 513,8) (n=8)	
VA	26,99	27,4	0.806	VA	13,6	22,8	0.294
(n=9)	(3.9; 40.1) (n=4)	(12.4; 92.3) (n=5)		(n=25)	(3.4; 39.2) (n=17)	(3.2; 40.9) (n=8)	

Abreviaturas: VA, razão VEGF/Angiostatina; VE, razão VEGF/Endostatina; VEGF, *Vascular Endothelial Growth Factor*; VCAM-1, *Vascular Cell Adhesion Molecule-1*; ICAM-1, *Intercellular Cell Adhesion Molecule-1*.

Valores apresentados em mediana (mínimo; máximo).

* Vinte e seis doentes sem envolvimento do tubo digestivo e 10 sem envolvimento da pele (= Pré-ES) no *baseline*.

Tabela 13- Evolução dos doentes sem envolvimento cardíaco no *baseline**

Variáveis	Sem envolvimento cardíaco no <i>follow-up</i>	Com envolvimento cardíaco no <i>follow-up</i>	Valor de <i>p</i>
VEGF (n= 27)	659.5 (143; 4479) (n=18)	691 (226; 1283) (n=9)	0.7
Endostatina (n=32)	18.4 (5.2; 178.8) (n=23)	33.4 (2.5; 44.0) (n=9)	0.173
Angiostatina (n=32)	53.3 (19.3; 115.6) (n=23)	46.1 (24.4; 101.1) (n=9)	0.586
ICAM-1 (n=26)	181.7 (132.5; 526.1) (n=18)	235.1 (155.3; 344.2) (n=8)	0.267
VCAM-1 (n=27)	1354.8 (237.3; 11.000) (n=18)	1206.4 (167.2; 2959.9) (n=9)	0.643
P seletina (n=25)	176.1 (141.3; 344.8) (n=18)	189.2 (130.5; 261.7) (n=8)	0.437
E seletina (n=25)	56.2 (21.1; 79.1) (n=18)	56.1 (43.99; 82.1) (n=8)	0.405
Endotelina-1 (n=28)	1.9 (0.20; 22.4) (n=18)	2.02 (1.0; 22.2) (n=9)	0.918
VE (n=27)	27.1 (3.8; 443.6) (n=18)	27.5 (8.9; 513.8) (n=9)	0.681
VA (n=27)	15 (3,4; 92.3) (n=18)	14.8 (3.2; 27.8) (n=9)	0.173

Abreviaturas: VA, razão VEGF/Angiostatina; VE, razão VEGF/Endostatina; VEGF, *Vascular Endothelial Growth Factor*; VCAM-1, *Vascular Cell Adhesion Molecule -1*; ICAM-1, *Intercellular Cell Adhesion Molecule-1*.

Valores apresentados em mediana (mínimo; máximo).

* Trinta e dois doentes sem envolvimento cardíaco no *baseline*.

Dos 52 doentes com envolvimento vascular periférico com necessidade de terapêutica no *baseline*, dos 38 com envolvimento pulmonar, dos 17 com alterações cardíacas e dos 38 com alterações digestivas, 11, 10, 2 e 1 respetivamente, agravaram no *follow-up*. Nenhum doente com envolvimento da pele no *baseline* agravou o seu score durante o estudo.

Os doentes com envolvimento vascular no *baseline* que agravaram durante o *follow-up* tinham valores significativamente mais baixos de VEGF do que aqueles que não agravaram; os

doentes com envolvimento pulmonar que agravaram durante o *follow-up* tinham valores significativamente mais elevados de endostatina no *baseline* do que aqueles que não agravaram (tabela 14).

Tabela 14 – Evolução dos doentes com envolvimento vascular e pulmonar no *baseline**

Variáveis	Sem agravamento vascular no <i>follow-up</i> (n=41)	Com agravamento vascular no <i>follow-up</i> (n=11)	Valor de p	Variáveis	Sem agravamento pulmonar no <i>follow-up</i> (n=28)	Agravamento pulmonar no <i>follow-up</i> (n=10)	Valor de p
VEGF (n= 43)	711.5 (340;4479) (n=32)	511 (143;1283) (n=11)	0.047	VEGF (n= 30)	767 (143;3902) (n=22)	659.5 (340-1592) (n=8)	0.673
Endostatina (n=52)	20.9 (0.0; 178.8) (n=41)	19.98 (2.5; 36.8) (n=11)	0.946	Endostatina (n=38)	16.27 (0.0; 44.0) (n=28)	27.81 (9.81; 178.78) (n=10)	0.037
Angiostatina (n=52)	48.52 (23.5; 126.5) (n=41)	53.3 (19.3; 85.4) (n=11)	0.413	Angiostatina (n=38)	46.11 (19.26; 126.48) (n=28)	62.78 (21.30; 81.30) (n=10)	0.486
ICAM-1 (n=42)	205.1 (130.2; 697.4) (n=31)	185.2 (144.97; 385.5) (n=11)	0.852	ICAM-1 (n=29)	237.96 (132.50; 526.08) (n=21)	292.79 (143.37; 488.98) (n=8)	0.526
VCAM-1 (n=43)	1468.5 (167.2; 11.000) (n=32)	1247.3 (273.3; 3495.9) (n=11)	0.824	VCAM-1 (n=30)	1468.5 (167.2; 11.000) (n=22)	1378.99 (709.97; 4146.5) (n=8)	0.808
P seletina (n=42)	180.6 (122.7; 609.5) (n=31)	187.7 (130.5; 241.9) (n=11)	0.92	P seletina (n=29)	187.44 (125.99; 344.84) (n=21)	175.06 (141.29; 203.62) (n=8)	0.495
E seletina (n=42)	54.6 (21.1; 90.2) (n=31)	57.9 (25.9; 82.1) (n=11)	0.943	E seletina (n=29)	52.35 (31.73; 90.15) (n=21)	50.08 (21.06; 82.46) (n=8)	0.435
Endotelina-1 (n=42)	1.85 (0.20; 22.4) (n=30)	2.7 (0.9; 10.1) (n=11)	0.626	Endotelina-1 (n=30)	2.3 (0.20; 22.4) (n=22)	1.1 (0.20; 16.1) (n=8)	0.222
VE (n=42)	28.5 (3.8; 443.6) (n=31)	17 (7.9; 513.8) (n=11)	0.258	VE (n=29)	47.11 (7.93; 459.31) (n=21)	25.85 (3.79; 87.14) (n=8)	0.143
VA (n=43)	13.5 (4.3;92.3) (n=32)	9.5 (3.2; 31.98) (n=11)	0.106	VA (n=30)	15.64 (3.19; 38.71) (n=22)	15.52 (4.25; 40.94) (n=8)	0.945

Abreviaturas: VA, razão VEGF/Angiostatina; VE, razão VEGF/Endostatina; VEGF, *Vascular Endothelial Growth Factor*; VCAM-1, *Vascular Cell Adhesion Molecule -1*; ICAM-1, *Intercellular Cell Adhesion Molecule-1*.

Valores apresentados em mediana (mínimo; máximo).

* Cinquenta e dois doentes com envolvimento vascular e 38 com envolvimento pulmonar no *baseline*.

Relação entre a evolução clínica e populações linfocitárias

Globalmente, e no que diz respeito à relação entre as células estudadas e o envolvimento orgânico de novo ou o seu agravamento no *follow-up*, encontramos apenas uma relação entre o número de células NK CD46+fraco e o aparecimento de alterações digestivas de novo no *follow-up*.

Os doentes que não tinham envolvimento gastrointestinal no *baseline* mas que o desenvolveram durante o *follow-up*, tinham valores de NK CD56+fraco marginalmente mais altos (mediana: 181; 74-310; $p=0.095$) do que aqueles que não desenvolveram essas alterações (mediana:136; 41-568) (resultados não mostrados).

Discussão

No nosso estudo, tal como descrito por outros autores [7], o subtipo limitado foi o mais prevalente e associou-se, maioritariamente, à presença de anticorpos ACA e o subtipo difuso à presença de anti-Scl70 [8].

A extensão do pregueamento cutâneo relacionou-se com o atingimento orgânico, sendo que os doentes com *score* mais elevado tiveram uma percentagem global de órgãos atingidos maior, o que também está de acordo com o publicado por outros autores [52]. Por outro lado, 2 dos 6 doentes que faleceram tinham subtipo difuso, *score* cutâneo mais elevado (≥ 2), encontravam-se em fases mais precoces da doença, tiveram atingimento multiorgânico grave e não melhoraram o seu *score* ao longo do *follow-up*. Apesar de ser um número muito pequeno, aponta no sentido dos resultados descritos na literatura [4].

Dos restantes doentes que mantiveram o mesmo *score* cutâneo, 10 fizeram terapêutica imunossupressora por patologia orgânica associada, mas a maioria tinha o subtipo limitado e/ou já se encontrava em estádios tardios da doença, onde a recuperação cutânea é menos esperada.

Relativamente aos doentes que melhoram de *score* cutâneo, a maioria pertencia ao subtipo difuso mas já se encontrava numa fase tardia da doença (apenas 1 na fase intermédia) e só 37.5% efetuaram terapêutica imunossupressora, o que vem demonstrar que a melhoria espontânea do pregueamento cutâneo pode ocorrer em fases mais avançadas da doença.

Todos os doentes com Pré-ES tinham anticorpos específicos de doença e padrão capilaroscópico típico, sendo que 6/10 (60%) evoluíram para ES limitada ao longo do *follow-up*. Uma vez que a duração média do diagnóstico nestes doentes era de cerca de 4 anos à data de inclusão no estudo, a taxa de progressão para ES situa-se ligeiramente abaixo do descrito na literatura (65.9 a 72.7% aos 5 e 10 anos, respetivamente) [12].

Mais de 50% dos doentes tiveram úlceras digitais ao longo do estudo e 31.6% do total de doentes tiveram úlceras recorrentes, o que está de acordo com o publicado na literatura [16]. Os doentes com úlceras ativas no início do estudo tiveram uma recorrência mais elevada de úlceras ao longo do *follow-up* do que o descrito por outros autores (81.8% vs 31-71%) [15,53].

As úlceras digitais foram mais prevalentes no subtipo difuso do que no limitado o que está de acordo com outros estudos [21,8] e, também em consonância com outros autores [22-26], foram mais frequentes em doentes com anticorpos anti-Scl 70 positivos do que naqueles com ACA positivos.

Tal como para Caramaschi *et al.* [27], o padrão capilaroscópico tardio foi o mais revalente nos doentes com úlceras digitais ao longo do estudo (43.8%), seguido do padrão ativo (40.6%).

A presença de UD quer no início da investigação quer durante o seu decurso, e de forma semelhante ao publicado por outros autores, associou-se a envolvimento de outros órgãos [54].

Ao longo do estudo, 68.6% dos doentes tiveram atingimento digestivo, valor que se situa abaixo do descrito na literatura [5,32,33]. Uma explicação para este facto poderá residir, em parte, na forma como os doentes foram avaliados (só valorização sintomática) e na utilização ou não de exames para comprovar o envolvimento do tubo digestivo. Na nossa investigação, a confirmação da dismotilidade gastrointestinal foi obrigatória através do uso de exames auxiliares considerados mais adequados para cada um dos segmentos envolvidos. Por outro lado, os sintomas relacionados com a orofaringe, não fazendo parte da escala de Medsger e podendo-se associar a outras entidades sobrepostas (ex: doença de Sjogren) não foram incluídos no estudo, o que também poderá ter contribuído para os resultados encontrados.

De acordo com Thoua *et al.*, o esófago foi o órgão mais frequentemente envolvido [34].

Embora vários autores não tenham encontrado diferenças na prevalência do envolvimento digestivo entre os subtipos limitado e difuso [22,34], nem relação com os autoanticorpos [22,34], exceto quando o envolvimento esofágico é grave [55,22], ou há atingimento gástrico associado [35], no nosso estudo as alterações encontradas foram mais frequentes no subtipo difuso e em doentes com anticorpos anti-Scl70.

Todos os doentes com dismotilidade esofágica foram tratados com procinéticos e/ou inibidores da bomba de prótons. Sempre que necessário foram suspensos fármacos que pudessem interferir na motilidade/sintomatologia. O doente com dismotilidade gastroesofágica grave, para além de vários fármacos procinéticos associados a inibidores da bomba de prótons, fez ciclofosfamida endovenosa, sem resultado, e teve necessidade de colocar gastrostomia, para dieta fracionada não culinária, por perda de peso. Por ausência de melhoria foi submetido a transplante autólogo de medula óssea com resolução completa dos sintomas e recuperação da motilidade gastrointestinal. O doente com proliferação bacteriana do delgado foi submetido a vários ciclos de antibioterapia associados a procinéticos. Apesar do transplante de medula óssea não se ter mostrado eficaz no tratamento de doentes com dismotilidade gastroesofágica [56] e apesar de apenas termos experiência com um doente, esta terapêutica deverá ser tomada em conta na abordagem de um doente com dismotilidade grave, refratária às medidas clássicas [57].

Ao longo do estudo, 53.1% dos doentes tiveram envolvimento cardíaco e 6.1% disfunção sistólica do ventrículo esquerdo o que, comparando estes resultados com os de outros autores com metodologias semelhantes [16,58], é claramente superior ao descrito na literatura. No entanto, a prevalência da disfunção ventricular parece estar subestimada pois,

segundo alguns autores, quando avaliada durante o exercício aumenta para cerca de 46% [38,59].

O envolvimento cardíaco foi mais frequente nos doentes com anticorpos anti-Scl70 positivos do que naqueles com ACA positivos, o que também está de acordo com os resultados relatados por outros autores [37].

Embora o envolvimento cardíaco ocorra em ambos os subtipos, há estudos que apontam, tal como o nosso, para uma maior incidência no subtipo difuso [16,41] e, outros, no limitado [42].

Dezoito doentes (32.7%), tiveram alterações *major* na TAC do tórax de alta resolução. No entanto, quando associamos as alterações *minor*, a frequência sobe para 47.3% e, se excluirmos da análise a pré-ES, aumenta para 53.2%, o que se aproxima do publicado na literatura [49,50].

O envolvimento intersticial pulmonar foi mais frequente nos doentes com anticorpos anti-Scl70 (86.7%) do que naqueles com ACA (35.3%) e no subtipo difuso (76.9%) do que no limitado (35.3%) o que está de acordo com o descrito por outros autores [8].

Por outro lado, e também de acordo com outros autores, 2 dos 3 doentes que desenvolveram HTAP pertenciam ao subtipo limitado e todos tinham o anticorpo anticentrómero [60]. A HTAP não respondeu à terapêutica vasodilatadora múltipla efetuada e os 3 doentes vieram a falecer.

Dos doentes com alterações *major* na TAC no início do estudo, 3 fizeram terapêutica imunossupressora, tendo 2 melhorado de *score*. Ao longo do *follow-up*, 6 doentes agravaram e iniciaram terapêutica imunossupressora.

A taxa de sobrevivência dos nossos doentes foi superior ao publicado na literatura [61]. Entre os 10 e os 40 anos a taxa de sobrevivência diminuiu a uma média de 1.9 pontos percentuais por ano. O cálculo das taxas de sobrevivência é mais exato se realizado após o falecimento dos doentes, no entanto, consideramos que a metodologia utilizada no presente estudo não terá tido uma influência relevante nos resultados obtidos.

Os resultados de sobrevivência nos dois subtipos são sobreponíveis, sendo que os valores referentes às taxas de sobrevivência em doentes com mais anos de evolução devem ser interpretados de forma cautelosa devido ao reduzido número da amostra.

A causa de morte foi atribuída a patologia cardíaca em metade dos casos e a patologia pulmonar na outra metade (embora pudessem ter patologias associadas), o que é diferente do relatado em outros estudos, em que a patologia cardíaca é responsável por cerca de 26% da mortalidade [44]. Nenhum dos doentes com Pré-ES faleceu durante o estudo.

Na nossa investigação, encontramos uma relação entre níveis mais baixos de endostatina e o aparecimento de alterações cutâneas, ou seja, o desenvolvimento de ES limitada em doentes que no *baseline* tinham Pré-ES. Apesar deste grupo de doentes ser pequeno, a diferença de valores é estatisticamente significativa ($p=0.011$), o que vem reforçar

a necessidade de confirmar estes dados em estudos posteriores com um maior número de doentes. Estes resultados vêm de encontro à hipótese por nós colocada previamente, sobre a possibilidade da endostatina poder funcionar como um marcador precoce da doença e ter valor preditivo na evolução de Pré-ES para ES [62].

Por outro lado, valores mais baixos de endostatina no *baseline* relacionaram-se com o desenvolvimento de alterações gastrointestinais no *follow-up* e, tanto quanto é do nosso conhecimento, não há estudos prévios que relatem esta associação.

Em ambas as situações, a razão VEGF/endostatina encontrava-se significativamente elevada, o que não aconteceu com o VEGF de forma isolada, pelo que esta razão parece exercer um papel importante no envolvimento destes órgãos.

Apesar de não termos conseguido demonstrar previamente uma relação entre a endostatina e o envolvimento pulmonar ou a sua gravidade [62], o que está de acordo com outros estudos publicados na literatura, verificamos que valores mais elevados de endostatina no *baseline* se relacionaram com o agravamento das lesões pulmonares pré-existentes durante o *follow-up*. Estes resultados vêm apoiar outros estudos que relacionam os níveis de endostatina com a gravidade do atingimento pulmonar [63].

Na nossa investigação, estabeleceu-se uma relação entre valores mais baixos de VEGF no *baseline* e o aparecimento de lesões vasculares mais graves no *follow-up*, levantando a hipótese, tal como Distler *et al.* [64], desta molécula poder exercer um papel protetor deste tipo de agravamento orgânico.

Relativamente às células T e NK, não encontramos uma relação estatisticamente significativa entre os vários subtipos celulares e atingimento orgânico ou sua gravidade durante o *follow-up*.

Pontos a destacar:

- 1) As manifestações orgânicas relacionaram-se com os subtipos da doença e autoanticorpos existentes;
- 2) A mortalidade foi inferior à descrita na literatura mas com uma responsabilidade cardíaca superior;
- 3) O transplante de medula óssea deverá ser considerado na abordagem da dismotilidade digestiva grave, refratária às medidas clássicas;
- 4) Valores mais baixos de endostatina e uma razão VEGF/endostatina mais alta, relacionaram-se com a evolução de Pré-ES para ES;
- 5) Valores mais baixos de endostatina e mais elevados de VEGF/endostatina relacionaram-se com o desenvolvimento de alterações gastrointestinais;
- 6) Valores mais elevados de endostatina relacionaram-se com a gravidade das lesões pulmonares;

- 7) Valores mais baixos de VEGF relacionaram-se com uma maior gravidade das lesões vasculares;
- 8) Tamanho da amostra como ponto fraco do estudo, condicionando uma interpretação mais conclusiva dos resultados. No entanto, é de salientar a relevância deste estudo dado que o tamanho amostral deve ser valorizado considerando tratar-se de uma doença rara na qual se verifica escassez de trabalhos relativamente a diversas relações estudadas.

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CAPÍTULO 5 |

DISCUSSÃO E CONCLUSÕES

CONTEÚDOS

Discussão Global

Relação entre os biomarcadores vasculares e imunológicos e os padrões capilaroscópicos

Relação entre os biomarcadores vasculares e imunológicos e os subtipos da doença esclerodérmica e tempo de evolução da ES

Relação entre os biomarcadores vasculares e imunológicos e o atingimento orgânico e sua gravidade

Conclusões

Referências

Reflexão sobre os resultados da investigação

Referências

Aplicabilidade dos resultados à prática clínica

5.1 – DISCUSSÃO GLOBAL

Neste estudo observacional prospetivo, definiram-se as seguintes questões de investigação:

- 1) A lesão endotelial é precoce e precede a desorganização microvascular?
- 2) Existe relação da lesão endotelial e alterações da angiogénese (avaliadas pelos marcadores serológicos) com as alterações na microvasculatura (avaliadas por capilaroscopia)?
- 3) Existe relação das lesões vasculares (avaliadas por capilaroscopia) com a ativação das células T, o desequilíbrio Th1/Th2, a diferenciação T citotóxica e as células NK?
- 4) Existe relação entre as alterações vasculares (avaliadas pelos marcadores serológicos) e imunológicas e o subtipo da doença, tempo de evolução, atingimento orgânico e sua severidade?
- 5) Os biomarcadores avaliados têm interesse na prática clínica?

A lesão endotelial, avaliada neste estudo pelo aumento dos níveis séricos de várias moléculas de adesão (VCAM-1, ICAM-1, E-seletina e P-seletina) e da ET-1, estava presente nos doentes com Pré-ES e na fase inicial da ES sendo, portanto, precoce (vejam-se as subsecções 4.2.3 e 4.2.4 e o Quadro 1).

Quadro 1 – Biomarcadores de lesão endotelial na Pré-ES e fase precoce da ES

Clínica	ET-1	VCAM-1	ICAM-1	P-seletina	E-seletina
Pré-ES	↑	↑	↓	↑	↑
Fase precoce da ES	↔	↑	↔	↑	↔

Abreviaturas: ES, Esclerodermia, ET-1, Endotelina 1; VCAM-1, *Vascular Cell Adhesion Molecule 1*; ICAM-1, *Intercellular Adhesion Molecule 1*.

↑ Aumento significativo dos níveis séricos; ↑ Aumento marginalmente significativo dos níveis séricos; ↓ Diminuição significativa dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos (por comparação com os observados em indivíduos normais).

Relação entre os biomarcadores vasculares e imunológicos e os padrões capilaroscópicos

A lesão endotelial foi observada no padrão capilaroscópico precoce, caracterizado pela existência de um pequeno número de capilares dilatados/gigantes e microhemorragias, ausência de áreas avasculares e uma distribuição capilar relativamente bem preservada, no padrão ativo, em que existem megacapilares e micro-hemorragias, perda capilar moderada e alteração discreta da arquitetura vascular, e no padrão tardio que se caracteriza pela presença de áreas avasculares extensas, capilares ramificados abundantes e desorganização da arquitetura vascular [1,2]. No padrão precoce observamos aumento da P-seletina e da VCAM-1 e, nos padrões ativo e tardio, aumento dos níveis séricos de várias moléculas de adesão (VCAM-1, E-seletina e P-seletina) e da ET-1 (vejam-se as sub-seções 4.2.3 e 4.2.4 e o Quadro 2), pelo que podemos dizer que a lesão endotelial precede e acompanha a desorganização microvascular.

Tal como no nosso estudo, Valim *et al.* [3] relacionaram os níveis de E-seletina com a deleção capilar e Sulli *et al.* [4] os níveis de ET-1 com os padrões ativo e tardio da capilaroscopia. Por outro lado, encontramos pela primeira vez, tanto quanto é do nosso conhecimento, aumento dos níveis séricos de VCAM-1 e P-seletina e diminuição dos níveis de ICAM-1 em todos os padrões capilaroscópicos. Uma explicação possível para a diminuição da ICAM-1 poderá residir na existência de anticorpos anti-ICAM-1 como já referido anteriormente [5].

Quadro 2 – Relação entre biomarcadores de lesão endotelial e padrão capilaroscópico

Padrão capilaroscópico	ET-1	VCAM-1	ICAM-1	Ps	Es
Precoce	↔	↑	↓	↑	↔
Ativo	↑	↑	↓	↑	↑
Tardio	↑	↑	↓	↑	↑

Abreviaturas: ET-1, Endotelina 1; VCAM-1, *Vascular Cell Adhesion Molecule 1*; ICAM-1, *Intercellular Adhesion Molecule 1*; Es, E-seletina; Ps, P-seletina; ↑ Aumento significativo dos níveis séricos; ↑ Aumento marginalmente significativo dos níveis séricos ↓ Diminuição significativa dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos (por comparação com os observados em indivíduos normais).

Simultaneamente ao aumento da expressão dos biomarcadores de lesão endotelial, verificamos que os biomarcadores de angiogénese estavam presentes em todos os padrões

capilaroscópicos, sendo que a endostatina já se encontrava aumentada e a razão VEGF/endostatina diminuída em doentes com alterações *minor* (fase mais precoce) (vejam-se as sub-secções 4.2.1 e 4.2.2 e o Quadro 3).

Estes dados apontam para a existência de um papel preponderante quer dos fatores angiogénicos quer angiostáticos, assim como da relação entre eles, na formação das lesões microvasculares.

O VEGF, um dos fatores angiogénicos mais potentes [6], encontrava-se significativamente aumentado no padrão ativo, altura em que a formação de vasos aberrantes (megacapilares) predomina; no padrão tardio, em que a desorganização microvascular é dominante, associada à perda de capilares e capilares ramificados abundantes (tentativa final de compensação de perda de vasos), o desequilíbrio entre fatores angiogénicos e angiostáticos parece ter um papel dominante, com predomínio dos angiostáticos (vejam-se as sub-secções 4.2.1 e 4.2.2 e o Quadro 3).

Na nossa investigação, e ao contrário do descrito por Distler *et al.* [7], encontramos um aumento da endostatina em todos os padrões capilaroscópicos; por outro lado, de acordo com os resultados publicados pelo mesmo autor, constatamos uma relação entre níveis decrescentes de VEGF e os diferentes padrões capilaroscópicos, do precoce até ao tardio, embora só tenhamos obtido diferenças significativas no padrão ativo.

No nosso estudo, verificamos também existir uma diminuição da razão VEGF/endostatina em todos os padrões capilaroscópicos e um aumento da angiostatina nos padrões ativo e tardio. Não encontramos descrita na literatura nenhuma relação entre os níveis de angiostatina e das razões VEGF/endostatina e VEGF/angiostatina e os vários padrões capilaroscópicos.

Quadro 3 – Relação entre os biomarcadores de angiogénese e o padrão capilaroscópico

Padrão capilaroscópico	VEGF	A	E	VEGF/A	VEGF/E
Precoce	↔	↔	↑	↔	↓
Ativo	↑	↑	↑	↔	↓
Tardio	↔	↑	↑	↔	↓

Abreviaturas: VEGF, *Vascular Endothelial Growth Factor*; A, Angiostatina; E, Endostatina; VEGF/A, razão VEGF / Angiostatina; VEGF/E, razão VEGF / Endostatina; ↑Aumento significativo dos níveis séricos; ↓Diminuição significativa dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos (por comparação com os observados em indivíduos normais).

Para além das alterações acima referidas, nos padrões ativos e tardios dos doentes com perfil esclerodérmico, constatamos uma diminuição dos linfócitos T do sangue periférico, nomeadamente dos linfócitos T CD8, T CD8 ativados CD45RO+, e dos linfócitos T citotóxicos CD56+ (veja-se a sub-secção 4.1.2 e o Quadro 4). Tal facto sugere que estas células possam ter um papel relevante no desenvolvimento das lesões microvasculares em fases mais avançadas. Não conseguimos, neste estudo, evidenciar uma relação entre um desequilíbrio Th1/Th2, avaliado pelo perfil de citocinas no soro, e a existência das lesões microvasculares.

Tanto quanto é do nosso conhecimento, não existem estudos publicados que relacionem estas células com os vários padrões capilaroscópicos.

Ao contrário de Cutolo *et al.* [8], que descreveram uma relação entre a presença de anticorpos anti Scl-70 e os padrões ativos e tardios da capilaroscopia, não encontramos relação significativa entre estes anticorpos e os diferentes padrões capilaroscópicos.

Quadro 4 – Relação entre os linfócitos T e as células NK e as alterações capilaroscópicas

Padrão capilaroscópico	LT	LT CD8	LT CD8 CD45RO+	LT CD8 HLA-DR+	LT CD56+	CNK	CNK CD56+ fraco	CNK CD56+ forte
Precoce	↔	↔	↔	↔	↔	↔	↔	↔
Ativo	↓	↓	↓	↔	↓	↔	↔	↔
Tardio	↓	↓	↓	↔	↓	↔	↔	↔

Abreviaturas: LT, Linfócitos T; CNK, Células NK.

↓ Diminuição significativa dos valores séricos; ↓ Diminuição marginalmente significativa dos valores séricos; ↔ Sem alteração significativa dos valores séricos (por comparação com os observados em indivíduos normais).

Relação entre os biomarcadores vasculares e imunológicos e os subtipos da doença esclerodérmica e tempo de evolução da ES

Em todos os subtipos de doença e em todas as fases evolutivas da ES, existia ativação endotelial mantida e alteração da angiogénese, evidenciadas pela alteração de vários biomarcadores séricos (vejam-se as sub-secções 4.2.1 a 4.2.4 e os Quadros 5 e 6).

No que diz respeito aos biomarcadores de lesão endotelial, verificamos que a ET-1 se encontrava marginalmente elevada na Pré-ES, enquanto no subtipo limitado e difuso, tal como o descrito por outros autores [4,9-10], esta se encontrava significativamente elevada.

Não encontramos estudos que relacionassem os níveis séricos de ET-1 com a Pré-ES.

Relativamente às moléculas de adesão, e contrariamente aos resultados descritos por Valentini *et al.* [11], notamos um aumento da VCAM-1 e uma diminuição da ICAM-1 nos doentes com Pré-ES. Por outro lado, e de acordo com os resultados descritos pelo mesmo autor, encontramos um aumento, ainda que marginalmente significativo, da E-seletina nestes doentes. Verificamos, ainda, e tanto quanto é do nosso conhecimento, pela primeira vez na literatura, a existência de um aumento da P-seletina nos doentes com Pré-ES. Constatamos, também, e de acordo com outros autores [12-13], níveis aumentados de VCAM-1 e de E-seletina (aumento marginalmente significativo) nos subtipos limitado e difuso.

Não encontramos estudos que relacionassem os níveis séricos da ICAM-1 e da P-seletina com o subtipo limitado e difuso da doença que, na nossa investigação, se apresentavam diminuídos e aumentados, respetivamente.

Relativamente aos biomarcadores de angiogénese, não verificamos, da mesma forma que Hummers *et al.* [14], uma relação significativa entre os valores de VEGF e os subtipos limitado e difuso. Tal como Distler *et al.* [7], encontramos um aumento dos valores de VEGF nos doentes com Pré-ES.

No que diz respeito à razão VEGF/endostatina, e ao contrário de Bartkowiak *et al.* [15], constatamos existir uma diminuição dos valores da mesma nos subtipos limitado e difuso e não apenas no subtipo difuso. Não encontramos descrita nenhuma relação entre a razão VEGF/endostatina e a Pré-ES assim como entre a razão VEGF/angiostatina e os diferentes subtipos da doença.

Tal como Bartkowiak *et al.* [15], observamos níveis aumentados de endostatina nos subtipos limitado e difuso mas, tanto quanto é do nosso conhecimento, descrevemos pela primeira vez um aumento desta molécula na Pré-ES e um aumento da angiostatina nos subtipos limitado e difuso.

No nosso estudo, a angiostatina e a ET-1 eram mais elevadas nos doentes com subtipo limitado que tinham CREST do que naqueles sem CREST, o que nos fez levantar a hipótese de estas moléculas poderem estar envolvidas em mecanismos patogénicos responsáveis pelo desenvolvimento do CREST. Não registamos outros estudos que estabelecessem uma relação semelhante.

Quadro 5 – Relação entre os biomarcadores vasculares e subtipos da doença esclerodérmica

Subtipo da doença Esclerodérmica	VEGF	A	E	VEGF /A	VEGF /E	ET-1	VCAM-1	ICAM-1	Es	Ps
Pré-ES	↑	↔	↑	↑	↓	↑	↑	↓	↑*	↑
ES-difusa	↔	↑	↑	↔	↓	↑	↑	↓	↑	↑
ES-limitada	↑	↑	↑	↔	↓	↑	↑	↓	↑	↑

Abreviaturas: VEGF, *Vascular Endothelial Growth Factor*; A, Angiostatina; E, Endostatina; VEGF/A, razão VEGF / Angiostatina; VEGF/E, razão VEGF / Endostatina; ET-1, Endotelina 1; VCAM-1, *Vascular Cell Adhesion Molecule 1*; ICAM-1, *Intercellular Adhesion Molecule 1*; Es, E-seletina; Ps, P-seletina.

↑ Aumento significativo dos níveis séricos; ↑ Aumento marginalmente significativo; ↓ Diminuição significativa dos níveis séricos;

↔ Sem alteração significativa dos níveis séricos (por comparação com os observados em indivíduos normais). * p=0.050.

Relativamente aos biomarcadores de lesão endotelial, verificamos que, tal como em outros estudos [4,10], a ET-1 se encontrava elevada em todos os estádios da doença, embora com significado estatístico apenas na fase tardia da ES. Contudo, quando avaliamos em conjunto a fase precoce com a intermédia existia uma diferença estatisticamente significativa relativamente aos controlos, pelo que as diferenças encontradas poderão dever-se ao baixo número de doentes incluídos em cada uma das fases.

Relativamente às moléculas de adesão, constatamos um aumento significativo da VCAM-1 e da P-seletina em todas as fases evolutivas. A ICAM-1 apesar de se encontrar elevada na fase precoce relativamente aos controlos, o que está de acordo com os resultados publicados por Hasegawa *et al.* [16], embora sem diferenças significativas, tornou-se significativamente inferior nas fases intermédia e tardia da ES. Uma explicação possível para a diminuição da ICAM-1 poderá residir na existência de anticorpos anti-ICAM-1 como anteriormente referido [5]. Quanto à E-seletina, apesar não de termos verificado uma diferença significativa relativamente aos controlos nas várias fases evolutivas da doença, observamos que o aumento se tornou marginalmente significativo (e com valores de p progressivamente mais baixos) à medida que o número de doentes incluídos nas diferentes fases foi aumentando. Mais uma vez, os resultados obtidos, nomeadamente nas fases precoce e intermédia, poderão relacionar-se com o tamanho das sub-amostras.

Contrariamente aos nossos resultados, Valim *et al.* [3] e Hasegawa *et al.* [16] que estudaram esta molécula em doentes com menos de 3 e 4 anos de evolução, respetivamente, e que incluíram, portanto, doentes com ES em fase precoce e intermédia, constataram um aumento da E-seletina nestes doentes relativamente aos controlos. Já no que diz respeito à P-seletina, os nossos resultados estão em consonância com aqueles publicados pelos mesmos autores. Não encontramos na literatura relação entre as várias moléculas de adesão e estádios mais tardios da doença.

No que diz respeito aos biomarcadores de angiogénese, em oposição ao descrito por Distler *et al.* [7], a endostatina encontrava-se elevada em todas as fases evolutivas da ES e, de acordo com Farouk *et al.* [17], tinha níveis significativamente mais elevados na fase intermédia do que na precoce.

No que concerne ao VEGF, ao contrário de Distler *et al.* [7], os valores não se relacionaram com as diferentes fases evolutivas, pois muito embora na fase tardia se tenha assistido a um aumento marginalmente significativo relativamente aos controlos, a diferença de valores entre os doentes com ES e os controlos já era também marginal.

Tanto quanto é do nosso conhecimento, estabelecemos, pela primeira vez, uma relação entre valores mais elevados de angiostatina e as fases intermédia e tardia da ES.

Quadro 6 – Relação entre os biomarcadores vasculares e as fases evolutivas da Esclerose Sistémica

Fase evolutiva da Esclerodermia	VEGF	A	E	VEGF /A	VEGF /E	ET-1	VCAM-1	ICAM-1	ES	PS
Fase precoce	↔	↔	↑	↔	↓	↔	↑	↔	↔	↑
Fase intermédia	↔	↑	↑	↔	↓	↑	↑	↓	↑	↑
Fase tardia	↑	↑	↑	↔	↓	↑	↑	↓	↑*	↑

Abreviaturas: VEGF, *Vascular Endothelial Growth Factor*; A, Angiostatina; E, Endostatina; VEGF/A, razão VEGF / Angiostatina; VEGF/E, razão VEGF / Endostatina; ET-1, Endotelina 1; VCAM-1, *Vascular Cell Adhesion Molecule 1*; ICAM-1, *Intercellular Adhesion Molecule 1*; ES, E-seletina; PS, P-seletina.

↑ Aumento significativo dos níveis séricos; ↓ Diminuição significativa dos níveis séricos; ↑ Aumento marginalmente significativo dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos (por comparação com os observados em indivíduos normais).

* p=0.050.

As alterações dos linfócitos no sangue periférico assumem particular importância nos subtipos limitado e difuso, nas fases iniciais e tardias da ES mas não na Pré-ES (veja-se a sub-secção 4.1.2 e os Quadros 7 e 8). Os resultados encontrados na literatura sobre os linfócitos no sangue periférico, nomeadamente sobre os T CD8+ e sobre as células NK são díspares, provavelmente devido às diferenças das características das populações estudadas (os subtipos celulares encontrados podem divergir nos vários subtipos e nos diferentes estádios da doença) e à forma como são expressos os resultados (percentagem vs valor absoluto). Ercole *et al.* [18] relacionaram a existência de uma maior percentagem de linfócitos T CD8+ e uma menor percentagem de células NK no sangue periférico com o subtipo difuso e com as fases tardias da doença. Apesar dos resultados não serem comparáveis, no nosso estudo, encontramos um número mais baixo de linfócitos T CD8+ nos subtipos limitado e

difuso e nas fases precoces e tardias da ES e só observamos diferenças significativas nas células NK nas fases precoces da ES, independentemente do subtipo.

Não encontramos estudos que tenham relacionado as restantes células estudadas na nossa investigação com os vários subtipos da doença esclerodérmica e com os diferentes estádios da ES.

Quadro 7 – Relação entre populações linfocitárias T e NK e os subtipos da doença esclerodérmica

Subtipo de doença esclerodérmica	LT	LT CD8	LT CD8 CD45RO+	LT CD8 HLA-DR+	LT CD56+	CNK	NK CD56+ <i>fraco</i>	NK CD56+ <i>forte</i>
Pré-ES	↔	↔	↔	↓	↔	↔	↔	↔
ES Limitada	↓	↓	↓	↔	↓	↓	↔	↓
ES Difusa	↓	↓	↓	↓	↓	↔	↔	↔

Abreviaturas: LT, Linfócitos T; CNK, Células NK; ES, Esclerodermia.

↓ Diminuição significativa dos valores séricos; ↓ Diminuição marginalmente significativa dos valores séricos; ↔ Sem alteração significativa dos valores séricos (por comparação com os observados em indivíduos normais).

Quadro 8 - Relação entre populações linfocitárias T e NK e as fases evolutivas da Esclerose Sistémica

Fase evolutiva da ES	LT	LT CD8	LT CD8 CD45RO+	LT CD8 HLA-DR+	LT CD56+	CNK
Fase precoce	↓	↓	↓	↓	↓	↓
Fase intermédia	↔	↓	↔	↔	↓	↔
Fase tardia	↓	↓	↓	↓	↓	↔

Abreviaturas: LT, Linfócitos T; CNK, Células NK; ES, Esclerodermia.

↓ Diminuição significativa dos valores séricos; ↓ Diminuição marginalmente significativa dos valores séricos; ↔ Sem alteração significativa dos valores séricos (por comparação com os observados em indivíduos normais).

Os anticorpos ACA foram os mais prevalentes no subtipo limitado (61.8%), enquanto os anticorpos anti-Scl-70 foram os mais prevalentes no subtipo difuso (53.8%), o que está de acordo com o descrito por outros autores [19] (veja-se a sub-secção 4.1.1). Os anticorpos anti-Ro52 foram positivos em 82.6% dos doentes em que foram pesquisados, conforme o descrito por Hudson *et al.* [20], não se tendo relacionado a sua presença com a sobreposição de outras patologias, nomeadamente com o Síndrome de Sjogren (veja-se a sub-secção 4.1.1). Por outro lado, e tanto quanto é do nosso conhecimento, pela primeira vez na literatura,

encontramos uma forte associação entre estes últimos anticorpos e o subtipo limitado (veja-se a sub-secção 4.1.1)

Relação entre os biomarcadores vasculares e imunológicos e o atingimento orgânico e sua gravidade

Como já referido, a classificação do envolvimento orgânico baseou-se no ponto de corte utilizado pela escala de gravidade de Medsger que, simultaneamente, serviu de base para calcular a gravidade da doença avaliada órgão a órgão [21].

Relativamente aos biomarcadores de lesão endotelial e de angiogénese (vasculares), e também aos imunológicos, foram definidas as seguintes questões:

- 1) Podem, alguns dos biomarcadores avaliados, ser considerados protetores ou de risco de um determinado atingimento orgânico e/ou da sua gravidade?
- 2) Podem, alguns dos biomarcadores avaliados, prever a evolução de um determinado atingimento orgânico?

No seu conjunto, estas duas questões pretendem estudar o interesse destes biomarcadores na prática clínica.

Ao longo desta discussão, utilizamos o termo “biomarcador de risco/ protetor inicial” para referir as relações clínico-laboratoriais pertinentes no início do estudo e “biomarcador de risco evolutivo/ de evolução” para as relações encontradas no desenvolvimento do mesmo. Embora não seja objetivo deste trabalho a definição de valores a usar como pontos de corte (dado que se assume que estas relações se manifestam num contínuo), deverão ser alvo de especial atenção doentes que apresentem valores superiores ou inferiores, conforme os casos, aos pontos médios do intervalo entre a mediana dos doentes que não evoluíram ou que não agravaram e a mediana daqueles que evoluíram ou agravaram um determinado atingimento orgânico durante o estudo.

Pele

Biomarcadores vasculares

Na nossa investigação, encontramos uma relação entre o VEGF e a razão VEGF/angiostatina com o envolvimento cutâneo no início do estudo e entre a endostatina e a razão VEGF/endostatina com o desenvolvimento de alterações cutâneas ao longo do mesmo.

O facto de os doentes sem atingimento cutâneo no início do estudo (Pré-ES) apresentarem valores de VEGF tendencialmente mais elevados (resultado marginalmente significativo) e uma razão VEGF/angiostatina significativamente superior à dos doentes com

atingimento cutâneo (ES), embora sem diferenças significativas nos níveis de angiostatina, pode ser interpretado como indicador de que quer o VEGF, quer o equilíbrio entre o VEGF e a angiostatina possam desempenhar um papel protetor no desenvolvimento deste tipo de atingimento orgânico (vejam-se as sub-secções 4.2.1 e 4.2.2 e Quadro 9).

Quadro 9 – Biomarcadores protetores de atingimento orgânico ou sua gravidade

	VEGF	A	E	VA	VE	ET-1	VCAM-1	ICAM-1	Es	Ps
Pele	↑	↔	↔	↑	↑	↔	↔	↔	↔	↔
Vascular	↔	↔	↔	↔	↔	↓	↔	↔	↔	↔
Pulmão	↔	↓	↔	↔	↔	↓	↔	↓	↓	↔
Pulmão interstício	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔
Pulmão vascular	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Coração	↔	↔	↔	↔	↔	↔	↔	↓	↔	↔
Gastrointestinal	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔

Abreviaturas: VEGF, *Vascular Endothelial Growth Factor*; A, Angiostatina; E, Endostatina; VA, razão VEGF / Angiostatina; VE, razão VEGF / Endostatina; ET-1, Endotelina 1; VCAM-1, *Vascular Cell Adhesion Molecule 1*; ICAM-1, *Intercellular Adhesion Molecule 1*; Es, E-seletina; Ps, P-seletina.

↑ Aumento significativo dos níveis séricos; ↑ Aumento marginalmente significativo dos níveis séricos; ↓ Diminuição significativa dos níveis séricos; ↓ Diminuição marginalmente significativa dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos.

Por outro lado, dado que os doentes com Pré-ES que evoluíram para ES durante o estudo tinham valores significativamente mais baixos de endostatina e mais altos de VEGF/endostatina (vs. os que não evoluíram para ES), embora sem diferenças significativas do VEGF, faz-nos levantar a hipótese de que quer a endostatina quer o desequilíbrio entre o VEGF e a endostatina possam funcionar como “biomarcadores de risco evolutivo” e, simultaneamente, preditores de atingimento cutâneo futuro (veja-se a sub-secção 4.3 e o Quadro 10).

Quadro 10 – Biomarcadores preditivos de atingimento ou agravamento orgânico

	VEGF	A	E	VEGF/A	VEGF/E	ET-1
Pele	↔	↔	↓	↔	↑	↔
Vascular	↓	↔	↔	↔	↔	↔
Pulmão	↔	↔	↑	↔	↔	↔
Pulmão interstício	↔	↔	↔	↔	↔	↔
Pulmão vascular	↔	↔	↔	↔	↔	↔
Coração	↔	↔	↔	↔	↔	↔
Gastrointestinal	↔	↔	↓	↔	↑	↔

Abreviaturas: VEGF, *Vascular Endothelial Growth Factor*; A, Angiostatina; E, Endostatina; VEGF/A, razão VEGF / Angiostatina; VEGF/E, razão VEGF / Endostatina; ET-1, Endotelina 1.

↑ Aumento significativo dos níveis séricos; ↓ Diminuição significativa dos níveis séricos; ↔ Sem alteração significativa dos níveis séricos.

Assim, doentes com Pré-ES e com valores mais baixos de endostatina e mais altos de VEGF/endostatina terão maior probabilidade de evoluírem para ES (Quadro 10 e capítulo 4.3). Deverão ser alvo de especial atenção doentes que apresentem valores superiores ao ponto médio do intervalo entre a mediana dos doentes que não evoluíram e a mediana daqueles que evoluíram para ES durante o estudo.

Ao contrário dos nossos resultados, Distler *et al.* [7], com uma amostra com tamanho sobreponível à nossa, relacionaram valores mais elevados de VEGF com o desenvolvimento de ES em doentes com Pré-ES ao fim de 1 ano, não tendo encontrado qualquer relação com os valores de endostatina.

Tanto quanto é do nosso conhecimento, nenhum dos estudos publicados relacionou os valores da endostatina, VEGF ou das razões VEGF/endostatina e VEGF/angiostatina com a proteção ou risco de envolvimento cutâneo.

Apesar de alguns estudos terem encontrado uma relação entre os valores de endostatina [17, 22] e do VEGF [23] e a gravidade do envolvimento cutâneo, outros [7, 17], tal como o nosso, não conseguiram estabelecer essa relação. Não encontramos nenhum estudo que tenha relacionado as razões VEGF/endostatina e VEGF/angiostatina com a gravidade do envolvimento cutâneo.

Embora as diferenças dos resultados encontrados se possam relacionar, pelo menos em parte, com o tamanho e a heterogeneidade das amostras, fica claro que o equilíbrio ou desequilíbrio entre os biomarcadores de angiogénese (pro-angiogénicos e angiostáticos) tem

um papel preponderante no desenvolvimento e gravidade das alterações cutâneas, possivelmente através de mais do que um mecanismo.

Biomarcadores imunológicos

Na nossa investigação encontramos uma associação entre o número de linfócitos T CD8, de linfócitos T CD8 ativados e de células NK CD56+ forte e o envolvimento cutâneo (veja-se a sub-secção 4.1.2 e o Quadro 11). Por outro lado, e de acordo com o descrito por outros autores [19, 24], foi encontrada uma associação entre os anticorpos ACA e o menor atingimento cutâneo (veja-se a sub-secção 4.1.1).

O número de linfócitos T CD8+, de linfócitos T CD8+ ativados e de células NK CD56+forte foi mais elevado nos doentes sem envolvimento cutâneo (Pré-ES) do que naqueles com esse envolvimento (ES) no início do estudo, indicando que a diminuição destes linfócitos possa ser um “marcador de risco inicial” para este tipo de atingimento orgânico (veja-se a sub-secção 4.1.2 e o Quadro 11).

Tanto quanto é do nosso conhecimento, nenhum dos estudos publicados relacionou este tipo de células com o envolvimento cutâneo ou a sua gravidade.

Quadro 11 – Biomarcadores protetores de atingimento orgânico e sua gravidade

	LT CD8+	LT CD8 CD45RO+	LT CD8 HLADR+	LT CD56+	CNK	CNK CD56+ fraco	CNK CD56+ forte
Pele	↑	↑	↑	↔	↑	↑	↑
Vascular	↑	↑	↑	↔	↑	↑	↑
Pulmão	↔	↔	↔	↑	↔	↔	↑
Pulmão interstício	↔	↔	↔	↑	↔	↔	↑
Pulmão vascular	↔	↔	↔	↔	↔	↔	↔
Coração	↔	↔	↔	↔	↔	↔	↔
Gastrointestinal	↔	↔	↔	↔	↔	↔	↔

Abreviaturas: LT, Linfócitos T; CNK, Células NK.

↑ Aumento significativo dos valores séricos; ↑ Aumento marginal dos valores séricos; ↔ Sem alteração significativa dos valores séricos.

Vascular Periférico

Biomarcadores vasculares

Na nossa investigação encontramos uma relação entre a ET-1 e a gravidade das lesões vasculares no início do estudo e entre VEGF e o agravamento das alterações vasculares ao longo do mesmo.

De acordo com os resultados apresentados por outros autores [4,25], a ET-1 parece ser um “marcador de risco inicial” para a gravidade do atingimento vascular, uma vez que se mostrou significativamente mais elevada nos doentes com úlceras digitais do que naqueles com envolvimento vascular periférico menos grave. Para além disso, o VEGF parece funcionar com um “marcador de risco evolutivo” (e, mais uma vez, simultaneamente preditor) para o agravamento das lesões vasculares periféricas, dado que os doentes que precisavam de terapêutica para este atingimento orgânico no início do estudo (*score* de Medsger ≥ 1) e que agravaram o seu atingimento ao longo do mesmo, tinham valores de VEGF mais baixos (vejam-se as sub-secções 4.2.1 a 4.2.3 e 4.3 e Quadros 9 e 10). Estes resultados estão em consonância com aqueles apresentados por Distler *et al.* que relacionaram o aparecimento de úlceras digitais com valores mais baixos de VEGF [7].

Assim, e não obstante as considerações atrás apresentadas, doentes com valores de VEGF acima da média das medianas de doentes com *score* vascular de Medsger ≥ 1 que não agravaram e das medianas daqueles que agravaram durante o estudo, merecerão particular atenção em termos de possível agravamento vascular.

Biomarcadores imunológicos

Os doentes sem envolvimento vascular periférico (ou sem necessidade de terapêutica para o Raynaud) tiveram, no início do estudo, valores mais elevados de linfócitos T CD8+, T CD8+ ativados, células NK, células NK CD56+fraco e NK CD56+forte do que doentes com envolvimento vascular mais grave. Estes resultados apontam para que valores mais baixos destes tipos celulares possam funcionar como “biomarcadores de risco inicial” para um envolvimento vascular periférico de maior gravidade (veja-se a sub-secção 4.1.2 e o Quadro 11). Não encontramos estudos que tenham relacionado este tipo de células com o atingimento vascular periférico ou com a sua gravidade.

Pulmão

Biomarcadores vasculares

Na nossa investigação encontramos uma relação entre os valores de angiostatina, VEGF, ET-1, ICAM-1 e E-seletina e o envolvimento pulmonar e/ou a sua gravidade no início do estudo e entre os valores de endostatina e o agravamento das lesões pulmonares durante o mesmo.

Em consonância com os resultados apresentados por outros autores [16, 26-27], a ICAM-1 parece funcionar como um “marcador de risco inicial” de envolvimento pulmonar, já que se encontrava mais elevada (embora com aumento marginalmente significativo) nos doentes que no início do estudo tinham envolvimento pulmonar do que naqueles sem esse envolvimento. Por outro lado, valores mais elevados de angiostatina, ET-1, E-seletina (esta última com elevação marginalmente significativa) e mais baixos de VEGF, ao relacionarem-se com a gravidade do atingimento pulmonar, poderão representar “biomarcadores de risco inicial” para a existência de lesões pulmonares mais graves. Uma vez que os doentes com atingimento pulmonar no início do estudo e com valores mais altos de endostatina foram os que agravaram ao longo do mesmo, poderemos considerar esta molécula como “marcador de risco evolutivo” e com valor preditivo para o agravamento das lesões pulmonares. Estes resultados estão em consonância com aqueles apresentados por outros autores que relacionaram valores elevados de endostatina com a gravidade das PRF [17] e da PSAP [14]. Tal como referido para aspetos anteriores, valores médios dos intervalos inter-medianas podem ter utilidade clínica na vigilância destes doentes (vejam-se as sub-secções 4.2.2 a 4.2.4 e 4.3 e Quadros 9 e 10).

Alguns estudos, tal como o nosso, relacionaram a gravidade do atingimento pulmonar com níveis elevados de E-seletina [28,29] e outros relacionaram a gravidade do atingimento pulmonar avaliado pela PSAP com níveis elevados de ET-1 [10]. Apesar de termos relacionado níveis mais elevados de ET-1 com a gravidade do atingimento pulmonar no início do estudo e com os valores da DLCO, não encontramos relação da ET-1 com a PSAP, FVC ou alterações na TAC, o que poderá estar relacionado com o tamanho da amostra.

Na nossa investigação, encontramos, ainda, uma relação entre níveis mais elevados de angiostatina e a gravidade do atingimento pulmonar, assim como valores mais baixos de VEGF e de FVC (contrariamente aos resultados apresentados Kikuchi *et al.* [30]) no início do estudo. Não encontramos estudos que tenham estabelecido uma relação entre a angiostatina e a gravidade do envolvimento pulmonar.

Biomarcadores imunológicos

Em consonância com os resultados descritos por Domagala- Kulawik *et al.* [31], que descreveram um aumento dos linfócitos T CD56+ no LBA de doentes com ES e atingimento pulmonar grave, no nosso estudo, e tanto quanto é do nosso conhecimento, pela primeira vez na literatura, o valor destas células foi mais baixo no sangue periférico de doentes com doença pulmonar mais grave do que naqueles com atingimento pulmonar menos grave, nomeadamente em doentes com alterações das PFR.

Encontramos, ainda, uma relação entre um número mais baixo de células NK CD56+ forte no sangue periférico e a existência de alterações mais graves na TAC do tórax de alta resolução. Não registamos estudos que tenham estabelecido esta relação.

Desta forma, valores mais baixos destas células parecem funcionar como “biomarcadores de risco inicial” deste tipo de envolvimento orgânico e sua gravidade (veja-se a sub-secção 4.1.2 e o Quadro 11).

Por outro lado, e de acordo com o publicado por outros autores [19,24], doentes com anticorpo ACA tiveram menor atingimento do interstício pulmonar do que doentes sem este anticorpo, pelo que o ACA parece funcionar como marcador protetor deste tipo de atingimento orgânico (veja-se a sub-secção 4.1.1).

Tubo digestivo

Biomarcadores vasculares

Nesta investigação encontramos uma relação entre valores mais baixos de endostatina e mais elevados da razão VEGF/endostatina com o envolvimento do tubo digestivo ao longo do estudo.

Desta forma, estes biomarcadores poderão representar “marcadores de risco evolutivo” (e preditivos) deste tipo de atingimento orgânico (veja-se a sub-secção 4.3 e o Quadro 10).

Assim, doentes com valores de endostatina e de VEGF/endostatina situados acima do ponto médio do intervalo das medianas dos valores de doentes sem envolvimento gastrointestinal e dos valores daqueles que evoluíram para esse atingimento ao longo do estudo, terão maior probabilidade de terem envolvimento digestivo.

Não encontramos estudos que tenham relacionado estes biomarcadores com este envolvimento orgânico.

Mais uma vez, e à semelhança do descrito para o atingimento cutâneo, o desequilíbrio entre os biomarcadores de angiogénese (pro-angiogénicos e angiostáticos) parece ter um papel preponderante no desenvolvimento de alterações digestivas.

Biomarcadores imunológicos

Encontramos uma relação, embora marginalmente significativa, entre valores mais elevados de células NK CD56+ fraco e o desenvolvimento de alterações digestivas, o que levanta a hipótese de estas células poderem funcionar como “biomarcadores de risco de evolução” deste tipo de atingimento orgânico (veja-se a sub-secção 4.3).

Tal como Walker *et al.* [19], encontramos uma relação entre a presença do anticorpo anti-Scl70 e o envolvimento digestivo, pelo que este parece funcionar como um marcador de risco inicial deste tipo de atingimento orgânico (veja-se a sub-secção 4.1.1)

Coração

Biomarcadores vasculares

Valores mais elevados de ICAM-1 no início do estudo relacionaram-se, ainda que de forma marginalmente significativa, com o envolvimento cardíaco e sua gravidade, o que nos leva a considerar que esta molécula possa funcionar como “marcador de risco inicial” do referido atingimento e sua gravidade (veja-se a sub-secção 4.2.4 e Quadro 9).

Não encontramos estudos que tenham demonstrado a existência de uma relação entre este marcador e este tipo de envolvimento orgânico.

Conclusões

Na nossa investigação demonstramos que a lesão/ ativação endotelial e as alterações da angiogénese estavam presentes em todos os subtipos da doença, em todas as fases evolutivas e em todos os padrões capilaroscópicos.

Verificamos, também, que algumas moléculas ou a razão entre elas, aumentaram ou diminuíram precocemente e acompanharam a evolução da doença (endostatina, VEGF/ endostatina, P-seletina, VCAM-1) enquanto outras predominaram em fases mais precoces, ainda sem fibrose cutânea (VEGF), outras em fases mais tardias da ES (angiostatina, ET-1, E-seletina) e outras pareceram ter impacto na progressão da doença e na patogénese do envolvimento de vários órgãos (ET-1, ICAM-1, E-seletina, angiostatina, endostatina, VEGF, VEGF/ angiostatina, VEGF/ endostatina).

A angiotatina e a ET-1 poderão estar envolvidas em mecanismos patogénicos responsáveis pelo desenvolvimento do CREST em doentes com o subtipo limitado, uma vez que os seus valores se encontravam mais elevados nestes doentes.

Demonstramos, também, que a diminuição, no sangue periférico, das células T CD8+, nomeadamente das ativadas, das T CD56+ e das células NK e seus subtipos, é uma característica da ES. As alterações imunológicas celulares assumiram importância fundamentalmente nas fases precoces e tardias da ES, em doentes com padrões capilaroscópicos mais avançados (ativo e tardio) e parecem participar na patogénese de vários envoltimentos orgânicos.

Os auto-anticorpos específicos da doença (ACA e anti-Scl70) relacionaram-se com os subtipos limitado e difuso e associaram-se ao envolvimento orgânico. Os anticorpos anti-Ro52, embora inespecíficos, foram muito prevalentes na ES, fundamentalmente no subtipo limitado.

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5.2 – REFLEXÃO SOBRE OS RESULTADOS DA INVESTIGAÇÃO

Existem vários estudos publicados que relacionam diferentes moléculas, anticorpos, citocinas e tipos celulares com diversos aspetos da doença esclerodérmica, como foi referido nos diferentes capítulos desta tese.

Tanto quanto sabemos, este é o primeiro estudo que simultaneamente analisa várias moléculas tradutoras de lesão endotelial e de angiogénese, vários tipos celulares, anticorpos e citocinas e que os relaciona com vários aspetos da doença esclerodérmica, desde as fases mais iniciais (Pré-ES) até fases mais tardias da ES e durante um período de 4 anos de *follow-up* clínico.

Corroboramos resultados já descritos na literatura, contrariamos outros e, sobretudo, apresentamos dados novos.

A divergência de resultados poderá dever-se em parte, como já referido, a diferenças nas metodologias utilizadas e nas características amostrais.

Um dos aspetos a realçar prende-se com a forma como é medido o tempo de evolução da doença: uns estudos, tal como o nosso e de acordo com o defendido por Medsger [1], contabilizam o início da doença a partir do momento em que surge o primeiro sinal ou sintoma atribuível à mesma, independentemente de ser ou não o Fenómeno de Raynaud, enquanto outros atribuem o início da doença ao aparecimento de outro sinal ou sintoma. A nossa opção prendeu-se com o facto de o Fenómeno de Raynaud surgir em 90% a 100% dos doentes com ES e de esta ser frequentemente a primeira manifestação da doença [2].

Outros pontos a destacar relacionam-se com a subdivisão do tempo de evolução da doença (fase precoce e tardia vs. precoce, intermédia e tardia [1]) e com a classificação das alterações do preguiamento cutâneo que não são uniformes entre os estudos.

A nossa opção de dividir os doentes em três fases acabou por se traduzir em menores tamanhos sub-amostrais, limitando a rejeição da hipótese nula na estatística inferencial. A análise conjunta das várias fases da doença, independentemente do subtipo da ES, constituiu, um ponto fraco do estudo já que seria preferível, se as amostras fossem maiores, analisa-las separadamente. Por outro lado, a escolha da subdivisão do preguiamento cutâneo de acordo com Le Roy [3], deveu-se ao facto de ser a mais utilizada entre os estudos publicados, de os subtipos limitado e difuso se associarem a características clínicas, laboratoriais e serológicas diferentes [1] e de não existir evidência de as outras subdivisões propostas na literatura serem superiores ou de se associarem às características acima referidas [1].

O método de avaliação dos diferentes atingimentos orgânicos também não é uniforme entre os estudos. Apesar das limitações já referidas nesta tese, optamos por usar a escala de gravidade de Medsger e utilizar o ponto de corte da mesma para classificar o atingimento de

um determinado órgão e sua gravidade, embora com as adaptações que consideramos necessárias relativamente à utilização de exames auxiliares de diagnóstico.

Na nossa investigação, tal como na maioria dos estudos existentes, avaliamos as moléculas e as células estudadas apenas num único momento, o que constitui um ponto fraco do estudo na medida em que pode existir uma variação temporal das mesmas. No entanto, ao contrário da maioria dos estudos, relacionamo-las com vários parâmetros clínicos, o que constitui um ponto forte da nossa investigação.

Já foi realçado o tamanho da amostra como ponto fraco do estudo, condicionando interpretações mais conclusivas dos resultados. No entanto, é de salientar a relevância desta investigação, dado que, embora reduzido em termos absolutos, o tamanho amostral deve ser valorizado considerando tratar-se de uma doença rara na qual se verifica escassez de trabalhos relativamente a diversas relações estudadas.

Outros estudos serão necessários, com um número mais elevado de doentes, para confirmar os nossos resultados.

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5.3- APLICABILIDADE DOS RESULTADOS À PRÁTICA CLÍNICA

Os resultados apresentados nesta investigação relativamente aos biomarcadores vasculares (de lesão endotelial e de angiogénese) e imunológicos poderão ter interesse quer no seguimento de doentes com Pré-ES quer no seguimento de doentes com ES, se confirmados em estudos posteriores, como já referido.

Isto é particularmente verdade no que diz respeito à monitorização dos diferentes atingimentos orgânicos (quadro 1), fundamental na decisão de instituição de terapêutica nestes doentes (capítulo 2.2).

Quadro 1 - Biomarcadores vasculares e imunológicos com interesse potencial no seguimento dos doentes com Pré-ES e ES

Orgão	Biomarcadores vasculares	Biomarcadores imunológicos
Pele	Endostatina; razão VEGF/endostatina; VEGF; razão VEGF/angiostatina.	LT CD8+; LT CD8 CD45RO+; LT CD8 HLA-DR+; CNK; CNK CD56+ fraco; CNK CD56+ forte; ACA
Vascular periférico	VEGF, ET-1.	LT CD8+; LT CD8 CD45RO+; LT CD8 HLA-DR+; CNK; CNK CD56+ fraco; CNK CD56+ forte
Pulmão	Endostatina; Angiostatina; VEGF; ET-1; ICAM-1; E-seletina;	LT CD56+; CNK CD56+ forte ACA
Coração	E-seletina.	-
Digestivo	Endostatina; razão VEGF/Endostatina	Scl-70

Abreviaturas: ACA, anticorpos anti-centrómero; Scl-70, anticorpo anti-topoisomerase; ICAM-1, *Intercellular Adhesion Molecule 1*; VEGF, *Vascular Endothelial Growth Factor*; ET-1, endotelina 1; LT, linfócitos T; CNK, células NK.

Uma vez que no início do estudo os doentes já apresentavam vários tempos de evolução de doença, os biomarcadores que chamamos “de risco/protetores iniciais” devem ser interpretados de forma cautelosa e o significado atribuído ao risco/proteção de um determinado envolvimento orgânico ou sua gravidade, de acordo com os resultados

encontrados, deve ser aproximado daquele conferido aos biomarcadores a que chamamos de “risco evolutivo/ de evolução”. Desta forma, parece fazer sentido olhar para os biomarcadores iniciais e evolutivos como um todo e justificado o seu uso para a avaliação do envolvimento orgânico e sua gravidade, num determinado momento e em relação ao futuro, tendo, portanto, valor prognóstico.

Assim, se um doente com determinado atingimento orgânico tiver desvio no sentido positivo ou negativo de um biomarcador com interesse para a gravidade desse órgão, esse resultado poderá ajudar-nos a ser mais ou menos agressivos do ponto de vista terapêutico (por exemplo, endostatina e atingimento pulmonar; ET-1 e atingimento vascular periférico) e a ponderar, nomeadamente, a referenciação mais precoce de um doente para transplante de medula óssea.

Pelo contrário, se um doente não tiver atingimento de um determinado órgão, o desvio dos valores de biomarcadores relacionados com o envolvimento do mesmo, no sentido positivo ou negativo, poderá ajudar-nos na decisão sobre a periodicidade de observação de um doente ao longo do tempo (por exemplo, pele e endostatina).

ANEXOS|