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ANDRÉA TAVARES DANTAS

AVALIAÇÃO DO PERFIL DE CITOCINAS E QUIMIOCINAS EM PACIENTES COM ESCLEROSE SISTÊMICA: CORRELAÇÃO COM MANIFESTAÇÕES CLÍNICAS E COM RESPOSTA AO TRATAMENTO

Recife

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Orientadora: Profa. Dra. Maira Galdino da Rocha Pitta
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O Tempo

A vida é o dever que nós trouxemos para fazer em casa.
Quando se vê, já são seis horas!
Quando se vê, já é sexta-feira!
Quando se vê, já é natal...
Quando se vê, já terminou o ano...
Quando se vê, perdemos o amor da nossa vida.
Quando se vê, passaram 50 anos!
Agora é tarde demais para ser reprovado...
Se me fosse dado um dia, outra oportunidade, eu nem olhava o relógio.
Seguiria sempre em frente e iria jogando pelo caminho a casca dourada e inútil das horas...
Seguraria o amor que está à minha frente e diria que eu o amo...
E tem mais: não deixe de fazer algo de que gosta devido à falta de tempo.
Não deixe de ter pessoas ao seu lado por puro medo de ser feliz.
A única falta que terá será a desse tempo que, infelizmente, nunca mais voltará.

(Mário Quintana)

RESUMO

DANTAS, A. T. Avaliação do perfil de citocinas e quimiocinas em pacientes com esclerose sistêmica: correlação com manifestações clínicas e com resposta ao tratamento *in vitro*. 2016. 225f. Tese (Doutorado). Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

A esclerose sistêmica (ES) é uma doença multissistêmica do tecido conjuntivo, de etiologia desconhecida, caracterizada essencialmente por fibrose progressiva da pele e órgãos internos. Os mecanismos fisiopatogênicos envolvem alterações vasculares e desregulação autoimune, com participação de múltiplas citocinas. Atualmente, as poucas opções de tratamento para a doença, sobretudo para as manifestações inflamatórias e fibróticas, apresentam eficácia limitada. Sendo assim, o objetivo desse trabalho foi avaliar o perfil de citocinas e quimiocinas produzidas por pacientes com ES e avaliar, *in vitro*, a resposta ao tratamento com metilprednisolona (MP) e com um novo derivado tiazolidínico LPSF/CR-35. Foram avaliados 67 pacientes com diagnóstico de ES e 64 voluntários saudáveis, pareados por sexo e por idade. Para avaliação da resposta *in vitro*, foi realizada cultura de células mononucleares do sangue periférico (PBMC). A quantificação de citocinas e quimiocinas no soro e no sobrenadante de culturas foi realizada através de *cytometric bead array* (CBA) e *enzyme linked immunosorbent assay* (ELISA). Em comparação com controles saudáveis, pacientes com ES apresentaram níveis séricos aumentados de IFN-γ, TGF-β, CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG, CXCL10/IP-10, os quais foram associados à presença de manifestações clínicas específicas. Descrevemos também, aumento dos níveis séricos de IL-35 e sua associação com a presença de fibrose pulmonar, assim como elevação dos níveis de IL-29/IFN-λ1 e sua correlação com níveis de IFN-γ em pacientes com ES. No intuito de melhor caracterizar a associação entre citocinas e manifestações clínicas, foram mensurados os níveis de citocinas e quimiocinas no sobrenadante das culturas de PBMC não estimuladas e/ou submetidas ao estímulo com anti-CD3/CD28. Observou-se associação entre produção de IL-10 e a extensão do envolvimento cutâneo, IL-2 e dismotilidade esofageana, IL-2 e IL-4 e fibrose pulmonar e IL-4 e IL-10 e a presença de úlceras digitais. Com relação às quimiocinas, foi detectado aumento do nível sérico de CCL5/RANTES em pacientes

com fibrose pulmonar. No sobrenadante de cultura de PBMC, verificou-se maior concentração de CCL5/RANTES e de CXCL8/IL-8 em pacientes com úlceras digitais e maior produção de CXCL9/MIG e CXCL10/IP-10 em pacientes com doença precoce. Com o objetivo de verificar a resposta *in vitro* ao tratamento com corticosteroides, as condições de cultura estimuladas com anti-CD3/CD28 foram tratadas com MP 100μM e, após 48 horas, foi observada significativa redução dos níveis de CCL2/MCP-1, CCL5/RANTES e CXCL8/IL-8 em pacientes com ES. Nesses mesmos pacientes, as citocinas IL-2, IL-4, IL-6, IL-10, IL-17A, TNF e IFN-γ não sofreram modulação após o tratamento com MP. Por fim, foi avaliada a resposta *in vitro* ao tratamento com o LPSF/CR-35. O composto inibiu significativamente a produção de IL-4, IL-10, IL-17A, CCL2/MCP-1 e CXCL8/IL-8 e aumentou a de TNF em pacientes com ES. Dessa forma, o presente estudo demonstrou que pacientes com ES apresentam um perfil diferente de produção de citocinas e quimiocinas em comparação com indivíduos saudáveis e esse achado pode ajudar na melhor caracterização dos diversos fenótipos de pacientes. O tratamento da doença permanece um desafio e a avaliação dos efeitos *in vitro* de novas ou de estabelecidas moléculas fornecem subsídios para o futuro desenvolvimento de ensaios clínicos.

Palavras-chave: Esclerose sistêmica. Citocinas. Quimiocinas. Glucocorticoides. Tiazolidinedionas.

ABSTRACT

DANTAS, A. T. Cytokines and chemokines profile in systemic sclerosis patients: correlation with clinical manifestations and response to *in vitro* treatment. 2016. 225f. Thesis (Doctorate). Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.

Systemic sclerosis (SSc) is a multisystem connective tissue disease of unknown etiology, characterized primarily by progressive fibrosis of the skin and internal organs. The pathophysiological mechanisms involve vascular abnormalities and autoimmune deregulation, with participation of multiple cytokines. Currently, there are few treatment options for the condition, especially for inflammatory and fibrotic manifestations, and they have limited effectiveness. Thus, the aim of this study was to evaluate the profile of cytokines and chemokines produced by SSc patients and assess the response to *in vitro* treatment with methylprednisolone (MP) and a novel thiazolidine LPSF/CR-35. We evaluated 67 patients with SSc and 64 healthy volunteers, matched for sex and age. To evaluate the *in vitro* response to the treatments proposed, culture of peripheral blood mononuclear cells (PBMC) was obtained. Cytokines and chemokines quantification in serum and culture supernatant was performed by enzyme linked immunosorbent assay (ELISA) and cytometric bead array (CBA). Compared to healthy controls, SSc patients showed increased serum levels of IFN-γ, TGF-β, CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG, CXCL10/IP-10, which were associated with the presence of specific clinical manifestations. We describe also an increase in serum levels of IL-35 and its association with pulmonary fibrosis, as well as elevation of IL-29/IFN-λ1 levels and its correlation with IFN-γ levels in SSc patients. In order to further characterize the association between cytokines and clinical manifestations, levels of cytokines and chemokines were measured in supernatant of PBMC cultures unstimulated and/or stimulated with anti-CD3/CD28. There were associations between IL-10 production and cutaneous involvement, IL-2 and esophageal dysmotility, IL-2 and IL-4 and lung fibrosis, and IL-4 and IL-10 and digital ulcers. Regarding chemokines, it was detected increased serum CCL5/RANTES level in patients with pulmonary fibrosis. In PBMC culture supernatant, there was a higher concentration of CCL5/RANTES and CXCL8/IL-8 in patients with digital ulcers and an increased

production of CXCL9/MIG and CXCL10/IP-10 in patients with early disease. In order to verify the *in vitro* response to corticosteroid treatment, the culture conditions stimulated with anti-CD3/CD28 were treated with MP 100μM, and after 48 hours, there was significant reduction in the levels of CCL2/MCP-1, CCL5/RANTES and CXCL8/IL-8 in patients with SSc. In the same patients, the cytokines IL-2, IL-4, IL-6, IL-10, IL-17A, TNF and IFN-γ not undergone modulation after treatment with MP. Finally, *in vitro* response to treatment with LPSF/CR-35 was evaluated. The compound significantly inhibited IL-4, IL-10, IL-17A, CCL2/MCP-1 and CXCL8/IL-8 and increased TNF in SSc patients. Thus, the present study demonstrated that patients with SSc present a different profile of cytokines and chemokines compared to healthy subjects and this finding may help in better characterization of the different phenotypes of patients. The treatment of the disease remains a challenge and assess *in vitro* effects of new or established molecules provide information for the future development of clinical trials.

Keywords: Systemic sclerosis. Cytokines. Chemokines. Glucocorticoids. Thiazolidinediones.

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LISTA DE ABREVIATURAS E SIGLAS

α-SMA	*Alpha-smooth muscle actin* (actina do músculo liso-alfa)
ACE	*angiotensin I converting enzyme* (enzima conversora de angiotensina I)
ACR	*American College Of Rheumatology*
Akt	*Protein kinase B*
ANA	Anticorpos antinucleares
AngII	Angiotensina II
Anti-SCl70	Anticorpo anti-DNA topoisomerase I
AR	Artrite reumatoide
ARA	Anticorpo anti-RNA polimerase
BANK1	*B-cell scaffold protein with ankyrin repeats 1*
BCC	Bloqueador de Canal de Cálcio
BMP-7	*Bone morphogenetic protein-7* (proteína óssea morfogenética-7)
BRA	Bloqueador do receptor de angiotensina
CBA	*Cytometric bead array*
CBP	*CREB-binding protein*
CD	Cutânea Difusa
CL	Cutânea Limitada
CTGF	*Connective tissue growth factor* (fator de crescimento do tecido conectivo)
DPI	Doença Pulmonar Intersticial
EBI3	Gene 3 induzido pelo vírus Epstein-Barr
ELISA	*Enzyme linked immunosorbent assay*
EMT	*Epitelial mesenchymal transition* (transição epitelial-mesenquimal)
ES	Esclerose Sistêmica
ET-1	*Endothelin-1* (Endotelina-1)
EULAR	*European League Against Rheumatism*
EUSTAR	*European League Against Rheumatism Scleroderma Trials And Research*
EVA	Escala Visual Analógica

FAK	*Focal adhesion kinase*
FGF	*Fibroblast growth fator* (Fator de crescimento do fibroblasto)
FRy	Fenômeno de Raynaud
GC	Glucocorticoide
HAP	Hipertensão Arterial Pulmonar
HAQ	*Health Assessment Questionaire*
HC	Hospital das Clínicas
HGF	*Hepatocyte growth factor* (fator de crescimento do hepatócito)
IBP	Inibidor de Bomba de Prótons
ICAM	*Intercellular Adhesion Molecule 1* (molécula de adesão intercelular)
IECA	Inibidor da Enzima Conversora de Angiotensina
IFN-γ	Interferon gama
IFN-λ	Interferon lambda
IL	Interleucina
IP-10	*Interferon gamma-induced protein* (proteína induzida por interferon gama)
IRF5	*Interferon regulatory factor 5*
iTreg	T Regulatórias Induzidas
JNK	*C-Jun N-Terminal Kinases*
LAP	*Latency-associated propeptide* (pró-peptídeo associado à latência)
LBA	Lavado Broncoalveolar
LINAT	Laboratório de imunomodulação e novas abordagens terapêuticas
LLC	*Large Latent Complex* (grande complexo latente)
LOX	*Lysyl oxidase enzyme*
LPSF	Laboratório de Planejamento e Síntese de Fármacos
LTBP	*Latent TGF-ß -binding protein* (Proteína ligante do TGF-β latente)
MAPK	*Mitogen activated protein kinases* (proteinas-quinases ativadas por mitógenos)
MCF	Metacarpofalangeanas
MCP	*Monocyte chemoattractant protein* (proteína quimiotática de monócitos)
MEC	Matriz extracelular

MIG	*Monokine induced by interferon gamma* (monocina induzida por interferon gama)
MIP-1α	*Macrophage inflammatory protein*-1α (proteína inflamatória de macrófagos)
MMP	Metaloproteinases de matriz
MP	Metilprednisolona
NK	*Natural Killer*
NK-κB	Fator nuclear kappa B
NOS	*nitric oxide synthase* (óxido nítrico sintase)
nTreg	T Regulatórias Naturais
NUPIT-SG	Núcleo de pesquisa em inovação terapêutica Suely Galdino
PAI-1	*Plasminogen activator inhibitor-1* (inibidor do ativador de plasminogênio-1)
PBMC	*Peripheral blood mononuclear cells* (células mononucleares do sangue periférico)
PDGF	*Platelet-derived growth factor* (fator de crescimento derivado de plaquetas)
PGE2	Prostaglandina E2
PGI2	Prostaciclina
PGZ	Pioglitazona
PI3K	*Phosphoinositide 3-Kinase*
PPARγ	*Peroxisome Proliferator-Activated Receptor* (receptor ativado por proliferadores de peroxissoma gama)
PPRE	*Peroxisome proliferator response elements* (elementos de resposta do PPAR)
PTEN	*Phosphate and tensin homologue*
PTPN22	*Protein tyrosine phosphatase, non-receptor type 22*
RANTES	*Regulated Upon Activation and Normal T Cells Expressed and Secreted* (Regulada sob Ativação, Expressa e Secretada por Células T Normais)
RGZ	Rosiglitazona
RNAm	Ácido Ribonucleico mensageiro
RORC	*Retinoic acid receptor-related orphan receptor*

RXR	Receptor de Retinoide X
SBE	*Smad*-b*inding element* (elemento ligador de Smad)
SFB	Soro Fetal Bovino
SHAQ	*Scleroderma Health Assessment Questionnaire*
SIL-6R	Receptor solúvel da IL-6
SLC	*Small latent complex* (pequeno complexo latente)
STAT	*Signal transducer and activator of transcription*
TAK-1	*TGFβ-activated kinase*
TC	Tomografia Computadorizada
TCLE	Termo de Consentimento Livre e Esclarecido
TGF-β	*Transforming Growth Fator Beta* (fator transformador do crescimento beta)
TGZ	Troglitazona
Th	T Helper
TIMP	*Tissue Inhibitor of Metalloproteinase* (inibidor tecidual de metaloproteinases)
TNF-α	Fator de Necrose Tumoral Alfa
Treg	T Regulatória
TZD	Tiazolidinedionas
UFPE	Universidade Federal de Pernambuco
VEGF	*Vascular endothelial growth factor* (fator de crescimento endotelial vascular)

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1 INTRODUÇÃO

A Esclerose Sistêmica (ES) é uma doença do tecido conjuntivo, de etiologia desconhecida, caracterizada essencialmente por fibrose progressiva da pele e órgãos internos. Apesar de ser uma doença relativamente rara, é considerada de extrema importância na reumatologia, tendo em vista seu importante impacto na qualidade de vida do indivíduo acometido e seu prognóstico reservado.[1; 2] A presença de manifestações clínicas graves associada à ausência de tratamento efetivo se traduzem em uma alta taxa de mortalidade, cerca de 3,5 vezes maior que a estimada para indivíduos do mesmo sexo e idade.[3]

Os mecanismos fisiopatogênicos são complexos e ainda pouco compreendidos. Entre os eventos patológicos implicados incluem-se lesão endotelial, com alterações vasculares; desregulação imune, com liberação de citocinas, mediadores inflamatórios e produção de autoanticorpos; e ativação de fibroblastos. Esse processo resulta em produção excessiva de constituintes da matriz extracelular, com o aparecimento do fenótipo fibrótico.[4]

Exames histológicos de lesões de pele em pacientes com doença inicial demonstram a presença de um infiltrado inflamatório com predomínio de células T precedendo o desenvolvimento da vasculopatia e da fibrose, indicando a participação dessas células na fisiopatologia da ES.[5] Sugere-se uma polarização da resposta imune no desenvolvimento da doença, com predomínio de citocinas pró-inflamatórias T helper (Th)1 e Th17 em estágios mais precoces, evoluindo posteriormente com participação mais acentuada da resposta Th2.[6] Estudos têm demonstrado uma associação entre manifestações clínicas da ES e níveis séricos e expressão tissular de citocinas, embora os resultados sejam muitas vezes contraditórios.[6; 7]

Tendo em vista a fisiopatologia da doença, os principais objetivos terapêuticos no tratamento da ES incluem: limitar os danos provocados pela inflamação e autoimunidade, sobretudo em fases mais precoces da doença; re-estabelecer a homeostase vascular e evitar o dano estrutural provocado pela fibrose. Devido à complexidade da ES e à grande heterogeneidade clínica da doença, com importante variação no padrão de envolvimento de órgãos, a abordagem do tratamento é programada de acordo com as necessidades de cada paciente, direcionada para os

órgãos acometidos e muitas vezes com o propósito sintomático.[8] Os imunossupressores tradicionais, como ciclofosfamida, metotrexato, azatioprina e micofenolato de mofetila, representam hoje os principais medicamentos utilizados no tratamento das manifestações inflamatórias e fibróticas da ES, porém com eficácia limitada.[9]

Os glucocorticoides (GC), fármacos amplamente utilizados no tratamento de doenças inflamatórias e autoimunes em geral, apresentam indicação restrita na ES. Embora os estudos clínicos sejam insuficientes para demonstrar eficácia da monoterapia ou do uso de altas doses de GC no tratamento da doença[10; 11; 12] e o seu uso esteja associado ao risco de desenvolvimento de crise renal esclerodérmica[13], na prática clínica, um percentual significativo dos pacientes com ES é tratado com GC, geralmente em associação com outros imunossupressores.[10] O mecanismo de ação e os efeitos moleculares dessa classe de fármacos no tratamento da ES são ainda indefinidos.

Neste contexto, fica clara a necessidade de estudos que visem à descoberta de novos alvos terapêuticos e de novos fármacos a serem utilizados no tratamento da ES. As tiazolidinedionas (TZD) correspondem a uma classe de compostos derivados da tiazolidina que apresentam diversos efeitos biológicos, os quais se traduzem em aplicações terapêuticas.[14] Essas moléculas agem essencialmente através da ligação aos receptores ativados por proliferadores de peroxissoma gama (PPARγ), que são fatores de transcrição dependentes de ligantes.[15] Estudos recentes têm sugerido um papel antifibrótico para os agonistas do PPARγ. Em fibroblastos normais, a ativação do PPARγ resultou em inibição da expressão do gene do colágeno e da transdiferenciação de miofibroblastos.[16] Em modelos animais, o tratamento com o agonista sintético rosiglitazona (RGZ) diminuiu de forma significativa a fibrose dérmica e a deposição de colágeno induzida por bleomicina.[17] Em pacientes com ES, foi demonstrada diminuição da expressão do PPARγ em fibroblastos cutâneos.[18; 19] Nesse sentido, derivados tiazolidínicos agonistas do PPARγ podem apresentar um potencial terapêutico na ES.

Tendo em vista a necessidade de melhor caracterização da fisiopatologia da doença e a busca de potenciais biomarcadores, no presente estudo, avaliamos o perfil de citocinas e quimiocinas presentes no sangue periférico de pacientes com ES e suas associações com as principais manifestações clínicas da doença. Adicionalmente,

com o objetivo de verificar estratégias terapêuticas para a doença, investigamos a resposta *in vitro* ao tratamento com a metilprednisolona (MP) e com o LPSF/CR-35, um novo derivado tiazolidínico sintetizado pelo Laboratório de Planejamento e Síntese de Fármacos (LPSF) da Universidade Federal de Pernambuco (UFPE).

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral desta proposta foi avaliar o perfil de citocinas e quimiocinas em pacientes com esclerose sistêmica e a resposta ao tratamento *in vitro* com o derivado tiazolidínico LPSF/CR-35.

2.2 Objetivos Específicos

- Descrever as características demográficas, clínicas e terapêuticas dos pacientes com ES;
- Determinar o perfil de citocinas e quimiocinas em pacientes com ES e suas associações com manifestações clínicas da doença;
- Verificar o efeito *in vitro* da metilprednisolona na produção de citocinas e quimiocinas em pacientes com ES;
- Avaliar, *in vitro*, o efeito do LPSF/CR35 na produção de citocinas e quimiocinas em pacientes com ES.

3 REVISÃO DA LITERATURA

3.1 Esclerose Sistêmica

A esclerose sistêmica é uma doença multissistêmica do tecido conjuntivo, de etiologia desconhecida, caracterizada por fibrose progressiva da pele e órgãos internos, associada a microangiopatia fibroproliferativa e presença de autoanticorpos.[5; 20] Corresponde a uma doença rara, com prevalência e incidência variando de acordo com a região geográfica e com os critérios empregados para seu o diagnóstico.[21; 22; 23] Avanços recentes com relação ao tratamento, com impacto na redução da mortalidade e melhora da sobrevida, têm contribuído para um aumento da prevalência da ES ao longo dos anos, variando, nas séries mais recentes, entre 38 casos/milhão de habitantes no Japão até 296 casos/milhão de habitantes na Argentina.[23] No Brasil, dados referentes a incidência e prevalência da ES não são conhecidos.

A ES predomina no sexo feminino (6:1), com pico de incidência dos 40 aos 50 anos.[24; 25] Com relação à raça, embora seja mais frequente em caucasianos, nos indivíduos afro-americanos a doença se apresenta de forma mais precoce e mais grave.[23; 25] Dados brasileiros, obtidos a partir do estudo epidemiológico conduzido pela Sociedade Brasileira de Reumatologia, que avaliou 1139 pacientes em 28 diferentes centros, evidenciou predominância do sexo feminino (87,4%) e da raça caucasiana (65%), com doença mais grave no gênero masculino e em afro-brasileiros.[26]

3.1.1 Etiopatogênese

A etiopatogenia da ES é ainda desconhecida e acredita-se que seja multifatorial, tendo sido estudada a contribuição de fatores genéticos e ambientais. Fatores ambientais associados a risco aumentado e gravidade da doença incluem exposição a sílica, resinas epoxi e solventes aromáticos. Além disso, também tem sido sugerida a associação entre agentes infecciosos, como o parvovírus B19, vírus *Epstein-Barr* e citomegalovírus, e o desenvolvimento da doença.[27]

Diversas evidências sugerem uma predisposição genética ao desenvolvimento da ES.[28; 29; 30; 31] O risco da doença é aumentado em parentes de primeiro grau em comparação com a população geral.[28] Além disso, estudos com gêmeos monozigóticos, apesar de não terem demonstrado concordância com relação à expressão clínica da doença, evidenciaram-na para a presença de anticorpos antinucleares (ANA) e para o perfil de expressão gênica de fibroblastos dérmicos cultivados.[29; 30] Estudos de genes candidatos têm identificado três principais subgrupos, os quais refletem as áreas de expressão da doença: fibrose, resposta imune e vasculopatia. Com relação à fibrose, os genes candidatos são responsáveis por codificar proteínas que compõem ou regulam a matriz extracelular, fatores de crescimento e citocinas, como o gene do fator de crescimento do tecido conectivo (CTGF).[32; 33] Entre os genes candidatos relacionados à inflamação e autoimunidade destacam-se *B-cell scaffold protein with ankyrin repeats 1* (BANK1), receptor da interleucina (IL)-23, *interferon regulatory factor 5* (IRF5), *signal transducer and activator of transcription* 4 (STAT4) e *protein tyrosine phosphatase, non-receptor type 22* (PTPN22), e entre aqueles relacionados ao comprometimento vascular incluem-se *nitric oxide synthase* (NOS), *angiotensin I converting enzyme* (ACE), *vascular endothelial growth factor* (VEGF) e *endothelin 1* (ET1).[31; 34]

3.1.2 Formas clínicas

De acordo com a extensão e localização do acometimento cutâneo, os pacientes são classificados em duas formas clínicas: a cutânea limitada (CL) e a cutânea difusa (CD). A CL é a forma mais frequente e é caracterizada pelo acometimento da pele restrito à face, ao pescoço e à região distal aos cotovelos e joelhos.[25; 35] A forma CD, por sua vez, apresenta um envolvimento mais rápido e generalizado da pele, com comprometimento precoce de órgãos internos.[35]

Uma importante diferença entre esses dois grupos refere-se à velocidade de progressão da doença. A forma CL tende a apresentar um início mais insidioso, geralmente precedido pela presença do fenômeno de Raynaud (FRy) durante anos. O comprometimento vascular da ES é mais proeminente nessa forma clínica, caracterizada por uma maior ocorrência de hipertensão arterial pulmonar (HAP) e

úlceras digitais. Essa forma também está associada a uma maior frequência do anticorpo anticentrômero.[36]

Por outro lado, pacientes com a forma CD apresentam uma evolução mais rápida, com comprometimento cutâneo muitas vezes simultâneo ao aparecimento do FRy. Além disso, o comprometimento visceral tende a ser mais precoce, sobretudo a doença pulmonar intersticial, e observa-se uma maior frequência de anticorpos anti-topoisomerase I e anti-RNA polimerase I e III. [37; 38] Apesar de as duas formas serem potencialmente graves, a CD está geralmente associada a um pior prognóstico, com maiores taxas de morbidade e mortalidade.[39]

3.1.3 Manifestações Clínicas

O comprometimento cutâneo, representado principalmente por um endurecimento progressivo da pele (Figura 1A e B), consiste na manifestação mais característica da doença, presente em mais de 90% dos pacientes.[37] A extensão do comprometimento cutâneo pode ser quantificada através do escore modificado de Rodnan[13] e tem sido considerada um marcador de gravidade da doença e preditor independente de mortalidade.[40] Além do espessamento cutâneo, outras alterações frequentemente encontradas na ES incluem telangiectasias, alterações da pigmentação (leucomelanodermia) (Figura 1A e B), diminuição da abertura oral (microstomia) e calcinose. [37]

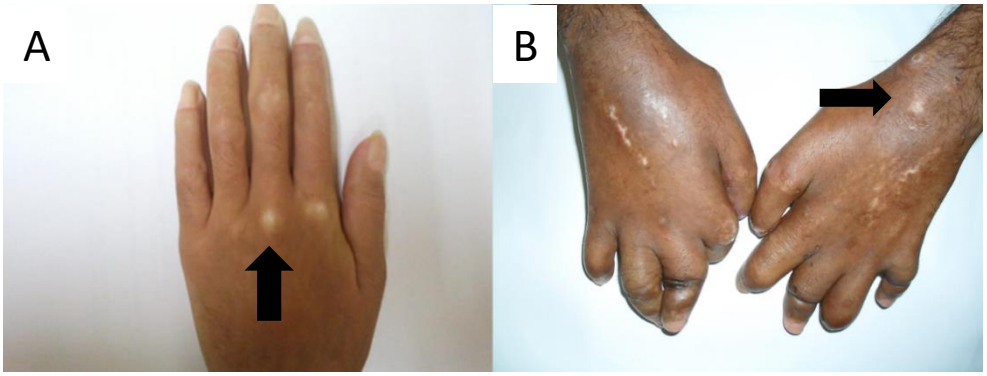

Figura 1. Espessamento cutâneo e alteração da pigmentação (setas) em mãos de pacientes com ES (banco de imagens do serviço de Reumatologia do Hospital das Clínicas - HC-UFPE).

O FRy representa a manifestação mais comum, presente em até 95% dos pacientes, e pode preceder em meses a anos as demais manifestações clínicas, sendo, portanto, um sinal de alerta para o diagnóstico precoce da doença.[25; 41] É caracterizado por uma resposta vasoconstrictora anormal ao frio e/ou estresse, que provoca espasmo das arteríolas cutâneas, manifestando-se, classicamente, sob a forma de palidez seguida por cianose e eritema, mais comumente observado nos dedos das mãos e pés [41] (Figura 2A).

Outra manifestação de origem vascular são as úlceras digitais, que ocorrem em até um terço dos pacientes e representam uma importante causa de dor e de incapacidade funcional. Úlceras crônicas constituem um importante local de infecção secundária, podendo evoluir com necrose e amputação (Figura 2B). A etiologia das úlceras é multifatorial, estando primordialmente associada à isquemia decorrente da vasculopatia, mas também a microtraumas repetitivos, ao espessamento cutâneo decorrente da fibrose e à presença de calcinose.[42]

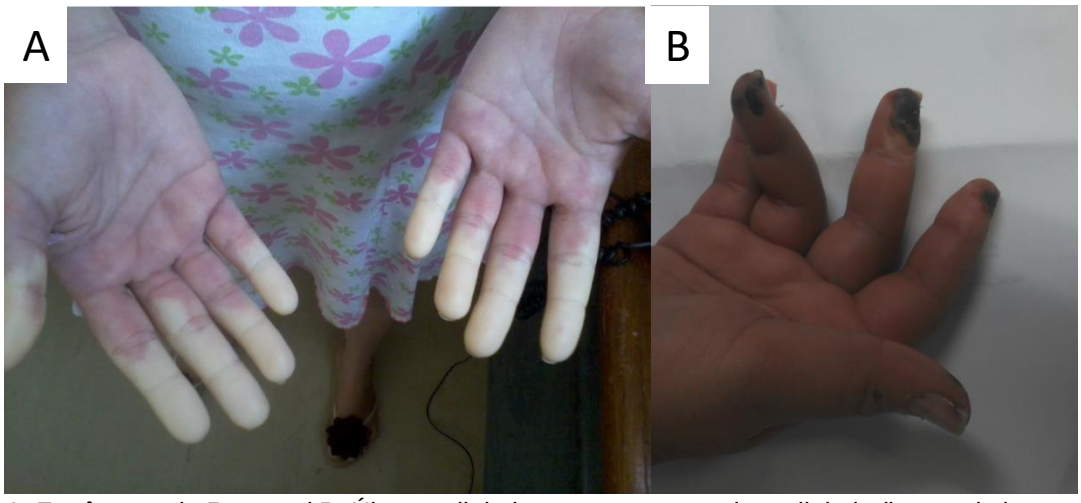

Figura 2. A. Fenômeno de Raynaud B. Úlceras digitais e necrose em polpas digitais (banco de imagens do serviço de Reumatologia do HC-UFPE).

O comprometimento vascular pulmonar, na forma de HAP, é descrito em 10 a 21% dos pacientes, sendo mais comum na forma cutânea limitada.[25; 43; 44] Manifesta-se com dispneia progressiva aos esforços, podendo evoluir com insuficiência cardíaca e respiratória. Representa atualmente uma das principais causas de mortalidade, com uma sobrevida estimada em 1 e 3 anos de 78% e 47%, respectivamente.[2]

A doença pulmonar intersticial (DPI) é, assim como a HAP, uma das principais causas de mortalidade nos pacientes com ES. A fibrose pulmonar, avaliada através de tomografia computadorizada (TC) de alta resolução, é encontrada em mais da metade dos pacientes, sendo mais comum na forma cutânea difusa. A apresentação clínica inclui dispneia progressiva, tosse seca e dor torácica, sendo importante o diagnóstico diferencial com HAP, embora as duas condições possam coexistir.[25; 45]

O comprometimento do trato digestivo é descrito como a complicação visceral mais frequente, sendo encontrada em até 80% dos pacientes.[2; 46] As alterações vasculares e fibróticas da doença podem comprometer qualquer porção do trato gastrintestinal, provocando distúrbios de motilidade, digestão e absorção. Como consequência, os pacientes podem apresentar disfagia, refluxo gastroesofágico, hemorragia digestiva, diarreia ou constipação, manifestações que têm importante impacto na qualidade de vida e podem estar, direta ou indiretamente, relacionadas à mortalidade.[46]

Previamente descrita como a principal causa de morte em pacientes com ES, a crise renal esclerodérmica tem mostrado uma incidência decrescente ao longo dos anos, atribuída à introdução de um tratamento clínico efetivo, os inibidores da enzima conversora de angiotensina (IECA). É caracterizada pelo início súbito de hipertensão arterial e insuficiência renal e é mais frequente nos indivíduos com a forma cutânea difusa, nos primeiros anos da doença e em usuários de corticosteroides.[2; 47]

Por fim, semelhante a outras doenças reumatológicas inflamatórias, o acometimento músculo-esquelético é também frequente em pacientes com ES, com uma prevalência de até 97%. Manifestações articulares, na forma de artralgia, artrite ou contraturas, estão associadas a importante comprometimento da capacidade funcional e da qualidade de vida. Além disso, a presença de miopatia, evidenciada através de mialgia, déficit de força e/ou elevação de enzimas musculares, está associada a doença mais grave.[48]

3.1.4 Autoanticorpos

Além das manifestações clínicas descritas anteriormente, a ES caracteriza-se pela presença de autoanticorpos em 80 a 95% dos pacientes[25; 38; 49], os quais podem

ter associação com manifestações clínicas específicas da doença. Muitos dos autoanticorpos descritos na ES têm como alvo antígenos nucleares, com frequência de 85 a 99%.[38] Entre os anticorpos considerados mais específicos para a doença, destacam-se o anticentrômero, o anti-DNA topoisomerase I (anti-Scl70) e anti-RNA polimerase (ARA) I e III. Embora o papel patogênico desses autoanticorpos não esteja definido, eles têm sido considerados como biomarcadores da doença.[38]

O anti-Scl70 é encontrado em 40 a 70% dos pacientes com a forma cutânea difusa e em cerca de 10% a 18% daqueles com a forma limitada. Sua presença é associada a um pior prognóstico, com maior frequência de comprometimento pulmonar, cardíaco e muscular, além de maior mortalidade.[38; 49]

Anticorpos anticentrômero são mais frequentes na forma CL, ocorrendo em 55 a 80% desses pacientes. Embora considerado marcador de melhor prognóstico em relação ao anti-Scl70, sua presença está associada a risco aumentado de HAP.[38; 49]

Anticorpos anti-RNA polimerase I, II e III são encontrados na ES, embora os tipos I e III sejam mais específicos para a doença.[38; 49] A presença de ambos está associada ao acometimento cutâneo difuso e ao maior risco de crise renal.[38]

3.1.5 Diagnóstico

O diagnóstico da ES é estabelecido a partir das manifestações clínicas, associadas a alterações laboratoriais. Em um paciente que se apresenta com FRy e manifestações viscerais características, o diagnóstico é claro, porém geralmente tardio e, dessa forma, muitas vezes sem possibilidade de intervenção terapêutica efetiva. Esforços têm sido empreendidos para uma identificação mais precoce da doença, com o objetivo de estabelecer um tratamento em uma fase mais inicial e tentar prevenir o desenvolvimento de danos irreversíveis. Entretanto, até o momento ainda não foram validados critérios diagnósticos para a ES.[50]

Formulados essencialmente para fins de estudos clínicos, os critérios classificatórios são frequentemente utilizados na prática clínica como ferramenta auxiliar de diagnóstico (Tabela 1). Contudo, são parâmetros estabelecidos para a identificação de doença definida, com o objetivo de permitir uma comparação adequada em estudos observacionais e seleção dos indivíduos em ensaios clínicos.

Nesse sentido, apesar de terem alta especificidade, esses critérios muitas vezes não são sensíveis o suficiente para a identificação precoce da doença.[51]

Tabela 1. Critérios classificatórios para esclerose sistêmica

	CRITÉRIOS ACR, 1980 [52]	**ES PRECOCE, 2001** [53]	**ES MUITO PRECOCE, 2009** [54]	**ACR/EULAR, 2013** [55]
Maiores	Espessamento cutâneo proximal às MCF	FRy	FRy Autoanticorpos (ANA, anticentrômero, anti-Scl70) Capilaroscopia alterada	Espessamento cutâneo proximal às MCF (9)
Menores	Esclerodactilia Microcicatrizes digitais ou perda de substância Fibrose pulmonar bibasal	Capilaroscopia alterada com padrão SD Autoanticorpos ES-específicos (anticentrômero, anti-Scl70, ARA I ou ARA III, anti-fibrilarina, anti-PM-Scl)	Calcinose *Puffy fingers* Úlceras digitais Disfunção do esfíncter esofágico inferior Telangiectasias TC de tórax com padrão de vidro fosco	*Puffy fingers* (2) ou Esclerodactilia (4) Úlceras digitais (2) ou Microcicatrizes de polpa digital (3) Telangiectasias(2) Capilaroscopia alterada(2) HAP(2) ou DPI(2) FRy (3) Autoanticorpos (3)
Definição	**1 critério maior ou 2 critérios menores**	**FRy objetivo + 1 critério menor OU FRy subjetivo + 2 critérios menores**	**3 critérios maiores OU 2 maiores + 1 menor**	**Pelo menos 9 pontos no somatório**

ACR= *American College of Rheumatology*, ANA= anticorpos antinucleares, ARA= anticorpo anti-RNA polimerase, DPI= doença pulmonar intersticial, EULAR= *European League Against Rheumatism*, ES= esclerose sistêmica, FRy= fenômeno de Raynaud, HAP= hipertensão arterial pulmonar, MCF= metacarpofalangeanas, TC= tomografia computadorizada.

3.1.6 Prognóstico

Estudos recentes demonstraram um aumento progressivo da sobrevida da doença, atribuído a um diagnóstico mais precoce e melhora no tratamento de algumas de suas complicações. Entretanto, permanecem ainda altas as taxas de morbimortalidade, sendo considerada a mais grave doença do tecido conjuntivo.[23] O risco de morte em pacientes com ES é 3,5 vezes maior, quando comparado ao risco esperado para indivíduos do mesmo sexo e idade.[56] Levantamento realizado pelo *European League against Rheumatism Scleroderma Trials and Research* (EUSTAR) avaliou 5860 pacientes em diversos centros no mundo, com registro de 284 óbitos. Entre as causas dos óbitos, 55% foram diretamente relacionadas à doença, com

destaque para a fibrose pulmonar (19%) e hipertensão arterial pulmonar (14%). Entre as causas não-relacionadas à ES, predominaram as infecções (13%), neoplasias (13%) e doença cardiovascular (12%), as quais muitas vezes estão diretamente relacionadas a complicações decorrentes da ES ou do seu tratamento[57]. No Brasil, avaliação de 168 óbitos entre 947 pacientes também encontrou a ES como causa direta da morte na maioria dos casos (65,5%), predominando a doença pulmonar intersticial e HAP, e entre as causas não-diretamente relacionadas, infecção (41,4%), doença cardiovascular (13,8%) e neoplasia (13,8%). [58]

3.2 Fisiopatologia da Fibrose e da Esclerose Sistêmica

A patogênese da ES é complexa e ainda pouco entendida, sendo caracterizada por três pilares principais: dano vascular, autoimunidade e fibrose. De uma maneira geral, a vasculopatia compreende proliferação fibrointimal de pequenos vasos e eventos vasoespásticos, os quais levam à isquemia. A desregulação autoimune inclui ativação de linfócitos, levando à produção de autoanticorpos, liberação de citocinas e quimiocinas e alteração do sistema imune inato. A fibrose, por sua vez, decorre da ativação de fibroblastos e síntese excessiva de matriz extracelular (MEC), provocando distorção da arquitetura normal do tecido. A heterogeneidade clínica da doença reflete exatamente a contribuição relativa de cada um desses três mecanismos.[4; 59]

A fibrose representa a via final comum na patogênese da ES, e é responsável por grande parte do prognóstico atribuído à doença. É caracterizada histologicamente pela substituição da arquitetura normal por tecido conjuntivo denso, composto por colágeno e outras proteínas da matriz extracelular. A MEC é formada por elementos celulares (células residentes e infiltrado de células oriundas da circulação) e tecido conjuntivo (composto por macro e micromoléculas, como colágenos, proteoglicanos, elastinas, fibrilinas e moléculas de adesão), além de servir como reservatório de fatores de crescimento como o *transforming growth fator beta* (TGF-β) e proteínas como CTGF, os quais regulam a diferenciação, proliferação e função das células residentes. Acúmulo excessivo de tecido conjuntivo resulta da superprodução por células mesenquimais ativadas e/ou déficit na degradação e *turnover* da MEC.[60; 61]

3.2.1 Fibroblastos

Os fibroblastos representam as células efetoras mais importantes no processo de fibrose e são responsáveis pela síntese e degradação da MEC. Sob a influência de sinais extracelulares específicos, essas células proporcionam síntese de colágeno e outras macromoléculas da MEC; promovem adesão e contração do tecido conjuntivo; secretam fatores de crescimento, citocinas e quimiocinas ou expressam receptores de superfície para eles; e sofrem transdiferenciação para miofibroblastos. Essas propriedades permitem aos fibroblastos participar da efetiva cicatrização de feridas, processo que, em condições fisiológicas, é autolimitado e efetivamente controlado. Por outro lado, é demonstrado que na fibrose patológica ocorre ativação ampliada e sustentada de fibroblastos.[62]

A ativação dos fibroblastos é estimulada por citocinas e outras moléculas secretadas, pela interação célula-célula e célula-matriz e por hipóxia.[4] Existem mecanismos para controlar a síntese de MEC e a ativação de fibroblastos, dessa forma prevenindo o acúmulo excessivo de matriz. Os fibroblastos possuem moléculas endógenas que reprimem a expressão de genes da MEC e a estimulação do TGF-β, como o bloqueio da transdução de sinal do TGF-β através da Smad-7. Outras moléculas também parecem funcionar como repressores celulares endógenos da síntese de colágeno, como Fli-1, p53 e o PPARγ. Estudos recentes mostraram que, na ES, parece haver um *déficit* funcional da Smad-7 e defeito na expressão, regulação e função desses inibidores endógenos.[16; 63; 64; 65; 66] (Figura 3)

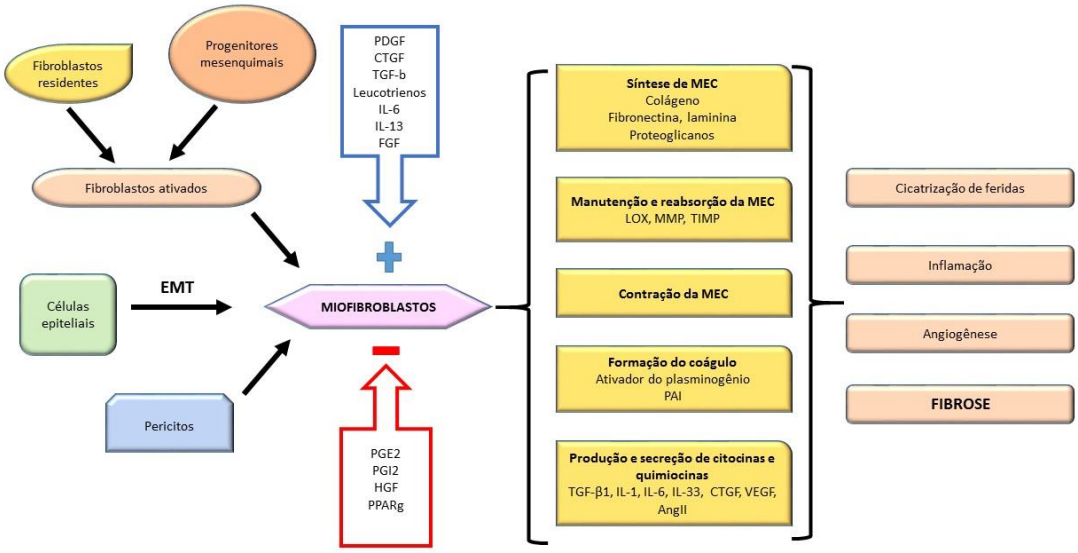

Figura 3. Representação esquemática da participação dos fibroblastos/miofibroblastos no processo de fibrose. Ang II= angiotensina II; CTGF=*connective tissue growth factor*; EMT = transição epitelial-mesenquimal; FGF =*fibroblast growth factot*; HGF=*hepatocyte growth factor*; IL= interleucina; LOX *lysyl oxidase enzyme*; MEC= matriz extracelular; MMP = metaloproteinases de matriz; PAI= *plasminogen activator inhibitor*; PDGF= *platelet derived growth factor*; PGE2= prostaglandina E2; PGI2= prostaciclina; PPARg= receptor ativado por proliferadores de peroxissoma;TGF-β= *transforming growth factor*-β;TIMP= *tissue inhibitor of metalloproteinase*; VEGF= *vascular endotelial growth factor*.

Na ES, os fibroblastos apresentam alterações fenotípicas caracterizadas por síntese aumentada de colágeno e outros componentes da MEC; produção aumentada de TGF-β, CTGF, IL-6, endotelina-1, PAI-1 e proteína quimiotática de monócitos (MCP-1); resistência ao interferon gama (IFN-γ) e a sinais inibitórios provenientes de células T; expressão alterada de integrinas e receptores para TGF-β, PDGF, MCP-1; expressão aumentada do coativador de transcrição p300; e estímulo à transdiferenciação para miofibroblastos.[5; 61]

No processo de fibrose, além da proliferação dos fibroblastos residentes, observa-se também um influxo aumentado de progenitores de células mesenquimais provenientes da medula óssea e a transdiferenciação de outras linhagens celulares, aumentando assim o estoque de células que contribuem para o acúmulo e remodelação da MEC.[4] Os miofibroblastos são células especializadas e funcionalmente ativas, que se caracterizam pela expressão de *alpha-smooth muscle actin* (α-SMA), por apresentar propriedades contráteis, maior taxa de síntese e secreção de componentes da MEC e resistência à apoptose.[62] Essas células sintetizam colágeno, TIMP e outros componentes da MEC e constituem a principal

fonte de TGF-β ativado durante a resposta fibrótica. Em processos patológicos, os miofibroblastos persistem no tecido acometido, promovendo contração excessiva da MEC, aspecto característico das cicatrizes crônicas.[62]

Os miofibroblastos podem se originar a partir de diferentes tipos celulares, além dos fibroblastos.[62] Sob determinadas condições, células epiteliais podem sofrer transformação para fibroblastos, caracterizando a transição epitelial-mesenquimal (EMT). Nesse processo, as células epiteliais perdem sua capacidade de adesão célula-célula, sofrem remodelação do citoesqueleto e exibem marcadores de superfície específicos, passando a apresentar um fenótipo mesenquimal.[67] Esse fenômeno pode ser induzido pelo TGF-β e suprimido pela proteína óssea morfogenética-7 (BMP-7) e tem participação na fisiopatologia do câncer, fibrose renal e fibrose pulmonar idiopática, embora seu papel na ES não esteja definido.[60; 61; 68] Outro elemento celular importante nesse processo são os pericitos, células mesenquimais normalmente presentes em microvasos, em íntimo contato com o endotélio, e que desempenham um papel na manutenção da homeostase vascular. Na ES, observa-se uma hiperplasia dos pericitos e expressão aumentada de receptores de PDGF; além disso, pericitos ativados podem sofrer transdiferenciação em fibroblastos produtores de colágeno e miofibroblastos, o que demonstra uma ligação entre injúria microvascular e fibrose.[69]

3.2.2 TGF-β

Das moléculas implicadas na patogênese da ES, o TFG-β é considerado o principal regulador da fibrogênese fisiológica e da fibrose patológica. O TGF-β apresenta atividades pleiotrópicas sobre diversos tipos celulares, incluindo aqueles envolvidos na fisiopatologia da ES. Faz parte da superfamília de proteínas de sinalização e compreende três isoformas, codificadas por diferentes genes: TGFB1, TGFB2 e TGFB3.[70]

O TGF-β é secretado por monócitos, linfócitos, plaquetas e fibroblastos e sintetizado como um pró-peptídeo biologicamente inativo. É clivado no aparelho de Golgi, originando um peptídeo maduro dimérico, o qual é mantido na sua forma latente

através de uma ligação não-covalente ao pró-peptídeo associado à latência (LAP), originando o pequeno complexo latente (SLC). O SLC liga-se a proteínas ligantes do TGF-β latente (LTBP), formando o grande complexo latente (LLC) (Figura 4). Sob condições específicas, essa forma latente é convertida a sua forma biologicamente ativa, e essa ativação é mediada por α_v-integrinas, trombospondina-1 e proteases, como a plasmina e metaloproteinases de matriz.[71; 72]

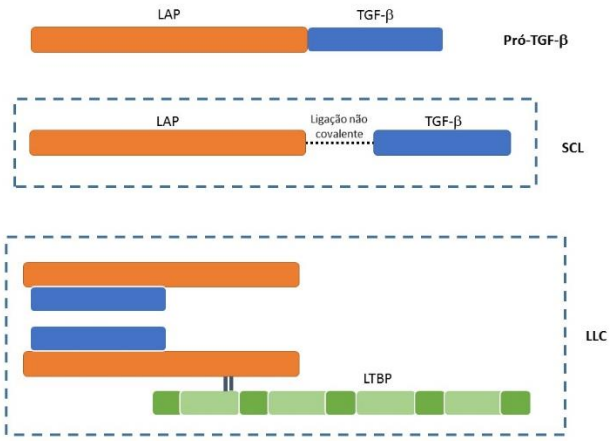

Figura 4. Representação esquemática da molécula do TGF-β na sua forma inativa. LAP= pró-peptídeo associado à latência; SLC= pequeno complexo latente; LTBP= proteínas ligantes do TGF-β latente; LLC= grande complexo latente.

A principal via de transdução de sinal desencadeada pelo TGF-β envolve um grupo de proteínas de sinalização intracelular denominadas Smads. O TGF-β liga-se ao receptor tipo II, promovendo o recrutamento, ligação e fosforilação do receptor tipo I, o que estimula a sua atividade de proteína-quinase.[70] Esse complexo promove a fosforilação da Smad2/3, permitindo a formação de um heterocomplexo com a Smad4, o qual se transloca para o núcleo. Em seguida, o complexo Smad ativado liga-se a uma sequência específica de DNA denominada elemento ligador de Smad (SBE) e ocorre o recrutamento de cofatores de transcrição. No caso do gene do colágeno tipo I, um importante alvo regulado pelo TGF-β/Smad3, o cofator necessário para a estimulação da transcrição é o p300.[60; 70; 73] A transdução de sinal através da via Smad é fortemente controlada por inibidores endógenos como Smad7, cuja expressão é também regulada pelo TGF-β[71] (Figura 5).

Embora a via Smad seja descrita como a principal envolvida na sinalização intracelular a partir do TGF-β, outras vias alternativas (não-Smad) têm sido descritas, incluindo *mitogen activated protein kinases* (MAPK) p38 e *c-Jun n-terminal kinases*

(JNK), *focal adhesion kinase* (FAK), *TGF-ß-activated kinase*-1(TAK1), *phosphoinositide 3-kinase* (PI3K), calcineurina fosfatase dependente de cálcio e a tirosina-quinase c-Abl.[60; 74] (Figura 5)

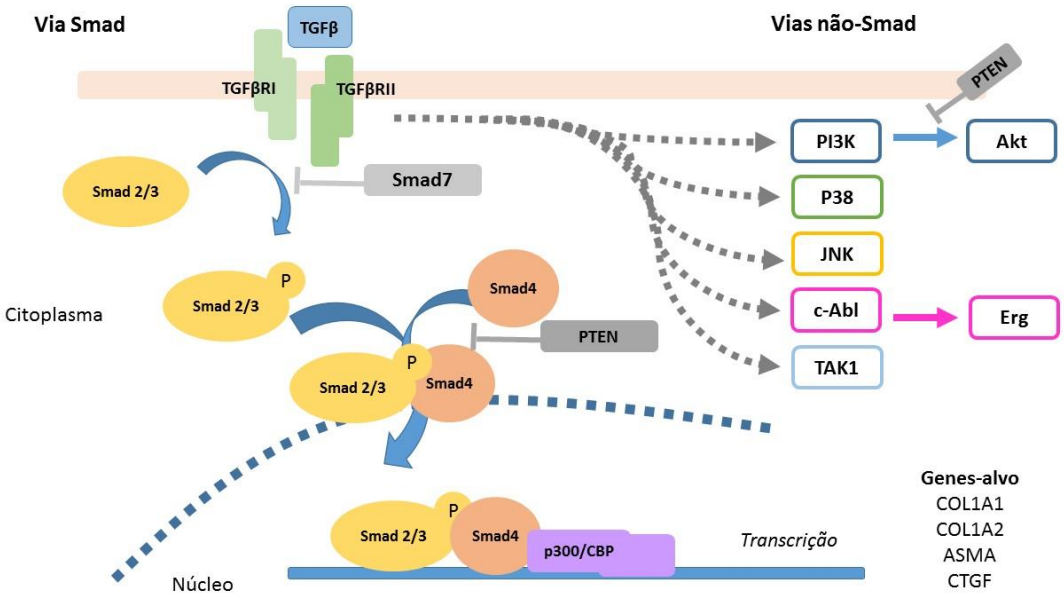

Figura 5. Vias de sinalização do TGF-β. Akt= *protein kinase B*; CBP=*CREB-binding protein*; JNK=*c-Jun n-terminal kinases*; PI3K= *phosphoinositide 3-kinase*; PTEN= *phosphate and tensin homologue*; TAK= *TGF-β-activated kinase*; TGF-β= *transforming growth factor*-β.

O TGF-β liga-se a receptores específicos expressos na superfície das células-alvo e sua ação depende do tipo celular e do microambiente envolvido. Nas células mesenquimais, age como um potente indutor da síntese de colágeno fibrilar; estimula a proliferação, migração, adesão e transdiferenciação de fibroblastos; e suprime a produção de metaloproteinases que degradam a matriz. [60; 71; 72] (Tabela 2) O TGF-β também promove a expressão de mediadores secundários, como CTGF, PAI-1, ET-1, os quais podem apresentar ação profibrótica direta ou promover um *feedback* positivo sobre a ativação do TGF-β.[72] Além disso, tem sido demonstrada ação do TGF-β sobre o sistema imune, promovendo uma diminuição da ativação de células T, o que leva a uma menor diferenciação de linfócitos Th1 e Th2 e induz diferenciação de células T regulatórias (Treg); por outro lado, na presença de outras citocinas como a IL-6, o TGF-β promove a diferenciação de células Th17.[72]

Tabela 2. Ações profibróticas do TGF-β[60; 72]

Recrutamento de monócitos
Estimula a síntese de colágeno, fibronectina, proteoglicanos, elastina e TIMP
Inibe a produção de metaloproteinases
Estimula proliferação e quimiotaxia de fibroblastos
Induz a produção de citocinas fibrogênicas (CTGF)
Bloqueia a síntese e atividade do IFN-γ
Estimula a produção de ET-1
Induz a resposta mitogênica de fibroblastos ao PDGF
Promove diferenciação fibroblasto-miofibroblasto e monócito-fibrócito
Promove a transição epitelial-mesenquimal
Inibe apoptose de fibroblastos

CTGF= *connective tissue growth factor*; ET-1= endotelina-1; IFN-γ= interferon gama; PDGF= *platelet-derived growth factor*; TIMP= *tissue inhibitor of metalloproteinase*

A expressão de genes regulados pelo TGF-β está aumentada em tecidos de pacientes com ES e tem associação com doença mais grave, sugerindo que esta citocina seja um mediador central no seu desenvolvimento.[71; 72] Estudos preliminares evidenciaram expressão aumentada de TGFB na pele de indivíduos com ES[75; 76; 77] e níveis séricos elevados em comparação com indivíduos saudáveis.[78] Além disso, também foi demonstrado que células mononucleares do sangue periférico (PBMC), sobretudo macrófagos/monócitos, de pacientes com ES apresentam uma maior produção espontânea de TGF- β em comparação com controles.[79] Entretanto, esses resultados são considerados ainda controversos, uma vez que não foram confirmados em outros estudos.[80; 81]

3.2.3 Linfócitos T e Citocinas

Além dos fibroblastos, os linfócitos T representam um outro grupo celular com importante participação na fisiopatologia da ES. Exames histológicos de lesões precoces na ES demonstram que um infiltrado inflamatório precede o desenvolvimento de fibrose e da vasculopatia, sugerindo-se que células inflamatórias, particularmente células T, desencadeiem o estímulo que direciona a superprodução de colágeno pelos fibroblastos.[5; 82] Os linfócitos T interagem com os fibroblastos de forma direta ou por ação parácrina, liberando citocinas que estimulam a síntese de colágeno, além de serem necessários para a produção de autoanticorpos característicos da ES, como o anti-Scl70.[83]

Várias subpopulações de linfócitos T (Th1, Th2, Th17, Th22, Treg) foram descritas até o momento, com funções distintas, e, na fisiopatologia das doenças reumáticas autoimunes, ocorre uma complexa interação entre esses grupos.[84] Níveis séricos aumentados de citocinas Th1 e Th2 são descritos em pacientes com ES quando comparados com indivíduos saudáveis.[6] Os níveis de citocinas Th2 tendem a diminuir a medida em que a esclerose cutânea regride e um estudo sugere que um desvio da resposta Th2 para Th1 se correlaciona com a melhora da fibrose cutânea na ES.[5; 85] Dessa forma, defende-se a existência de uma polarização Th1/Th2 na fisiopatologia da ES.[86] Acredita-se que as citocinas pró-inflamatórias Th1 e Th17 participem mediando um processo inflamatório em fases precoces da doença, enquanto as Th2 promovam ativamente a resposta fibrótica, correlacionando-se com a extensão da fibrose e com o comprometimento visceral.[6] (Tabela 3)

Tabela 3. Citocinas implicadas na patogênese da esclerose sistêmica

Molécula	Origem	Função em fibroblastos normais	Evidências de participação na ES
IL-4 [87; 88; 89; 90]	Linfócitos Th2, mastócitos, macrófagos	↑ quimiotaxia de fibroblastos ↑ proliferação de fibroblastos ↑ síntese de colágeno ↑ transdiferenciação miofibroblastos ↑ produção de TGF-β, CTGF e TIMP	↑ níveis séricos e do número de linfócitos produtores de IL-4; ↑ expressão de IL-4 e seu RNAm em fibroblastos cultivados e provenientes de biópsia de pele.
IL-6 [91]	Monócitos, linfócitos B e T, fibroblastos, células endoteliais	↑ proliferação de fibroblastos ↑ síntese de colágeno e TIMP-1	↑ níveis no soro e LBA ↑ expressão de IL-6 em fibroblastos cutâneos de ES; Correlação com o comprometimento cutâneo.
IL-13 [83; 88; 92; 93]	Linfócitos Th2, mastócitos	↑ produção de TFG-β por macrófagos ↑ proliferação de fibroblastos ↑ síntese de colágeno	↑ níveis séricos em pacientes com ES; Produção desregulada de IL-13 por linfócitos CD8(+) efetores de pacientes com ES Correlação com formas mais graves da doença cutânea; Inibição de IL-13 atenua a fibrose pulmonar induzida por bleomicina.
IL-17 [94; 95].	Linfócitos Th17	↑ proliferação de fibroblastos ↑ secreção de citocinas pro-inflamatórias, como IL-6 e IL-8 ↑ expressão de ICAM-1	↑ níveis séricos em pacientes com ES e possível associação com doença precoce; ↑ células Th17 no sangue periférico de indivíduos com ES.
IL-23 [96]	Linfócitos	Indução da via Th17, na presença de IL-6 e TGF-β	↑ níveis séricos Associação com doença precoce e comprometimento pulmonar.
IL-10 [97]	Monócitos ativados, linfócitos	↓ expressão do gene do colágeno tipo I em fibroblastos dérmicos ↑ expressão de MMP-1 e MMP-3 ↑ produção de IL-4 e IL-13	↑ níveis no LBA de pacientes com ES e doença pulmonar; ↑ níveis séricos em pacientes com doença difusa e correlação com escore cutâneo .
IFN-γ [91; 97; 98; 99]	Linfócitos Th1	↓ proliferação de fibroblastos ↓ transdiferenciação de miofibroblastos ↓ síntese de colágeno ↓ expressão do gene do colágeno	Fibroblastos resistentes aos efeitos inibitórios do IFN-γ Níveis séricos elevados ou diminuídos em pacientes com ES (resultados conflitantes) Associação com a forma cutânea limitada e com a presença de autoanticorpos
TNF [91; 97; 100; 101; 102]	Monócitos, macrófagos Células endoteliais Linfócitos Th1	↑ quimiotaxia de fibroblastos ↓ produção de colágeno ↑ síntese de colagenase	↑ níveis elevados no soro e no LBA Associação com progressão da doença pulmonar
IL-12 [85; 103]	Monócitos, macrófagos Células dendríticas Linfócitos B		↑ níveis séricos em pacientes ES e associação com progressão da doença

TGF-β = *transforming growth factor-β;* TIMP= *tissue inhibitor of metalloproteinase*; ICAM= *intercelular adhesion molecule 1*; IFN-γ = interferon-γ; ET-1 = endotelina 1; CTGF=*connective tissue growth factor;* PDGF= *platelet derived growth factor*; IL-6 = interleucina 6; MMP= metaloproteinases de matriz; LBA = lavado broncoalveolar; TNF = fator de necrose tumoral.

Linfócitos Th1 originam-se a partir da estimulação de células Th *naive* pela IL-12 e IFN-γ e caracterizam-se pela expressão dos fatores STAT3 e T-Bet e pela produção de citocinas que participam da resposta imune celular, como o IFN-γ e fator de necrose tumoral (TNF). [84; 104] Na ES, as citocinas do tipo Th1 são consideradas pró-inflamatórias e antifibróticas.[6] Por exemplo, IFN-γ é capaz de inibir a expressão de procolágeno em fibroblastos humanos e em modelo experimental de fibrose.[105; 106] Avaliação dos níveis séricos de IFN-γ em pacientes com ES mostraram resultados conflitantes [91; 98], porém houve uma associação da maior expressão com a forma cutânea limitada [99] e com a presença de autoanticorpos.[91] (Figura 6)

Com relação ao TNF, foi sugerida ação prófibrótica do TNF solúvel ao promover a transição da inflamação pulmonar para a fase fibrótica em modelo animal [107]. Em pacientes com ES e também em modelos animais, foi descrito aumento da expressão e dos níveis séricos e cutâneos do TNF e do seu receptor [108 91; 102; 109; 110; 111], além de associação com a presença de fibrose pulmonar [112]. Ademais, em linfócitos pré-ativados de pacientes com ES, a coestimulação com TNF proporcionou um aumento da secreção de moléculas profibróticas IL-6 e IL-6R.[111]

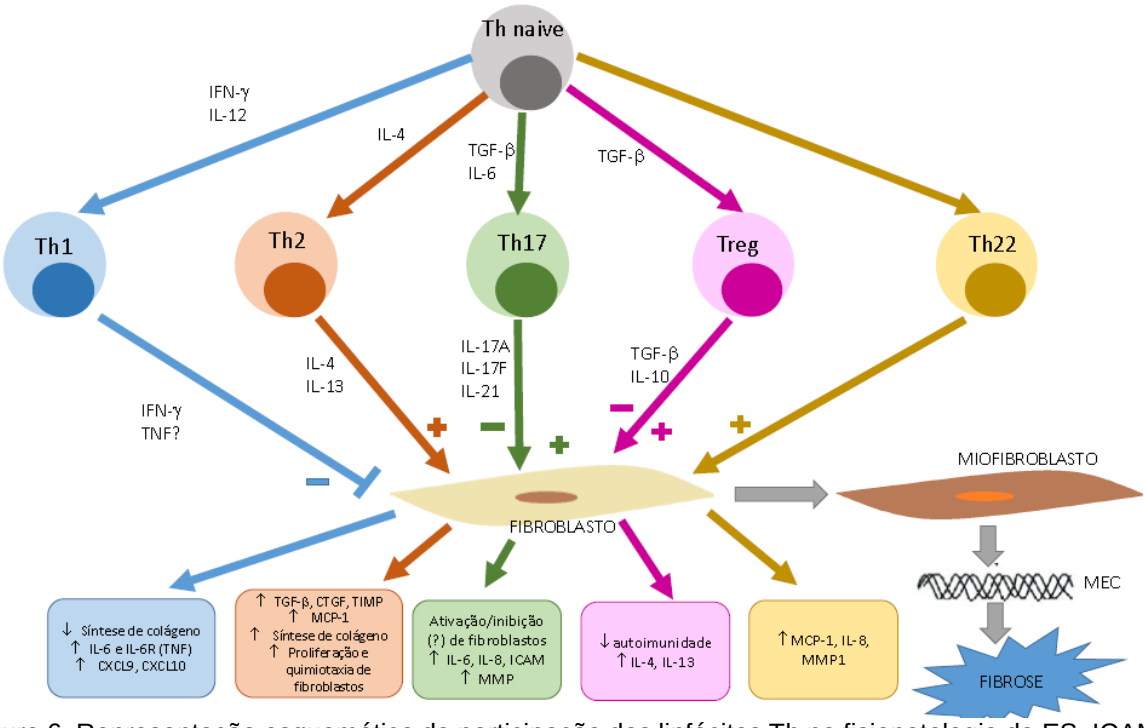

Figura 6. Representação esquemática da participação dos linfócitos Th na fisiopatologia da ES. ICAM= *intercelular adhesion molecule;* IFN-γ= interferon-γ; IL=interleucina; IL-6R= receptor da IL-6; MCP-1= *monocyte chemoattractant protein-1;* MEC= matriz extracelular; TGF-β= *transforming growth factor-β;* TIMP= *tissue inhibitor of metalloproteinase;*TNF= fator de necrose tumoral; Th= t helper; Treg= T regulatória;

A diferenciação dos linfócitos Th2 ocorre a partir da exposição da célula Th *naive* à IL-4 e caracteriza-se pela expressão de STAT6 e GATA3, sendo essa população responsável pela produção de IL-4 e IL-13.[84; 104] Essas citocinas têm demonstrado propriedades profibróticas e pro-angiogênicas.[5] Em fibroblastos normais, a IL-4 estimula a proliferação, quimiotaxia, síntese de colágeno e produção de TGF-β, CTGF e TIMP.[87] Em pacientes com ES foram identificados níveis séricos elevados de IL-4, assim como aumento no número de linfócitos T produtores de IL-4.[88; 89; 108] Além disso, também foi detectada expressão aumentada de IL-4 e seu ácido ribonucleico mensageiro (RNAm) em fibroblastos cutâneos.[90]

Com relação aos efeitos profibróticos da IL-13, destacam-se a estimulação da produção de TGF-β por macrófagos e a estimulação direta da proliferação de fibroblastos e da síntese de colágeno.[83] Também foram detectados níveis séricos aumentados de IL-13 em pacientes com ES [88; 108] e uma produção desregulada de IL-13 por linfócitos CD8+ efetores de pacientes com ES, apresentando uma correlação com formas mais graves da doença cutânea.[83]

Estudos também têm sugerido um papel das citocinas da via Th17, como IL-17A e F e IL-23, na patogênese da fibrose na ES. As células Th17 expressam o fator de transcrição *retinoic acid receptor-related orphan receptor C* (RORC) e sua diferenciação ocorre na presença de IL-6 e TGF-β.[84] A IL-17A tem sido implicada na ativação de fibroblastos, estimulando-os a secretar citocinas pró-inflamatórias, como IL-6 e CXCL8/IL-8, além de aumentar a expressão de ICAM-1 e estimular a secreção de IL-6 por células endoteliais.[94] Pacientes com ES apresentam uma maior frequência de células Th17 no sangue periférico em comparação com controles.[5; 113] Um outro estudo demonstrou que células Th17 inibiam a ativação de miofibroblastos na pele de pacientes com ES, sugerindo um papel antifibrótico da via Th17.[114] Também foram demonstrados níveis séricos elevados de IL-23 [96] e de IL-17 em pacientes com ES e sua possível associação com doença precoce.[94; 95] Entretanto, esses resultados são controversos, uma vez que outros autores evidenciaram níveis séricos diminuídos dessas citocinas em pacientes com ES.[91; 115] Acredita-se, portanto, que em humanos as células Th17, apesar de terem propriedades pró-inflamatórias, atuam também limitando o estímulo fibrótico.[7]

Células T regulatórias (Treg) são classificadas em naturais (nTreg) e induzidas ou adaptativas (iTreg). As nTreg (células T CD25+CD4+) expressam o fator de

transcrição Foxp3, estão naturalmente presentes no sistema imunológico normal e a sua deficiência favorece o aparecimento de doenças autoimunes.[116] As Tregs adaptativas incluem células T reguladoras 1 (Tr1) secretoras de IL-10, células Th3 secretoras de TGF-β, células T CD8+CD28- e células T CD4-CD8- que expressam receptor de células T γ/δ.[6] Na ES, tem sido descrita uma diminuição quantitativa e uma redução da atividade supressora das células Treg.[86; 117; 118] Identificou-se também diminuição de células Tr1 produtoras de IL-10 e dos níveis séricos circulantes de IL-10 em pacientes com ES.[86; 117]

O TGF-β parece contribuir para a estimulação desses dois subgrupos celulares funcionalmente opostos (Th17 e Treg) e seu efeito final depende da presença ou não de citocinas pró-inflamatórias, como IL-1 e IL-6. Ou seja, a presença simultânea de TGF-β e IL-6 favoreceria a geração de células Th17 em detrimento de células Treg, dessa forma alterando o equilíbrio homeostático.[119] Nesse contexto, estudo publicado recentemente demonstrou polarização da resposta imune para a via Th17 em relação ao fenótipo Treg.[119] Aumento nos níveis séricos e locais de IL-1, IL-6, TGF- β e IL-23 em pacientes com ES, citocinas implicadas na diferenciação e expansão da via Th17, são achados que respaldam essa alteração na relação Th17/Treg.[7]

A IL-6 é uma citocina produzida por monócitos, linfócitos T, fibroblastos e células endoteliais, com propriedades pró-inflamatória e pró-fibrótica. Atua estimulando a diferenciação de miofibroblastos, síntese de colágeno, TIMP-1, além de promover a polarização para resposta imune Th2 e Th17.[120] Níveis séricos de IL-6 estão elevados na ES e parecem se correlacionar com a forma cutânea difusa e com a extensão do comprometimento cutâneo.[91; 98; 108] Além disso, observou-se uma produção espontânea elevada de IL-6 e do seu receptor solúvel (sIL-6R) por células mononucleares do sangue periférico (PBMC) de pacientes com ES em comparação com controles saudáveis.[121]

Os linfócitos Th22 caracterizam-se pela produção de IL-22, na ausência de IL-17 e IFN-γ. Entretanto, a IL-22 também pode ser produzida por células Th17. Poucos estudos avaliaram o papel da IL-22 no desenvolvimento da fibrose e na fisiopatologia da ES. Nesse sentido, demonstrou-se aumento da frequência de células Th22 (células IL-17A-IL-22+IFNg-IL4-) circulantes em pacientes com ES e uma associação com doença pulmonar intersticial.[5] Foi também observada expressão aumentada de IL-22 e células IL-22+ na pele de pacientes com ES e uma correlação positiva com o escore

de Rodnan modificado.[118; 122; 123] Mais recentemente, foi sugerida a participação da IL-22 no desenvolvimento de fibrose ao se demonstrar uma resposta aumentada de fibroblastos ao TNF, com aumento da produção de CCL2/MCP-1, CXCL8/IL-8 e MMP1, após exposição à IL-22.[123] Embora ainda preliminares, esses resultados sugerem uma ação pró-fibrótica da IL-22.

Em adição à avaliação das citocinas já bem estabelecidas mencionadas anteriormente, destaca-se ainda a descoberta progressiva de novas citocinas e de seus papeis na fisiopatologia de doenças autoimunes, além de potencial uso como biomarcadores, motivando a investigação em pacientes com ES. Nesse sentido, um estudo avaliou a IL-9, tendo demonstrado níveis séricos elevados e associação com menor frequência e gravidade do comprometimento pulmonar em pacientes com ES.[124]

A família da IL-12 inclui as citocinas IL-12, IL-23, IL-27 e IL-35 e correspondem a um grupo de citocinas heterodiméricas com efeitos biológicos na regulação do sistema imune. A IL-12 é composta pelas subunidades p35 e p40 e participa da diferenciação Th1.[125] A IL-23 é formada pelas subunidades p40 e p19 e faz parte da via Th17 referida anteriormente. A IL-27 apresenta as subunidades EBI3 (gene 3 induzido pelo vírus Epstein-Barr) e a p28 e possui um papel na resposta imune ainda não completamente definido, com descrição de propriedades pró- e anti-inflamatórias.[126] Em pacientes com ES, um estudo demonstrou níveis séricos elevados da IL-27 e associação positiva com extensão do comprometimento cutâneo e fibrose pulmonar.[127] A IL-35 foi descrita mais recentemente e caracteriza-se pela presença das subunidades EBI3 e p35.[128] Estudos em modelos animais sugeriram propriedades anti-inflamatórias, com diminuição da atividade de células T efetoras e redução da progressão de doenças inflamatórias e autoimunes, como artrite induzida por colágeno e colite experimental.[129; 130; 131] Seu papel na ES ainda não está definido e dois estudos recentes apresentaram resultados contraditórios com relação a propriedades fibróticas.[132; 133]

A IL-29 (interferon lambda1 ou IFN-λ1) pertence à família dos interferons tipo III, juntamente com IFN-λ2 (IL-28A) e IFN-λ3 (IL-28B), e é produzida por PBMC e células dendríticas.[134] Foi demonstrado que a IL-29 promove uma supressão da resposta Th2, através da inibição da secreção de IL-4, IL-5 e IL-13 por células T.[135; 136] Embora a IL-29 não tenha sido avaliada na ES, esses achados fornecem subsídios para a

investigação de uma possível desregulação dessa citocina na fisiopatologia da doença.

3.2.4 Quimiocinas

Quimiocinas são citocinas pró-inflamatórias, inicialmente implicadas no recrutamento e ativação de neutrófilos, monócitos e linfócitos, mas que apresentam também importante papel na indução de angiogênese e síntese de MEC. São classificadas de acordo com os dois resíduos iniciais de cisteína em: CC (os primeiros dois resíduos de cisteína são imediatamente adjacentes na estrutura primária), CXC (os primeiros dois resíduos de cisteína são separados por um aminoácido), CX3C (separação entre os resíduos de cisteína envolve três aminoácidos) e C (apresenta um resíduo de cisteína a menos).[97; 137] (Tabela 4)

A proteína quimiotática de monócitos-1 (MCP-1) ou CCL2 é produzida por células mononucleares, mastócitos, células endoteliais e epiteliais, queratinócitos, fibroblastos e células musculares lisas e representa um importante agente quimiotático para monócitos e células T de memória ativadas.[97] CCL2/MCP-1 estimula a produção de colágeno por fibroblastos através do estímulo à produção de TGF-β.[138] Na pele acometida de pacientes com ES é descrita expressão aumentada de CCL2/MCP-1 em fibroblastos, queratinócitos, células endoteliais vasculares e células mononucleares perivasculares.[139; 140; 141] Também foram descritos níveis aumentados no soro e no LBA, além de associação com fibrose pulmonar e com a presença de autoanticorpos.[98; 142; 143; 144; 145; 146; 147; 148; 149] Em uma análise longitudinal, demonstrou-se diminuição dos níveis de CCL2/MCP-1 com a progressão da doença, associada a melhora da fibrose cutânea e pulmonar, sugerindo um papel dessa molécula como marcador sorológico de atividade da ES.[150]

Tabela 4. Quimiocinas implicadas na patogênese da fibrose na esclerose sistêmica[60]

Molécula	**Origem**	**Efeitos**	**Evidências de participação na Esclerose Sistêmica**
CCL2/MCP-1 [97; 98; 139; 140; 141; 142; 143; 145; 146; 148].	Células mononucleares, mastócitos, células endoteliais e epiteliais, queratinócitos, fibroblastos, células musculares lisas	Estimula produção de colágeno dependente de TGF-β	↑ expressão cutânea em fibroblastos, queratinócitos, células endoteliais vasculares e células mononucleares perivasculares ↑ níveis no soro e no LBA Associação com fibrose pulmonar e com a presença de autoanticorpos
CCL3/MIP-1α [142; 151; 152]	Células T ativadas, células B, monócitos e mastócitos	Estimula produção de TGF-β por macrófagos	↑ níveis no soro e LBA Associação com fibrose pulmonar
CCL7/MCP-3 [153; 154; 155]	Monócitos, fibroblastos	Estimula produção de colágeno dependente de TGF-β	↑ expressão em fibroblastos cutâneos ↑ níveis séricos Associação com quadro cutâneo e pulmonar
CCL5/RANTES[148; 149; 156; 157]	Linfócitos, T, fibroblastos, células endoteliais, queratinócitos		↑ expressão cutânea ↑ níveis séricos e no LBA
CXCL8/IL-8[91; 101; 149; 156; 158; 159; 160; 161; 162]	Monócitos, linfócitos T, neutrófilos, células NK, células endoteliais, fibroblastos, células epiteliais	Estimula quimiotaxia de fibroblastos	↑ expressão em fibroblastos cutâneos ↑ níveis no soro e no LBA Associação com envolvimento pulmonar, HAP, anti-Scl70
CXCL10/IP-10[145; 149; 150; 163; 164]	Macrófagos, linfócitos T, neutrófilos, células endoteliais, fibroblastos, queratinócitos, pré-adipócitos	Propriedade antiangiogênica Quimiotaxia linfócitos Th1	↑ níveis no soro ↑ expressão cutânea Associação com doença renal e pulmonar e escore de Rodnan
CXCL9/MIG[149; 150; 164]	Macrófagos, linfócitos T, neutrófilos, células endoteliais, fibroblastos, queratinócitos, pré-adipócitos	Propriedade antiangiogênica Quimiotaxia linfócitos Th1	↑ níveis no soro ↑ expressão cutânea

CTGF= *connective tissue growth factor*; HAP= hipertensão arterial pulmonar; IL= interleucina; IP-10= *IFN-γ induced protein*; LBA = lavado broncoalveolar; MCP= *monocyte chemoattractant protein*; MIG= *monokine induced by IFN-γ*; MIP-1α= *macrophage inflammatory protein*-1α; PDGF= *platelet-derived growth factor*; RANTES= *regulated upon activation and normal T cells expressed and secreted*; TGF-β= *transforming growth factor*- β.

Outra quimiocina implicada na fisiopatologia da fibrose é a proteína inflamatória de macrófagos-1α (MIP-1α) ou CCL3. Estudo com modelo animal de fibrose evidenciou elevação dos níveis de CCL3/MIP-1α precedendo o desenvolvimento da

fibrose cutânea e pulmonar.[152] Sugere-se que essa citocina estimule a produção de TGF-β por macrófagos, promovendo assim a expressão de colágeno tipos I e III.[151] Em pacientes com ES, foram demonstrados níveis séricos e pulmonares aumentados de CCL3/MIP-1α e associação com fibrose pulmonar.[142; 147; 148; 156]

A MCP-3 ou CCL7 é produzida por monócitos e, em menor quantidade, por fibroblastos e apresenta importante efeito quimiotático para monócitos, linfócitos, células dendríticas, granulócitos e células *natural killer* (NK).[155; 165] Utilizando modelos animais e pacientes com ES, foi detectada expressão aumentada de CCL7/MCP-3 em fibroblastos cutâneos[153] e no soro de pacientes, além de associação com extensão da fibrose cutânea e com a gravidade da doença pulmonar.[154]

A quimiocina regulada sob ativação, expressa e secretada por células T normais (RANTES) ou CCL5 também apresenta efeito quimiotático para macrófagos, eosinófilos, basófilos, mastócitos e linfócitos T.[157] Evidências da sua participação na fisiopatologia da ES incluem expressão aumentada em queratinócitos de pacientes[157], além de níveis elevados no soro[148; 149] e no LBA.[156]

A IL-8 (CXCL8) é um fator quimiotático para neutrófilos. Macrófagos pulmonares, além de fibroblastos cutâneos e pulmonares apresentam secreção aumentada dessa citocina.[159; 166] Diversos estudos demonstraram níveis aumentados de CXCL8/IL-8 em fibroblastos cutâneos [158; 159], no soro [149; 160] e no LBA [101; 156; 161] de pacientes com ES, além de associação com comprometimento pulmonar[101; 156; 160; 161], HAP[162] e positividade para o anti-Scl70.[91] Apesar dessas associações descritas, ainda não está definido o real papel da CXCL8/IL-8 nos mecanismos de fibrose, sugerindo-se um efeito indireto ao promover, por exemplo, a quimiotaxia de fibroblastos.[167]

A proteína induzida por IFN-γ (IP-10) ou CXCL10 e a monocina induzida por IFN-γ (MIG) ou CXCL9 são quimiocinas produzidas por diversos tipos celulares (linfócitos T, neutrófilos, monócitos, esplenócitos, células endoteliais, fibroblastos, queratinócitos e pré-adipócitos) sob a influência de citocinas específicas, como IFN-γ e TNF.[168] Apresentam função quimioatrativa para linfócitos produtores de IFN-γ (linfócitos Th1) e propriedades antiangiogênicas.[145; 164; 168] Em pacientes com ES, foram identificados níveis séricos elevados e aumento da expressão cutânea de CXCL9/MIG e CXCL10/IP-10.[145; 149; 150; 163; 164] Além disso, CXCL10/IP-10 elevada

apresentou associação com comprometimento pulmonar e renal [163] e com extensão do comprometimento cutâneo.[169] Em um estudo longitudinal, observou-se níveis decrescentes de CXCL10/IP-10 com a progressão da doença, reforçando a hipótese de que a resposta Th1 é mais proeminente nas fases precoces da ES.[145]

3.3 Tratamento da esclerose sistêmica

De uma maneira geral, o tratamento da ES é determinado pela extensão e gravidade das manifestações clínicas. Até o momento, a maioria das terapias disponíveis são direcionadas para o alívio sintomático, havendo poucas opções consideradas realmente eficazes.[8]

Tendo em vista o desequilíbrio entre substâncias vasodilatadoras (prostaciclina e óxido nítrico) e vasoconstrictoras (endotelina) na fisiopatologia das manifestações vasculares, o tratamento dessas complicações tem sido realizado essencialmente com vasodilatadores. Com relação ao FRy e úlceras digitais, o tratamento inclui inicialmente o uso de bloqueadores do canal de cálcio (BCC) e, em casos de refratariedade ou de acordo com a gravidade, podem ser utilizados análogos de prostaciclina, inibidores da fosfodiesterase e antagonistas da endotelina.[42; 170] Essas últimas classes de medicamentos também representam o grande avanço no tratamento da HAP nos últimos anos, apresentando impacto na diminuição da mortalidade desses pacientes.[43; 170] Quanto à crise renal esclerodérmica, o tratamento agressivo da hipertensão arterial é o principal objetivo e, para isso, os IECA representam os fármacos de primeira escolha.[170; 171]

3.3.1 Imunossupressores

Uma vez que a ativação do sistema imune e a presença de infiltrado de células inflamatórias são características das fases iniciais da doença, drogas imunossupressoras têm sido utilizadas no tratamento da ES. A ciclofosfamida é atualmente o tratamento de escolha para a doença pulmonar intersticial e em alguns

casos de doença cutânea precoce e difusa.[170; 172] Outras opções com imunossupressores não seletivos incluem metotrexato, azatioprina e micofenolato de mofetila.[172; 173] Entretanto, os efeitos do tratamento com esses imunossupressores tradicionais são considerados ainda parciais e beneficiam apenas uma pequena parcela de pacientes em fases iniciais da doença, com diminuição da eficácia à medida que a doença progride [174].

3.3.2 Corticosteroides

Embora os GC sejam considerados de essencial importância no manejo de diversas doenças reumáticas autoimunes devido a seus efeitos anti-inflamatório e imunomodulador, seu papel no tratamento da ES tem se mostrado menos significante. Entre os efeitos atribuídos aos GC inclui-se a diminuição de citocinas produzidas por macrófagos (IL-1 e TNF) e linfócitos (IL-2), através da inibição do fator de transcrição nuclear kappa B (NF-kB).[6] Entretanto, o efeito desses fármacos em PBMC e fibroblastos de pacientes com ES são ainda indefinidos.

Os estudos clínicos são insuficientes para demonstrar a eficácia da monoterapia ou do uso de altas doses no tratamento de manifestações fibróticas cutâneas ou pulmonares.[10; 11; 12] Além disso, o uso de doses altas e por período prolongado, sobretudo nos casos de doença precoce, tem sido associado a maior risco de desenvolver crise renal.[13] Entretanto, mesmo diante da ausência de evidências clínicas que respaldem o seu uso, na prática clínica mais de 40% dos pacientes com ES são tratados com corticosteroides, geralmente em associação com outros imunossupressores.[10] Entre as indicações melhor estabelecidas até o momento, destacam-se o tratamento da sinovite, miosite e como terapia acessória na doença pulmonar intersticial.[175]

3.3.3 Terapias promissoras

Estudos têm se voltado para o desenvolvimento de terapias antifibróticas efetivas, porém, até o momento, não há nenhum fármaco aprovado para esse fim no tratamento da ES. Diante dos avanços na elucidação da fisiopatologia da ES, esforços têm sido empreendidos para o desenvolvimento de fármacos com maior eficácia, melhor perfil de segurança e mais específicos para vias envolvidas na patogênese da ES.

Tendo em vista a reconhecida importância do TGF-β no processo de fibrose, as estratégias terapêuticas buscam o bloqueio de sua produção, atividade ou sinalização intracelular. Entretanto, o TGF-β também apresenta potente atividade anti-inflamatória e imunossupressora, importante na fisiologia normal e representando um mecanismo de proteção contra a autoimunidade. Sendo assim, uma estratégia antifibrótica ideal tendo como alvo o TGF-β deveria inibir sua resposta fibrótica, sem alterar seu efeito imunorregulador fisiológico.[17; 176] Um ensaio clínico que utilizou anticorpo monoclonal direcionado contra TGF-β1, apesar de ter apresentado um bom perfil de segurança, não demonstrou eficácia no tratamento da doença cutânea difusa.[177]

Inibidores da tirosina quinase, como o imatinibe, atuam interferindo em vias de sinalização de citocinas e fatores de crescimento, como TGF-β e PDGF, e demonstraram importante efeito antifibrótico em estudos *in vitro* e em modelos animais.[178; 179] Entretanto, ensaios clínicos randomizados com o imatinibe evidenciaram eficácia limitada, com aumento da frequência de efeitos colaterais.[180; 181]

Em séries de casos e estudos abertos, a depleção de células B através do uso do rituximabe, um anticorpo monoclonal quimérico anti-CD20, vem apresentando benefícios clínicos, com melhora da fibrose cutânea e pulmonar, sugerindo ser esta uma estratégia de tratamento promissora na ES.[182; 183] Adicionalmente, recentes estudos também têm avaliado o uso de anticorpos monoclonais direcionados contra citocinas potencialmente envolvidas do desenvolvimento da doença. O anti-receptor da IL-6 tocilizumabe demonstrou melhora de manifestações articulares em pacientes com ES[184] e atualmente um ensaio clínico randomizado está em andamento para

avaliar o efeito sobre a fibrose cutânea.[185] Outros potenciais alvos terapêuticos atualmente considerados incluem a IL-13, IL-17 e quimiocinas, como CCL2/MCP-1.

Por fim, estudos com o transplante autólogo de células tronco têm apresentado resultados promissores, com reversão da fibrose cutânea, melhora da função pulmonar e da qualidade de vida. A estratégia consiste em promover uma ablação do sistema imune disfuncional, com a erradicação de células B e T autoagressivas e aumento de linfócitos T regulatórios, tentando reestabelecer uma resposta imune normal.[186]

3.3.4 Derivados Tiazolidínicos

As tiazolidina-2,4-dionas correspondem a uma classe de compostos derivados da tiazolidina e caracterizados pela presença de um anel de 5 membros, contendo um átomo de enxofre na posição 1, um átomo de nitrogênio na posição 3 e dois grupos carbonila nas posições 2 e 4, podendo apresentar substituintes nas posições 3 e 5.[14; 26] (Figura 7)

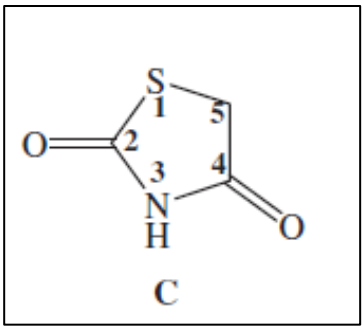

Figura 7. Estrutura do anel da tiazolidina-2,4-diona.

Fonte: Jair et al, 2013.

As TZD têm apresentado diversos efeitos biológicos, que se traduzem em aplicações terapêuticas. Tradicionalmente, o efeito mais intensamente explorado até o momento é sua atividade anti-hiperglicêmica, promovendo uma ação sensibilizadora da insulina. Essas moléculas agem principalmente através da ligação

ao PPARγ, que são fatores de transcrição ativados por ligantes e que pertencem à superfamília dos receptores nucleares de hormônios.[187]

Em células não estimuladas, o PPARγ está localizado no citoplasma formando complexos com os seus co-repressores. Após a ligação do agonista, o PPARγ heterodimeriza com o receptor de retinoide X (RXR) e co-ativadores como o p300 são recrutados. Este complexo é translocado para o núcleo onde se liga a uma sequência específica de DNA, o elemento de resposta do PPAR (PPRE), em promotores de genes alvo, promovendo sua transcrição.[15; 188] Na ausência do ligante, o complexo PPAR/RXR encontra-se ligado a co-repressores transcripcionais e histonas desacetilases, que impedem a sua ligação ao PPRE.[189]

A ativação do PPARγ e sua posterior ligação ao RXR, promove a transcrição de genes envolvidos no metabolismo glicídico, lipídico e na diferenciação celular. Entre os efeitos descritos, ocorre um aumento da captação de ácidos graxos circulantes por adipócitos, diminuindo seu efeito tóxico sobre células beta pancreáticas, hepatócitos e células musculares, além de promover diminuição de TNF e leptina, dessa forma, melhorando a sensibilidade periférica à insulina.[14]

Derivados tiazolidínicos agonistas do PPARγ apresentam propriedades anti-inflamatórias. Estudos *in vitro* demonstraram capacidade de modulação de citocinas pró-inflamatórias em PBMC de pacientes com artrite reumatoide (AR).[15] Ensaios clínicos preliminares em pacientes com AR têm demonstrado efeito na diminuição dos parâmetros de atividade da doença.[190; 191; 192] Mais recentemente, estudos com TZD têm evidenciado o potencial antifibrótico dessas moléculas, tendo sido demonstrado, *in vitro*, efeitos como diminuição da expressão de CTGF[193], inibição da diferenciação de miofibroblastos[16; 194; 195; 196] e indução da diferenciação adipogênica de fibroblastos cutâneos.[19]

Embora o mecanismo de ação dos derivados tiazolidínicos sejam principalmente mediados pela ativação do PPARγ[197], também tem sido demonstrado que essas moléculas podem atuar através de vias PPARγ-independentes, como inibição da via de sinalização NF-κB.[198] Com relação aos efeitos antifibróticos, alguns estudos também demonstraram mecanismo de ação independente da ativação do PPARγ.[195; 199; 200; 201] Ferguson et al.[200] demonstraram que um agonista sintético do PPARγ inibe a expressão de α-SMA através da desregulação de acetilação do coativador p300/CBP. Da mesma forma, Kulkarni et al.[201] mostraram que ligantes do PPARγ

inibem a fosforilação do Akt induzida por TGF-β, sem modificação do efeito com o uso do antagonista do PPARγ.

Derivados tiazolidínicos agonistas do PPARγ, incluindo a RGZ, pioglitazona (PGZ) e troglitazona (TGZ), foram originalmente aprovados para o tratamento do diabetes tipo 2, mas sua comercialização tem sido questionada em muitos países devido principalmente ao perfil de segurança cardiovascular, com descrição de exacerbação de insuficiência cardíaca.[202; 203; 204] Também foi relatado aumento do risco de câncer de bexiga[205] e do risco de fratura em mulheres.[206] Sendo assim, estudos são direcionados para a descoberta e síntese de novas moléculas que apresentem não só eficácia nos desfechos propostos, mas também um perfil de segurança mais adequado.

4 MÉTODOS

4.1 Tipo de estudo

O presente estudo foi desenvolvido em duas etapas, sendo a primeira um estudo transversal, descritivo, com componente analítico, para avaliação do perfil de citocinas nos pacientes com ES. A segunda etapa consistiu em estudo do tipo experimental translacional para avaliação *in vitro* do efeito do tratamento com LPSF/CR-35 ou metilprednisolona em pacientes com ES.

4.2 Local e período do estudo

O estudo foi realizado no período de março/2012 a janeiro/2016. A etapa de seleção e avaliação clínica dos pacientes foi realizada no ambulatório de Reumatologia do HC-UFPE e os procedimentos experimentais foram realizados no Laboratório de Imunomodulação e Novas Abordagens Terapêuticas (LINAT) do Núcleo de Pesquisa em Inovação Terapêutica (NUPIT-SG) da UFPE.

4.3 População de estudo

4.3.1 Recrutamento dos pacientes com ES

Foram selecionados e avaliados 67 pacientes com ES segundo os critérios de inclusão e exclusão.

Critérios de Inclusão

- Idade acima de 18 anos;

- Diagnóstico de ES de acordo com os critérios preliminares do ACR [52] ou os critérios de ES precoce [53];

- Acompanhamento regular no ambulatório de reumatologia do HC-UFPE.

Critérios de Exclusão

- Não consentimento em participar do estudo;

- Diagnóstico associado de outra doença reumatológica autoimune (síndrome de superposição) ou esclerodermia localizada [37];

- Gestantes;

- Presença de infecção aguda ou crônica no momento da avaliação;

- Diagnóstico prévio ou atual de neoplasia;

- Tratamento com derivado tiazolidínico;

- Impossibilidade de coleta de sangue periférico.

4.3.2 Recrutamento de voluntários saudáveis

O grupo controle foi formado por 64 voluntários saudáveis (62 mulheres, média de idade 46,8 ± 11,8 anos), pareados por sexo e idade, escolhidos aleatoriamente no HC-UFPE (acompanhantes dos pacientes ou funcionários do hospital). Esses indivíduos foram submetidos a uma entrevista clínica com o pesquisador principal e eram excluídos caso houvesse diagnóstico conhecido de outra doença reumatológica imunoinflamatória, imunodeficiências, infecção aguda ou crônica ou uso de tratamento imunossupressor e/ou corticoide e/ou derivado tiazolidínico.

4.4 Definição das variáveis clínicas

Para caracterização do comprometimento clínico dos pacientes foram utilizadas as seguintes definições [39; 207]:

- classificação da doença: cutânea limitada e cutânea difusa [35];

- tempo de doença (meses): tempo desde o início do primeiro sintoma, excluindo-se o FRy;

- fadiga: avaliada pela escala visual analógica (EVA) [208];

- comprometimento cutâneo: avaliação clínica quanto à presença de espessamento cutâneo;

- extensão do comprometimento cutâneo: avaliada pelo escore modificado de Rodnan [209];

- fenômeno de Raynaud objetivo: presença avaliada pelo médico[41; 53];

- comprometimento vascular: presença ou história de ulceração de polpas digitais ou superfícies extensoras ou história de necrose extensa e/ou amputação;

- comprometimento articular: presença de artrite evidenciada no exame físico;

- comprometimento muscular: fraqueza muscular acompanhada de elevação das enzimas musculares;

- comprometimento gastrintestinal: presença de dismotilidade esofageana avaliada pela cintilografia esofágica e/ou presença de esofagite evidenciada pela endoscopia digestiva alta;

- comprometimento pulmonar: presença de fibrose pulmonar na TC de tórax;

- hipertensão arterial pulmonar: pressão sistólica da artéria pulmonar ≥ 35 mmHg estimada pelo ecocardiograma transtorácico com doppler;

- crise renal esclerodérmica: registro no prontuário de insuficiência renal, com ou sem elevação da pressão arterial, atribuída à ES;

- medicações utilizadas pelo paciente: BCC, IECA, bloqueador do receptor de angiotensina (BRA), ácido acetilsalicílico, pentoxifilina, cilostazol, sildenafila, bosentana, corticosteroide, metotrexato, azatioprina, micofenolato de mofetila, ciclofosfamida (oral ou venosa), inibidor da bomba de prótons (IBP), procinéticos e anti-inflamatórios não-hormonais;

- perfil de autoanticorpos: positividade para anticorpos antinucleares, anticentrômero e/ou anti-Scl70, de acordo com registro no prontuário;

- peso, altura e pressão arterial sistólica e diastólica, avaliados no dia da consulta;

- avaliação da qualidade de vida através do *Health Assessment Questionnaire* (HAQ) e do *Scleroderma Health Assessment Questionnaire* (sHAQ) [210; 211].

4.5 Compostos avaliados

O composto avaliado LPSF/CR-35 [(3-(4-bromo-benzil)-5-(3-bromo-benzilideno)-tiazolidina-2,4-diona)] foi cedido pelo LPSF-UFPE (Figura 8). Trata-se de um derivado tiazolidínico cuja rota de síntese e citotoxicidade em PBMC já foram previamente descritas, apresentando 91,2% de viabilidade na dose de 100µM. [212] A metilprednisolona (Nova farma) foi utilizada na dose de 100µM.

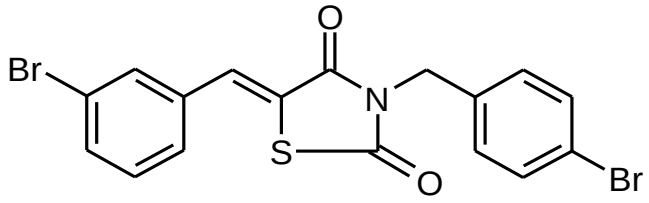

Figura 8. Estrutura química do LPSF/CR-35 [(3-(4-bromo-benzil)-5-(3-bromo-benzilideno)-tiazolidina-2,4-diona)]

4.6 Cultura de PBMC

As PBMC foram isoladas a partir do sangue por centrifugação com Ficoll PaqueTM Plus (GE Healthcare Bio-Sciences) e cultivadas (10^6 células/1000 µL) em meio RPMI 1640 (Gibco) suplementado com L-Glutamina, 10% de soro fetal bovino (SFB) (Lonza), 10mM de HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco) e 200 U/mL de Penicilina/Estreptomicina (Gibco). As PBMC foram estimuladas com anti-CD3 300ng/ml e anti-CD28 400ng/ml (Ebioscience), na presença de metilprednisolona 100 μM ou LPSF/CR35 100 μM (Tabela 5). Para a quantificação das citocinas, o sobrenadante das culturas foi recolhido 48 horas após o tratamento.[213]

Tabela 5. Condições utilizadas na cultura de PBMC de pacientes com esclerose sistêmica e controles saudáveis

Condições	Células	Estímulo	Composto
1	Células (10^6 céls/1000μL)		
2	Células (10^6 céls/1000μL)	Anti-CD3+Anti-CD28	
3	Células (10^6 céls/1000μL)	Anti-CD3+Anti-CD28	LPSF/CR35 100μM
4	Células (10^6 céls/1000μL)	Anti-CD3+Anti-CD28	MP 100μM
5*	Células (10^6 céls/1000μL)	Anti-CD3+Anti-CD28	GW9662 10μM
6*	Células (10^6 céls/1000μL)	Anti-CD3+Anti-CD28	GW9662 10μM +LPSF/CR35 100μM

* condições realizadas em PBMCs de controles; MP= metilprednisolona

Com o objetivo de avaliar se a ação do LPSF/CR-35 era mediada pela via do PPARγ, foi utilizado o antagonista sintético irreversível do PPARγ GW9662 (Sigma), na dose de 10 μM. Em duas condições das culturas de PBMC de indivíduos saudáveis, as células foram pré-tratadas com o antagonista, 30 minutos antes da administração do LPSF/CR-35. (Tabela 5)

4.7 Diferenciação adipogênica

Para verificar a atividade agonista de PPARγ do composto LPSF/CR-35, foi realizada a avaliação da indução de diferenciação adipogência. Para esse fim, foram utilizadas as células da linhagem de fibroblastos pluripotentes murinos 3T3-L1 (Banco de células do Rio de Janeiro), cultivadas no meio DMEM (Gibco) suplementado com soro de bezerro 10% (Gibco). Para o protocolo de diferenciação, as células foram cultivadas em placas de 24 poços e, após alcançar uma confluência acima de 80%, foi adicionado o meio de indução de diferenciação, composto por DMEM suplementado com 10% de SFB (Lonza) e 10μg/ml de insulina, 0,5 Mm de 3-isobutil1-metilxantina (Sigma) e 1 μM de dexametasona (Sigma), por 72 horas. Em seguida, as células foram cultivadas no meio de manutenção (DMEM suplementado com 10% SFB e 10μg/ml de insulina) por 7 dias até a completa diferenciação. Os tratamentos avaliados (LPSF/CR-35 e rosiglitazona) foram acrescentados no primeiro e no terceiro dia de indução.

A avaliação da diferenciação adipogênica foi realizada com a coloração Oil red O (Sigma) em microscópio óptico. Ao término do protocolo de indução, o meio de cultura foi removido, as células foram lavadas com PBS e fixadas com formalina a

10% por 60 minutos. Em seguida, os poços foram lavados com água destilada e incubados com isopropanol a 60% por 5 minutos e coradas com Oil-red O por 10 minutos. Em seguida, as células foram lavadas com água destilada e observadas no microscópio óptico para visualização do acúmulo de lipídeos corados em vermelho.

4.8 Dosagem de citocinas por ELISA

As citocinas presentes no sobrenadante de cultura e no soro dos pacientes e dos controles foram quantificadas por ELISA sanduíche humano, seguindo as instruções recomendadas pelos fornecedores. No soro dos pacientes foram avaliadas as seguintes citocinas: IL-4, IL-6, IL-10 e IFN-γ (BD Biosciences, San Jose, CA, USA); IL-8, IL-9, IL-17, IL-22, IL-27, IL-23, TGF-β, TNF-α, CCL-2, IL-29, IL-35, IL-2 e IL-13 (eBioscience, San Diego, CA, USA). No sobrenadante das culturas de PBMC foram avaliadas IL-6 e IL-4 (BD Biosciences, San Jose, CA, USA); IL-13, IL-8 e CCL2 (eBioscience, San Diego, CA, USA). A leitura da absorbância foi realizada em espectrofotômetro devidamente calibrado (Biotek). (Tabela 6)

Tabela 6. Citocinas avaliadas no soro através da técnica ELISA, fabricantes e limites de detecção

CITOCINA	FABRICANTE	LIMITES DE DETECÇÃO
IL-2	Ebioscience	1,5 – 250 pg/ml
IL-4	BD Biosciences	3,90 – 500 pg/ml
IL-6	BD Biosciences	4,69 – 300 pg/ml
IL-7	R&D	3,90 – 500 pg/ml
IL-8	Ebioscience	1,95 – 250 pg/ml
IL-9	Ebioscience	0,78 – 100 pg/ml
IL-10	BD Biosciences	3,90 – 500 pg/ml
IL-13	Ebioscience	3,90 – 500 pg/ml
IL-17	Ebioscience	3,90 – 500 pg/ml
IL-22	Ebioscience	7,82 – 1000 pg/ml
IL-23	Ebioscience	15,63 – 2000 pg/ml
IL-27	Ebioscience	62,5 – 8000 pg/ml
IL-29	Ebioscience	7,82 – 1000 pg/ml
IL-35	Ebioscience	0,78 – 100 pg/ml
CCL-2	Ebioscience	7,82 – 1000 pg/ml
IFN-γ	BD Biosciences	4,69 – 600 pg/ml
TGF-β	Ebioscience	7,82 – 1000 pg/ml
TNF-α	BD Biosciences	7,82 – 1000 pg/ml

4.9 Dosagem de citocinas por *cytometric bead array* (CBA)

Citocinas no soro de pacientes e sobrenadante de cultura de PBMC também foram avaliadas por CBA, de acordo com o protocolo recomendado pelo fabricante. Foram utilizados os kits Human Th1/Th2/Th17 Kit (Ref 560484, BD Bioscience) e Human Chemokine Kit (Ref 552990, BD Bioscience), cujos limites de detecção estão especificados na tabela 7. As aquisições foram obtidas pelo citômetro de fluxo BD™ Accuri C6 (EUA). A análise de quantificação das citocinas testadas foi realizada pelo BD™ FCAP Array Software v3.0 seguindo instruções do fabricante.

Tabela 7. Citocinas e quimiocinas avaliadas no soro e sobrenadantes de culturas através da técnica CBA, fabricantes e limites de detecção

CITOCINA	LIMITES DE DETECÇÃO
Kit Human Th1/Th2/Th17 Cytokine	
IL-2	2,60 - 5000 pg/ml
IL-4	4,90 – 5000 pg/ml
IL-6	2,40 – 5000 pg/ml
IL-10	4,50 – 5000 pg/ml
TNF	3,80 – 5000 pg/ml
IFN-γ	3,70 – 5000 pg/ml
IL-17A	18,9 – 5000 pg/ml
Kit Human Chemokine	
CXCL8/IL-8	0,20 – 2500 pg/ml
CCL5/RANTES	1,00 – 2500 pg/ml
CXCL9/MIG	2,50 – 2500 pg/ml
CCL2/MCP-1	2,70 – 2500 pg/ml
CXCL10/IP-10	2,80 – 2500 pg/ml

4.10 Fluxograma de coleta dos dados

Após a assinatura do termo de consentimento livre e esclarecido (TCLE) (Apêndice A), os pacientes foram submetidos a uma avaliação clínica detalhada, com coleta de dados demográficos e referentes à doença e ao tratamento, para preenchimento de ficha clínica específica (Apêndice B).

De acordo com a etapa da pesquisa a ser desenvolvida, os pacientes e controles foram submetidos a coleta de 4ml de sangue em tubo a seco com gel para realização da dosagem de citocinas no soro por ELISA ou CBA, além de coleta de 15ml de sangue em tubo contendo o anticoagulante heparina para a realização de cultura de PBMC.

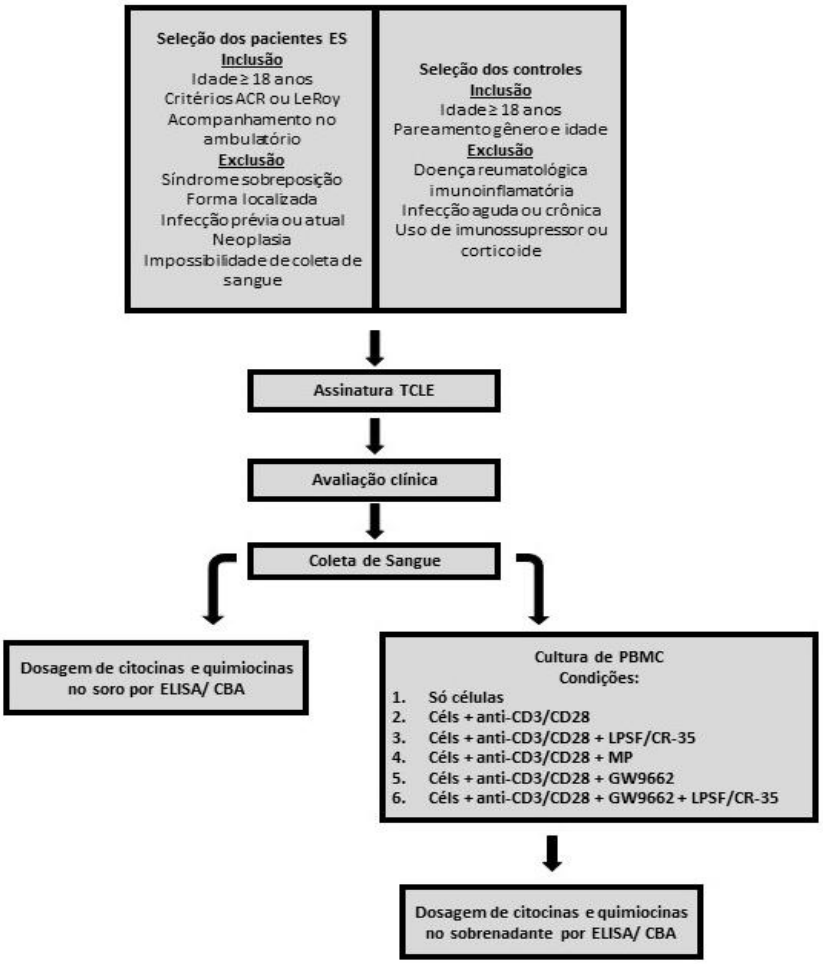

Figura 9. Fluxograma de coleta de dados e procedimentos experimentais.

4.11 Aspectos éticos

O projeto foi aprovado pelo comitê de ética em pesquisa em seres humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, sob o registro nº 529/11 (Anexo A).

4.12 Análise dos resultados

As análises foram realizadas com o software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, EUA) e o Stata SE 12.0 (StataCorp, Texas, EUA). O teste de D'Agostino foi utilizado para avaliação de normalidade da amostra. A expressão dos resultados das variáveis contínuas foi feita pelas médias e desvio-padrão ou erro-padrão para as amostras com distribuição normal e mediana com variação interquartil para aquelas com distribuição não-normal. Para verificação de possíveis diferenças entre médias, foi utilizado o teste "t" de *Student* para amostras independentes, quando a distribuição apresentou distribuição normal, ou o teste de Mann-Whitney nos casos de distribuição não-gaussiana. Para comparação dos níveis de citocinas entre duas condições foi utilizado o teste Wilcoxon's signed rank. Associação entre duas variáveis contínuas avaliadas pelo teste de Spearman, sendo considerada correlação fraca se $0<r\leq0.35$, moderada $0.35<r\leq0.67$ e forte se $0.67<r\leq1$.

As associações entre níveis de citocinas e quimiocinas e manifestações clínicas foram avaliadas através das análises univariada e multivariada. Na análise univariada foi utilizado o teste não paramétrico mencionado acima (teste de Mann-Whitney). Para avaliação da associação entre manifestações clínicas e as diferentes variáveis biológicas após ajuste para variáveis de interesse (idade, tempo de doença e tratamento realizado), foram realizadas análise de regressão linear múltipla (para variáveis contínuas) e análise de regressão logística múltipla (para variáveis categóricas). Valores de $p<0,05$ foram considerados significantes.

5 RESULTADOS

5.1 Determinação do perfil clínico dos pacientes com esclerose sistêmica

Foram avaliados 67 pacientes com diagnóstico de esclerose sistêmica, sendo a maioria do gênero feminino (95,5%), com média de idade de 46 anos (variação 19 a 79). Com relação à forma clínica, 50,7% dos pacientes apresentavam a forma cutânea limitada e 49,3%, a forma cutânea difusa. Quanto às manifestações clínicas, as mais frequentes foram as vasculares (fenômeno de Raynaud e úlceras digitais) e gastrointestinal (dismotilidade esofageana). A tabela 8 demonstra as principais características demográficas e clínicas dos pacientes avaliados.

Em seguida, realizamos a comparação entre os pacientes com as formas clínicas CD e CL. Os dois grupos foram semelhantes no que se refere à maioria das variáveis clínicas e demográficas avaliadas (tabela 8). O escore de Rodnan médio foi maior nos pacientes com a forma CD ($p<0,0001$). Além disso, foi observada uma maior frequência de esofagite no grupo CD (71,4% x 31,6%, $p=0,04$). Ressalta-se ainda que os pacientes com a forma cutânea difusa apresentaram escores mais altos na avaliação da qualidade de vida através dos questionários HAQ e SHAQ.

Tabela 8. Comparação das características clínicas entre as formas cutânea difusa e limitada de pacientes com Esclerose Sistêmica no HC-UFPE (n=67).

CARACTERÍSTICAS	**TOTAL (N=67)**	**CUTÂNEA DIFUSA (n=33)**	**CUTÂNEA LIMITADA (n=34)**	**p**
Idade (anos) media (± DP)	46 (±13,3)	48.6 (±12,0)	43,4 (±14,2)	0,11
Gênero feminino n(%)	64 (95,5)	31 (93,9)	33 (97,1)	0,61
Tempo de não-FRy (meses) mediana (AIQ)	120 (49,5-195)	84,0 (48,0-205,5)	121,0 (62,3-156,0)	0,92
Manifestações Clínicas n(%)				
Fenômeno de Raynaud	64 (95,5)	32 (96,7)	32 (94,1)	1,0
Úlceras digitais	39 (58,2)	21 (63,6)	18 (52,9)	0,46
Artrite	20 (29,8)	08 (24,2)	12 (35,3)	0,42
Miosite	17 (25,4)	10 (30,3)	07 (20,6)	0,41
Dismotilidade esofageana (n=56)	32 (57,1)	14/28 (50,0)	18/28 (64,3)	0,42
Esofagite (n=33)	16 (48,5)	10/14 (71,4)	6/19 (31,6)	**0,04**
Fibrose pulmonar	25 (37,3)	13 (39,4)	12 (35,3)	0,80
HAP (n=63)	09 (14,3)	6/31 (19,3)	3/32 (9,4)	0,30
Crise renal	02 (3,0)	02 (6,1)	0	-
Fadiga (EVA) mediana (AIQ)	46,5 (14,3-66,0)	43,5 (10,5-66,0)	47,0 (18,8-63,3)	0,67
Escore de Rodnan mediana (AIQ)	7,5 (2,8-15,3)	15 (6-23)	4 (2,0-9,5)	**<0,0001**
Sorologia n(%)				
ANA positivo (n=57)	54 (94,7)	24/25 (96,0)	30/32 (93,8)	1,0
Anti-Scl70 positivo (n=38)	17 (44,7)	10/16 (62,5)	7/22 (31,8)	0,10
Anticentrômero positivo (n=55)	08 (14,6)	4/24 (16,7)	4/31 (12,9)	0,72
HAQ mediana (AIQ)	1,375 (0,375-1,875)	1,75 (0,69-2,19)	0,75 (0,22-1,56)	**0,007**
SHAQ mediana (AIQ)	1,14 (0,53-1,515)	1,38 (0,60-1,64)	0,88 (0,48-1,27)	**0,01**
Tratamento n(%)				
Corticosteroide	23 (34,3)	13 (39,4)	10 (29,4)	0,45
Ciclofosfamida	07 (10,5)	03 (9,1)	04 (11,8)	1,0
Azatioprina	13 (19,4)	06 (18,2)	07 (20,6)	1,0
Micofenolato de mofetil	03 (4,5)	02 (6,1)	01 (2,9)	0,61
Metotrexato	06 (9,0)	02 (6,1)	03 (8,8)	1,0

AIQ=amplitude interquartil; ANA= anticorpos antinucleares; DP= desvio-padrão; EVA= escala visual analógica; FRy= fenômeno de Raynaud; HAP= hipertensão arterial pulmonar; HAQ= *Health Assessment Questionnaire*; SHAQ= *Scleroderma Health Assessment Questionnaire*.

5.2 Artigo I - Increased IL-35 serum levels in systemic sclerosis and association with pulmonary interstitial involvement

Artigo publicado na revista *Clinical Rheumatology*, vol 34 (9), 2015; doi: 10.1007/s10067-015-3006-y.

RESUMO

O objetivo desse estudo foi avaliar o nível sérico de IL-35 e sua associação com manifestações clínicas em pacientes com ES. Os níveis séricos de IL-35 foram medidos por ELISA em 56 pacientes com ES e 53 controles saudáveis. Níveis séricos de IL-35 foram significativamente maiores em pacientes com ES (5,08 ± 0,76 pg/ml) em comparação com indivíduos saudáveis (1,89 ± 0,69 pg/ml; p<0.0001). Pacientes com fibrose pulmonar apresentaram níveis de IL-35 mais elevados em comparação com pacientes sem essa manifestação (7,75 ± 1,36 e 3,08 ± 0,70 pg/ml, respectivamente, p=0.0022). Adicionalmente, pacientes em tratamento com azatioprina apresentaram concentrações de IL-35 mais elevadas (8,81±1,81 pg/ml) em comparação com aqueles que não tomavam (4,35± 0,82 pg/ml, p=0,02). IL-35 está elevada no soro de pacientes com ES e está associada com a presença de fibrose pulmonar. Nossos achados sugerem que essa citocina pode ter um papel em doenças fibróticas, porém estudos adicionais são necessários para avaliar o papel da IL-35 na patogênese da ES.

ABSTRACT

Objective. To assess the serum IL-35 level and its association with clinical manifestations in patients with systemic sclerosis (SSc). Methods. IL-35 serum levels were measured by ELISA from 56 patients with SSc and 53 healthy controls. Association of IL-35 serum levels were sought with clinical parameters. Results. Serum IL-35 levels were significantly higher in SSc patients (5.08 ±0.76 pg/ml) than in healthy individuals (1.89±0.69 pg/ml; p<0.0001). Patients with lung fibrosis had higher IL-35 levels than those without fibrosis (7.75 ±1.36 and 3.08 ± 0.70 pg/ml, respectively, p=0.0022). Conclusion. IL-35 is elevated in the serum of patients with SSc and is associated with lung fibrosis. Our findings suggest that this cytokine can have a role in

fibrotic diseases but further studies are needed to address the role of IL-35 in the pathogenesis of SSc.

Key Indexing Terms: interleukin-35, systemic sclerosis, pulmonary fibrosis, cytokine.

INTRODUCTION

The pathogenesis of systemic sclerosis (SSc) is still not fully understood. It is suggested that initial vascular damage leads to an inflammatory response that finally promotes the development of intense fibrosis. Previous studies have demonstrated that cytokines or growth factors stimulate the synthesis of extracellular matrix components and modulate leukocyte function. Studies about the Treg population in SSc patients have produced conflicting data. Some studies reported reduced frequencies and/or impaired function of CD4+CD25+ Treg in SSc patients [1]. However, Slobodin et al. showed increased numbers of Treg cells in patients with SSc and associations with activity and severity of the disease [2].

Interleukin IL-35 is a new member of the IL-12 family, which includes IL-12, IL-23 and IL-27. IL-35 is a heterodimeric cytokine consisting of Epstein-Barr virus–induced gene protein 3 (EBI3) and the p35 subunit of IL-12 [3, 4]. IL-35 is not constitutively expressed in human Treg cells and its expression occurs upon stimulation, being considered the characteristic factor of Treg cells [5, 6]. The gene encoding IL-35 is also transcribed by vascular endothelial cells, smooth muscle cells and monocytes after activation with proinflammatory cytokines and lipopolysaccharide [6]. IL-35 induces the transformation of CD4+ effector T cells into Treg cells that in turn express IL-35 but lack expression of Foxp3, TGF-β and IL-10 [7].

Studies in mice indicate that IL-35 is an important anti-inflammatory cytokine and can efficiently suppress T effector cell activity and reduce the progression of inflammatory diseases and autoimmune diseases, such as collagen-induced arthritis [8, 9] and experimental colitis [10]. However, its real role in the pathophysiology of human diseases is still unclear. Therefore, the purpose of this study was to assess the serum IL-35 level and its potential association with clinical manifestations in patients with SSc.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 56 SSc patients (53 women, 3 men) with a mean age of 45.1 years (range 19–79 yrs). All patients fulfilled the criteria for SSc of the American College of Rheumatology (ACR) [11]. Patients were classified as limited cutaneous SSc (lcSSc; n=30) or diffuse cutaneous SSc (dcSSc; n=26) [12]. The mean disease duration, calculated from the time of onset of the first non-Raynaud event, was 132.5 ± 119.9 months (Table 1). Fifty-three age- and gender- matched healthy individuals served as controls.

Patients' exams and treatment were recorded down from the medical charts. Lung involvement (ground glass pattern or bibasilar fibrosis) was observed using high-resolution computed tomography (HRCT); esophagus involvement was determined by scintilography or endoscopy; proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement were recorded. In addition, a pulmonary function test (PFT) was performed to examine the severity of pulmonary fibrosis; vital capacity < 80% of predicted was considered abnormal. Pulmonary artery pressure was estimated by Doppler echocardiogram; pressures above 35 mmHg were interpreted as pulmonary hypertension. The modified Rodnan total skin thickness score was used to evaluate skin fibrosis [13].

The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco, and informed consent was obtained from all patients.

ELISA

IL-35 serum levels were measured with specific ELISA kits (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 0.78 pg/ml.

Statistical analysis

Statistical analyses of the data were performed using GraphPad Prism (version 6.0). The results are expressed as the mean ± standard error (SE). D'Agostino test verified the normality of samples. The Mann-Whitney U test was used to compare IL-35 levels and the Fisher's exact probability test to compare frequencies. Spearman's rank correlation coefficient was used to examine the relationship between two

continuous variables. We considered correlation (R) strength as follows: $0<R\leq0.35$ = weak correlation; $0.35<R\leq0.67$ = moderate correlation; $0.67<R\leq1$ = strong correlation. A p-value < 0.05 was considered significant.

RESULTS

Serum IL-35 levels were significantly higher in SSc patients (5.08 ± 0.76 pg/ml) than in healthy individuals (1.89 ± 0.69 pg/ml; $P<0.0001$; Fig. 1). There were no differences in serum IL-35 levels between those with limited cutaneous SSc and those with diffuse cutaneous SSc (4.87 ± 1.07 and 5.32 ± 1.11 pg/ml, p=0.776), but levels were higher in all SSc groups than the control group ($p<0.0001$).

We classified patients with SSc into two groups according to the presence or absence of each organ involvement and compared serum IL-35 levels between these groups (Table 2). We found that patients with lung fibrosis had higher IL-35 levels than those without fibrosis (7.75 ±1.36 and 3.08 ± 0.70 pg/ml, respectively, p=0.0022). There was not any statistically significant difference in other clinical manifestations. Also, serum IL-35 levels did not correlate with disease duration nor was there association with the presence of anti-Scl70 antibody or anticentromere antibody. Regarding treatments, patients taking azathioprine had increased IL-35 levels (8.81 ± 1.81 pg/ml) compared to those not taking (4.35 ± 0.82 pg/ml, p=0.0220). No significant difference was found between IL-35 levels and other treatments.

DISCUSSION

This is the first report of elevated serum IL-35 levels in SSc patients. IL-35 levels were raised in both dSSc and lSSc, suggesting that this cytokine can be a serological marker for SSc. Furthermore, we found that IL-35 levels were higher in patients with lung fibrosis.

Although there are no studies about IL-35 in SSc, some reports are available for other rheumatic diseases. Ouyang et al demonstrated decreased IL-35 levels in patients with active systemic lupus erythematosus (SLE) compared with healthy controls and patients with inactive SLE in addition to a negative correlation with disease activity [14]. On the other hand, Qiu et al found that SLE patients have increased plasma levels of IL-12 family cytokines, including IL-35 [15]. In rheumatoid arthritis

(RA), there was increased IL-35 serum levels in patients with very early disease and a significant correlation with disease activity [16]. Furthermore, there were raised levels in the synovial fluid of RA patients [17]. These findings suggest that IL-35 could have a pro-inflammatory action.

IL-35 is upregulated in inflammation in response to proinflammatory cytokine stimulation such as tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), and IL-1β but its exact action mechanism is still not elucidated [6]. Elevated serum levels could be interpreted as a marker of inflammatory status. Even more intriguing is the possible action of IL-35 in fibrotic mechanisms, as suggested by increased IL-35 levels in patients with lung fibrosis but not with other inflammatory manifestations.

Since Treg cells are the main source of IL-35, increased levels of IL-35 could indicate an increase of Treg cells in peripheral blood. Although the Treg role in SSc pathogenesis is not still clear, a recent study described raised Treg cell frequency in SSc patients and a correlation with clinical phenotypes and clinical progression parameters [18]. However, despite the increased Treg levels in SSc patients, these cells lack their normal immune suppression function. Liu et al demonstrated that the main Treg compartment in SSc patients was composed of nonsuppressive CD4+CD25+FoxP3lowCD45RA- cells, a subset that secretes IL-17 [19]. Accordingly, Jiang et al showed increased frequency of Treg and Th17 cells in SSc patients and elevated Tregs were associated with interstitial lung disease and low carbon monoxide diffusing capacity [20]. Taken together, these data indicate that an imbalance between Treg and Th17 cells plays an important role in SSc pathogenesis.

In SLE patients, treatment with methylprednisolone in one study increased the serum IL-35 level and the percentage of CD4+EBI3+ T cells [14]; in another, Qiu et al demonstrated that prednisone treatment downregulated the expression of IL-35 [15]. In RA patients, treatment with methotrexate and low dose glucocorticoids decreased IL-35 serum levels [16]. We did not find differences on IL-35 levels according to corticosteroid use but, interestingly, patients taking azathioprine had higher IL-35 levels. It is not known whether this association is due to cause or effect. In the same way as corticosteroids, AZA is associated with IL-35 higher levels and this could be an important effect of the treatment. On the other hand, this association may only indicate that patients with fibrotic manifestations, with higher IL-35 levels, are also those using AZA. Furthermore, it is also necessary to consider the possibility of lung toxicity

induced by AZA, as previously described by Ishida et al. [21]. Longitudinal study of IL-35 serum levels in SSc will be required to clarify this point.

We realize that this preliminary report has some limitations such as the cross-sectional design of the study. A longitudinal study would be beneficial to better understand the IL-35 levels changes that are associated with the progression of the disease as well as the disease activity. Furthermore, IL-35 levels were measured in a heterogeneous SSc patient population and we were not able to identify, the IL-35 source as Treg cells.

In conclusion, we first demonstrated increased IL-35 serum levels in SSc patients. Moreover, IL-35 levels were higher in patients with lung fibrosis and in patients taking azathioprine. Further studies are required to clarify the role of this cytokine in SSc pathogenesis and its potential utility as a serological biomarker of the disease and as a new therapeutic target.

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TABLES

Table 1. Clinical and laboratory characteristics of SSc patients (n=56)

Characteristics	
Age (yrs)	
Mean ± SE (range)	45.1 ± 1.9 (19-79)
Gender	N(%)
Female	53 (94.6)
Disease duration (months)	
Mean ± SE (range)	130.5 ± 16.3 (8-696)
Clinical subgroups	N(%)
Diffuse cutaneous	26 (46.4)
Limited cutaneous	30 (53.6)
Clinical manifestations	**N (%)**
Raynaud phenomenon	28 (50)
Digital ulcer	33 (58.9)
Pitting scars	36 (64.3)
Telangiectasias	35 (62.5)
Esophageal dysfunction (n=47)	34 (60.7)
Lung fibrosis on thorax CT	24 (42.9)
Vital capacity < 80% on PFT (n=47)	29 (51.8)
Pulmonary arterial hypertension (n=54)	9 (16.1)
Muscle involvement	10 (17.9)
Rodnan score	
Mean (range)	9.9 (0-36)
Autoantibodies	N(%)
Positive ANA (n=49)	46 (93.9)
Positive anticentromere (n=30)	7 (23.3)
Positive Anti-SCl70 (n= 33)	14 (42.4)
Treatment	N(%)
Steroids	20 (35.7)
Azathioprine	10 (17.9)
Methotrexate	05 (8.9)
Cyclophosphamide	06 (10.7)
Mycophenolate mofetil	03 (5.4)

CT = computed tomography; PFT= pulmonary function test

Table 2. Associations of IL-35 levels with organ involvement in Systemic Sclerosis

Organ involvement	**Status**	**Mean ± SE**	**p**
Raynaud phenomenon	+	3.55 ± 0.86	0.0534
	-	6.62 ± 1.22	
Digital ulcer	+	5.28 ± 1.10	0.9305
	-	4.80 ± 1.01	
Pitting scars	+	5.80 ± 1.07	0.3849
	-	3.79 ± 0.91	
Telangiectasia	+	4.93 ± 1.02	0.5118
	-	5.34 ± 1.15	
Esophageal dysfunction	+	5.09 ± 0.87	0.2068
	-	3.42 ± 1.45	
Lung fibrosis	**+**	**7.75 ± 1.36**	**0.0022**
	-	**3.08 ± 0.70**	
Pulmonary arterial	+	5.81 ± 2.43	0.6618
hypertension	-	4.99 ± 0.83	
Muscle involvement	+	6.33 ± 1.67	0.2452
	-	4.90 ± 0.88	

Table 3. Clinical and laboratory characteristics of SSc patients with lung fibrosis (n=24)

Characteristics	
Age (yrs)	
Mean ± SE (range)	43.3 ± 15.5 (19-79)
Gender	N(%)
Female	23 (95.8)
Disease duration (months)	
Mean ± SE (range)	150.4 ± 119.9 (8-696)
Clinical subgroups	N(%)
Diffuse cutaneous	12 (50.0)
Limited cutaneous	12 (50.0)
Clinical manifestations	N (%)
Dyspnoea	15 (62.5)
Esophageal dysfunction (n=18)	16 (88.9)
Vital capacity < 80% on PFT (n=18)	14 (77.8)
Ground glass on thorax CT	10 (41.7)
Pulmonary arterial hypertension (n=23)	5 (21.7)
Rodnan score	
Mean (range)	10.7 (0-35)
Autoantibodies	N(%)
Positive ANA (n=22)	20 (90.9)
Positive anticentromere (n=14)	3 (21.4)
Positive Anti-SCl70 (n= 16)	8 (50.0)
Treatment	N(%)
Steroids	09 (37.5)
Azathioprine	06 (25.0)
Methotrexate	01 (4.2)
Cyclophosphamide	02 (8.3)
Mycophenolate mofetil	03 (12.5)

FIGURES

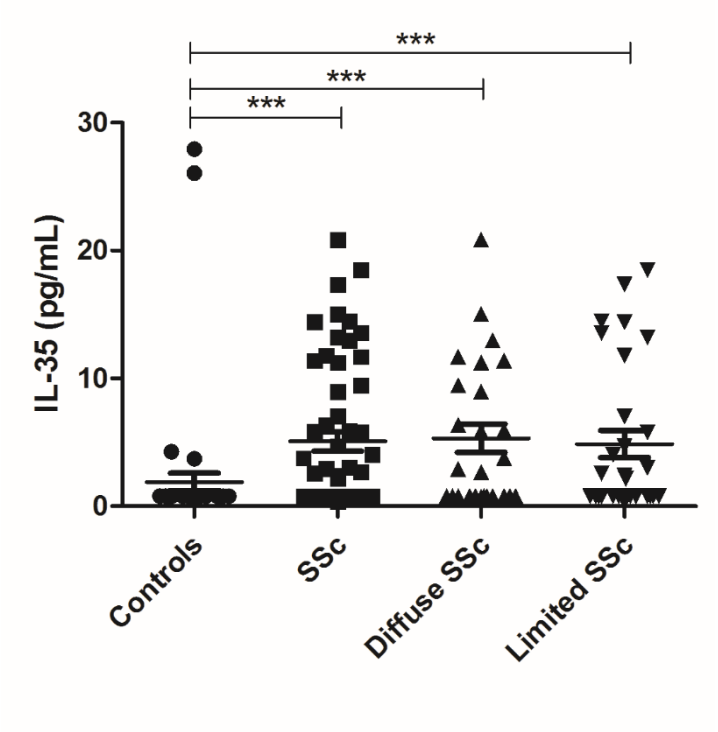

Figure 1. Serum IL-35 levels in controls, patients with SSc, diffuse SSc and limited SSc. Horizontal lines indicate the mean value in each group. (*** p<0.001)

5.3 Artigo II - Interferons and systemic sclerosis: correlation between interferon gamma and interferon-lambda 1 (IL-29).

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RESUMO

Introdução: Interferon (IFN)-λ1 é uma citocina recentemente descrita, membro da família dos interferons tipo III, conhecidos por sua atividade antiviral, antiproliferativa e antitumoral. Estudos recentes indicam que essa citocina tem também função imunorreguladora, mas seu papel na patogênese de doenças autoimunes ainda não é bem estabelecido.

Métodos: os níveis séricos de IFN-λ1 e IFN-γ foram medidos por ELISA em 52 pacientes com ES e 53 indivíduos saudáveis. Foi avaliada a associação entre os níveis séricos das citocinas e os parâmetros clínicos.

Resultados: os níveis séricos de IFN-λ1 e IFN-γ em pacientes com ES foram significativamente maiores que em indivíduos saudáveis (24,82 ± 8,78 e 11.04 ± 3.04 pg/ml, p<0.0001; 34,11 ± 8,11 e 10,73 ± 2,77 pg/ml, p<0.0001, respectivamente). Foi encontrada uma correlação positiva entre os níveis de IFN-λ1 e IFN-γ em pacientes com ES (p=0,0103, r=0,3526). Níveis elevados de IFN-γ foram associados à presença de miosite (p=0,0483).

Conclusão: o presente estudo identificou, de forma pioneira, níveis elevados de IFN-λ1 em pacientes com ES. Além disso, foi encontrada uma correlação entre os níveis de IFN-λ1 e IFN-γ e uma associação entre IFN-γ e comprometimento muscular. Estudos adicionais in vitro e in vivo são necessários para uma melhor compreensão do papel do IFN-λ1 na ES.

ABSTRACT

Backgroud. Interferon (IFN)-λ1 is a newly described cytokine, member of type III interferons family, that is known for its antiviral, anti-proliferative and antitumor activity. Recent studies indicated that this cytokine has also immune-regulatory function but its role in the pathogenesis of autoimmune diseases is not established yet. We evaluated

serum levels of IFN-λ1 in systemic sclerosis (SSc) patients and healthy controls and its association with IFN-γ and clinical manifestations.

Methods. IFN-λ1 and IFN-γ serum levels were measured by ELISA from 52 patients with SSc and 53 healthy controls. Association of cytokines serum levels were sought with clinical parameters.

Results. IFN-λ1 and IFN-γ levels in SSc patients were significantly higher than those in healthy individuals (24.82 ±8.78 and 11.04 ± 3.04 pg/mL, p <0.0001; 34.11 ±8.11 and 10.73 ±2.77 pg/mL, p<0.0001, respectively). We found a positive correlation between IFN-λ1 and IFN-γ levels in SSc patients (p=0.0103, r=0.3526). IFN-γ levels were associated with muscle involvement (p=0.0483).

Conclusion. We first showed raised IFN-λ1 levels in SSc patients. Furthermore, we found a correlation between IFN-λ1 and IFN-γ levels and an association between IFN-γ and myositis. Additional in vitro and in vivo studies are needed to understand IFN-λ1 role in systemic sclerosis.

INTRODUCTION

Systemic Sclerosis (SSc) is an autoimmune disease characterized by inflammation and extracellular matrix deposition that leads to vasculopathy and fibrosis of the skin and internal organs. Although the pathogenesis of SSc remains unclear, previous studies have suggested that some cytokines and growth factors are important events because they stimulate synthesis of extracellular matrix components, injure endothelial cells and modulate the function of leukocytes. (1)

In SSc, occurs differentiation towards Th2 response (2) and Th2 cytokines has demonstrated pro-fibrotic properties (3). As example, serum levels of IL-4 and IL-13 are significantly elevated in SSc patients compared with healthy individuals (4, 5) and IL-4 levels gradually decrease as skin sclerosis improves (6). In vitro studies showed increased expression of collagen in response to IL-4 and IL-13 stimulation (7, 8). On the other hand, Th1 and Th17 cytokines are believed to be involved in the inflammatory processes in the early stage and are implicated in an anti-fibrotic response, by inhibiting collagen deposition and enhancing MMP production (1, 3).

IFN-lambda 1 (IFNλ-1), also known as IL-29, belongs to the family of type III interferons (IFN), together with IFN-λ2 and IFN-λ3 (IL-28A and IL-28B, respectively).

IFN-λ receptor consists of a IL-28 receptor chain (IL-28RA, also known as IFNLR1) and IL-10R2 chain that is also the subunit of the receptor of IL-10, IL-22 and IL-26 (9). IL-29 can be expressed in multiple tissues and is produced by human peripheral blood mononuclear cells (PBMC) and dendritic cells (DC) (10, 11).

It was already described antiviral, anti-proliferative and antitumor effects of IFN-λ1, but immune-regulatory function is still gradually been elucidated (12). IFN-λ1 was able to inhibit human Th2 cell responses by diminishing secretion of IL-4, IL-5 and, mainly, IL-13 in T cells (13, 14). This cytokine also increased IL-6, IL-8 and IL-10 levels secretion by monocytes in a dose-dependent manner (13, 15).

In this study, we evaluated serum levels of IFN-λ1 in SSc patients and healthy controls and its association with IFN-γ and with clinical manifestations.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 52 SSc patients (49 women, 3 men) with a mean age of 44.8 years (range 19–79 yrs). All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR) (16). Patients with overlapping syndromes were excluded. Patients were classified into limited cutaneous SSc (lcSSc; n=28) and diffuse cutaneous SSc (dcSSc; n=24) groups (17). The mean disease duration, calculated from the time of onset of the first non-Raynaud event, was 130.1 ± 123.1 months (range 8–696 months). The characteristics of SSc patients are summarized in the Table 1. Fifty-three age- and gender- matched healthy individuals were included as controls.

Clinical and laboratorial manifestations were reviewed from the medical charts. Lung involvement (ground glass pattern and/or bibasilar fibrosis) was observed using high-resolution computed tomography (HRCT); esophagus involvement was determined by scintilography and/or endoscopy; proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement. In addition, a pulmonary function test (PFT) was evaluated to examine the severity of pulmonary fibrosis; vital capacity < 80% of the predicted normal values was considered abnormal. Pulmonary artery pressure was estimated by Doppler echocardiogram; mean pulmonary artery

pressure (mPAP) above 35 mmHg were interpreted as pulmonary hypertension. The modified Rodnan total skin thickness score was used to evaluate skin fibrosis (18).
The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

ELISA

IFN-λ1 and IFN-γ serum levels were measured with specific ELISA kits (eBioscience, San Diego, CA, USA and BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. The detection limits of this assays was 7.8125 and 4.6875pg/ml, respectively.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism (version 6.0) statistical program. The results are expressed as the mean ± standard error (SE). D'Agostino test verified the normality of samples. The Mann-Whitney U test was used to compare IFN-λ1 levels and the Fisher's exact probability test to compare frequencies. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. We considered correlation (R^2) strength as follows: $0<R^2\leq0.35$ = weak correlation; $0.35<R^2\leq0.67$ = moderate correlation; $0.67<R^2\leq1$ = strong correlation. A probability value of $p < 0.05$ was considered significant.

RESULTS

Serum IFN-γ and IFN-λ1 levels are higher in SSc patients than in healthy controls.

IFN-λ1 levels in patients with SSc were significantly higher than those in healthy individuals (24.82 ± 8.78 and 11.04 ± 3.04 pg/ml, respectively; $p <0.0001$). In addition, SSc patients had higher IFN-γ levels than controls (34.11 ± 8.11 and 10.73 ± 2.77 pg/ml, respectively; $p <0.0001$). There were no differences in serum IFN-λ1 and IFN-γ levels between those with limited cutaneous SSc and those with diffuse cutaneous SSc, but both levels were higher than control group ($p<0.0001$) (Figure 1).

Serum IFN-γ levels are associated with muscle involvement.

We classified patients with SSc into two groups according to the presence or absence of each organ involvement and compared serum IFN-λ1 and IFN-γ levels between these groups (Table 2). We found that patients with myositis had higher IFN-γ levels than those without muscle involvement (50.13 ± 19.33 and 29.99 ± 9.33 pg/ml, respectively, p=0.0483). No significant association was found between IFN-λ1 levels and clinical manifestations.

Association between IFN-λ1 and IFN-γ serum levels in SSc patients

Next, we investigate the association between IFN-λ1 and IFN-γ levels and we found a positive correlation between IFN-λ1 and IFN-γ levels in SSc patients (p=0.0103, r=0.3526). (Figure 2)

DISCUSSION

This is the first report of elevated serum IFN-λ1 levels in SSc patients. Increased IFN-λ1 levels were already demonstrated in other autoimmune rheumatic diseases. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients had significantly higher IFN-λ1 mRNA and serum protein levels than normal controls (11, 15). In SLE, IFN-λ1 levels were higher in patients with active disease and serum levels were positively correlated with anti-dsDNA antibody, C-reactive protein (CRP) and negatively correlated with C3. Also, IFN-λ1 expression were higher in SLE patients with renal involvement, arthritis and skin lesions compared with patients without these manifestations (15, 19). In RA patients, IFN-λ1 was highly expressed in PBMCs, serum, synovial fluid and synovium (20).

We also found increased IFN-γ levels in SSc patients. Systemic sclerosis has often been considered a Th2 cytokine disease. Th1 cytokines such as IFN-γ are considered have antifibrotic effects, while Th2 cytokines such as IL-4 and IL-13 have profibrotic effects (21). However, some studies support Th1 activation in the peripheral blood of SSc patients, with increased production of IFN-γ (22). On the other hand,

Scala et al demonstrated lower levels in SSc patients in comparison to normal controls (23) and Needleman et al reported no differences (4). The concept of polarization of Th1/Th2 cytokines by itself cannot explain the measurement of cytokine levels observed in SSc serum and should take in account that cytokines can be produced by other cell types than Th1/Th2 subtypes, including T CD8+, monocytes, macrophages, dendritic cells, fibroblasts, endothelial cells, Th17, regulator T and B cells (1).

Clinically, higher expression of IFN-γ in SSc patients has been associated with cutaneous limited subgroup (24) and antitopoisomerase or anticentromere positive patients (22). We found an association between IFN-γ levels and myositis. Muscle involvement is described in approximately 70% of SSc patients and can be a debilitating manifestation (25). Some earlier studies suggested IFN-γ role in pathogenesis of autoimmune myopathies (26-29) and this group of diseases share some clinical and pathological features with SSc.

The ability of IFN-λ1 to modulate human immune responses in vitro has been studied. Jordan et al demonstrated that IFN-λ1 alters the Th1/Th2 balance in human T-cells. IFN-λ1 inhibited IL-13 and enhanced IFN-γ secretion in human T cells and monocytes (13). Besides IL-13 effect, Srinivas et al also demonstrated downregulation of IL-4 and IL-5 by IFN-λ1 (14). Dai et al showed, in stimulated CD4+ human T cells, a late increase in IFN-γ production in response to IFN-λ1, which was attributed be secondary to Th2 cytokines inhibition (30). Thus, IFN-λ1 activity seems to be more strongly anti-Th2 than pro-Th1 (13, 14, 30). The present study detected a positive correlation between IFN-λ1 and IFN-γ levels in SSc patients but was unable to evaluate the association between IFN-λ1 and Th2 cytokines because the latter were undetectable in the serum of patients.

Jordan et al demonstrated that healthy PBMC, especially monocytes and macrophages, upregulated IL-6, IL-8 and IL-10 secretion in response to IFN-□1 exposition but this effect was modulated by IL-10, suggesting a negative feedback mechanism by this cytokine (31). In keratinocytes, IFN-λ1 treatment induced expression of proinflammatory cytokines IL-6, IL-8, CCL3, and CXCL9 and promoted recruitment PBMC (19). IFN-λ1 also upregulated IL-6 and IL-8 mRNA expression and induced IL-6 and IL-8 release from synovial fibroblasts of RA patients (20, 32) and, in PBMC of SLE patients, IFN-λ1 induced secretion of chemokines IP-10, MIG and IL-8 (15).

In conclusion, we first showed raised IFN-λ1 levels in SSc patients. Furthermore, we found a correlation between IFN-λ1 and IFN-γ levels and an association between IFN-γ and myositis. Additional in vitro and in vivo studies are needed to understand IFN-λ1 role in systemic sclerosis.

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TABLES

Table 1. Clinical and laboratory characteristics of SSc patients (n=52)

Characteristics	SSc (n=52)	lcSSc (n=28)	dcSSc (n=24)
Age (yrs)			
Mean ± SD (range)	44.8 ± 14.4 (19-79)	42.1 ±14.9 (19-79)	47.9 ±13.5 (24-73)
Gender - N (%)			
Female	49 (94.2)	27 (96.4)	22 (91.7)
Disease duration (months)			
Mean (range)	130.1 (8-696)	145.8 (15-696)	111.6 (8-331)
Rodnan score			
Mean (range)	9.6 (0-36)	5.3 (5-15)	14.7 (0-36)
Autoantibodies - N (%)			
Positive ANA (n=45)	42 (93.3)	24 (92.3)	18 (94.7)
Positive anticentromere (n=42)	5 (17.9)	2 (7.7)	3 (15.8)
Positive Anti-SCl70 (n= 32)	13 (40.6)	5 (26.3)	8 (61.5)
Treatment - N (%)			
Steroids	19 (36.5)	10 (35.7)	9 (37.5)
Azathioprine	09 (17.3)	6 (21.4)	3 (12.5)
Methotrexate	04 (7.7)	2 (7.1)	2 (8.3)
Cyclophosphamide	05 (9.6)	3 (10.7)	2 (8.3)
Mycophenolate mofetil	03 (5.8)	1 (3.6)	2 (8.3)

Table 2. Associations of IFN-λ1 and IFN-γ levels with organ involvement in Systemic Sclerosis

Organ involvement	**Status**	**IFN-λ1 mean ± SEM**	**p**	**IFN-γ mean ± SEM**	**p**
Raynaud	+	22.0 ± 10.5	0.47	30.8 ± 9.2	0.46
phenomenon	-	27.4 ± 14.0		37.2 ± 13.2	
Digital ulcer	+	8.9 ± 0.5	0.13	31.3 ± 10.4	0.85
	-	44.9 ± 19.3		37.6 ± 13.0	
Pitting scars	+	10.2 ± 1.5	0.15	30.6 ± 9.5	0.91
	-	48.2 ± 22.0		39.7 ± 14.8	
Telangiectasia	+	18.1 ± 8.2	0.63	38.0 ± 11.2	0.85
	-	35.7 ± 18.8		27.9 ± 11.3	
Esophageal	+	33.7 ± 14.5	0.86	36.6 ± 12.3	0.93
dysfunction	-	9.3 ± 1.0		29.3 ± 12.6	
Lung fibrosis	+	28.3 ± 12.3	0.58	38.5 ± 14.5	0.97
	-	21.9 ± 12.6		30.3 ± 8.8	
Pulmonary arterial hypertension	+	30.6 ± 21.0	0.18	27.1 ± 15.0	0.87
	-	24.7 ± 10.1		34.8 ± 9.5	
Muscle involvement	+	49.9 ± 35.0	0.11	50.1 ± 19.3	**0.048**
	-	19.1 ± 7.3		30.0 ± 9.3	

FIGURES

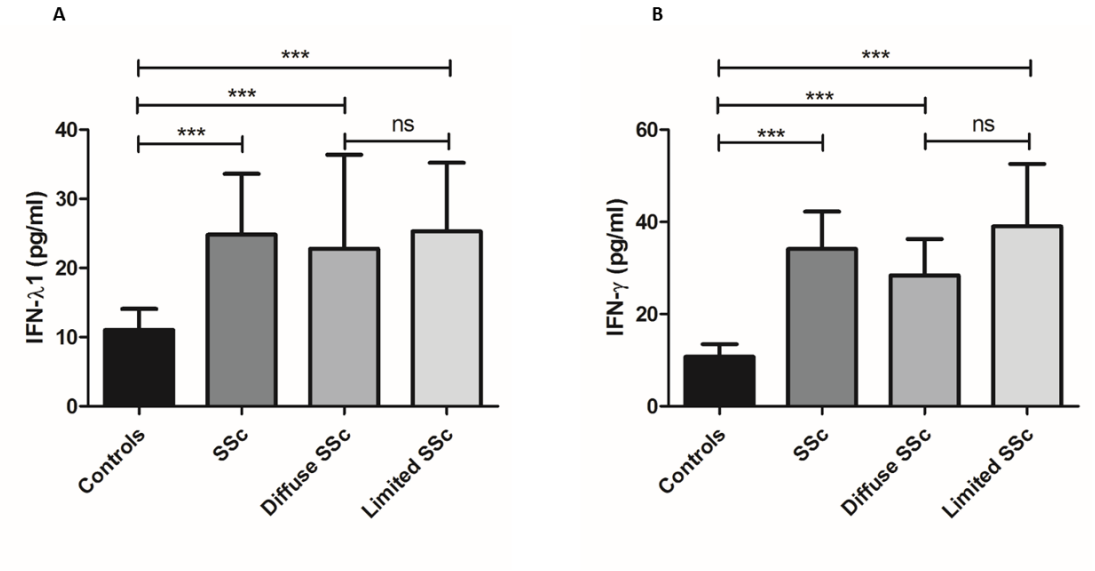

Figure 1. Serum IFN-λ1 and IFN-γ levels in SSc patients and healthy controls. (*** p<0.001; ns= not significant)

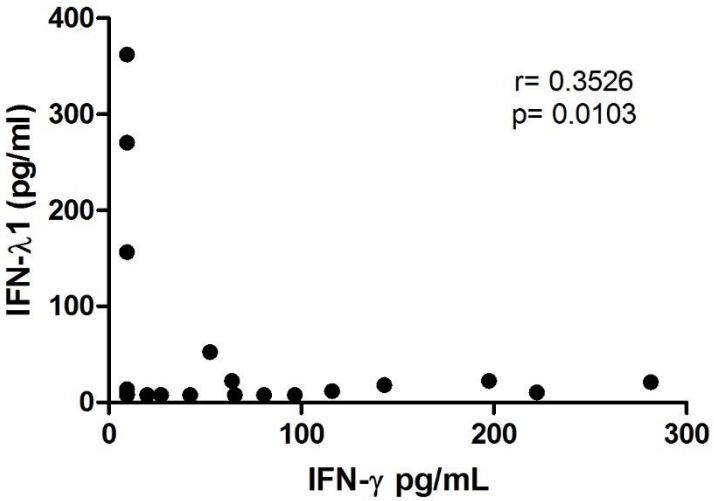

Figure 2. Correlation between IFN-λ1 and IFN-γ levels in SSc patients.

5.4 Artigo III - Reassessing the role of the active TGF-β1 as a biomarker in systemic sclerosis: association of serum levels with clinical manifestations

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RESUMO

Contexto. Embora o TGF-β apresente um papel central no desenvolvimento da fibrose e na fisiopatologia da ES, seu papel como biomarcador da doença permanece indefinido, uma vez que os estudos realizados apresentam resultados divergentes.
Objetivos. Determinar os níveis de TGF-β1 ativo em soro, pele e sobrenadante de cultura de PBMC e sua associação com manifestações clínicas em pacientes com ES.
Métodos. Foram avaliados os níveis séricos de TGF-β1 ativo em 56 pacientes com ES e 24 controles saudáveis. Em 20 pacientes, foi realizada cultura de PBMC e quantificada a produção espontânea de TGF-β1 ativo e após estímulo com anti-CD3/CD28. A mensuração dos níveis de TGF-β1 ativo foi realizada através de ELISA. Foi avaliada a expressão do mRNA de TGFB1 em 13 pacientes com ES e seis controles saudáveis através de RT-PCR.
Resultados. Os níveis séricos de TGF-β1 foram significativamente maiores em pacientes com ES em comparação com controles saudáveis ($p<0,0001$). Maiores níveis séricos do TGF-β1 foram associados à presença da forma cutânea difusa ($p=0,02$), úlceras digitais ($p=0,02$), fibrose pulmonar ($p<0,0001$) e positividade do anti-topoisomerase I ($p=0,03$), além de maior pontuação no escore de Rodnan modificado ($p=0,046$). A maioria dos sobrenadantes de cultura de PBMC apresentaram níveis indetectáveis de TGF-β1, mesmo após o estímulo com anti-CD3/CD28. Não foram detectadas diferenças significantes na expressão de TFGB1 entre pacientes e controles.
Conclusão. Níveis séricos elevados de TGF-β1 e sua associação com manifestações vasculares e fibróticas em pacientes com ES sugerem que essa molécula pode ser um marcador de pior prognóstico em pacientes com a doença.

ABSTRACT

Objective. To determine active TGF-β1 (aTGF-β1) levels in serum, skin and peripheral blood cell (PBMC) culture supernatants and to understand their associations with clinical parameters in systemic sclerosis (SSc) patients.

Methods. We evaluated serum samples from 56 SSc patients and 24 healthy controls (HC). In 20 SSc patients, we quantified spontaneous or anti-CD3/CD28 stimulated production of aTGF-β1 by PBMC. The aTGF-β1 levels were measured by ELISA. Skin biopsies were obtained from 13 SSc patients and six HC, and TGFB1 expression was analyzed by RT-PCR.

Results. TGF-β1 serum levels were significantly higher in SSc patients than in HC ($p<0.0001$). Patients with increased TGF-β1 serum levels were more likely to have diffuse subset ($p=0.02$), digital ulcers ($p=0.02$), lung fibrosis ($p<0.0001$), positive anti-topoisomerase I ($p=0.03$) and higher modified Rodnan score ($p=0.046$). Most of our culture supernatant samples had undetectable levels of TGF-β1. No significant difference of TGFB1 expression was observed in the SSc skin compared with HC skin.

Conclusion. Raised active TGF-β1 serum levels and its association with clinical manifestations in scleroderma patients suggest that this cytokine could be a marker of fibrotic and vascular involvement in SSc.

INTRODUCTION

Since the discovery of the potent profibrotic and immunomodulatory activities of transforming growth factor β (TGF-β), this cytokine has been considered a central player in the process of fibrogenesis in systemic sclerosis (SSc) and other fibrotic diseases. Its effects interfere with a wide array of cellular functions that are relevant to fibrosis, including differentiation of myofibroblasts, production of extracellular matrix molecules and decreased synthesis of collagen degrading metalloproteinases. These activities lead to upregulation of collagen and extracellular matrix synthesis and, consequently, fibrosis (1, 2).

There are three isoforms of TGF-β, each encoded by a distinct gene, and with a distinct expression pattern: TGF-β1, TGF-β2 and TGF-β3. Although increased expression of all the three isoforms has been observed in patients with SSc, TGF-β1 has been the most extensively studied. Secreted as a latent form, TGF-β has to be activated at the target site to induce cellular responses. Since activation of latent TGF-β is critical for the functioning of this growth factor, production levels of active TGF-β are likely to be more important than the levels of the latent form (3).

The blockade of TGF-β as a treatment strategy in SSc is currently under investigation, with conflicting results (4-6). The important role of TGF-β in tissue fibrosis suggests that measurements of its serum levels may reflect the activity of the fibrotic process. However, there have been discordant reports about concentrations of TGF-β in the peripheral blood of patients with SSc (7-12).

Therefore, the current study was conducted to explore whether relationships exist between active TGF-β1 levels and clinically relevant parameters. The study analyzed serum, culture supernatants from peripheral blood mononuclear cells (PBMC), and skin from systemic sclerosis patients.

METHODS

Study subjects

Patients were recruited from the out-patient clinic at the Hospital das Clínicas of Universidade Federal de Pernambuco (UFPE), Brazil. We included 56 SSc patients

(53 female patients, mean age 45.1 ± 14.3 years, range 19-79) and 24 healthy controls (22 female volunteers, mean age 45.2 ± 11.8 years, range 23-68). Patients fulfilled the preliminary criteria of the American College of Rheumatology (ACR) for the classification of SSc (13). Patients with overlapping diseases were excluded. The study was approved by the ethics committee of the UFPE (CEP/CCS/UFPE 529/11) and, according to the Declaration of Helsinki, written informed consent was obtained from each subject before being included in the study.

SSc patients were classified into two categories: diffuse cutaneous systemic sclerosis (dSSc) and limited cutaneous systemic sclerosis (lSSc) (14). Disease duration was defined as the time from onset of the first non-Raynaud's manifestation of SSc. Clinical and laboratorial manifestations were reviewed from the medical charts. Lung fibrosis was observed using high-resolution computed tomography (HRCT); esophagus involvement was determined by scintilography and/or endoscopy; proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement. Pulmonary artery pressure was estimated by Doppler echocardiogram; pressures above 35 mmHg were interpreted as pulmonary hypertension (15, 16). The modified Rodnan total skin thickness score (mRSS) was used to evaluate skin fibrosis (17). Clinical characteristics of the SSc patients are reported in Table 1.

PBMCs purification and culture

PBMCs were obtained from the heparinized blood samples of 20 SSc patients. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1×10^6 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 µg/ml) (Gibco, Carlsbad, CA, USA). Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA), and after 48h culture supernatant was collected for quantification of cytokines.

Measurement of serum TGF-β1 levels

Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at −70 °C prior to use. Active TGF-β1 concentrations in serum and culture supernatants were determined by specific ELISA kit according to the manufacturer´s recommendation (eBioscience, San Diego, CA, USA). Each sample

was tested in duplicate. The detection limit of the test according to the manufacturer was 8 pg. /ml and the standard curve covered a concentration range from 8-1000 pg. /ml.

RNA isolation and real-time polymerase chain reaction (RT-PCR)

Skin biopsies were obtained from 13 SSc patients. Skin samples of five healthy controls were obtained by plastic surgery from individuals without any other autoimmune diseases. All biopsies were taken from a lesional forearm site with a punch of 5 mm. Samples were stored in RNAlater (Qiagen, Hilden, Germany). Tissues were homogenized using *Tissue Ruptor* (QIAGEN). Total RNA was isolated from tissues with RNeasy Mini Kit (QIAGEN Sciences, Maryland, USA) or Trizol (Invitrogen), in both cases according to the manufacturers' instructions. For cDNA synthesis, 4-6 μg of total RNA was used. *High-Capacity cDNA Reverse Transcription Kit* (Applied Biosystems, Warrington, UK) was used according to manufacturer's protocol. TGFB1 mRNA levels were measured by quantitative real-time- Polymerase Chain Reaction (qRT-PCR) using 18S ribosomal gene as the internal standard. Standard TaqMan probe was Hs00998133_m1 for TGFB1. Real-time PCR reactions were performed on ABIPrism 7900HT sequence detection PCR machine (Applied Biosystem) according to the manufacturer's protocol. Data were analyzed with SDS version 2.3 software using the comparative C_t ($2^{-\Delta\Delta C_t}$) method.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) according to their distribution. The Mann-Whitney U test was used to compare serum cytokines levels. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. We considered correlation (R^2) strength as follows: $0<R^2\leq0.35$ = weak correlation; $0.35<R^2\leq0.67$ = moderate correlation; $0.67<R^2\leq1$ = strong correlation. The association between TGF-β1 levels and the different internal organs involved was studied by univariate and multivariate analyses. In univariate analysis,

we used the above mentioned test (Mann-Whitney U test) to compare variables in subjects with or without organ involvement. To evaluate the association between the organs involved and different biological variables that were present after simultaneously adjusting for the other variables of interest, we performed multiple linear regression analysis for the clinical variables with linear scores and multiple logistic regression for the clinical variables with dichotomous scores. A probability value of $p < 0.05$ was considered significant.

RESULTS

Serum TGF-β1 levels in SSc patients

TGF-β1 serum levels were significantly higher in SSc patients [90.38 (55.84-157.1) pg./mL] than in healthy control subjects [27.56 (15.63-51.16) pg./mL, p<0.0001)]. When SSc patients were classified into cases of lSSc and dSSc, both lSSc patients [81.94 (39.44-134.0)] and dSSc [(100.1 (58.50-191.5)] patients had significantly higher TGF-β1 levels than healthy controls (p=0.001 and p<0.0001, respectively). Among the SSc subsets, there was no significant difference in serum TGF-β1 levels between dSSc and lSSc patients. (Figure 1)

Increased serum TGF-β1 levels are associated with clinical manifestations

In order to evaluate the association between serum levels and clinical manifestations, we classified the patients based on the presence/absence of the main clinical parameters. In a univariate analysis, we found that serum TGF-β1 levels were higher in SSc patients with lung fibrosis (p=0.004), digital ulcer (p=0.02) and positive anti-topoisomerase I (p=0.04) compared to patients without these manifestations. (Table 2)

To confirm these associations, these variables were further subjected to multivariate logistic regression analysis adjusted for age, gender, disease duration and treatment. In this analysis, lung fibrosis and positive antitopoisomerase I were independently associated with TGF-β1 levels (p<0.0001 and p=0.03, respectively). Also, patients with higher circulating TGF-β1 levels were more likely to have diffuse cutaneous form (p=0.02) (Table 2). Finally, we performed a logistic regression analysis

for continuous variables also adjusted for age, gender, disease duration and treatment. By this analysis we found that TGF-β1 levels were associated with modified Rodnan score (regression coefficient = 0.03, p=0.046).

mRNA expression of TGFB1 in skin biopsies

Next, we evaluated TGFB1 mRNA expression in skin biopsies of 13 SSc patients and six healthy controls. TGFB1 mRNA was expressed in all SSc skin biopsies, but only in four healthy control biopsy samples. No significant difference of TGFB1 expression was observed in the SSc skin compared with HC (relative mRNA levels 1.3 ±0.3 and 1.2 ±0.8, p=0.24). There were no differences in TGFB1 skin expression between those with lSSc and those with dSSc (data not shown). No significant association was observed between mRNA levels and clinical manifestations (data not shown).

TGF-β1 levels in PBMC cultures

We next addressed spontaneous and stimulated (anti-CD3/CD28) TGF-β1 production by SSc PBMCs. However, among 20 SSc patients, only six presented detectable levels of active TGF-β1 in supernatants of stimulated and/or non-stimulated PBMCs cultures.

DISCUSSION

We used different biological sources to assess the role of active TGF-β1 as a biomarker in SSc. Although it is still a controversial issue in the literature, some studies have indirectly suggested the potential usefulness of TGF-β1 levels as a biomarker. This came to light when they evaluated the reduction of TGF-β1 after specific treatments (18, 19). Most of the studies have failed to establish a relationship between TGF-β1 serum levels and clinical features in SSc patients (7-9) but, in our study, we demonstrated a significant association between higher serum TGF-β1 levels and diffuse subset, digital ulcers, lung fibrosis, skin involvement and positive anti-topoisomerase I.

Previous studies have reported conflicting results on the utility of serum TGF-β1 (7-12). We observed significantly higher serum TGF-β1 levels in SSc patients. Our findings are in agreement with what had been suggested by other authors (7, 11, 12).

However, some studies found no differences of TGF-β1 levels between SSc patients and controls (8, 9) or even lower categories (10). These studies could have posted different results on account of differences in the study population (disease duration, treatment), techniques used and the molecules evaluated (total or active TGF-β1).

Although the univariate analysis has not identified differences in mean TGF-β1 serum levels between dSSc and lSSc subsets, after adjustment for age, gender, disease duration and treatment used, we found that patients with diffuse form were more likely to have higher active TGF-β1 levels. We also verified a positive correlation between TGF-β1 levels and the extent of skin fibrosis, as assessed by mRSS. Sato et al (8) found that serum TGF-β1 levels tended to be higher in patients with dSSc than in patients with lSSc but there was no significant correlation with mRSS. An opposite result was described by Dziadzio et al (10), where they demonstrated reduced levels of active TGF-β1 in dSSc and an inverse correlation with extent of skin fibrosis. This finding suggests that active TGF-β1 may be sequestered in involved SSc tissue. An important difference between this and our study was in the disease duration, since patients with established disease tend to have higher TGF-β1 levels compared with those in the early stages of disease (12). While in the study by Dziadzio et al patients with diffuse form had early disease (median 5 yrs.), our patients were in the later stages of disease (median 10 yrs.).

Despite the recognized participation of TGF-β1 in the development of pulmonary fibrosis, its role as biomarker of lung disease is undefined (20) as yet. Previous studies have identified increased TGF-β1 serum levels in patients with idiopathic pulmonary fibrosis (21, 22), but no previous association was observed in SSc patients. Furthermore, the data on the presence of increased expression of TGF-β in lung tissues of patients with SSc (23-25) are inconclusive. Our data showed that TGF-β1 levels were significantly higher in patients with lung fibrosis compared to healthy controls.

Corroborating our finding of an association between TGF-β1 levels and the diffuse cutaneous subset and pulmonary fibrosis, we observed higher TGF-β1 levels in patients with positive anti-topoisomerase I. Furthermore, the presence of scleroderma-associated autoantibodies is correlated with distinct clinical phenotypes of SSc. Anti-topoisomerase I antibodies are also associated with diffuse skin involvement and lung fibrosis and are correlated with a poor prognosis and SSc-related mortality (26).

We found higher TGF-β1 levels in patients with digital ulcers. Although TGF-β has been traditionally considered an important mediator of fibrosis, it can also contribute to vascular abnormalities in SSc. It was demonstrated that TGF-β induces the synthesis of endothelin, a potent vasoconstrictor implicated in ulcer pathogenesis in systemic sclerosis (27). It was found that TGF-β acts synergistically with endothelin (28). The vascular effect of TGF-β, through its interaction with the co-receptor endoglin, also results in activation of endothelial cells and vascular smooth muscle cells (29).

In order to better evaluate the association between clinical manifestations and TGF-β1 levels, we tried to measure TGF-β1 in other biological sources. Given that TGF-β1 is secreted by numerous cell types, including monocytes/macrophages and lymphocytes (30), we decided to investigate TGF-β1 spontaneous and stimulated production by PBMCs from SSc patients. Two previous studies had reported conflicting results on this line of investigation. Giacomelli et al (31) demonstrated that total TGF-β1 production by PBMC is normal in SSc. Hasegawa et al (32) showed increased spontaneous production of active TGF-β1 by PBMC from both lSSc and dSSc patients compared with controls. In this study, monocytes/macrophages were the main producing cells since secretion of TGF-β1 by T cells, B cells and NK cells was undetectable (32). Most of our samples were below the kit detection limit. As our patients are undergoing treatment with immunosuppressive and/or corticosteroids, it is possible that this may have affected the measurement. Furthermore, as the main cellular sources of TGF-β1 in peripheral blood are monocytes/macrophages, using a specific stimulus for T lymphocytes (anti-CD3/CD28) has not enabled increased production of TGF-β1.

We also could not demonstrate differences or clinical correlations in TGFB1 mRNA expression between skin biopsies of SSc patients and healthy controls. Matsushita et al (11) evaluated only diffuse SSc patients and showed enhanced skin TGFB1 expression in SSc patients with early disease. Probably, our small patients' number was not enough to detect these differences.

Taken together, our findings demonstrated raised active TGF-β1 serum levels in SSc patients. Furthermore, we showed a significant association between active TGF-β1 serum levels and vascular (digital ulcers) and fibrotic (lung and skin) manifestations in SSc patients, suggesting that raised levels could be evaluated as a marker of advanced disease.

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DECLARATION OF INTEREST

The authors report no declarations of interest.

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TABLES

Table 1. Demographic and clinical characteristics of the patients with systemic sclerosis (n=56)

Characteristic	
Age (yrs), mean ± SD (range)	45.1 ± 14.3 (19-79)
Gender female, n (%)	53 (94.6)
Disease duration (months), median (range)	120 (8-696)
Clinical subgroups, n (%)	
Diffuse cutaneous	26 (46.4)
Limited cutaneous	30 (53.6)
Rodnan score, median (range)	7.0 (0-36)
Clinical manifestations, n(%)	
Raynaud phenomenon	54 (96.4)
Digital ulcer	33 (58.9)
Esophageal dysfunction (n=48)	28 (58.3)
Lung fibrosis	24 (42.9)
Pulmonary arterial hypertension (n=54)	9 (16.7)
Arthritis	18 (32.1)
Muscle involvement	14 (25.0)
Autoantibodies, n (%)	
Positive ANA (n=49)	46 (93.9)
Positive anticentromere (n=44)	7 (15.9)
Positive antitopoisomerase I (n= 33)	14 (42.4)
Treatment, n (%)	
Glucocorticoids	20 (35.7)
Azathioprine	10 (17.9)
Methotrexate	4 (7.1)
Mycophenolate mofetil	3 (5.6)
Cyclophosphamide	6 (10.7)

Table 2. Associations of TGF-β1 levels with organ involvement in Systemic Sclerosis (n=56)

Organ involvement	**Status**	**TGF-β1 levels (pg/ml)**	**p value**			
		Median [IQR]	Unpaired t test	Model 1	Model 2	Model 3
Diffuse cutaneous subset	+	100.1 [58.5-191.5]	0.10	0.01	0.02	0.02
	-	81.9 [39.4-134.0]				
Digital ulcer	+	113.8 [66.0-187.3]	0.02	NS	NS	NS
	-	60.1 [44.4-125.1]				
Esophageal dysfunction	+	111.0 [55.4-161.8]	0.63	NS	NS	NS
	-	82.9 [59.0-118.0]				
Lung fibrosis	+	153.2 [81.3-180.2]	0.004	0.005	0.004	<0.0001
	-	64.7 [43.5-116.6]				
Pulmonary arterial hypertension	+	81.3 [55.4-134.8]	0.99	NS	NS	NS
	-	96.3 [55.4-163.8]				
Muscle involvement	+	106.9 [60.8-159.1]	0.48	NS	NS	NS
	-	82.9 [44.1-157.7]				
Arthritis	+	81.3 [43.2-151.1]	0.40	NS	NS	NS
	-	96.6 [58.5-163.0]				
Anti-topoisomerase I	+	100.1 [67.6-232.6]	0.04	0.03	NS	0.03
	-	57.6 [34.7-131.6]				

Model 1= adjusted for age and gender; Model 2= adjusted for age, gender, disease duration; Model 3= Adjusted for age, gender, disease duration, treatment. NS= not significant

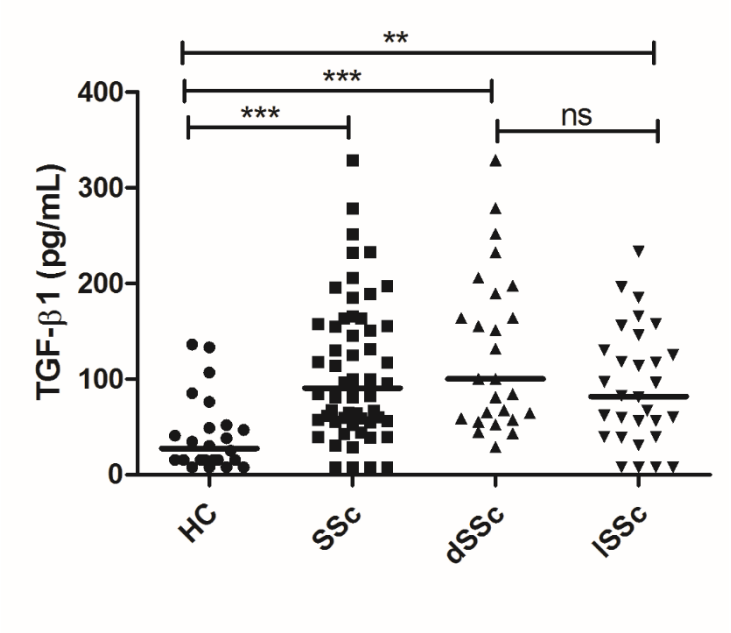

Figure 1. Serum levels of active TGF-β1 in healthy controls (HC), systemic sclerosis patients (SSc), diffuse cutaneous systemic sclerosis patients (dSSc) and limited cutaneous systemic sclerosis patients (lSSc). Short horizontal bars represent median. **p=0.001, ***P<0.0001, ns= not significant.

5.5 Artigo IV - Investigation of cytokines production by peripheral blood cells in systemic sclerosis patients and relationship with clinical manifestations

Artigo a ser submetido à revista Clinical Immunology

RESUMO

Desregulação imune representa um dos mecanismos centrais na patogênese da ES. Citocinas produzidas por linfócitos e monócitos são importantes mediadores e induzem dano tissular, recrutam células inflamatórias e promovem produção de matriz extracelular e fibrose. O presente estudo avaliou a expressão de citocinas no soro e no sobrenadante de cultura de PBMC de pacientes com ES e sua associação com manifestações clínicas. As amostras de soro foram obtidas de 56 pacientes e 56 controles saudáveis pareados por sexo e idade. Foram realizadas culturas de PBMC em 19 pacientes e oito controles. A quantificação dos níveis de IL-2, IL-4, IL-6, IL-10, IL-17A, TNF e IFN-γ foi realizada por ELISA ou CBA. Os níveis das citocinas séricas, com exceção da IL-17A, ficaram abaixo do limite de detecção do kit na maioria dos pacientes e controles. PAcientes com fibrose pulmonar apresentam maiores níveis séricos de IL-17A em comparação com aqueles sem essa manifestação (p=0,04). Na condição de cultura de PBMC não-estimulada, a concentração de IL-2 foi maior em pacientes com dismotilidade esofageana (p=0,04) e os níveis de IL-10 apresentaram uma correlação positiva com o escore de Rodnan modificado (p=0,03). Após a estimulação com anti-CD3/CD28, maior produção de IL-2 e IL-4 foi observada em pacientes com fibrose pulmonar (p=0,01 e p=0,006, respectivamente) e maiores níveis de IL-10 (p=0,04) e IL-4 (p=0,04) em pacientes com úlceras digitais. Pacientes com ES apresentam significantes diferenças no perfil de produção de citocinas em comparação com indivíduos saudáveis. Essa produção alterada foi associada com comprometimento de pele e de órgãos internos. Esses achados sugerem um potencial papel como biomarcador para essas citocinas.

ABSTRACT

Immune dysregulation is as a central process in the pathogenesis of systemic sclerosis (SSc). Cytokines produced by lymphocytes and monocytes are important mediators and induce tissue damage, recruit additional inflammatory cells, and promote extracellular matrix production and fibrosis. The current report sought to evaluate the expression of representative cytokines in serum and culture supernatants from peripheral blood mononuclear cells in SSc patients and their association with clinical manifestations. Serum samples were obtained from 56 SSc patients and 56 unrelated age- and gender- matched healthy individuals. Resting and anti-CD3/CD28 stimulated PBMC cultures were performed from 19 SSc patients and eight controls. IL-2, IL-4, IL-6, IL-10, IL-17A, TNF and IFN-γ levels were measured by ELISA or CBA. Serum cytokines, except IL-17A, were below the kit detection limit in most of patients and controls. Patients with lung fibrosis had higher IL-17A serum levels than those without this involvement ($p=0.04$). In unstimulated PBMC, IL-2 concentration was higher in patients with esophageal dysmotility ($p=0.04$) and IL-10 levels had a positive correlation with modified Rodnan score ($p=0.03$). After anti-CD3/CD28 stimulation, higher levels of IL-2 and IL-4 were observed in SSc patients with lung fibrosis ($p=0.01$ and 0.006 respectively) and higher levels of IL-10 ($p=0.04$) and IL-4 ($p=0.04$) in patients with digital ulcers. In conclusion we identified substantial changes in cytokines production in SSc compared to healthy subjects. This altered cytokine production was associated with cutaneous and internal organs involvement. These findings suggest a potential biomarker role for these cytokines.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by vasculopathy, autoantibody production and excessive collagen deposition resulting in skin and organ fibrosis. Immune dysregulation is as a central process in the pathogenesis of SSc. Lymphocytes and monocytes produce cytokines that induce tissue damage, recruit additional inflammatory cells, and promote extracellular matrix production and fibrosis. The Th1/Th2 cell immune response plays a pivotal role in the development of the disease, but it has also been demonstrated that

Th17 cells and Tregs participate in the pathogenesis of SSc. It is postulated that the imbalance between cytokines produced by these cell subsets drives inflammation in the early stages of disease (Th1 and Th17 predominant) and fibrosis in the later stages of scleroderma (Th2 predominant) (1, 2).

Several studies have been reported alterations in serum cytokine levels in SSc patients, with inconclusive results (1, 3-6). These discrepant results are explained by several aspects such as heterogeneity within SSc population, differences in disease duration, influence of systemic treatment, sample size and the quantification method used. Therefore, in this study, we evaluated the expression of representative cytokines in serum and culture supernatants from resting and stimulated peripheral blood mononuclear cells (PBMC) from SSc patients and their association with clinical manifestations.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 56 SSc patients (53 women, 3 men) with a mean age of 45.1 years (range 19–79 yrs). All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR) (7). Patients with overlapping syndromes were excluded. SSc patients were classified into limited cutaneous SSc (lcSSc; n=30) and diffuse cutaneous SSc (dcSSc; n=26) groups (8). The characteristics of SSc patients are summarized in the Table 1. Fifty-six unrelated age- and gender- matched healthy individuals were included as controls. Controls were excluded if they were in regular use of medication or history of alcohol consumption or smoking in the last 15 days.

Clinical and laboratorial manifestations were reviewed from the medical charts. Lung fibrosis was observed using high-resolution computed tomography (HRCT); esophagus involvement was determined by scintilography and/or endoscopy; proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement. Pulmonary artery pressure was estimated by Doppler echocardiogram; pressures above 35 mmHg were interpreted as pulmonary hypertension. The modified Rodnan total skin thickness score was used to evaluate skin fibrosis (9).

The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

Blood samples and PBMC culture

Peripheral venous blood samples were drawn into collection tubes without additives for serum analyzes from all participants. All samples were stored at –70°C prior to use. PBMCs were obtained from the heparinized blood of SSc patients and controls. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1×10^6 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 µg/ml) (Gibco, Carlsbad, CA, USA). Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) and after 48h culture supernatant was collected for cytokines quantification.

Cytokine and chemokine quantification

Cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17A) levels were quantified in serum and PBMC culture supernatants. Serum cytokines levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA for IL-4, IL-6, IL-10 e IFN- γ and eBioscience, San Diego, CA, USA for IL-17, TNF, IL-2). The lower detection limits for the ELISA analyses were as follows: 3.9 pg/ml for IL-17A, IL-4 and IL-10; 7.82 pg/ml for TNF-α; 4.69 pg/ml for IL-6; 9.37 pg/ml for IFN-γ; 1.5 pg/ml for IL-2.

Concentrations of cytokines in culture supernatants were determined by cytometric bead array (CBA), according to the manufacturer’s protocol (CBA, BD Biosciences). Briefly, 50 µL samples were subjected to analysis in duplicate using the cytometric bead array kit on a BD Accuri C6 cytometry. The concentration of serum cytokines were quantified using FCAP Array software v3.0. The detection limit for IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17 were 4.1 pg/ml, 3.7 pg/ml, 2.9 pg/ml, 3.3 pg/ml, 2.5 pg/ml, 3.3 pg/ml, and 4.2 pg/ml, respectively.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard error (SE) if they were in normal distribution or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann-Whitney U test was used to compare serum cytokines levels. Wilcoxon's signed rank test was used to compare differences in cytokine production of PBMCs. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. We considered correlation (R^2) strength as follows: $0<R^2\leq0.35$ = weak correlation; $0.35<R^2\leq0.67$ = moderate correlation; $0.67<R^2\leq1$ = strong correlation. A probability value of $p < 0.05$ was considered significant.

RESULTS

- **Serum cytokine levels in SSc patients and clinical associations**

Circulating cytokine levels were determined in serum from SSc patients and were compared with those from control subjects. In most of SSc patients and controls, serum levels of IL-2, IL-4, IL-10, IL-6 and TNF were below the lowest detection limit. For IL-17A, serum levels were higher in SSc patients compared with healthy control, but this difference was not statistically significant (p=0.06). We classified patients with SSc into two groups according to the presence or absence of each organ involvement and compared serum IL-17A levels between these groups. Patients were excluded from this analysis if the specific status was not known. We found that patients with lung fibrosis had higher IL-17A levels than those without this involvement (p=0.04) (data not shown). No significant association was found between IL-17A levels and other clinical manifestations.

- **Spontaneous cytokines production by PBMCs from systemic sclerosis patients and association with clinical manifestations**

Since the levels of most cytokines were undetectable in the serum, we decided to evaluate the production of these cytokines by PBMCs from scleroderma patients and healthy individuals. In the absence of exogenous stimuli, the basal production of

TNF (p=0.004), IL-10 (p=0.048), IL-2 (p<0.001) and IL-6 (p=0.01) was higher in culture PBMC of SSc patients than healthy controls (Figure 1). Furthermore, there was a spontaneous production of IFN-γ by scleroderma PBMC while this cytokine was below detection threshold in all controls. Few SSc patients and healthy subjects expressed spontaneous IL-4 and IL-17A production by PBMCs. (Table 2)

In this condition, there were a positive strong correlation between IFN-γ and TNF (r=0.89, p<0.0001). We also observe positive correlations between IL-6 and IFN-γ (r=0.93, p<0.0001), TNF (r=0.96, p<0.0001), IL-2 (r=0.71, p=0.01) and IL-10 (r=0.90, p=0.0002)(Table 3).

Next, we evaluated the association between supernatants cytokine levels and clinical manifestations. We found that IL-2 concentration was higher in patients with esophageal dysmotility (p=0.04) and IL-10 levels had a positive correlation with modified Rodnan score (r=0.49, p=0.03). (Figure 2)

- **Cytokines production by anti-CD3/CD28 stimulated PBMCs from systemic sclerosis patients and association with clinical manifestations**

After stimulation with anti-CD3/CD28 for 48h, there was a significant release of cytokines IL-17A, IFN-γ, TNF, IL-10, IL-4 and IL-2 from PBMC in normal control subjects and SSc patients compared with the spontaneous production of cytokines under basal condition. In SSc patients, increased IL-6 production was not significant after stimulation (p=0.41). However, when compared with stimulated PBMC from control subjects, scleroderma PBMCs stimulated with anti-CD3/CD28 had lower concentrations of TNF (p=0.009), IL-10 (p=0.018), and IL-2 (p=0.002). (Figure 3)

In this condition, it was maintained the correlation between IFN-γ production and TNF (r=0.89, p<0.0001) but we also observe positive correlations between IL-10 and TNF (r=0.65, p=0.003), IFN-γ (r=0.61, p=0.005) and IL-17A (r=0.79, p=0.006) (Table 3).

Higher levels of IL-2 and IL-4 produced by stimulated PBMC were observed in SSc patients with lung fibrosis (p=0.01 and 0.006 respectively) (Figure 4A and B). We also found significantly higher levels of IL-10 (p=0.04) and IL-4 (p=0.04) in patients with digital ulcer (Figure 4C and D). A positive correlation was observed between modified Rodnan score and IL-10 levels (r=0.49; p=0.031) as well as disease duration and IL-

17A (r=0.61, p=0.006), TNF (r=0.58, p=0.009) and IFN-γ (r=0.49, p=0.033) (data not shown).

DISCUSSION

Systemic sclerosis is a rheumatic systemic disease characterized by vascular and immunological abnormalities, leading to progressive fibrosis of the skin and other visceral organs. Defective T cells and monocytes regulation has been postulated to play a crucial role in its pathogenesis and in the disease manifestations. In this study we demonstrated that SSc patients differed markedly from healthy controls in cytokine production before and after stimulation with mitogen. In addition, this altered cytokine production is associated with some clinical manifestations of the disease.

In the present study, most of serum cytokines evaluated were below the kit detection limit. Cytokines serum measurements are influenced by several factors such as low circulating levels and presence of soluble receptors, anti-cytokine antibodies and receptor antagonists (10). For these reasons, in addition to measuring serum cytokines, we decided to perform the evaluation of the *in vitro* cytokines production by PBMCs, since a stimulated cytokine production can reflect the *in vivo* spontaneous production of this molecule (11).

The concentrations of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF secreted by unstimulated and anti-CD3/CD28 stimulated PBMC from scleroderma patients and healthy individuals were determined and compared. Spontaneous *in vitro* release of cytokines by PBMCs may serve as a measure of their activation *in vivo* (12). Several studies demonstrated that, in healthy subjects, unstimulated cells produce cytokine proteins at very low levels (12-14). In our study, unstimulated production of all evaluated cytokines, except IL-17A, was higher in SSc patients compared to HC, which may reflect a state of persistent activation *in vivo*. Some previous studies evaluated spontaneous cytokines production by SSc PBMC (15-18). They described enhanced TNF (15), IL-2, IL-4, IFN-γ (16), IL-6 (19, 20), IL-10 (21) and IL-17 (17, 18) spontaneous expression in scleroderma peripheral blood cells, but our study showed a simultaneous increase of all these cytokines. This non-specific increase in cytokine production by SSc PBMC may reflect a generalized activation state, with stimulating production of pro as well as anti-fibrogenic cytokines, as a regulatory mechanism.

Although both patients and controls have experienced an increase in production of the studied cytokines in response to anti-CD3/CD28 stimulation, the magnitude of this response was lower in SSc patients. Compared to healthy controls, scleroderma patients had a markedly lower capacity to produce TNF, IL-2 and IL-10 after mitogen stimulation. Greater efficiency in cytokines production by the control group could be related to impairment or the lack of ability of SSc T lymphocytes to produce these cytokines in response to the stimuli we used. It remains to be better elucidated whether the lower cytokine production by PBMC from scleroderma patients is due to decreased cytokine production per cell, or to a decrease in the number of cytokine-producing cells in PBMC.

We found a strong positive correlation between IFN-γ and TNF production by scleroderma PBMCs, both as unstimulated or after stimulation with anti-CD3 / CD28. It has been postulated that both cytokines present important role in SSc pathogenesis and they can act in a synergistic way. Although *in vitro* studies are not conclusive about the pro or anti-fibrotic effects of these molecules individually (22-24), combination of IFN-γ and TNF induced a stronger secretion of the pro-fibrotic molecules IL-6 and CCL2 in SSc fibroblasts (25). Consistent with these results, we also observed a strong positive association between IL-6 production and IFN-γ and TNF levels in unstimulated scleroderma PBMC. In addition, we detected positive association between spontaneous PBMC production of IL-6 and IL-2, IL-4 and IL-10. These cytokines have been implicated in fibrosis development (26-28) and in SSc pathogenesis, with interdependent regulatory mechanisms (5, 6, 29). As example, it was demonstrated a positive correlation between IL-6 and IL-10 serum levels (29) as well as between levels of TNF and IL-10 and IL-6 (5).

Specific clinical manifestations of SSc were associated with alterations in distinct cytokine levels in serum and supernatant of PBMC culture. We found that patients with lung fibrosis had higher IL-17A serum levels compared to patients without this manifestation. Corroborating our findings, previous studies described higher expression of IL-17 in lung tissue (17) and an association between serum levels and the extent of lung damage (30). However, we did not find association between IL-17A spontaneous or stimulated production by scleroderma PBMC and lung fibrosis or other clinical involvement. In our samples, many culture conditions exhibit IL-17A levels below the detection limit and the remaining small number of patients evaluated may

have been insufficient to identify this association. Besides IL-17A, our group previously described increased IFN-γ serum levels and its association with the presence of myositis in patients with SSc (31).

We also verified that IL-10 spontaneous PBMC production showed a positive correlation with modified Rodnan score, similar to that reported by Sato et al (29). Higher IL-2 levels were produced by unstimulated PBMCs from SSc patients with esophageal dysmotility. Although some studies have described higher serum levels of IL-2 in patients with SSc and association with disease activity (32, 33), relationship with gastrointestinal impairment has not been demonstrated. Enhanced IL-4 levels were found in patients with lung fibrosis. Reinforcing the role of IL-4 as a marker of pulmonary fibrosis, some studies have described IL-4 enhanced expression in the lung, as well as increased number of IL-4-producing CD4+ T cells in scleroderma patients with lung disease (34-36). However, as far as we know, association between IL-2 and lung disease, as well as between IL-4 and IL-10 and digital ulcers had not been identified previously.

Finally, we observed a positive correlation between the disease duration and levels of TNF, IFN-γ and IL-17A produced by stimulated PBMC. Gourh et al (6) also described higher IFN-γ levels in patients with disease duration > 10 years. However, contrary to our findings, they showed increased TNF serum levels in patients with early disease (6) and Kurosawa *et al* (17) showed that IL-17 was overproduced also in early stage of disease. While alterations in cytokine levels were associated with specific clinical manifestations of SSc, longitudinal studies are needed to determine the real role of measuring these cytokines levels as biomarker or predictor of prognosis.

We realize that this report has some limitations such as the cross-sectional design of the study. Cytokines levels were measured in a heterogeneous SSc patient population, most of them with chronic disease and under treatment. There is a possibility of selection bias in the cytokine production analysis by using supernatants from a subset of subjects, selected based on sample availability. Furthermore, we were not able to identify the cytokines cells source.

In conclusion we identified substantial changes in cytokines production in SSc compared to healthy subjects. This altered cytokine production was associated with cutaneous and involvement of internal organs. Our findings support the view that chronic cellular activation occurs in SSc which leads to persistent release of cytokines

involved in its pathogenesis. Taken together, these data bring to light the complex immunopathogenesis of SSc and the clinical heterogeneity that is seen within disease.

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TABLES

Table 1. Clinical and laboratory characteristic of the patients with systemic sclerosis

Characteristics	Serum patients (N=56)	PBMC patients (n=19)
Age (yrs)		
Mean ± SD (range)	45.1 ± 14.3 (19-79)	42.3 ± 8.8 (24-63)
Gender N(%)		
Female	53 (94.6)	19 (100)
Disease duration (months)		
Mean ± SD (range)	131.8 ± 118.9 (8-696)	105.8 ± 78.3 (8-232)
Clinical subgroups N(%)		
Diffuse cutaneous	26 (46.4)	12 (63.2)
Limited cutaneous	30 (53.6)	7 (36.8)
Clinical manifestations N (%)		
Digital ulcer	33 (58.9)	11 (57.9)
Esophageal dysfunction	28/48 (58.3)	9/16 (56.3)
Lung fibrosis on thorax CT	24 (42.9)	5 (26.3)
Pulmonary arterial hypertension	9 (16.1)	2 (10.5)
Arthritis	18 (32.1)	5 (26.3)
Muscle involvement	14 (25.0)	7 (36.8)
Rodnan score		
Median (range)	7.0 (0-36)	4.0 (2-21)
Autoantibodies N(%)		
Positive ANA	46/49 (93.9)	17/18 (94.4)
Positive anticentromere	7/47 (14.9)	2/16 (12.5)
Positive Anti-SCl70	14/33 (42.4)	5/9 (55.6)
Treatment N(%)		
Steroids	20 (35.7)	10 (52.6)
Azathioprine	10 (17.9)	3 (15.8)
Methotrexate	4 (7.1)	3 (15.8)
Cyclophosphamide	6 (10.7)	2 (10.5)
Mycophenolate mofetil	3 (5.4)	1 (5.3)

Table 2. Cytokines levels in supernatants produced by unstimulated PBMC from systemic sclerosis patients (SSc) and healthy controls (HC)

	SSc patients (n=19)	Healthy controls (n=8)	p
IFN-γ	494.3 (253.3-899.2)	< 3.7	-
TNF	11.9 (6.4-24.4)	3.8 (3.8-4.3)	**0.004**
IL-10	16.0 (8.4-40.8)#	5.8 (4.7-28.8)	**0.048**
IL-2	105.3 (48.4-213.5)	2.6 (2.6-8.0)	**<0.001**
IL-6	3036 (1768-4604)▪	476.7 (217.4-1717) ◦	**0.010**

Results are expressed as median [IQR]. Cytokines levels are in pg/ml. Statistical significance comparing patients versus controls. #n=18; ▪n=11; ◦n=6 (patients were excluded in which both unstimulated and stimulated conditions were above or below the detection limit)

Table 3. Correlations of cytokines production by unstimulated and anti-CD3/CD28 stimulated PBMCs from SSc patients

	IL-2	IL-6	IL-10	TNF
Unstimulated				
IL-2	-			
IL-6	**R=0.71**	-		
	P=0.01			
IL-10	R=0.18	**R=0.90**	-	
	P=0.46	**P=0.0002**		
TNF	**R=0.65**	**R=0.96**	R=0.38	-
	P=0.003	**P<0.0001**	P=0.11	
IFN-γ	**R=0.85**	**R=0.93**	R=0.17	**R=0.89**
	P<0.0001	**P<0.0001**	P=0.49	**P<0.0001**
Stimulated				
IL-2	-			
IL-6	R=0.23	-		
	P=0.50			
IL-10	R= -0.22	R=0.21	-	
	P=0.38	P=0.53		
TNF	R= -0.27	R= 0.08	**R=0.65**	-
	P=0.27	P=0.81	**P=0.003**	
IFN-γ	R= -0.17	R= 0.13	**R=0.61**	**R=0.89**
	P=0.49	P=0.70	**P=0.005**	**P<0.0001**

FIGURES

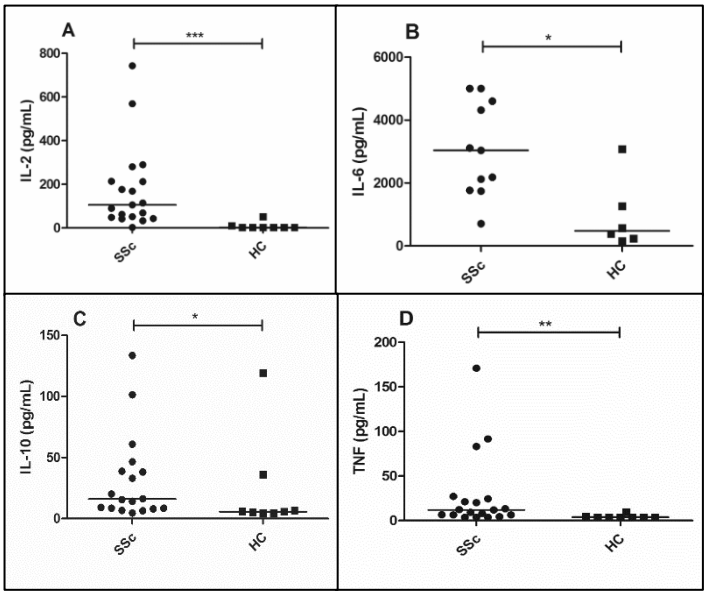

Figure 1. Cytokines levels in supernatants produced by unstimulated PBMCs from systemic sclerosis patients (SSc) and healthy controls (HC). (A) Spontaneous production of IL-2 is higher in PBMCs from SSc patients than HC. The same pattern was observed for IL-6 (B), IL-10 (C) and TNF (D). *p<0.05, **p<0.01, ***p<0.001. Short horizontal bars represent median. SSc= systemic sclerosis, HC= healthy controls.

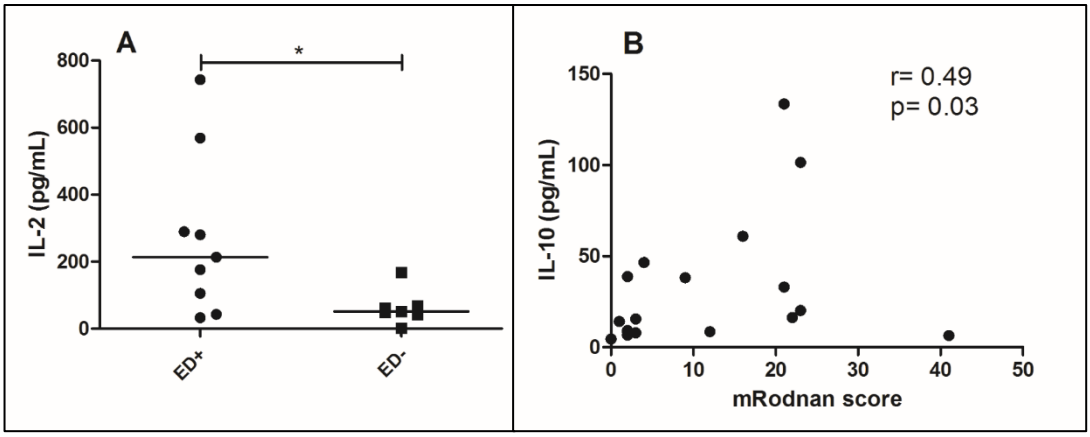

Figure 2. Associations of cytokines levels in unstimulated supernatant from SSc PBMCs and clinical manifestations. (A) IL-2 levels were higher in patients with esophageal dysmotility. (B) IL-10 levels had a positive correlation with modified Rodnan score. *p<0.05. ED= esophageal dysmotility, mRodnan score= modified Rodnan score.

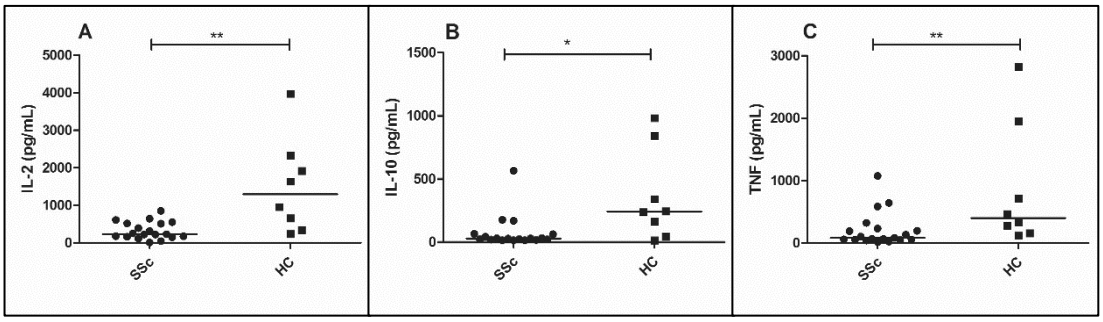

Figure 3. Cytokines levels produced by anti-CD3/CD28 stimulated PBMCs from systemic sclerosis patients (SSc) and healthy controls (HC). (A) Stimulated production of IL-2 is higher in PBMCs from HC than SSc patients. The same pattern was observed for IL-10 (B) and TNF (C). *p<0.05, **p<0.01. Short horizontal bars represent median. SSc= systemic sclerosis, HC= healthy controls.

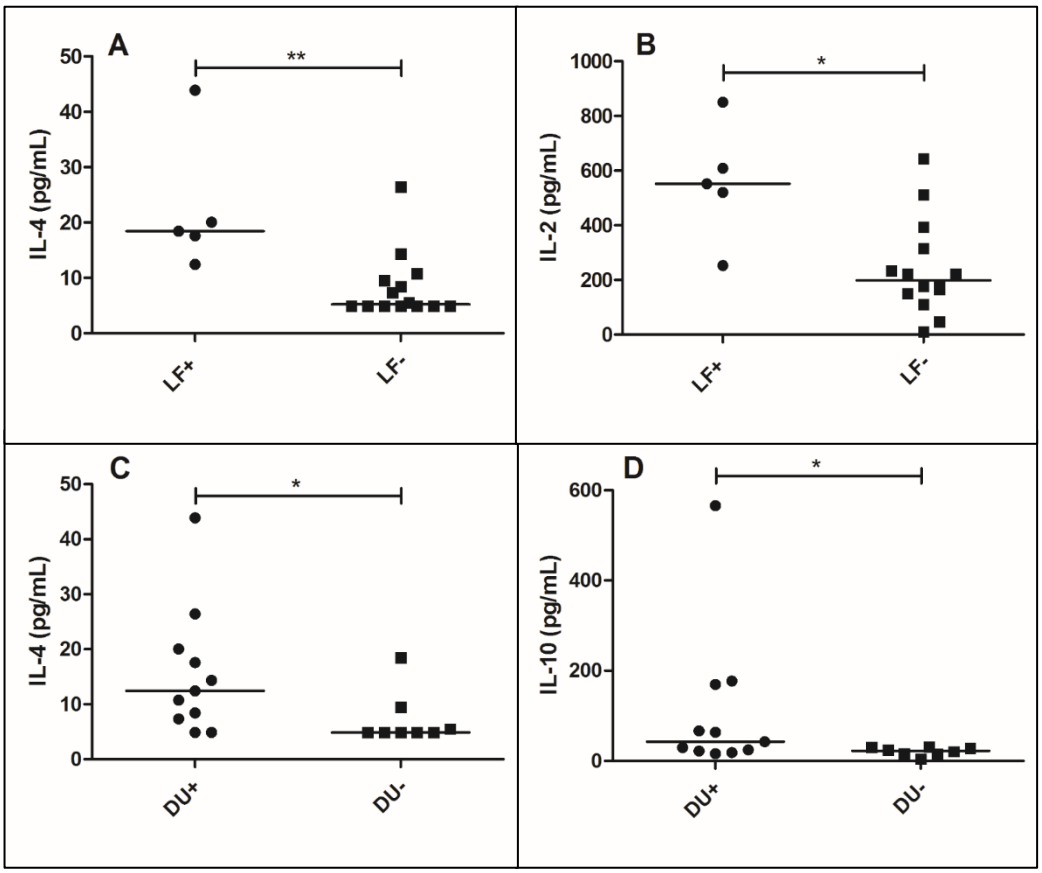

Figure 4. Associations of cytokines levels in stimulated supernatant from SSc PBMCs and clinical manifestations. Patients with lung fibrosis had higher levels of IL-4 (A) and IL-2 (B). Patients with digital ulcers had higher levels of IL-4 (C) and IL-10 (D). *p<0.05, **p<0.01. LF= lung fibrosis, DU= digital ulcer.

5.6 Artigo V – Investigation of chemokine profile in Brazilian systemic sclerosis patients reveals CCL5 and CXCL8 as biomarkers of clinical manifestations

Artigo a ser submetido è revista Human Immunology

RESUMO

O objetivo do presente estudo foi investigar os níveis séricos e a produção *ex vivo* de quimiocinas em pacientes brasileiros com ES e associações com manifestações clínicas. Amostras de soro foram obtidas de 35 pacientes e 25 controles saudáveis pareados por sexo e por idade. Os níveis de quimiocinas no soro e no sobrenadante de cultura de PBMC foram quantificados por CBA. Os níveis de CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG e CXCL10/IP-10 foram significativamente maiores em pacientes com ES em comparação com indivíduos saudáveis. Houve uma associação entre os níveis séricos de CXCL8/IL-8 e CXCL10/IP-10 e a presença de miosite (p=0,003 e 0,03, respectivamente). Além disso, níveis séricos de CCL5/RANTES foram maiores em pacientes com fibrose pulmonar (p=0,02) e úlceras digitais (p=0,04). No sobrenadante de cultura de PBMC, foram observadas maiores concentrações de CCL5/RANTES (p=0,03) e CXCL8/IL-8 (p=0,04) em pacientes com úlceras digitais. Pacientes com ES níveis de quimiocinas estão aumentados em pacientes com ES e apresentam associação com importantes manifestações clínicas, sugerindo que sejam marcadores úteis para avaliação da doença.

ABSTRACT

The purpose of this study was to investigate chemokines serum levels and *ex vivo* production by PBMC from a Brazilian population of systemic sclerosis (SSc) patients and their associations with clinical manifestations. Serum samples were obtained from 35 SSc patients and 25 age- and gender- matched healthy controls (HC). Chemokines levels in serum and supernatant of PBMC culture were measured by CBA. CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG and CXCL10/IP-10 serum levels were significantly increased in SSc compared with HC. There was an association between CXCL8/IL-8 and CXCL10/IP-10 serum levels and myositis(p=0.003 and p=0.03, respectively). Besides, CCL5/RANTES serum levels were higher in patients with lung fibrosis(p=

0.02) and digital ulcer (p=0.04). In supernatants from PBMC cultures, we found higher levels of CCL5/RANTES (p=0.03) and CXCL8/IL-8 (p=0.04) in patients with digital ulcers. In conclusion, chemokines levels are increased in SSc patients and they are associated with important clinical manifestations, suggesting that they are candidates for disease biomarkers.

INTRODUCTION

Systemic sclerosis is a rare autoimmune disease characterized by excessive collagen production which results in fibrosis of the skin and visceral organs. Although the pathogenesis remains incompletely defined, T-cell activation and associated cytokines and chemokines released are thought to play a pivotal role through stimulating fibroblasts to promote fibrosis (1, 2).

Chemokines are a large family of small cytokines that share sequence homology and similar tertiary structures. They present potent chemoattractant effects on specific leukocyte subsets, but can also induce a variety of cellular responses such as angiogenesis or matrix protein synthesis. Because of these effects, chemokines are likely to be important contributors to the pathogenesis of SSc. In SSc, preceding the characteristic vasculopathy and fibrosis, inflammatory cell infiltrates, mainly consisting of activated T cells and macrophages, are often seen in perivascular areas in early skin lesions (3, 4).

Previous studies demonstrated enhanced chemokines serum levels in SSc patients, suggesting their participation in the pathogenesis (5-14). Some chemokines showed pro-fibrotic action. As an example, CCL2/MCP-1 stimulates the production of collagen by a specific subset of fibroblasts, promotes migration of inflammatory cells to involved tissue and induces Th2 differentiation (15). Besides, chemokines can present proangiogenic (CXCL8/IL-8) or anti-angiogenic (CXCL9/MIG and CXCL10/IP-10) effects, and, in this way, they can participate in vascular abnormalities found in SSc (14, 16). Despite these evidences, studies are still inconclusive about their participation in SSc development and their roles as disease markers.

For a better understanding of the role of these chemokines as potential biomarkers in SSc, we investigated serum levels of CCL2, CCL5, CXCL8, CXCL9 and

CXCL10 and *ex vivo* production by peripheral blood mononuclear cells (PBMC) from a Brazilian population of SSc patients and associations with clinical manifestations.

METHODS

Patients

Serum samples were obtained from 35 female SSc patients with a mean age of 45.0 years (range 24-75 yrs). All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR) (17). Patients with overlapping syndromes were excluded. SSc patients were classified into limited cutaneous SSc (lcSSc; n=14) and diffuse cutaneous SSc (dcSSc; n=21) groups (18). The mean disease duration, calculated from the time of onset of the first non-Raynaud event, was 115.6 ± 81.1 months (range 8–331 months). Early disease was defined as disease duration <2 years. The characteristics of SSc patients are summarized in the Table 1. Twenty-five unrelated age- and gender- matched healthy controls (HC) were included as controls.

Clinical and laboratorial manifestations were reviewed from the medical charts. Lung fibrosis was observed using high-resolution computed tomography (HRCT); esophagus involvement was determined by scintilography and/or endoscopy; proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement. Pulmonary artery pressure was estimated by Doppler echocardiogram; pressures above 35 mmHg were interpreted as pulmonary hypertension. The modified Rodnan total skin thickness score was used to evaluate skin fibrosis (19).

The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

PBMC purification and culture

PBMCs were obtained from the heparinized blood of patients and controls. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1×10^6 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 µg/ml) (Gibco, Carlsbad, CA, USA).

Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) and after 48h culture supernatant was collected for cytokines quantification.

Chemokine quantification

Chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10) levels were quantified in serum and PBMC culture supernatants by cytometric bead array (CBA), according to the manufacturer's protocol (CBA, BD Biosciences). Briefly, 50 µl serum samples were subjected to analysis in duplicate using the cytometric bead array kit on a Accuri C6 Flow Cytometer (BD, Biosciences). The concentration of serum cytokines was quantified using FCAP Array software v3.0. The detection limits were CXCL8/IL-8 0.2 pg/ml, CCL5/RANTES 1.0 pg/ml, CXCL9/MIG 2.5 pg/ml, CCL2/MCP-1 2.7 pg/ml and CXCL10/IP-10 2.8 pg/ml.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard error (SE) if they were in normal distribution or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann-Whitney U test was used to compare serum cytokines levels. Wilcoxon's signed rank test was used to compare differences in cytokine production of PBMCs. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. We considered correlation (R^2) strength as follows: $0<R^2\leq0.35$ = weak correlation; $0.35<R^2\leq0.67$ = moderate correlation; $0.67<R^2\leq1$ = strong correlation. A probability value of $p < 0.05$ was considered significant. The association between the cytokine and chemokine levels and the different internal organs involved was studied in univariate and multivariate analysis. In univariate analysis, we used the above mentioned test (Mann-Whitney U test) to compare variables in subjects with or without organ involvement. To evaluate the association between organs involved and different biological variables was present after simultaneously adjusting for the other variables of interest (age and treatment), we performed multiple linear regression analysis for the clinical variables with linear scores and multiple logistic regression for the clinical variables with dichotomous scores.

RESULTS

Serum chemokines levels and clinical correlations in SSc patients

We found that CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG and CXCL10/IP-10 serum levels were significantly increased in SSc compared with HC (Figure 1). No statistically significant difference was observed in CXCL8/IL-8 serum levels between patients and controls. No differences in the levels of any of these cytokines were noted between SSc subtypes (diffuse vs limited). Serum levels were similar in patients who had been taking glucocorticoids and/or other immunosuppressive drugs and patients who had not taking them. We also evaluated the correlations between each serum chemokine level in SSc. We verified a positive correlation between CCL2 and levels of CXCL9 ($r=0.40$, $p=0.01$) and CXCL10 ($r=0.60$, $p=0.0001$) and between CXCL9 and CXCL10 levels ($r=0.54$, $p=0.0009$). (Figure 2)

Next, we examined associations between serum levels of these cytokines and the main clinical parameters in SSc patients. We found that patients with myositis had increased CXCL8/IL-8 levels compared to patients without this manifestation ($p=0.02$). We also observed that patients with early disease had higher serum levels of CCL2/MCP-1 ($p=0.04$), CXCL10/IP-10 ($p=0.01$) and CXCL9/MIG ($p=0.04$) and, inversely, it was a positive correlation between disease duration and CCL5/RANTES levels ($r=0.45$, $p=0.01$).

To better understand the above relationship of clinical characteristics of SSc with cytokine levels, we undertook a multivariate analysis, while simultaneously adjusting for age, corticosteroid and immunosuppressive treatment. We found that CCL5/RANTES serum levels were independently associated with lung fibrosis ($p=0.02$) and digital ulcer ($p=0.04$) and maintained a positive correlation with disease duration ($p=0.01$). Furthermore, patients with myositis were more likely to have higher CXCL8/IL-8 ($p=0.003$) and CXCL10/IP-10 ($p=0.03$) levels. (Table 2)

Spontaneous and stimulated chemokines production by PBMCs from systemic sclerosis patients

Since chemokines levels in serum may not adequately reflect the producing potential of immune cells, we decide also evaluate spontaneous and stimulated chemokine production by PBMCs from SSc patients (Table 3). Initially, we investigated

the correlation between chemokine levels in PBMCs supernatants with clinical manifestations, we did not find any association in the unstimulated condition. However, in stimulated supernatants, we found that patients with digital ulcers had higher levels of CCL5/RANTES (p=0.03) and CXCL8/IL-8 (p=0.04). Lastly, patients with disease duration <2 years had higher CXCL9/MIG and CXCL10/IP-10 levels (p=0.04 and p=0.02, respectively).

Regarding the correlation between each chemokines levels produced by unstimulated and stimulated PBMCs (Table 4). In the non-stimulated condition, we observed a strong positive correlation between CXCL9/MIG and CXCL10/IP-10 levels (r=0.93, p<0.0001), CCL5/RANTES and CXCL9/MIG (r=0.67, p=0.002), and CCL5/RANTES and CXCL10/IP-10 (r=0.72, p=0.0005). After anti-CD3/CD28 stimulation, the positive correlation between CXCL9/MIG and CXCL10/IP-10 levels (r=0.97, p<0.0001) was maintained. There were also positive correlations between CXCL8/IL-8 and CCL2/MCP-1 (r=0.73, p=0.0004), CCL5/RANTES (r=0.75, p=0.0002) and CXCL9/MIG (r=0.50, p=0.03).

DISCUSSION

Our results showed that the serum levels of inflammatory chemokines CXCL10/IP10, CXCL9/MIG, CCL2/MCP-1 and CCL5/RANTES were significantly higher in SSc patients than in healthy individuals. Besides, we demonstrated significant associations between clinical manifestations and chemokines levels in serum and PBMCs supernatants from SSc patients.

CCL2/MCP-1 can be produced by a large variety of cells including fibroblasts, epithelial cells, mononuclear cells, endothelial cells and smooth muscle cells (15). Notably, CCL2/MCP-1 is strongly expressed in scleroderma cells and tissues, especially in the early phase of the disease (5, 20, 21). It has been reported that CCL2/MCP-1 might predominantly chemoattract Th2 cells and further participate in the fibrotic process by inducing chemotactic activity for PBMC, promoting myofibroblasts differentiation and stimulating fibroblast collagen synthesis and the release of IL-4 from T cells (20-25). Similar to that reported in other studies, we found increased CCL2/MCP-1 serum levels in SSc patients, suggesting a role of disease biomarker for this chemokine (5, 8-13, 26, 27). Besides, we detected higher serum concentrations in

patients with early disease. This finding was also observed by some authors (5, 28) and this could suggest a participation of this cytokine in early stages of disease. However, Antonelli et al (13) did not find changes in the scleroderma serum concentrations of CCL2/MCP-1 over time and Vettori et al (27) has described higher levels in patients with definitive disease. Many studies reported association between CCL2/MCP-1 levels and organ involvement, such as lung fibrosis (5), renal and cardiac disease (8), extension of skin involvement (12) and diffuse subset (12, 29), but we could not demonstrate any clinical association.

In addition to this Th2 chemotactic chemokine, previous studies suggested a role of Th1 chemotactic chemokines in the development of SSc (7). CXCL10/IP10, CCL5/RANTES, and CXCL9/MIG are considered chemoattractants for Th1 lymphocytes. Similar to previous reports, we found increased levels of CXCL9/MIG and CXCL10/IP-10 in serum from SSc patients (7, 10, 11, 13, 14, 30). Furthermore, these chemokines levels were higher in patients with early disease, finding consistent with that previously described by other studies (11, 13). Because they are closely related cytokines, their levels showed significant correlations with each other in serum and in supernatant culture from unstimulated and stimulated PBMCs (11, 31). However, it is not possible to conclude the real functional role of elevated levels of CXCL9/MIG and CXCL10/IP-10 circulating, since it was demonstrated decreased expression of their common receptor in SSc patients (14). It is noteworthy that CXCL10/IP-10 has been implicated in preventing the development of lung fibrosis through their angiostatic properties and their ability to inhibit the recruitment of fibroblasts (32-34).

We detected elevated CCL5 serum levels in SSc patients. In addition to serum, increased expression of CCL5/RANTES was also described in skin keratinocytes in previous studies with SSc patients (11, 12, 35, 36). In a murine model of scleroderma, increased expression of CCL5/RANTES preceded the development of dermal and pulmonary fibrosis and, because of this, it is believed that this chemokine should participate in early stages of SSc pathogenesis (37). However, we found that raised CCL5/RANTES levels were associated to higher disease duration, suggesting that this cytokine can also influence chronic disease. Nonetheless, the role of CCL5/RANTES in the pathogenesis of fibrosis, other than recruitment of inflammatory cells into tissue, remain undetermined. An interesting finding of our study was the relationship between presence of lung fibrosis and CCL5/RANTES levels. Although its presence has been

associated with interstitial lung disease of other etiologies (38, 39), its relationship with pulmonary fibrosis in SSc patients had not yet been described.

CXCL8/IL-8 is a potent chemoattractant and activator for neutrophils that have been shown to infiltrate mainly the lung in SSc. Also it induces migratory phenotype of fibroblasts and promotes fibroblast chemotaxis and stimulates angiogenesis (40). Higher serum IL-8 concentrations have been more frequently detected in SSc patients than in controls (6, 11, 27, 41) and some studies reported association with lung disease (6) and pulmonary arterial hypertension (9, 41, 42). Our study failed to detect elevation of serum IL-8 levels but we found significant correlation between the levels of CXCL8/IL-8 and other important chemokines in SSc such as CCL2/MCP-1, CCL5/RANTES and CXCL9/MIG.

CCL5/RANTES and CXCL8/IL-8 present pro-angiogenic effects (43, 44). We showed that patients with digital ulcers had higher levels of CXCL8/IL-8 levels in PBMC culture and CCL5/RANTES levels in both serum and in the culture supernatant. Dysregulated angiogenesis plays a role in the development of scleroderma vascular disease and an increase in the levels of pro-angiogenic growth factors may represent an ongoing attempt at angiogenic tissue repair or may reflect growth factor release from damaged tissues (45). We also detected higher CXCL8/IL-8 and CXCL10/IL-10 levels in patients with myositis. Although this association has not been previously described in patients with SSc, some studies have suggested an association between these cytokines levels and inflammatory myopathies, especially in patients with interstitial lung disease (46, 47).

Although it is the first study to evaluate the profile of chemokines in Brazilian patients with SSc as far as we know, we recognize that it has some important limitations. Our sample size is limited and our findings warrant further study in larger validation cohorts.

In summary, we have shown that chemokines levels are increased in SSc patients and they are associated with important clinical manifestations. Specifically, higher CCL5/RANTES levels were found in patients with lung fibrosis and digital ulcer as well as increased CXCL8/IL-8 levels were detected in SSc patients with myositis and digital ulcer. These findings suggest that these chemokines are candidates for disease biomarkers. Further longitudinal studies may clarify the real utility of chemokine levels as prognostic indicators in SSc patients.

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TABLES

Table 1. Clinical and laboratory characteristic of the patients with systemic sclerosis

Characteristics	Serum patients (N=35)	PBMC patients (n=19)
Age (yrs)		
Mean ± SD (range)	45.0 ± 12.0 (24-75)	42.3 ± 8.8 (24-63)
Disease duration (months)		
Mean ± SD (range)	115.6 ± 81.1 (8-331)	105.8 ± 78.3 (8-232)
Clinical subgroups N(%)		
Diffuse cutaneous	21 (60.0)	12 (63.2)
Limited cutaneous	14 (40.0)	7 (36.8)
Clinical manifestations N (%)		
Digital ulcer	22 (62.9)	11 (57.9)
Esophageal dysfunction	14/27 (51.8)	9/16 (56.3)
Lung fibrosis on thorax CT	13 (37.1)	5 (26.3)
Pulmonary arterial hypertension	5/33 (15.1)	2 (10.5)
Arthritis	11 (31.4)	5 (26.3)
Muscle involvement	11 (31.4)	7 (36.8)
Rodnan score		
Median (range)	10 (0-41)	4.0 (2-21)
Autoantibodies N(%)		
Positive ANA	30/32 (93.8)	17/18 (94.4)
Positive anticentromere	3/30 (10.0)	2/16 (12.5)
Positive Anti-SCl70	12/22 (54.5)	5/9 (55.6)
Treatment N(%)		
Steroids	13 (37.1)	10 (52.6)
Azathioprine	8 (22.9)	3 (15.8)
Methotrexate	4 (11.4)	3 (15.8)
Cyclophosphamide	3 (8.6)	2 (10.5)
Mycophenolate mofetil	3 (8.6)	1 (5.3)

Table 2. Associations of chemokines serum levels with organ involvement in systemic sclerosis patients (n=35)

Organ involvement	Cytokine	Odds ratio	95% confidence interval	p value
Myositis	CXCL10/IP-10	1.003	1.0004-1.006	0.03
	CXCL8/IL-8	1.008	1.003-1.014	0.003
Digital ulcer	CCL5/RANTES	1.002	1.0001-1.003	0.04
Lung fibrosis	CCL5/RANTES	1.002	1.0003-1.003	0.02

* Adjusted for age and treatment.

Table 3. Chemokines levels in supernatants produced by unstimulated and anti-CD3/CD28 PBMCs from systemic sclerosis patients (n=19).

	Unstimulated	**CD3/CD28**
CXCL10/IP-10	14.3 [7.2-59.4]	42.8 [12.7-72.3]
CCL2/MCP-1	392.1 [289.8-505.2]	468.7 [348.5-665.3]
CXCL9/MIG	132.7 [76.2-304.6]	273.8 [116.6-536.3]
CCL5/RANTES	327.1 [224.9-476.8]	360.4 [244.5-438.5]
CXCL8/IL-8	1057 [865.6-1488]	1379 [1025-2306]

Results are expressed as median [IQR]. Chemokines levels are in pg/ml. There are no statistical significant differences between levels in unstimulated versus anti-CD3/CD28 cells.

Table 4. Correlation between chemokines levels in PBMC supernatant from SSc patients

	CCL2/MCP-1	**CCL5/RANTES**	**CXCL8/IL-8**	**CXCL9/MIG**
Unstimulated PBMC				
CCL5/RANTES	R=0.30	-		
	P=0.22			
CXCL8/IL-8	**R=0.53**	**R=0.51**	-	
	P=0.02	**P=0.02**		
CXCL9/MIG	R=0.36	**R=0.67**	R=0.39	-
	P=0.13	**P=0.002**	P=0.10	
CXCL10/IP-10	R=0.40	**R=0.72**	R=0.34	**R=0.93**
	P=0.09	**P=0.0005**	p=0.15	**p<0.0001**
Anti CD3/CD28 stimulated PBMC				
CCL5/RANTES	**R=0.62**	-		
	P=0.0044			
CXCL8/IL-8	**R=0.73**	**R=0.75**	-	
	P=0.0004	**P=0.0002**		
CXCL9/MIG	R=0.44	R=0.31	**R=0.50**	-
	P=0.06	P=0.20	**P=0.03**	
CXCL10/IP-10	R=0.42	R=0.32	R=0.43	**R=0.97**
	P=0.07	P=0.18	P=0.07	**P<0.0001**

FIGURES

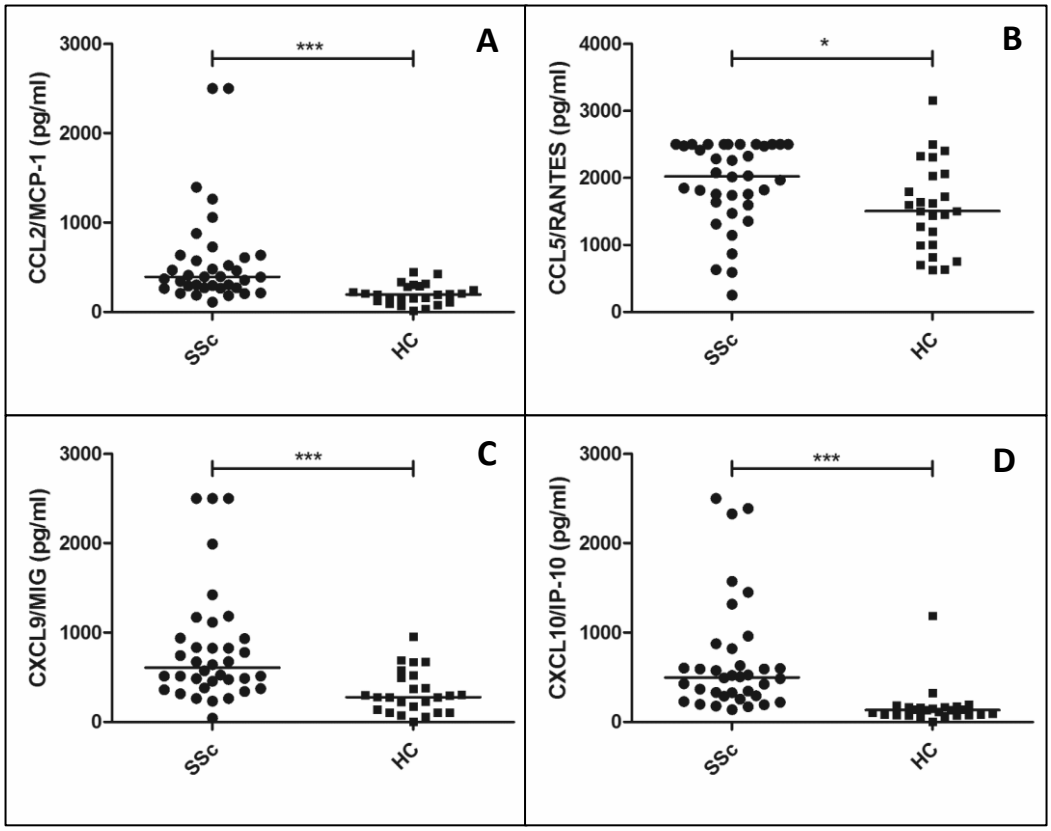

Figure 1. Serum levels of chemokines in SSc patients and healthy controls (HC). A) CCL2/MCP-1, B) CCL5/RANTES, C) CXCL9/MIG, D) CXCL10/IP-10. Serum levels are expressed in pg/ml. Horizontal bars indicate median. *p<0.01, ***p<0.0001, ns= not significant

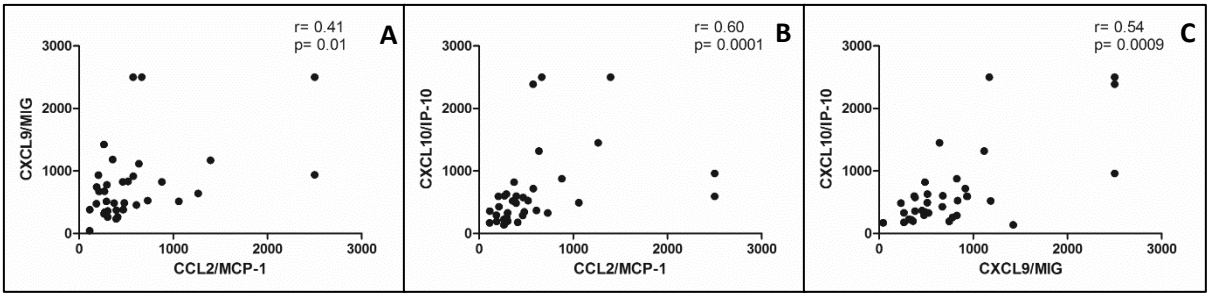

Figure 2. Correlations between serum chemokines levels in SSc patients. A) CCL2/MCP-1 and CXCL9/MIG, B) CCL2/MCP-1 and CXCL10/IP-10, C) CXCL9/MIG and CXCL10/IP-10. Serum levels are expressed in pg/ml.

5.7 Artigo VI – Methylprednisolone inhibits CCL2, CXCL8 and CCL5 production in PBMC from systemic sclerosis patients

Artigo a ser submetido para publicação na revista *Autoimmunity*

RESUMO

Introdução. Embora os glucocorticoides (GC) sejam um importante tratamento para a maioria das doenças reumáticas autoimunes, seu papel na esclerose sistêmica (ES) ainda é controverso. Tendo em vista a que uma complexa rede de citocinas e quimiocinas podem contribuir para a patogênese da ES, torna-se importante entender os efeitos dos GC na modulação dessas moléculas.

Métodos. Foram avaliados os níveis séricos de citocinas e quimiocinas em 52 pacientes com ES, classificados de acordo com o tratamento ou não com GC. A resposta ao tratamento com metilprednisolona (MP) em células mononucleares do sangue periférico (PBMC) foi avaliada em pacientes com ES (n=15) e indivíduos saudáveis (n=8), após o estímulo com anti-CD3/CD28. Citocinas (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) e quimiocinas (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1 and CXCL10/IP-10) foram quantificadas no soro e no sobrenadante de culturas de PBMCs através de *cytometric bead array* (CBA) ou *enzyme-linked immunosorbent assay* (ELISA).

Resultados. Não foram observadas diferenças significantes nos níveis séricos de citocinas/quimiocinas entre pacientes que tomavam ou não GC sistêmicos. Após o estímulo com anti-CD3/CD28, PBMCs tratadas com MP (100μM) apresentaram uma redução significante dos níveis de CCL2/MCP-1 (p=0,001), CCL5/RANTES (p=0,04) e CXCL8/IL-8 (p=0,003) em pacientes com ES. Em PBMCs de controles saudáveis, foi verificada a diminuição dos níveis de IFN-γ, TNF, IL-2 e IL-10 após o tratamento com MP, em comparação com a condição estimulada (p<0,01 para todas as citocinas), porém, em pacientes com ES, não houve diminuição significante nos níveis de nenhuma dessas citocinas avaliadas.

Conclusão. CCL2/MCP-1, CCL5/RANTES e CXCL8/IL-8 são quimiocinas potencialmente moduladas por corticosteroides *in vitro* em pacientes com ES mas nenhum efeito foi observado na produção das demais citocinas. Esses achados podem refletir a experiência clínica de limitada eficácia dos GC no tratamento da ES.

ABSTRACT

Background. Given that a complex network of cytokines and chemokines may contribute to the pathogenesis of systemic sclerosis (SSc), it is important to understand the effects of glucocorticoids (GC) treatment on modulation of these molecules.

Methods. We evaluated cytokines and chemokines levels in serum samples from 52 SSc patients taking or not systemic glucocorticoids. Peripheral blood mononuclear cell (PBMC) responses to methylprednisolone (MP) were examined from 15 SSc patients and 8 healthy control subjects following PBMC stimulation with anti-CD3/CD28. Cytokines (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1 and CXCL10/IP-10) levels were quantified in serum and in PBMC culture supernatants by CBA or ELISA.

Results. Compared with patients not taking corticosteroids, we did not observe any significant differences in cytokines/chemokines serum levels in patients using systemic corticosteroids. After stimulation with anti-CD3/CD28, PBMCs treated with MP (100 μM), showed a significant reduction of CCL2/MCP-1($p=0.001$), CCL5/RANTES($p=0.04$) and CXCL8/IL-8($p=0.003$) levels in scleroderma patients. In PBMC from healthy controls we observed decreased IFN-γ, TNF, IL-2 and IL-10 levels after MP treatment compared with stimulated condition ($p<0.01$ for all) but in SSc patients we did not find any significant reduction in these cytokines levels after MP treatment.

Conclusion. CCL2/MCP-1, CCL5/RANTES and CXCL8/IL-8 are chemokines potentially modulated by corticosteroids *in vitro* in SSc patients but no effect was observed on IL-2, IL-4, IL-6, IL-10, IL-17A, TFN and IFN-γ secretion. These findings may reflect the well-known clinical experience of limited effectiveness of GC in the treatment of SSc.

INTRODUCTION

Several studies indicate that systemic sclerosis (SSc) presents desregulated production of cytokines implicated in vascular damage and fibrosis. Since it has been known that Th1 cytokines, such as IFN-γ, have antifibrotic and anti-angiogenic effects, while Th2 cytokines, such as IL-4 and IL-13, have profibrotic and pro-angiogenic effects, SSc has often been considered a Th2 cytokine disease (1, 2). However, other mechanisms are also involved, such as imbalance between Th17 and Treg cells, although their real role in SSc pathogenesis are not fully elucidated (3). Chemokines

are also involved in SSc development. In addition to their function as chemoattractants of T cells and inflammatory cells into tissues, they play a role in angiogenesis and fibrosis regulation. Some studies demonstrated enhanced expression of CCL2/MCP-1, CXCL8/IL-8, CXCL9/MIG, CXCL10/IL-10 and CCL5/RANTES in serum, lung and skin of SSc patients (4-6). Given that the complex network of cytokines and chemokines may contribute to the pathogenesis of SSc, it is important to understand the effects of different treatments on modulation of these molecules.

Glucocorticoids (GC) have anti-inflammatory and immunosuppressive properties. They exert most of their biological effects through a genomic action, due to the interaction between their cytosolic receptor and the target genes, resulting in increased expression of regulatory proteins (transactivation) or decreased production of proinflammatory proteins (transrepression) (7-9). Previous studies demonstrated a downregulation of type 1 cytokines and subsequent increased production of type 2 cytokines in healthy human peripheral blood mononuclear cells (PBMC) after dexamethasone treatment (10, 11). I*n vitro* studies verified that dexamethasone treatment promoted suppression of pro-inflammatory cytokines and chemokines production, such as TNF, IL-1b, IL-6 and IL-8, in healthy subjects (12, 13).

Although glucocorticoids are the mainstay treatment for most rheumatic autoimmune diseases, their role in systemic sclerosis is still controversial. There is no randomized controlled study addressing the real efficacy of corticosteroids monotherapy in SSc. Despite the lack of evidence for their effectiveness, recent meta-analysis demonstrated the widespread use of glucocorticoids in SSc (14). About 40% of SSc patients were treated with corticosteroids, with or without immunosuppressive drugs, mostly in those with the diffuse SSc (14, 15). Currently, experts share the opinion that glucocorticoids may be indicated only in patients with inflammatory myositis, synovitis or alveolitis (16). On the other hand, long-term, high-dose glucocorticoids have been implicated in precipitating renal crisis, a serious disease complication (17).

Corroborating this controversy about clinical use of corticosteroids in SSc treatment, experimental studies assessing in vitro effects of GC in PBMCs from SSc patients are lacking. Therefore, the aims of this study were to investigate the effects of current corticosteroids treatment on serum cytokine and chemokine profile of SSc patients compared to healthy controls and to determine GC effects on cytokine production from stimulated PBMCs obtained from these patients.

METHODS

Study Subjects

We recruited 52 patients (49 female) for serum analysis, classified according to American College of Rheumatology criteria for SSc (18). Patients were classified as limited cutaneous SSc (lcSSc; n=28) or diffuse cutaneous SSc (dcSSc; n=24) (19). The mean disease duration, calculated from the time of onset of the first non-Raynaud phenomenon event, was 131.5 ± 122.2 months. The characteristics of SSc patients are summarized in the Table 1. From this group, we randomly selected 35 patients for serum chemokines analysis and 15 patients for PBMC culture. Eight age- and gender-matched healthy individuals served as controls for PBMC culture. (Table 1)

The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

PBMC purification and culture

PBMCs were obtained from the heparinized blood of patients and controls. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1×10^6 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 µg/ml) (Gibco, Carlsbad, CA, USA). Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) in the presence or absence of methylprednisolone 100µM (Pfizer, New York, NY, USA). Cells were incubated at 37°C in a humidified 5% CO2 incubator. Culture supernatant was collected after 48h for cytokines quantification.

Cytokine and chemokine quantification

Cytokines (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10) levels were quantified in serum and PBMC culture supernatants. Concentrations of cytokines in culture supernatants and chemokines in serum and culture supernatants were determined by cytometric bead array (CBA), according to the manufacturer's protocol (CBA, BD Biosciences). Briefly, 50 µl serum samples were subjected to analysis in

duplicate using the cytometric bead array kit on a Accuri C6 Flow Cytometer (BD, Biosciences). The concentration of serum cytokines was quantified using FCAP Array software v3.0.1. The detection limits were IFN-γ 4.1 pg/ml, TNF 3.7 pg/ml, IL-2 2.9 pg/ml, IL-4 3.3 pg/ml, IL-6 2.5 pg/ml, IL-10 3.3 pg/ml and IL-17 4.2 pg/ml. For chemokines, the limits of detection were CXCL8/IL-8 0.2 pg/ml, CCL5/RANTES 1.0 pg/ml, CXCL9/MIG 2.5 pg/ml, CCL2/MCP-1 2.7 pg/ml and CXCL10/IP-10 2.8 pg/ml.

Serum cytokines levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA for IL-4, IL-6, IL-10 e IFN- γ and eBioscience, San Diego, CA, USA for IL-17, TNF-α, IL-2). The lower detection limits for the ELISA analyses were as follows: 3.9 pg/ml for IL-17A, IL-4 and IL-10; 7.82 pg/ml for TNF; 4.69 pg/ml for IL-6; 9.37 pg/ml for IFN-γ; 1.5 pg/ml for IL-2.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard error (SE) if they were in normal distribution or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann-Whitney U test was used to compare serum cytokines levels. Wilcoxon's signed rank test was used to compare differences in cytokine production of PBMCs. A probability value of $p < 0.05$ was considered significant.

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RESULTS

- **Cytokines and chemokines serum levels in patients taking glucocorticoids**

To assess the effects of GC treatment on cytokines and chemokines serum levels, we first evaluated 52 SSc patients, of whom 19 (36.5%) were on systemic glucocorticoids treatment with prednisone. Mean dose of prednisone treatment was 3.8 mg/d (range 0-30 mg/d). Patients taking GC showed higher frequency of arthritis compared with patients not taking ($p<0.0001$). There were not statistically significant differences in other clinical manifestations. (Table 2)

In most of SSc patients, serum levels of IL-2, IL-4, IL-10, TNF and IL-6 were below the lowest detection limit. For IFN-γ and IL-17A, there was no differences in

serum levels between patients taking or not corticosteroids. Also, we did not observe any significant differences in chemokines serum levels in patients taking corticosteroids compared to patients not taking (Table 3). In order to rule out the influence of systemic treatment, we evaluated separately the patients who were not taking any immunosuppressive treatment. There were not differences on serum levels of cytokines and chemokines between these group of patients (data not shown). We also assessed serum cytokines levels in patients taking only GC compared with patients treated with immunosuppressive drugs, but there was no significant difference (data not shown).

- **Effects of methylprednisolone treatment on chemokine production by PBMC from SSc patients and healthy controls after stimulation with anti-CD3/CD28**

Next, we evaluated effects of MP on chemokine production by stimulated PBMC in healthy controls. We observed a significant increase in CXCL9 and CXCL10 supernatant levels and decrease in CCL2 levels after MP treatment. (Figure 1 and supplementary table S1)

In scleroderma PBMCs, MP treatment also caused a significant reduction in CCL2 levels ($p=0.001$) but we could not observe any effect on CXCL9 or CXCL10 levels. Besides, we detected a significant decrease in CCL5 and CXCL8 levels after MP treatment ($p=0.04$ and 0.003, respectively) (Figure 1 and supplementary table S1). When we evaluated only SSc patients not taking steroids, we also observed a significant reduction in CCL2, CCL5 and CXCL8 levels ($p=0.02$, 0.008 and 0.008 respectively). However, in patients who were treated with systemic steroids, these effects were not detected, except for CCL2 which also showed a significant decrease ($p=0.03$) (Data not shown).

- **Effects of methylprednisolone treatment on cytokine production by PBMC from SSc patients and healthy controls after stimulation with anti-CD3/CD28**

Following treatment of anti-CD3/CD28 stimulated PBMCs with methylprednisolone at 100 µM, we observed a significant reduction of IFN-γ, TNF, IL-2 and IL-10 levels in healthy controls compared with stimulated condition ($p<0.01$ for all). IL-4 levels in supernatant cultures from healthy controls became undetectable after treatment with MP which also indicate *in vitro* inhibition. Only IL-17A showed no

significant reduction after MP treatment (p=0.27) in controls. (Figure 2 and supplementary table S2)

Interestingly, in SSc patients we did not find any significant reduction in cytokines levels after MP treatment. Decreases in the mean levels of IFN-γ, IL-10 and IL-4 were not statistically significant (p= 0.23, 0.08 and 0.13, respectively). Still, IL-17A, IL-2 and TNF have higher mean levels after MP treatment, also not statistically significant. (Figure 2 and supplementary table S2).

In order to rule out the influence of systemic treatment with corticosteroids, we evaluated separately the patients who were treated with prednisone or not, but the results have not changed (data not shown). We also assessed the response to corticosteroids according to clinical form (diffuse vs limited) but there was no modulation of cytokines after MP treatment in any of the subgroups (data not shown).

DISCUSSION

We demonstrate that, although corticosteroids did not have immunomodulatory effect on IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A production in PBMC from scleroderma patients, they promoted downregulation of CCL2, CCL5 and CXCL8 production, important chemokines involved in the SSc pathogenesis.

We did not observe different levels of serum cytokines and chemokines in patients who were treated with corticosteroids compared with patients not taking GC. This finding may not explain the real *in vivo* effect of GC on modulation of these molecules as serum levels could not reflect the action of these chemokines *in situ*. Moreover, our patients taking GC were using low doses, which may not be sufficient to inhibit the production of these molecules. To better understand this effect, we recognize that the ideal would be to conduct a longitudinal study with pre and post treatment observations.

Few previous small or non-randomized trials suggested some usefulness of corticosteroid treatment for early diffuse SSc (20-22) but its use is mainly limited by the risk of developing renal crisis (23, 24). Currently, glucocorticoids have been associated with other immunosuppressive drugs such as cyclophosphamide, azathioprine, mycophenolate mofetil and methotrexate for the treatment of some scleroderma manifestations, such as skin sclerosis and lung disease. There are no recommendations for use of GC in the treatment of gastrointestinal and vascular

manifestations of SSc, including Raynaud phenomenon, digital ulcers and pulmonary arterial hypertension (14). Knowing the main effects of corticosteroids on modulating secretion of some molecules involved in SSc pathophysiology may help understanding the real benefit of this class of drugs to treat the disease.

Previous studies showed that serum, skin and lung expression of CCL2/MCP-1, CCL5/RANTES and CXCL8/IL-8 are significantly increased in SSc patients (25-28). Besides, experimental studies demonstrated their involvement in the recruitment of immune cells and development of fibrosis, suggesting a role of these molecules in SSc pathogenesis (4, 29). In our study, MP treatment was able to significantly inhibit CCL2 production in healthy subjects and SSc patients, while reduction of CCL5 and CXCL8 levels was observed only in SSc patients. It has been demonstrated that corticosteroids can inhibit the production of CCL2, CCL5 and CXCL8 in other inflammatory diseases and different cell types (12, 30, 31). Inhibition of CCL2 signalling had anti-fibrotic effects in several animal models of SSc and thus it has been suggested that inhibition of CCL2 and its receptor CCR2 may be a potential therapeutic target (25, 32). It has been also demonstrated that CCL2 and/or CCL5 were also downregulated in patients treated with alprostadil, iloprost or bosentan, medications used in treatment of vascular manifestations of SSc (27, 28).

On the other hand, in healthy controls, MP enhanced production of CXCL9 and CXCL10, molecules classified as Th1 chemokines, but this effect was not observed in SSc patients. These chemokines have shown anti-fibrotic effects, such as inhibition of bleomycin-induced lung fibrosis by CXCL10 (33) and regulation of TGF-β1-induced epithelial-mesenchymal transition by CXCL9 (34). CXCL10 levels are significantly elevated in early-stage disease, with a progressive decline with disease evolution (35, 36). Most of our patients had chronic disease, which is associated with lower levels of CXCL10, and this could explain the absence of MP modulating effect on this cytokine.

Previous i*n vitro* and *in vivo* studies have suggested that glucocorticoids inhibit the production of IL-12, IFN-γ and TNF, but upregulate the production of IL-4, IL-10 and IL-13 in healthy subjects, thus promoting a shift to Th2 response rather than generalized immunosuppression (11, 37). In contrast, our results showed significant suppression of IFN-γ, TNF, IL-10, IL-2, IL-4 and IL-6 production in PBMCs from healthy subjects indicating a not selective effect of corticosteroids. Curiously, we did not observe reduction in IL-17A levels. This lack of inhibition of IL-17A is consistent with studies that demonstrated the existence of a specific subset of Th17 cells refractory to

glucocorticoid (38) and upregulation of Th17 pathway in lupus patients treated with GC (39).

Regarding the effect of MP on cytokine production by SSc PBMCs, our results showed that patients with systemic sclerosis have diminished corticosteroid sensitivity of their circulating PBMCs when compared with healthy controls. To our knowledge, no study has evaluated *in vitro* the effect of corticosteroids on the cytokines modulation in systemic sclerosis. Studies with inflammatory diseases have demonstrated that *in vitro* response could be used as an indicator of clinical corticosteroid responsiveness (40-42). As example, supported by proven clinical efficacy, glucocorticoids suppressed expression of proinflammatory cytokines in PBMCs and fibroblast-like rheumatoid synoviocytes in rheumatoid arthritis (43, 44). Prior treatment with corticosteroids was unlikely to have influenced PBMC responsiveness because we found no difference in MP response among the patients who took regular oral prednisone in comparison to those that did not take oral corticosteroids. These findings may explain, in part, the missing or partial response to glucocorticoids observed in SSc patients treatment in clinical practice.

In conclusion, we demonstrated that CCL2, CCL5 and CXCL8 are chemokines potentially modulated by corticosteroids in SSc patients. However, no effect was observed on other cytokines secretion in scleroderma PBMCs treated with methylprednisolone. These findings may reflect the well-known clinical observation of limited effectiveness of corticosteroids in the treatment of SSc.

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TABLES

Table 1. Clinical and laboratory characteristics of SSc patients.

Characteristics	Serum patients (n=52)	PBMC patients (n=15)
Age (yrs)		
Mean ± SD (range)	44.8 ± 14.4 (19-79)	42.3 ± 9.5 (24-63)
Gender N(%)		
Female	49 (94.2)	15 (100)
Disease duration (months)		
Mean ± SD (range)	131.5 ± 122.2 (8-696)	101.2 ± 82.0 (8-232)
Clinical subgroups N(%)		
Diffuse cutaneous	24 (46.1)	09 (60.0)
Limited cutaneous	28 (53.9)	06 (40.0)
Clinical manifestations N (%)		
Digital ulcer	29 (55.8)	09 (60.0)
Esophageal dysfunction	25 (48.1)	08 (53.3)
Lung fibrosis on thorax CT	24 (46.2)	03 (20.0)
Pulmonary arterial hypertension	07 (13.5)	02 (13.3)
Arthritis	17 (32.7)	04 (26.7)
Muscle involvement	13 (25.0)	06 (40.0)
Rodnan score		
Median (range)	6.0 (0-36)	4.0 (0-41)
Autoantibodies N(%)		
Positive ANA	42/45 (93.3)	13/14 (92.9)
Positive anticentromere	5/43 (11.6)	01/13 (7.7)
Positive Anti-SCl70	13/32 (40.6)	03/07 (42.9)
Treatment N(%)		
Glucocorticoids	19 (36.5)	07 (46.7)
Azathioprine	09 (17.3)	02 (13.3)
Methotrexate	05 (9.6)	03 (20.0)
Cyclophosphamide	05 (9.6)	02 (13.3)
Mycophenolate mofetil	03 (5.8)	01 (6.7)

ANA= antinuclear antibodies; CT = computed tomography; PBMC= peripheral blood mononuclear cells

Table 2. Clinical and laboratory characteristics of SSc patients treated or not with systemic glucocorticoids

Characteristics	Glucocorticoids + (n=19)	Glucocorticoids – (n=33)	p
Age (yrs)			
Mean ± SD (range)	42.5 ± 14.4	46.1 ± 14.5	0.38
Gender N(%)			
Female	19 (100)	30 (90.9)	0.29
Disease duration (months)			
Mean ± SD (range)	90.7 ± 69.7	153.7 ± 139.0	0.06
Clinical subgroups N(%)			
Diffuse cutaneous	9 (47.4)	15 (45.5)	1.00
Limited cutaneous	10 (52.6)	18 (54.5)	
Clinical manifestations N (%)			
Digital ulcer	9 (47.4)	20 (60.6)	0.40
Esophageal dysfunction	6 (31.6)	19 (57.6)	0.09
Lung fibrosis on thorax CT	9 (47.4)	15 (45.5)	1.00
Pulmonary arterial hypertension	2 (10.5)	5 (15.2)	1.00
Arthritis	7 (36.8)	10 (30.3)	**<0.0001**
Muscle involvement	7 (36.8)	6 (18.2)	0.19
Rodnan score			
Median (range)	5.0 (0-31)	8.0 (0-36)	0.77
Autoantibodies N(%)			
Positive ANA	16/18 (88.9)	26/27 (96.3)	0.56
Positive anticentromere	3/16 (18.8)	2/27 (7.4)	0.34
Positive Anti-SCl70	7/12 (58.3)	6/20 (30.0)	0.15
Treatment N(%)			
Azathioprine	02 (10.5)	05 (15.2)	1.00
Methotrexate	01 (5.3)	03 (9.1)	1.00
Cyclophosphamide	-	05 (15.2)	-
Mycophenolate mofetil	01 (5.3)	02 (6.1)	1.00

ANA= antinuclear antibodies; CT = computed tomography

Table 3. Cytokines and chemokines serum levels in SSc patients taking or not glucocorticoids (GC)

	GC +	GC -	p
IFN-γ[#]	9.4 [9.4-9.4]	9.4 [9.4-31.0]	0.53
IL-17A[#]	3.9 [3.9-7.9]	3.9 [3.9-8.3]	0.72
CXCL10/IP-10*	604.4 [242.6-1098]	489.8 [319.2-594.8]	0.42
CCL2/MCP-1*	295.2 [239.3-650]	437.5 [300.8-638.9]	0.38
CXCL9/MIG*	677.1 [409.6-1016]	520.6 [380.3-933.9]	0.88
CCL5/RANTES*	2285 [1475-2745]	2147 [1728-2658]	0.88
CXCL8/IL-8*	27.6 [2.9-73.8]	32.6 [14.1-124.2]	0.23

Results are expressed as median [IQR]. [#]n=52; *n=35

Table S1. Effects of methylprednisolone (MP) treatment on chemokines production from anti-CD3/CD28 stimulated PBMC of SSc patients.

	SSc patients (n=14)				**Healthy controls (n=8)**			
	Unstimulated	**CD3/CD28**	**CD3/CD28 + MP**	**p**	**Unstimulated**	**CD3/CD28**	**CD3/CD28 + MP**	**p**
CXCL10	14.3 [7.2-59.4]	42.8 [12.7-72.3]	34.3 [7.8-74.7]	0.33	97.0 [70.8-333.2]	3.8 [2.8-16.9]	136.4 [115.5-485.5]	**0.001**
CCL2	392.1 [289.8-505.2]	468.7 [348.5-665.3]	317.2 [234.6-386.6]	**0.001**	715.3 [486.9-931.7]	1715 [988.1-1950]	139.1 [37.3-413.9]	**0.01**
CXCL9	132.7 [76.2-304.6]	273.8 [116.6-536.3]	204.3 [109.3-382.6]	0.22	620.6 [346.4-941.6]	2.5 [2.5-6.7]	734.4 [231.4-1380]	**0.01**
CCL5	327.1 [224.9-476.8]	360.4 [244.5-438.5]	299.2 [234.6-359.3]	**0.04**	500.0 [327.2-1038]	352.1 [301.8-1451]	641.8 [456.1-1281]	0.25
CXCL8	1057 [865.6-1488]	1379 [1025-2306]	977.6 [859.3-1392]	**0.003**	857.7 [516.4-1649]	757.4 [412.9-1902]	762.5 [295.9-2175]	0.94

Results are expressed as median [IQR]. Cytokines levels are in pg/ml. p = CD3/CD28 x CD3/CD28/MP

Table S2. Effects of methylprednisolone (MP) treatment on cytokines production from anti-CD3/CD28 stimulated PBMC of SSc patients.

	SSc patients (n=15)				**Healthy controls (n=8)**			
	Unstimulated	**CD3/CD28**	**CD3/CD28 + MP**	**p**	**Unstimulated**	**CD3/CD28**	**CD3/CD28 + MP**	**p**
IL-17A	18.9 [18.9-18.9] [#]	26.2 [18.9-96.2]	35.8 [18.9-81.6]	1.0	18.9 [18.9-18.9]	72.0 [20.8-159.9]	28.4 [19.8-66.3]	0.27
IFN-γ	520.3 [192.1-1102]	877.1 [595.1-3942]	652.5 [358.6-3438]	0.23	< 9.37	1556 [621.5-3245]	52.8 [12.3-213.0]	**0.01**
TNF	12.2 [4.3-27.1]	104.4 [57.5-323.8]	38.2 [9.1-300.8]	0.30	3.8 [3.8-4.3]	399.0 [189.0-1639]	4.4 [3.8-9.4]	**0.01**
IL-10	20.1 [8.6-46.6]	29.9 [20.8-66.7]	19.8 [7.5-61.7]	0.08	5.8 [4.7-28.8]	243.1 [75.9-718.6]	64.1 [23.1-107.9]	**0.01**
IL-2	114.0 [51.9-280.2]	253.2 [176.3-551.9]	224.4 [96.5-450.5]	0.45	2.6 [2.6-8.0]	1297 [415.1-2218]	44.7 [9.8-86.4]	**0.01**
IL-4	4.9 [4.9-4.9] *	11.6 [7.6-18.2]	4.9 [4.9-9.6]	0.13	< 3.9	20.9 [12.3-29.4]	< 3.9	-
IL-6	3677 [1747-4901] **	4266 [2405-5000]	2940 [1150-4888]	0.38	572.9 [239.5-3076]***	5000 [4288-5000]	2450 [1084-3598]	**0.02**

Results are expressed as median [IQR]. Cytokines levels are in pg/ml. #n=10, *n=12, **n=8, ***n=7; p = CD3/CD28 x CD3/CD28/MP

FIGURES

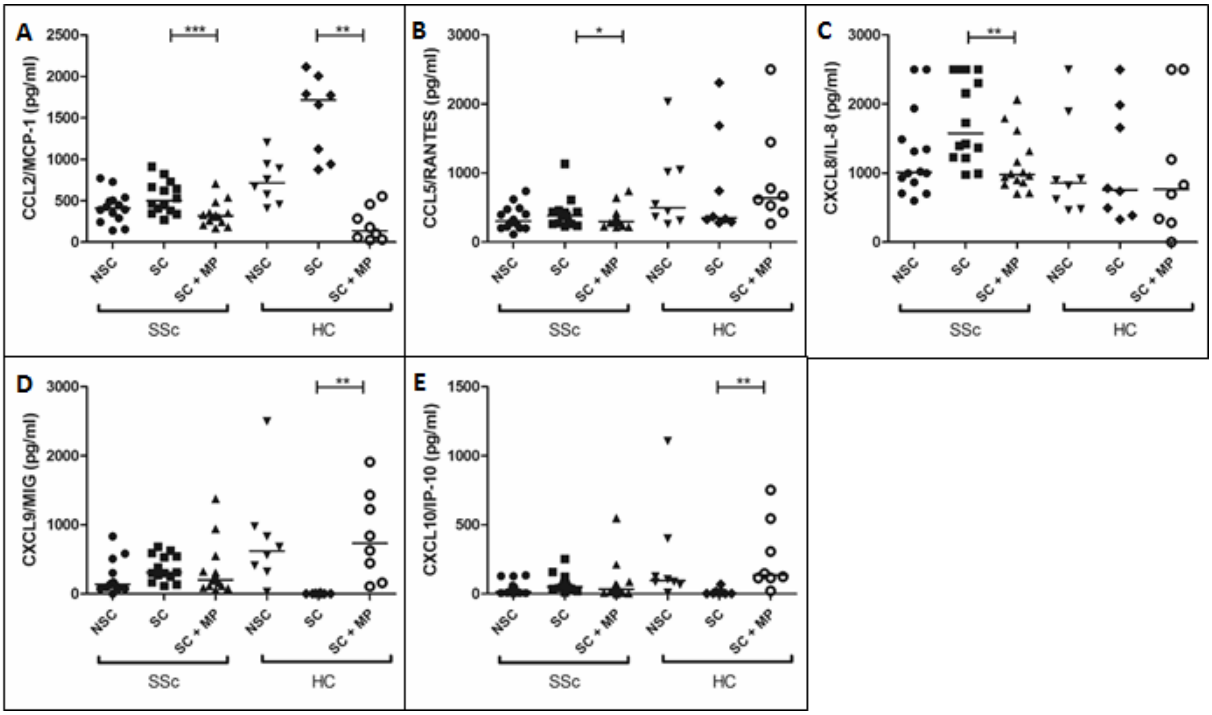

Figure 1. Evaluation of methylprednisolone effect on chemokine production in PBMC cultures from scleroderma patients and healthy controls. (A) Methylprednisolone significantly decreased CCL2/MCP-1 levels in SSc patients and HC. MP reduced CCL5/RANTES (B) and CXCL8/IL-8 (C) production in PBMCs from SSc patients. MP decreased CXCL9/MIG (D) and CXCL10/IP-10 (E) secretion in PBMCs from HC. *p<0.05, **p<0.01, ***p<0.001. SSc= systemic sclerosis, HC= healthy controls, NSC= non stimulated cell, SC= stimulated cell, MP= methylprednisolone.

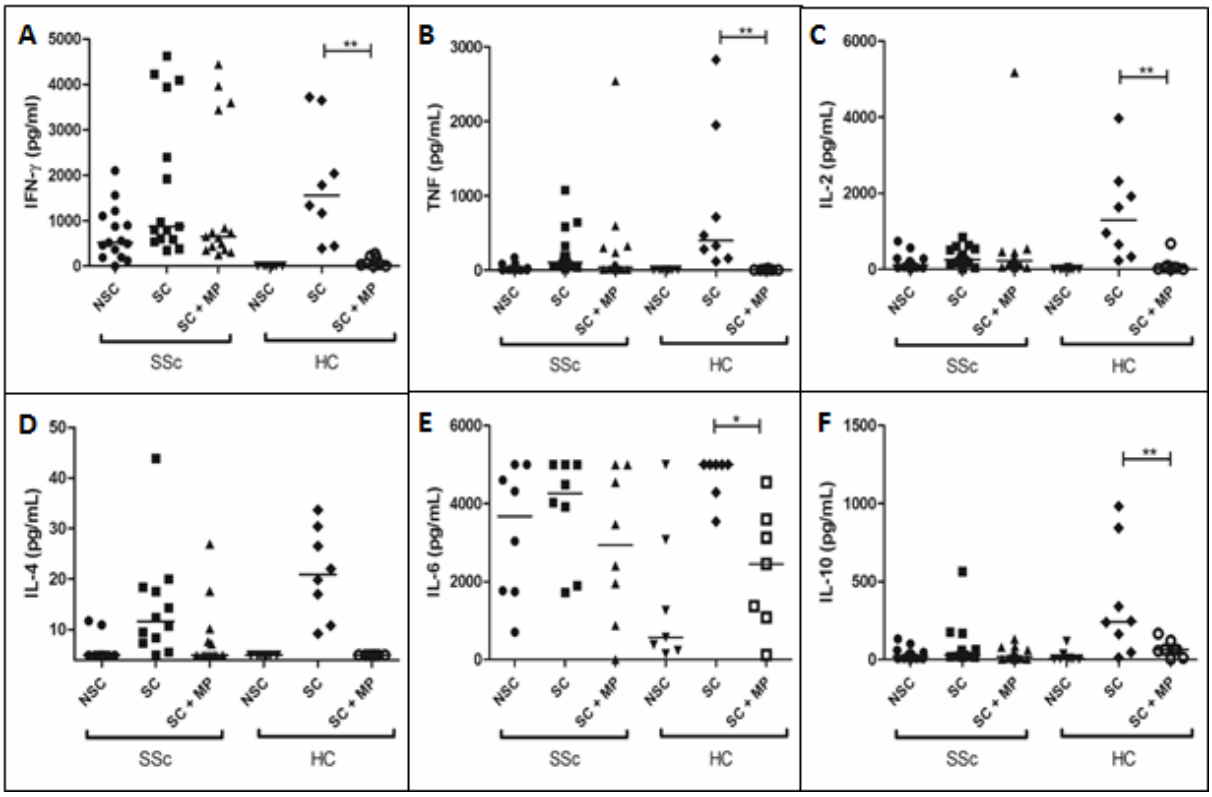

Figure 2. Evaluation of methylprednisolone effect on cytokine production in PBMC cultures from scleroderma patients and healthy controls. (A) Methylprednisolone significantly decreased IFN-γ levels in HC but not in SSc patients. The same pattern was observed for TNF (B), IL-2 (C), IL-4 (D), IL-6 (E) and IL10 (F). *p<0.05, **p<0.01. SSc= systemic sclerosis, HC= healthy controls, NSC= non stimulated cell, SC= stimulated cell, MP= methylprednisolone.

5.8 Artigo VII – A novel thiazolidinedione derivative modulates cytokines and chemokines production in PBMC from systemic sclerosis patients

Artigo a ser submetido à revista *Journal of Rheumatology*

RESUMO

Introdução. Tendo em estudos prévios demonstraram que derivados tiazolidínicos (TZD) apresentam efeitos anti-inflamatórios e antifibróticos, o presente estudo teve por objetivo avaliar a atividade imunomoduladora de uma nova TZD (LPSF/CR-35) em PBMC de pacientes com esclerose sistêmica.

Métodos. A resposta ao tratamento com LPSF/CR-35 (100µM) foi verificada no sobrenadante de cultura de PBMC estimulada com anti-CD3/CD28 de 19 pacientes com ES e oito controles saudáveis. Concentrações de citocinas (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and quimiocinas (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1 and CXCL10/IP-10) foram quantificadas por CBA. O antagonista seletivo do PPARγ GW9662 foi utilizado para avaliar se a atividade do LPSF/CR-35 é mediada pelo PPARγ. Para verificar se o LPSF/CR-35 apresenta atividade agonista do PPARγ, foi avaliada a capacidade do composto de induzir diferenciação adipogênica.

Resultados. Após o tratamento com LPSF/CR-35, foi observada uma redução significativa dos níveis de IL-17A (p=0.02), IL-10 (p=0.001) e IL-4 (p=0.04) e um aumento da produção de TNF (p=0.004) no sobrenadante de cultura de PBMC em pacientes com ES. Adicionalmente, o composto também reduziu a concentração de CCL2/MCP-1 (p=0.003) e CXCL8/IL-8 (p=0.03). Os efeitos promovidos pelo LPSF/CR-35 não foram antagonizados pelo GW9662. O composto não foi capaz de promover diferenciação de adipócitos.

Conclusão. O novo derivado tiazolidínico LPSF/CR-35 foi capaz de modular a secreção de citocinas e quimiocinas em pacientes com ES por um mecanismo independente do PPARγ. Estudos adicionais são necessários para definir seu papel no desenvolvimento de fibrose e seu real mecanismo de ação.

ABSTRACT

Objective. We aimed to evaluate the immunomodulatory activity of a new thiazolidinedione (TZD) analogue (LPSF/CR-35) in peripheral blood mononuclear cells (PBMC) from SSc patients.

Methods. PBMC response to LPSF/CR-35 (100µM) was examined from SSc patients (n=19) and healthy control subjects (n=8) following PBMC stimulation with anti-CD3/CD28. Cytokines (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1 and CXCL10/IP-10) levels were quantified in PBMC culture supernatants by cytometric bead array (CBA). The selective PPARγ antagonist GW9662 was used to explore the potential role of PPARγ in mediating LPSF/CR-35 modulatory effects on cytokines production. Adipogenic differentiation activity of LPSF/CR-35 was investigated.

Results. We observed a significant reduction in IL-17A (p=0.02), IL-10 (p=0.001) and IL-4 levels (p=0.04) and an increase in TNF secretion (p=0.004) after LPSF/CR-35 treatment in SSc patients. Also, LPSF/CR-35 decreased CCL2/MCP-1 (p=0.003) and CXCL8/IL-8 (p=0.03) levels in scleroderma patients. Even though LPSF/CR-35 is a thiazolidine derivative, its effects were not mediated by PPARγ activation.

Conclusion. We demonstrated that the new thiazolidinedione derivative modulates cytokines and chemokines involved in SSc pathogenesis by a mechanism PPARγ independent. Additional studies are necessary to investigate its role on fibrosis development and its real mechanism of action.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease whose pathophysiology is characterized by vasculopathy, immune dysregulation and fibrosis. This immune dysregulation comprises autoantibodies production, T cell and macrophage/monocyte proliferation and synthesis of profibrotic cytokines. These events contribute to fibroblasts activation and trans-differentiation into myofibroblasts, resulting in excessive deposition of extracellular matrix. From a molecular point of view, several profibrotic mediators such as cytokines, chemokines and growth factors contribute to

the pathogenesis of SSc by increasing the fibrogenic cellular response, but their complex interrelationships are not fully elucidated (1, 2).

There is no curative treatment for SSc and the available treatment options have limited efficacy. In general, SSc treatments are determined by the extent and severity of disease manifestations. At present, there are few efficacious therapeutic options for SSc, with most current therapies being targeted towards symptom relief. Therefore, interest exists in identifying new targets to address this unmet medical need (3, 4). Given that the complex interplay of cytokines and chemokines may contribute to the pathogenesis of SSc, it is important to understand the effects of different treatments on modulation of these molecules.

Thiazolidine-2,4-dione (TZD) is a well-characterized synthetic ring that acts primarily as PPARγ agonist with multiple pharmacological effects. Varied substituents on the thiazolidine-2,4-dione nucleus have provided a wide spectrum of biological activities, such as anti-hyperglycemic, anti-cancer, anti-inflammatory and anti-microbial effects (5, 6). *In vitro* and *in vivo* studies have shown that TZD have properties of inhibiting Th1 cytokines production, such as IL-2, IL-12, TNF and IFN-γ (7, 8), as well as, suppressing Th17 differentiation and expression (8, 9). In addition to downregulating proinflammatory cytokines, TZD can modulate Th2 cytokines, promoting enhanced or reduced expression of IL-4, IL-5 and IL-13, according to evaluated disease (8, 10, 11). In different cells types and animal models, TZD inhibited expression of chemokines CCL2/MCP-1 and CXCL8/IL-8 (12-14), CXCL-10 (15-17), CCL5/RANTES (15) and CXCL9/MIG (18). All these effects described above suggest that TZD may be promising molecules for the treatment of SSc.

In most cases, the anti-inflammatory and antifibrotic effects of the TZD have been reported to involve both PPARγ-dependent mechanisms (8, 19). This mechanism involves ligand binding to PPARγ, heterodimerization with retinoid X receptor (RXR) and its ligand, nuclear translocation, binding to the PPARγ response elements (PPRE), and induction of gene transcription (20). However, there are emerging data that they can mediate some of their biological activities via a PPARγ-independent pathway (21-24).

Currently approved for the treatment of type 2 diabetes mellitus, recent concerns regarding the safety of TZD have prompted renewed interest in more selective PPARγ modulators (25). Considering that the PPARγ ligands TZD have

potential anti-inflammatory and anti-fibrotic effects, which may be useful in the treatment of SSc, this work aimed to evaluate the immunomodulatory activity of a new TZD analogue (LPSF/CR-35) in peripheral blood mononuclear cells (PBMC) from SSc patients.

MATERIALS AND METHODS

Study population

Nineteen female SSc patients (mean age 42.3 ± 8.8 years) classified according to American College of Rheumatology criteria for SSc (26) were recruited from the Rheumatology Division at the Hospital das Clínicas-Universidade Federal de Pernambuco. Demographic, clinical and laboratorial data were collected from all patients by questionnaire and from hospital records. The characteristics of SSc patients are summarized in the Table 1. After exclusion of any rheumatic disease, eight age- and gender- matched healthy individuals were recruited as controls (HC). Controls were excluded if they were in regular use of medication or history of alcohol consumption or smoking in the last 15 days.

The study protocol was approved by ethics comittee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

Compound

LPSF/CR-35 [3-(4-bromo-benzyl)-5-(3-bromo-benzylidene)-thiazolidine-2,4-dione] were supplied by the Laboratory of Planning and Synthesis of Drugs of Federal University of Pernambuco (Recife, Brazil) (Figure 1). Such compounds were dissolved in DMSO (Sigma Chemical), at a final concentration of 0.1% DMSO. Chemical synthesis and cytotoxicity of the compound were previously described by our group (data unpublished).

PBMC purification and culture

PBMCs were obtained from the heparinized blood of patients and controls. PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1

× 10^6 cells/ml) were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 µg/ml) (Gibco, Carlsbad, CA, USA). Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) in the presence or absence of LPSF/CR-35 100 µM. In selected experiments, cultures were pretreated with the PPARγ irreversible antagonist GW9662 (10µM) for 30 min (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated at 37°C in a humidified 5% CO2 incubator. Culture supernatant was collected after 48h to cytokines quantification.

Cytokines and chemokines quantification

Concentrations of cytokines (Human Th1/Th2/Th17 Cytokine BD Bioscience) and chemokines (Human Chemokine BD Bioscience) in culture supernatants were determined by cytometric bead array (CBA), according to the manufacturer's protocol. Briefly, 50 µL samples were subjected to analysis in duplicate using the cytometric bead array kit on a BD Accuri C6 cytometry. The concentration of serum cytokines was quantified using FCAP Array software v3.0. The detection limit for IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17 were 4.1 pg/ml, 3.7 pg/ml, 2.9 pg/ml, 3.3 pg/ml, 2.5 pg/ml, 3.3 pg/ml, and 4.2 pg/ml, respectively. For chemokines, the limits of detection were CXCL8/IL-8 0.2 pg/ml, CCL5/RANTES 1.0 pg/ml, CXCL9/MIG 2.5 pg/ml, CCL2/MCP-1 2.7 pg/ml and CXCL10/IP-10 2.8 pg/ml.

IL-13 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The lower detection limits for IL-13 analyses was 3.9 pg/ml.

Adipocyte differentiation

The mouse embryonic fibroblast cell line 3T3-L1 was obtained from the Banco de Células do Rio de Janeiro. The cells were incubated in a culture medium consisting of DMEM, 10 % fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO2 in air.

For differentiation protocol, cells were seeded in a 24-well plate at 2 x 10^4 cells per well with culture medium described above. After reaching 80-90% confluence, the cells were incubated for 72 hours in the induction medium (DMEM, 10% fetal bovine serum, 10 µg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 1.0 µM dexamethasone,

100 units/mL penicillin and 100 µg/mL streptomycin). Thereafter, cells were maintained in adipocyte maintenance medium (DMEM with 10% FBS and 10 µg/ml insulin) for 7 days. Treatments with LPSF/CR-35, rosiglitazone and/or GW was started at day 1 and 3 of induction.

Adipogenic differentiation of these cells was evaluated by Oil Red O stain (Sigma-Aldrich, St. Louis, MO). Briefly, cells were washed two times with phosphate-buffered saline (PBS) and fixed with 10% formalin at room temperature for 60 min. After fixation, cells were washed with qsp water and incubated with 60% isopropanol and stained with filtered Oil Red O solution for 30 min. Subsequently, Oil Red O staining solution was removed and the plates were rinsed with water to remove unbound dye. The stained lipid droplets were viewed with an optical microscope (Leica microsystems, Germany) and images were captured with a digital camera.

Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard error (SE) if they were in normal distribution or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann-Whitney U test was used to compare serum cytokines levels. Wilcoxon's signed rank test was used to compare differences in cytokine production of PBMCs. A probability value of $p < 0.05$ was considered significant.

RESULTS

LPSF/CR-35 inhibits IL-4, IL-17A and IL-10 and enhances TNF secretion in SSc patients

To investigate the effect of LPSF/CR-35 treatment on cytokines production, we stimulated PBMCs from HC and SSc patients with anti-CD3/CD28 in the presence of LPSF/CR-35 100 µM and collected supernatants after 48h. There was a significant decrease in IFN-γ ($p=0.008$), IL-2 ($p=0.008$) and IL-4 ($p=0.04$) in PBMC from healthy subjects treated with LPSF/CR-35 when compared with anti-CD3/CD28 stimulated cells (Table 2). In SSc patients, we observed a significant reduction in IL-17A ($p=0.02$),

IL-10 (p=0.001) and IL-4 levels (p=0.04) and an increase in TNF secretion (p=0.004) after LPSF/CR-35 treatment (Table 2). Reduction in IL-13 levels after LPSF/CR-35 treatment was not significant (p=0.256) (data not shown).

LPSF/CR-35 reduces CCL2/MCP-1 and CXCL8/IL-8 production in SSc patients

Next, we determined the effects of LPSC/CR-35 on chemokines secretion in HC and SSc patients. In healthy controls, there was a significant increase in CXCL9/MIG and CXCL10/IP-10 levels (p=0.008 for both) (Table 3). Differently, in SSc patients, we observed a significant decrease in CCL2/MCP-1 and CXCL8/IL-8 levels after LPSF/CR-35 treatment (p=0.0035 and 0.0294, respectively) (Table 3).

Modulation of cytokines production by LPSF/CR-35 is PPARγ independent

Since LPSF/CR-35 is thiazolidine derivative, we sought to explore the potential role of PPAR-γ in mediating its modulatory effects on cytokines production by PBMCs. To this end, GW9662, a selective PPARγ antagonist, was used. Preincubation of normal PBMCs with GW9662 failed to rescue stimulated production of cytokines in the presence of LPSF/CR-35. GW9662 by itself had no effect on anti-CD3/CD28-induced stimulation (Figure 2).

LPSF/CR-35 does not stimulate adipogenesis

Since the cytokine modulating activity of the LPSF/CR-35 was not mediated via the PPARγ, we sought to examine if LPSF/CR-35 induces adipogenic differentiation in preadipocytes cells. For this purpose, mouse embryonic fibroblats cells 3T3-L1 were incubated with LPSF/CR-35, or the synthetic PPARγ ligand (rosiglitazone) in parallel, for up to 7 days. Phase-contrast microscopy showed a substantial time-dependent accumulation of cytosolic oil droplets induced by rosiglitazone but not by LPSF/CR-35 in these cells. These results demonstrate that LPSF/CR-35, despite being a thiazolidine derivative, do not have PPARγ agonist activity (Figure 3).

DISCUSSION

The results presented here showed that a novel compound LPSF/CR-35 was able to reduce IL-4, IL-17A, IL-10, CCL2/MCP-1 and CXCL8/IL-8 and enhance TNF production from stimulated PBMC in SSc patients. In addition, even though LPSF/CR-35 is a thiazolidine derivative, its effects were not mediated by PPARγ activation.

Cytokines play a major role in regulating ECM deposition by fibroblasts (27). Studies demonstrated that scleroderma patients present higher IL-4 serum levels as well as enhanced IL-4-producing T cells (28-30). In vitro, IL-4 has pro-fibrotic properties, since it induces proliferation of fibroblasts, myofibroblasts differentiation and stimulates collagen biosynthesis (31). SSc individuals also have increased expression of IL-17 in serum and skin and a higher frequency of Th17 cells in their peripheral blood than healthy controls. Unlike IL-4, IL-17 has demonstrated the ability to down-regulate the fibrosis process in human studies. On the other hand, this cytokine promotes production of pro-inflammatory chemokines such as CXCL8/IL-8 and CCL2/MCP-1, suggesting an indirect pro-fibrotic effect (32). These findings suggest that scleroderma may benefit from inhibition of IL-4 and IL-17.

In some studies, SSc patients presented raised IL-10 serum levels (28, 33, 34) and these increased levels were associated with lung and skin fibrosis and with the presence of myositis (29). IL-10 has a general anti-inflammatory effect by inhibiting proinflammatory cytokine synthesis in monocyte/macrophages (35). However, its role in the development of fibrosis has not been clear. While IL-10 overexpression induced lung fibrosis by a mechanism CCL2/CCR2 dependent (36), the treatment with IL-10 reduced gene expression of extracellular matrix proteins in TGF-β induced skin fibroblasts (37). Thus, it is uncertain to speculate whether reduction of IL-10 production in patients with SSc will translate into clinical benefit.

In contrast to above results, the levels of TNF in the same culture supernatants increased after LPSF/CR-35 treatment. Several previous *in vitro* studies have demonstrated that TNF has anti-fibrotic effects (38-41) and clinical trial with anti-TNF drugs have failed to show benefit in the treatment of SSc (42). Besides, there have been some case reports of developing of morphea, a localized form of scleroderma, after treatment with TNF inhibitors (43-45). Although the increase in TNF levels appears to be a useful effect in SSc treatment based on the above data, this data should be interpreted with caution since *in vitro* studies have also shown that TNF

stimulated the production of pro-fibrotic cytokines by T lymphocytes and fibroblasts in SSc (46, 47).

A remarkable finding was the ability of LPSF/CR-35 to inhibit CCL2/MCP-1 and CXCL8/IL-8 production in SSc patients. In the last years, blocking of chemokine signaling has gained growing interest as a potential new therapeutic strategy. CCL2/MCP-1 is a predominant monocyte chemoattractant and activator of mononuclear cells, promoting migration of inflammatory cells from the blood into involved tissues. In addition to its chemoattractant activities, this chemokine induces a Th2 differentiation and stimulates synthesis of extracellular matrix proteins in SSc fibroblasts (48, 49). Because of these effects, this chemokine emerges as a potential therapeutic target in SSc. Although the results with monoclonal human anti-MCP-1 antibody and CCR2 antagonist treatment in rheumatoid arthritis have not been encouraging (50, 51), these molecules have not been evaluated in SSc patients. Furthermore, the discovery of new molecules able to modulate the CCL2/MCP-1 production in ES may be an important finding.

CXCL8/IL-8 is a potent chemoattractant and activator for neutrophils but it also induces migratory phenotype of fibroblasts, promotes fibroblast chemotaxis and stimulates angiogenesis (52). Higher serum IL-8 concentrations have been more frequently detected in SSc patients than in controls (53-56) and some studies reported association with lung disease (53) and pulmonary arterial hypertension (54, 57, 58), but its real participation in SSc pathogenesis is still undefined. Given these findings, although the IL-8 seems to present a secondary role in the development of fibrosis in SSc, its modulation can also be beneficial in the treatment of these patients.

The LPSF/CR-35 effects in PBMCs from healthy controls were a little different from those in SSc. We also observed a decrease in IL-4, IL-10 and IL-17A production in HC, but not significant in the last two. There was also an important decrease in INF-γ and IL-2 production, but no changes in TNF levels. It is interesting to note that the effects on cytokines production described above seem to be disease-specific since they were different from those observed in healthy controls. While in SSc patients we verified a reduction in CCL2/MCP-1 and CXCL8/IL-8 levels, in HC there was an increase in CXCL9/MIG and CXCL10/IP-10 production. This study was not able to determine the cell source of the studied cytokines, which could help to better understand the effects observed in their production.

Although the LPSF/CR-35 is a thiazolidinedione derivative, its effects were not mediated by activation of PPARγ. Furthermore, the inability to promote adipocyte differentiation suggests that this molecule is in fact not a PPARγ agonist. Mounting evidence indicates that TZDs derivatives can interfere with multiple signaling mechanisms, independently of PPARγ activation (59, 60), such as phosphoinositide 3-kinase (PI3K-c) and cyclooxygenase (COX-2) pathways (6). Therefore, the mechanism of action of LPSF/CR-35 needs to be further elucidated.

In conclusion, we demonstrated that the new thiazolidinedione derivative modulates cytokines and chemokines involved in SSc pathogenesis by a mechanism PPARγ independent. Additional studies are necessary to investigate its role on fibrosis development and its real mechanism of action.

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TABLES

Table 1. Clinical and laboratory characteristics of SSc patients (n=19)

Characteristics	
Age (yrs)	
Mean ± SD (range)	42.3 ± 8.8 (24-63)
Disease duration (months)	
Mean ± SD (range)	105.8 ± 78.3 (8-232)
Clinical subgroups	
Diffuse cutaneous	12 (63.2)
Limited cutaneous	07 (36.8)
Clinical manifestations	
Digital ulcer	11 (57.9)
Esophageal dysfunction	9/16 (56.3)
Lung fibrosis on thorax CT	5 (26.3)
Pulmonary arterial hypertension	2/17 (11.8)
Arthritis	5 (26.3)
Muscle involvement	7 (36.8)
Rodnan score	
Mean (range)	10.9 (0-41)
Autoantibodies	
Positive ANA	17/18 (94.4)
Positive anticentromere	2/16 (12.5)
Positive Anti-SCl70	5/9 (55.6)
Treatment	
Steroids	10 (52.6)
Azathioprine	03 (15.8)
Methotrexate	03 (15.8)
Cyclophosphamide	02 (10.5)
Mycophenolate mofetil	01 (5.3)

CT = computed tomography

Table 2. Effects of LPSF/CR-35 treatment on cytokines production from anti-CD3/CD28 stimulated PBMC of SSc patients and healthy controls.

	SSc patients (n=19)			Healthy controls (n=8)		
	CD3/CD28	CD3/CD28 + LPSF/CR-35	p	CD3/CD28	CD3/CD28 + LPSF/CR-35	p
IL-17A	32.6 [24.2-96.2] #	19.1 [18.9-28.0]	**0.02**	93.5 [26.3-164.7] ***	18.9 [18.9-46.9]	0.09
IFN-γ	857.9 [574.5-2748]	1042 [407.8-2313]	0.39	1556 [621.5-3245]	226.5 [74.2-717.0]	**0.008**
TNF	83.9 [56.1-234.3]	202.1 [83.9-853.5]	**0.004**	399.0 [189.0-1639]	262.8 [98.3-1000]	0.94
IL-10	28.1 [18.5-63.7]	8.0 [6.1-23.8]	**0.001**	243.1 [76.0-718.6]	15.0 [5.2-59.4]	0.08
IL-2	233.2 [165.6-520.2]	253.3 [86.4-604.0]	0.82	1297 [415.1-2218]	205.5 [122.1-624.1]	**0.008**
IL-4	11.6 [6.9-18.8] *	8.2 [5.2-12.4]	**0.04**	20.9 [12.3-29.4]	5.8 [4.9-15.0]	**0.04**
IL-6	4084 [1856-4975] **	3432 [1863-4190]	0.32	5000 [3915-5000] ##	3377 [1796-5000]	0.31

#n=10, *n=14, **n=10, ***n=7, ##n=5. Results are expressed as median [IQR]. Cytokines levels are in pg/ml.
p = CD3/CD28 x CD3/CD28/CR35

Table 3. Effects of LPSF/CR-35 treatment on chemokines production from anti-CD3/CD28 stimulated PBMC of SSc patients and healthy controls.

	SSc patients (n=19)			Healthy controls (n=8)		
	CD3/CD28	CD3/CD28 + CR-35	p	CD3/CD28	CD3/CD28 + CR-35	p
CXCL10/IP-10	42.8 [12.7-72.3]	51.9 [22.7-78.3]	0.31	3.8 [2.8-16.9]	276.5 [108.0-492.9]	**0.008**
CCL2/MCP-1	468.7 [348.5-665.3]	339.2 [222.7-437.9]	**0.003**	1715 [988.1-1950]	1482 [1251-1814]	0.64
CXCL9/MIG	273.8 [116.6-536.3]	212.8 [154.9-435.2]	0.73	2.5 [2.5-6.7]	1401 [483.2-2188]	**0.008**
CCL5/RANTES	360.4 [244.5-438.5]	357.9 [294.3-425.5]	0.86	352.1 [301.8-1451]	655.7 [545.4-1834]	0.08
CXCL8/IL-8	1374 [1017-2196] #	1182 [920.3-1370]	**0.03**	757.4 [412.9-1902]	0.2 [0.2-1875]	0.46

#N=18. Results are expressed as median [IQR]. Cytokines levels are in pg/ml.
p = CD3/CD28 x CD3/CD28/CR35

FIGURES

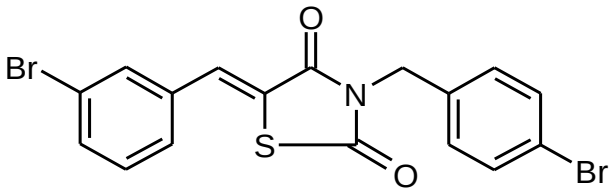

Figure 1. Chemical structure of 3-(4-Bromo-benzyl)-5-(3-bromo-benzylidene)-thiazolidine-2,4-dione (LPSF/CR-35)

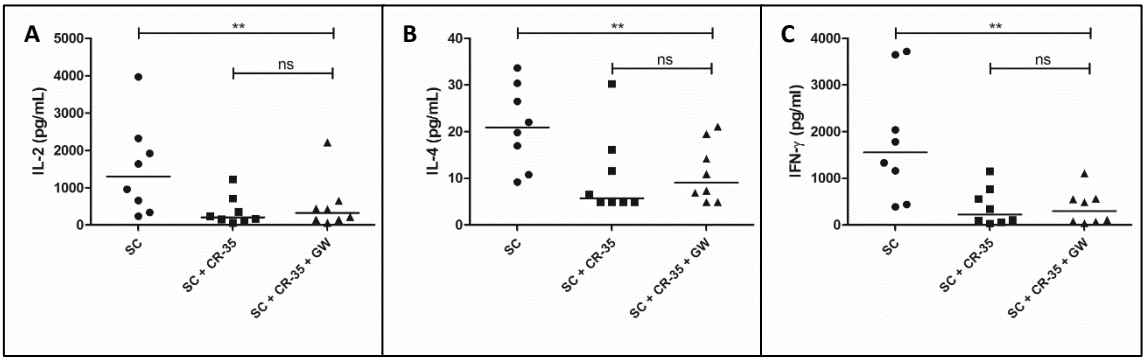

Figure 2. Inhibition of cytokines production is PPARγ independent. LPSF/CR-35 effect on IL-2 (A), IL-4 (B) and IFN-γ (C) production was not reversed by preincubation of cells with the PPARγ irreversible antagonist GW9662 at 10 µM. **p<0.01. SC= stimulated cell

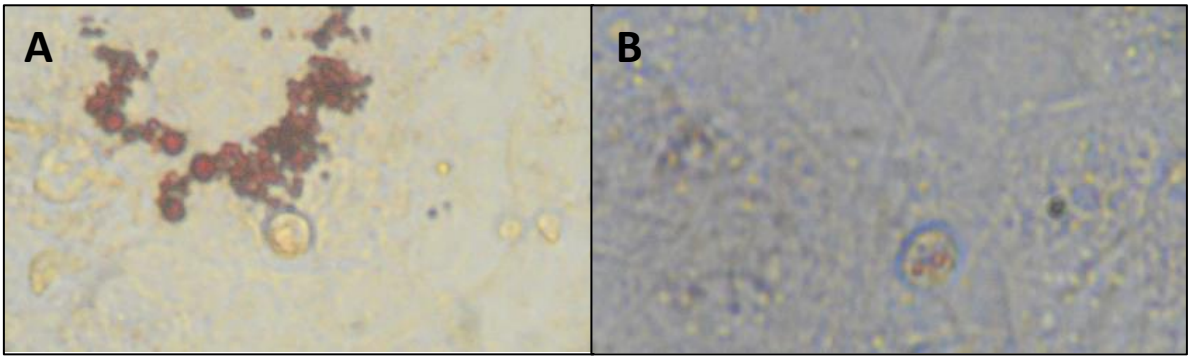

Figure 3. LPSF/CR-35 did not promote adipogenesis. Confluent 3T3-L1 preadipocytes were incubated with LPSF/CR-35 or rosiglitazone for 7 days. Optical microscopy showed a substantial time-dependent accumulation of cytosolic oil droplets stained by Oil red O in rosiglitazone treated cells (A) but not in LPSF/CR-35 treated cells.

6. DISCUSSÃO

A produção alterada de citocinas tem sido implicada como possível mecanismo patogênico em uma variedade de doenças autoimunes.[214] Na ES, o aumento da expressão de mediadores pró- e anti-inflamatórios e pró- e antifibróticos no soro e em órgãos-alvo comprometidos pela doença reforçam a forte interação existente entre os mecanismos de inflamação e de fibrose que caracterizam a fisiopatologia da doença.[6; 104] A disfunção vascular, com lesão endotelial e comprometimento da angiogênese, parece ser o evento inicial, podendo preceder em anos o envolvimento de outros órgãos. A progressão dos eventos não é necessariamente sequencial, havendo disfunção simultânea em vários mecanismos regulatórios.[215; 216] Como característica histológica da doença, sobretudo nas fases mais iniciais, identifica-se a presença de um infiltrado inflamatório com predomínio de linfócitos CD4+ e monócitos, sendo essas células as principais responsáveis pela liberação de citocinas, quimiocinas e fatores de crescimento que, entre outros efeitos, promoverão a ativação dos fibroblastos e consequente aumento da produção de matriz extracelular.[215; 216; 217]

A complexidade da fisiopatologia se traduz em uma grande diversidade de manifestações clínicas. Pacientes com ES podem apresentar variados fenótipos, que diferem quanto à gravidade dos órgãos acometidos, evolução natural, resposta ao tratamento e prognóstico, tornando a população de pacientes bastante heterogênea. Embora, em uma proporção significativa dos casos, a doença curse com desenvolvimento de alterações fibróticas irreversíveis, a instituição de um tratamento precoce poderia evitar a instalação de uma fibrose extensa ou lesão vascular grave. Sendo assim, estratégias se voltam para a identificação de fatores preditores de atividade, gravidade e resposta ao tratamento, na tentativa de individualizar e oferecer uma melhor assistência a cada paciente. Idealmente, a avaliação de tecidos afetados, como a pele ou os pulmões, proporcionariam a identificação de biomarcadores mais fidedignos. Como a realização de procedimentos invasivos, como biópsias seriadas, é impraticável, atualmente, os autoanticorpos específicos ou relacionados à ES são os biomarcadores mais utilizados para o diagnóstico, classificação e para tentar predizer o desenvolvimento de manifestações clínicas específicas.[218; 219] Entretanto, além de apresentarem limitações quanto ao potencial de diagnóstico e classificação por terem, em geral, baixa sensibilidade, os autoanticorpos não fornecem informações

quanto à resposta ao tratamento.[49; 220] Nesse sentido, justifica-se a necessidade de investigação de novas moléculas que preencham essas lacunas.

Nosso estudo avaliou os níveis séricos de 23 diferentes citocinas/quimiocinas em pacientes com ES. Em comparação com controles saudáveis, identificamos elevação nas concentrações séricas de IL-35, IFN-λ1/IL-29, IFN-γ, TGF-β, CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG e CXCL10/IP-10, as quais foram associadas à presença de importantes manifestações clínicas. Esses achados, além de sugerirem a participação dessas moléculas na fisiopatologia da ES, indicam sua potencial utilidade como biomarcadores da doença.

O presente estudo descreveu o aumento dos níveis séricos de IL-35 e sua associação com a presença de fibrose pulmonar em pacientes com ES, introduzindo a hipótese de que essa citocina pode estar associada à fisiopatologia da doença. Simultaneamente à nossa publicação, dois recentes estudos avaliaram a participação da IL-35 no desenvolvimento da fibrose.[132; 133] Tomcik et al.[132] demonstraram aumento da expressão das subunidades da IL-35 (EBI3 e p35) na pele e em fibroblastos dérmicos, além de níveis séricos aumentados em pacientes com ES. Nesse estudo também foi observado que o TGF-β induziu a expressão e liberação de IL-35 por fibroblastos cutâneos de pacientes com ES, e que a IL-35 promoveu a diferenciação de miofibroblastos e aumentou a síntese de colágeno, sugerindo propriedades pró-fibróticas para essa molécula.[132] Resultados opostos foram obtidos por Kudo et al.[133], que evidenciaram diminuição da expressão de colágeno em fibroblastos normais tratados com IL-35 ou com a sua subunidade EBI3. Da mesma forma, EBI3 provocou diminuição do colágeno em fibroblastos de pacientes com ES e reduziu a fibrose dérmica em modelo animal de fibrose induzida por bleomicina.[133] Esse mesmo estudo evidenciou ainda diminuição da expressão de EBI3 em queratinócitos e células Treg da pele de pacientes com ES, embora não tenha evidenciado diferenças nos níveis séricos em comparação com controles saudáveis.[133] Esses estudos preliminares e contraditórios são ainda insuficientes para elucidar o papel da IL-35 na ES.

Identificamos também níveis séricos elevados de IFN-λ1/IL-29 em pacientes com ES. Embora estudos prévios tenham relatado aumento da concentração sérica dessa citocina em outras doenças reumatológicas autoimunes, como lúpus e artrite reumatoide[221; 222], essa foi a primeira descrição dessa alteração em pacientes com ES. O IFN-λ1/IL-29 parece apresentar ação imunomoduladora sobre linfócitos T e

monócitos, promovendo inibição da produção de citocinas Th2 e, consequentemente, aumento da secreção de citocinas Th1, como IFN-γ.[135; 136; 223] Em concordância com esses achados, nós descrevemos uma correlação positiva entre os níveis séricos de IFN-λ1/IL-29 e IFN-γ. IFN-λ1/IL-29 também parece promover aumento da expressão de outras citocinas/quimiocinas como IL-6, IL-10, CXCL8/IL-8, CXCL9/MIG e CXCL10/IP-10.[222; 224; 225; 226; 227] Tendo em vista o nosso achado de níveis séricos elevados do IFN-λ1/IL-29 em pacientes com ES e a descrição prévia de seus efeitos *in vitro* na modulação de moléculas envolvidas na fisiopatologia da doença, estudos adicionais são necessários para avaliar a participação dessa citocina na ES.

Diversos estudos *in vitro* e *in vivo* vêm demonstrando o papel do TGF-β como mediador central na fisiopatologia da ES, participando de mecanismos de regulação não apenas da fibrose, como também da inflamação e da homeostase vascular.[71; 72; 228] A avaliação dos níveis séricos apresenta resultados discordantes na literatura, explicados pelas diferenças nas moléculas de TGF-β dosadas (ativa x total), a sensibilidade das técnicas utilizadas e a população examinada.[78; 81; 85; 98; 108; 229] No nosso estudo, optamos por avaliar a fração ativa do TGF-β1, que corresponde à molécula funcional da isoforma mais prevalente do TGF-β[230] e encontramos níveis séricos significativamente mais elevados em pacientes com ES. Entretanto, nosso principal achado foi a associação dos níveis do TGF-β1 com marcadores de pior prognóstico da doença, como a forma cutânea difusa, a presença de anti-Scl70, fibrose pulmonar e úlceras digitais.

Algumas limitações estão associadas à dosagem sérica de citocinas. Os resultados podem ser afetados por aspectos inerentes ao procedimento, como coleta do material biológico, processamento e armazenamento da amostra, técnica escolhida para quantificação das citocinas, bem como fatores relacionados ao paciente, como idade, gênero, ritmo circadiano, ingestão de alimentos e atividade física.[231] Além disso, essas moléculas, em condições normais, estão presentes no sangue periférico em concentrações muito baixas e podem sofrer influência da presença de receptores solúveis, proteínas de ligação e autoanticorpos.[232] Mesmo assim, a dosagem de citocinas no soro tem sido reconhecida como um procedimento válido para identificação de biomarcadores em diversas doenças autoimunes ou não.[231; 233; 234; 235] A identificação de níveis elevados em situações não-fisiológicas sugere ativação da via da citocina em questão e fornece subsídios para uma melhor compreensão da

fisiopatologia das doenças e para desenvolvimento de estratégias diagnósticas e terapêuticas.[236]

Embora tenhamos identificado maiores concentrações séricas de IL-17A em pacientes com fibrose pulmonar e de IFN-γ em pacientes com comprometimento muscular, um percentual significativo das citocinas avaliadas no soro permaneceram abaixo do limite de detecção dos kits utilizados. Sendo assim, com o intuito de melhor caracterizar a associação entre citocinas e manifestações clínicas, foram também mensurados os níveis de citocinas no sobrenadante das culturas de PBMCs não estimuladas e/ou submetidas ao estímulo policlonal para células T com anti-CD3 e anti-CD28. Observou-se associação entre a produção de IL-10 e a extensão do envolvimento cutâneo, IL-2 e dismotilidade esofageana, IL-2 e IL-4 e fibrose pulmonar e IL-4 e IL-10 e a presença de úlceras digitais. Com relação às quimiocinas no sobrenadante de cultura de PBMCs, verificou-se maior concentração de CCL5/RANTES e de CXCL8/IL-8 em pacientes com úlceras digitais e maior produção de CXCL9/MIG e CXCL10/IP-10 em pacientes com doença precoce.

A doença pulmonar intersticial é uma complicação grave e descrita em todas as formas de ES, sendo atualmente a principal causa de mortalidade desses pacientes.[57] Para o diagnóstico dessa complicação, são utilizados exames de imagem, mais comumente a tomografia computadorizada, e testes de função pulmonar. Entretanto, além do custo e dificuldade de acesso a esses exames, não estão bem definidos os parâmetros de avaliação de atividade fibrótica e de resposta ao tratamento.[237] Sendo assim, estratégias de pesquisa são direcionadas para a identificação de um biomarcador como parâmetro de avaliação adicional. No presente estudo, foram identificados níveis séricos elevados de IL-17A e CCL5/RANTES em pacientes com fibrose pulmonar, em comparação com pacientes sem essa complicação, enquanto no sobrenadante das culturas de PBMCs estimuladas, essa associação foi detectada em pacientes com maiores concentrações de IL-2 e IL-4. Estudos anteriores descreveram maior expressão de IL-17 em biópsia pulmonar de pacientes com ES, bem como uma relação entre os níveis séricos e a extensão do comprometimento pulmonar.[94; 115] IL-4 foi identificada em LBA e no condensado do ar exalado de pacientes com ES e correlacionou-se negativamente com parâmetros de função pulmonar.[101; 238] Aumento de número de células T CD4+ produtoras de IL-4 foi descrito no sangue periférico [239] e no LBA de pacientes com ES, com subsequente diminuição após o tratamento com imatinibe.[181] Esses achados reforçam a importância da IL-4 na

fisiopatologia da doença e ilustram a utilização dessa citocina como parâmetro de avaliação de resposta ao tratamento. Com relação à CCL5/RANTES, embora tenha sido descrita uma associação com doença pulmonar intersticial de outras etiologias[240; 241], essa relação não havia ainda sido identificada em ES.

A presença de úlceras digitais, decorrente de desregulação vasomotora e alterações histológicas vasculares, é uma complicação frequente em pacientes com ES e está associada a importante comprometimento funcional, com impacto significativo na qualidade de vida.[42] Diversos mediadores vasculares estão desregulados, refletindo o dano endotelial e resultando em uma angiogênese deficiente.[242] No presente estudo, foram constatados níveis elevados de IL-4, IL-10, bem como CCL5/RANTES e CXCL8/IL-8, quimiocinas consideradas pró-angiogênicas, em pacientes com úlceras digitais. O aumento da expressão sérica ou cutânea de moléculas pró-angiogênicas, como o VEGF, é bem documentado em pacientes com ES e pode representar um mecanismo compensatório ou refletir a liberação de fatores de crescimento a partir de tecido lesionados.[243; 244] Por outro lado, embora o papel da IL-4 e da IL-10 nos distúrbios vasculares não esteja bem definido, alguns estudos prévios sugeriram propriedades anti-angiogênicas para ambas as moléculas.[245; 246; 247]

Além das associações clínicas descritas acima, a detecção de níveis elevados de diferentes citocinas e a identificação de importantes correlações entre eles ilustram a complexa rede de mediadores inflamatórios envolvidos na fisiopatologia da ES. Nesse sentido, estratégias de tratamento com enfoque na modulação de citocinas/quimiocinas têm sido investigadas como opções terapêuticas na doença.

Atualmente, as poucas opções de tratamento para a doença, sobretudo para as manifestações inflamatórias e fibróticas, apresentam eficácia limitada. Embora uma proporção significativa de pacientes com ES seja tratada com glucocorticoides, estudos clínicos não demonstraram benefício relevante no tratamento de manifestações fibróticas cutâneas ou pulmonares.[10; 11; 12]

No nosso estudo, 36,5% dos pacientes avaliados usavam GC sistêmicos. Não foram identificadas diferenças dos níveis séricos de citocinas e quimiocinas entre pacientes que tomavam ou não essa medicação. Este achado pode não explicar o verdadeiro efeito *in vivo* dos GC na produção dessas citocinas, uma vez que os níveis séricos podem não refletir a ação das citocinas *in situ*. A modulação de citocinas séricas pelo tratamento com GC é descrita em outras doenças autoimunes,

identificando pacientes responsivos ao tratamento sistêmico.[248; 249] Embora as quimiocinas tenham sido detectadas no soro de todos os pacientes avaliados, das demais citocinas apenas o IFN-γ e a IL-17A conseguiram apresentar concentrações suficientes para serem dosadas por ELISA. Sendo assim, não é possível inferir conclusões com relação ao efeito dos GC nos níveis séricos das demais citocinas. Além disso, a dose média de GC utilizada pelos nossos pacientes foi baixa e, dessa forma, pode não ter sido suficiente para provocar modulação dos níveis dessas citocinas.

Estudos com doenças inflamatórias demonstraram que a resposta *in vitro* pode ser usada como um indicador da resposta clínica ao GC.[250; 251; 252] Com o objetivo de verificar a resposta *in vitro* ao tratamento com corticosteroides, as condições de cultura estimuladas com anti-CD3/CD28 foram tratadas com MP. Foi observada significativa redução dos níveis de CCL2/MCP-1, CCL5/RANTES e CXCL8/IL-8 em pacientes com ES. Como discutido anteriormente, essas quimiocinas têm sido implicadas na fisiopatologia da doença e sua redução poderia se traduzir em benefício clínico. Especificamente, a CCL2/MCP-1 tem sido sugerida como alvo terapêutico, tendo em vista seus efeitos pró-fibróticos, como estímulo à secreção de IL-4 por linfócitos T e, indiretamente, à produção de colágeno por fibroblastos.[139; 253; 254; 255] Entretanto, ainda não há evidência clínica do benefício da inibição da CCL2/MCP-1 em outras doenças.[256; 257] Por outro lado, embora o tratamento *in vitro* com MP tenha provocado redução da produção de IFN-γ, TNF, IL-10, IL-2, IL-4 e IL-6 em PBMC de controles saudáveis, nenhuma dessas citocinas sofreram modulação após o tratamento com MP em pacientes com ES. Esses achados poderiam justificar, pelo menos parcialmente, a limitada eficácia observada na prática clínica do tratamento de pacientes esclerodérmicos com GC.

Por fim, foi avaliada a resposta *in vitro* ao tratamento com o LPSF/CR-35. O composto inibiu significativamente a produção de IL-4, IL-10, IL-17A, CCL2/MCP-1 e CXCL8/IL-8 e aumentou a de TNF em pacientes com ES. Como mencionado acima, CCL2/MCP-1 e CXCL8/IL-8 representam potenciais alvos terapêuticos na ES tendo em vista suas ações pró-inflamatórias e pró-fibróticas.[155; 167] Adicionalmente, a inibição da IL-4, uma citocina com reconhecidas propriedades fibrogênicas, também pode apresentar um benefício clínico em pacientes com ES.[87; 258; 259] Em concordância com essa teoria, um estudo clínico demonstrou que a melhora/estabilização da fibrose

pulmonar após o tratamento com imatinibe foi associada à diminuição do número de células T produtoras de IL-4.[181]

Sabe-se que A IL-17A pode induzir a produção de citocinas pró-inflamatórias, como IL-6 e CXCL8/IL-8 [260], e de TGF-β1 [261], além de estudos com modelos animais terem demonstrado sua participação no desenvolvimento de fibrose pulmonar induzida por bleomicina.[262] Diante desses achados, a inibição da IL-17A também pode representar uma estratégia terapêutica na ES. Com relação à IL-10, os estudos são inconclusivos quanto ao seu papel no desenvolvimento de fibrose. Enquanto o aumento da expressão de IL-10 induziu o aparecimento fibrose pulmonar em modelos animais, por um mecanismo dependente da CCL2/MCP-1[222], a exposição à essa citocina reduziu a expressão de proteínas da MEC em fibroblastos cutâneos.[141] Sendo assim, não é possível concluir quais seriam os reais benefícios da inibição dessa citocina em pacientes com ES.

O LPSF/CR-35 é um novo derivado tiazolidínico caracterizado pela presença do substituinte bromobenzilideno, o que lhe confere uma maior eletronegatividade, proporcionando teoricamente uma ligação mais forte ao receptor do PPARγ e, consequentemente, uma maior ação anti-inflamatória (dados não publicados). Embora o principal mecanismo de ação descrito das TZDs seja via ligação e ativação do PPARγ[197], têm sido reconhecidas outras potenciais proteínas-alvo, caracterizando efeitos PPARγ-independentes.[263] Os efeitos do LPSF/CR-35 sobre a produção de citocinas por PBMC não foram reestabelecidos com a utilização do antagonista do PPARγ GW9665, sugerindo que esses efeitos foram independentes da ativação do PPARγ. Ademais, o tratamento de pré-adipócitos com o LPSF/CR-35 não induziu a sua diferenciação, aparentemente indicando que esse composto não apresenta atividade agonista do PPARγ, apesar de ser um derivado tiazolidínico. Estudos complementares são necessários para confirmar o real mecanismo de ação do composto.

Conjuntamente, os achados descritos nesse estudo caracterizam o perfil de citocinas e quimiocinas em pacientes com ES atendidos em um serviço de referência de Pernambuco. Apesar de ter avaliado uma população restrita de pacientes, não há registro de estudo semelhante em pacientes brasileiros. Adicionalmente, os dados obtidos com os estudos *in vitro* elucidam alguns dos efeitos moleculares dos GC em

pacientes com ES e revelam uma molécula promissora, o LPSF/CR-35, para o tratamento da doença.

7 CONCLUSÕES

- Pacientes com ES apresentaram um perfil de produção de citocinas e quimiocinas distinto do observado em controles saudáveis, sugerindo uma potencial utilidade dessas moléculas como biomarcadores da doença.

- Foi descrito, pela primeira vez, a elevação de níveis séricos de IL-35 em pacientes com ES, em comparação com indivíduos saudáveis, e sua associação com a presença de fibrose de pulmonar.

- Níveis séricos de IL-29/IFN-λ1 encontram-se aumentados em pacientes com ES e apresentaram uma correlação positiva com os níveis de IFN-γ. Além disso, níveis séricos de IFN-γ mostraram-se elevados em pacientes com comprometimento muscular.

- Pacientes com ES apresentaram níveis de TGF-β1 aumentados no soro em comparação com controles saudáveis e uma associação com a forma cutânea difusa, fibrose pulmonar e úlceras digitais e com a positividade ao anti-Scl70, indicando que essa molécula pode ser um marcador de pior prognóstico.

- Maiores níveis séricos de IL-17A foram encontrados em pacientes com fibrose pulmonar. Em sobrenadante de PBMCs de pacientes com ES, observou-se também associação entre a produção de IL-10 e a extensão do envolvimento cutâneo, IL-2 e dismotilidade esofageana, IL-2 e IL-4 e fibrose pulmonar e IL-4 e IL-10 e a presença de úlceras digitais.

- Avaliação do perfil de quimiocinas demonstrou aumento dos níveis séricos de CCL2/MCP-1, CCL5/RANTES, CXCL9/MIG e CXCL10/IP-10 em pacientes com ES e uma associação entre os níveis de CCL5/RANTES e fibrose pulmonar. No sobrenadante de cultura de PBMCs, verificou-se maior concentração de CCL5/RANTES e de CXCL8/IL-8 em pacientes com úlceras digitais e maior produção de CXCL9/MIG e CXCL10/IP-10 em pacientes com doença precoce.

- A metilprednisolona promoveu diminuição dos níveis de CCL2/MCP-1 e CXCL8/IL-8 em PBMCs de pacientes com ES, porém não interferiu na produção

de IL-2, IL-4, IL-6, IL-10, IL-17A, TNF e IFN-γ, indicando que os GC apresentam efeito limitado no tratamento da doença.

- O LPSF/CR35 promoveu a inibição da produção de IL-4, IL-10, IL-17A, CCL2/MCP-1 e CXCL8/IL-8 e aumentou a de TNF em PBMCs de pacientes com ES, sugerindo que esse composto apresenta potencial terapêutico na doença.

8 PERSPECTIVAS

- Investigar mecanismos fisiopatogênicos na ES através da avaliação do efeito das novas citocinas IL-35 e IL-29 (IFN-λ1) em PBMCs e fibroblastos de pacientes com ES e da modulação dos seus níveis após o tratamento com metilprednisolona, azatioprina e/ou novos compostos.
- Avaliar a resposta de PBMCs a diferentes estímulos mitogênicos (anti-CD3/CD28, PMA+Iono) em pacientes com ES em comparação com indivíduos saudáveis.
- Avaliar o efeito do LPSF/CR-35 na expressão gênica de citocinas e quimiocinas em PBMCs de pacientes com ES.
- Avaliar o efeito do LPSF/CR-35 na produção de citocinas e expressão de moléculas pró-fibróticas em fibroblastos de pacientes com ES e controles saudáveis.
- Elucidar o mecanismo de ação do LPSF/CR-35.

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ANEXOS

ANEXO A – Aprovação do Comitê de Ética em Pesquisa da UFPE

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE PERNAMBUCO
Comitê de Ética em Pesquisa
Av. da Engenharia, s/n – 1º Andar, Cid. Universitária, CEP 50740-600, Recife - PE.
Tel/fax: 81 2126 8588 - www.ufpe.br/ccs; e-mail: cepccs@ufpe.br

Of. Nº. 075/2012 - CEP/CCS

Recife, 01 de fevereiro de 2012

A
Doutoranda Andrea Tavares Dantas
Hospital das Clínicas – CCS/UFPE

Registro do SISNEP FR - 487479
CAAE – 0522.0.172.000-11
Registro CEP/CCS/UFPE Nº 529/11
***Título:* Avaliação da atividade Antifibrótica in vitro de Derivados Tiazolidínicos Agonistas PPAR em pacientes portadores de Esclerose Sistêmica**
Pesquisador Responsável: Andrea Tavares Dantas

Senhor (a) Pesquisador (a):

Informamos que o Comitê de Ética em Pesquisa Envolvendo Seres Humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco (CEP/CCS/UFPE) registrou e analisou de acordo com a Resolução N.º 196/96 do Conselho Nacional de Saúde, o protocolo de pesquisa em epígrafe, liberando-o para início da coleta de dados em 01 de fevereiro 2012.

Ressaltamos que a aprovação definitiva do projeto será dada após a entrega do relatório final, conforme as seguintes orientações:

a) Projetos com, no máximo, 06 (seis) meses para conclusão: o pesquisador deverá enviar apenas um relatório final;
b) Projetos com períodos maiores de 06 (seis) meses: o pesquisador deverá enviar relatórios semestrais.

Dessa forma, o ofício de aprovação somente será entregue após a análise do relatório final.

Atenciosamente

Prof. Geraldo Bosco Lindoso Couto
Coordenador do CEP/ CCS / UFPE

APÊNDICES

APÊNDICE A – Termo de Consentimento Livre e Esclarecido

« AVALIAÇÃO DA ATIVIDADE ANTIFIBRÓTICA *IN VITRO* DE DERIVADOS TIAZOLIDÍNICOS AGONISTAS DO PPARγ EM PACIENTES PORTADORES DE ESCLEROSE SISTÊMICA»

Nós o convidamos a participar de uma pesquisa que visa estudar o que acontece no corpo fazendo com que a esclerose sistêmica se desenvolva. Como pesquisadores responsáveis deste estudo, nosso objetivo é descobrir o que está desrregulado no seu corpo, levando ao desenvolvimento da doença. Nesta pesquisa só será realizado testes com as células presentes no seu sangue e/ou na sua pele. Deste modo, nenhum medicamento será administrado em você durante toda a pesquisa.

É importante ressaltar que:

1. Sua participação é inteiramente **voluntária;**
2. Você pode deixar de participar do estudo a qualquer momento que quiser;
3. Nós solicitaremos novamente sua aprovação (verbal) no momento de cada coleta;
4. Suas informações nunca serão divulgadas, ou seja, permanecerão confidenciais;
5. Faremos um banco de dados e caso a pesquisa traga bons resultados, você será um dos primeiros beneficiados.

A esclerose sistêmica é uma doença auto-imune, inflamatória crônica que acomete, principalmente, a pele, podendo comprometer também outros órgãos, como pulmão, coração e trato digestivo. Com o passar do tempo, os portadores de esclerose sistêmica podem desenvolver incapacidade para realização de suas atividades tanto de vida diária como profissional. Apesar de rara, a esclerose sistêmica é uma doença grave e ainda sem cura.

Nós solicitamos a sua colaboração e participação neste estudo porque você é **portador de esclerose sistêmica ou por você ser um voluntário não portador desta doença**. Este estudo inclui a participação de 100 indivíduos. Entre os quais, 30 são voluntários sadios e 70 são portadores da esclerose sistêmica.

Para este estudo, precisamos coletar algumas células de sangue. A coleta de sangue é feita no braço e a quantidade coletada é equivalente a duas colheres de sopa (10-15 ml). Antes de iniciar a coleta, nós limparemos o seu braço com álcool, e todo material usado na coleta é descartável. A coleta será feita por profissionais treinados e competentes e orientados para reduzir os riscos. Para este estudo também será necessário realizar uma pequena biópsia da pele. O objetivo é estudar também o que acontece na pele das pessoas que têm esclerose sistêmica, um dos órgãos mais comprometidos na doença. O procedimento será realizado por profissionais competentes devidamente treinados para reduzir os riscos para o paciente.

Os riscos envolvidos nesse projeto se referem à coleta de sangue, que pode ser desconfortável e o braço pode ficar um pouco dolorido e apresentar hematoma que é uma área arroxeada no local da coleta. A biópsia da pele pode ser desconfortável e deixar uma pequena cicatriz. Com relação aos benefícios, você será submetido a uma avaliação clínica completa e, caso seja detectada alguma alteração, será prescrito o tratamento adequado.

Os dados coletados pelo estudo ficarão armazenados em arquivos no serviço de Reumatologia, sob a responsabilidade da Dra Andrea Dantas. Os resultados deste estudo serão apresentados em congressos, conferências ou em revistas médicas, mas não aparecerá seu nome. Suas informações nunca serão divulgadas, permanecerão confidenciais.

Você tem alguma pergunta sobre a sua participação neste estudo?

Se você tiver alguma pergunta mais tarde, poderá conversar com um dos membros da nossa equipe, podendo contatar:

Dra. Andréa Tavares Dantas, Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco e Médica do Hospital Das Clínicas da Universidade Federal de Pernambuco. Av. Prof. Moraes Rego S/N - Cidade Universitária, CEP: 50.670-901 Recife – PE.E-mail: andreatdantas@uol.com.br. Telefone: 55 (81) 2126.3575.
Profa. Dra Angela Luzia Branco Pinto Duarte, Médica do Hospital das Clínicas da Universidade Federal de Pernambuco. Av. Prof. Moraes Rego S/N - Cidade Universitária, CEP: 50.670-901 Recife – PE .E-mail: aduarte@terra.com.br. Telefone/Fax: 55 (81) 3454.0155.
Profa. Dr. Suely Lins Galdino, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego S/N - Cidade Universitária, CEP: 50.670-901 Recife – PE. E-mail: suelylinsgaldino@gmail.com. Telefone/Fax: 55 (81) 2126-8346

Assim como, o coordenador do Comitê de Ética em Pesquisa - CEP/CCS, o **Professor Dr. Geraldo Bosco Lindoso Couto**, do Centro de Ciências da Saúde, da Universidade Federal de Pernambuco, situado na Avenida da Engenharia s/n – 1º Andar, Cidade Universitária, Recife-PE, CEP: 50740-600, Telefone/Fax: 55 (81) 2126-8588.

Você ficará com uma cópia deste documento.

Nome do responsável pela coleta de sangue: _____

Se você aceitar participar deste estudo, por favor, preencha o formulário abaixo.

Nome: _____

RG:_____ Data: _____

Endereço:_____

Assinatura: _____

Testemunha 1: _____

RG: _____ Data: _____

Testemunha 2: _____

RG: _____ Data: _____

Pesquisador Responsável: _____Data:_____

Assinatura: _____

APÊNDICE B – Ficha Clínica

Número da Ficha ☐☐☐	**Data de preenchimento** _____ / _____ / _____	**Sexo** 1. Masculino 2. Feminino ☐
Nome do paciente		
	7. Idade (anos) ☐☐	**Raça** 1. Branca 2. Negra 3. Parda 4. Indígena 5. Amarela ☐

CLASSIFICAÇÃO

16. Forma Clínica 1. Cutânea Limitada 2. Cutânea Difusa 3. Sine Escleroderma 4. Overlap (EXCLUIR) 5. Localizada (EXCLUIR) ☐	**17. Tempo de Doença** 1. < 2 anos 2. 2-5 anos 3. > 5 anos ☐

Comprometimento Sistemas

1. Pele
2. Raynaud
3. Úlceras
4. Doença pulmonar intersticial
5. Hipertensão arterial pulmonar
6. Musculo-esquelético
7. Comprometimento esofageano
8. Renal

AVALIAÇÃO ATUAL DA DOENÇA

COMPROMETIMENTO VASCULAR

Úlceras em atividade? 1. Sim **2.** Não ☐	**Número de úlceras em atividade** ☐

COMPROMETIMENTO MÚSCULO-ESQUELÉTICO

Artrite? **1.** Sim **2.** Não	**Número de articulações dolorosas** ☐	**Número de articulações edemaciadas** ☐
Déficit de força? **1. Sim** **2. Não**		**CPK** ☐

COMPROMETIMENTO CARDIO-PULMONAR

Dispneia? **1. Sim** **2. Não**	**Valor do CVF nos últimos 6 meses:**

Quadro 1 – Escala modificada de Borg usada no Brasil

0	Nenhuma
0,5	Muito, muito leve
1	Muito leve
2	Leve
3	Moderada
4	Pouco intensa
5	Intensa
6	
7	Muito intensa
8	
9	Muito, muito intensa
10	Máxima

Presença de vidro fosco em TAC tórax nos últimos 6 meses

1. **Sim**
2. **Não**

	DIREITO				ESQUERDO			
	0	1	2	3	0	1	2	3
Dedos								
Dorso das mãos								
Antebraço								
Braço								
Face								
Tórax anterior								
Abdome								
Coxa								
Perna								
Dorso dos pés								

ESCORE DE RODNAN:

MEDICAÇÕES (especificar a dose)

1. Sim- atual 2. Não- parou há < 30 dias 3. Não- parou há > 30 dias 4. Nunca usou 5. Não sabe informar

102. BCC
( ) Nifedipina
( ) Anlodipina
( ) Diltiazem
( ) Verapamil

103. IECA
( ) Captopril
( ) Enalapril

104. BRA
( ) Losartan
( ) Valsartan

105. AAS

106. Pentoxifilina

107. Sildenafil

108. Bosentana

109. Corticoide
( ) Prednisona
( ) Prednisolona
( )

110. Metotrexato

111. Azatioprina

112. Micofenolato

113. Ciclofosfamida VO

114. Ciclofosfamida EV

Data da última aplicação: ____/____/____

115. IBP
( ) Omeprazol
( ) Pantoprazol

116. Procinético
( ) Bromoprida
( ) Domperidona

117. AINE

118. OUTROS (Especificar):

EXAMES LABORATORIAIS (DATA:)			
Hb : **Ht:**	**C3:** **C4:**	**CPK:** **Creatinina:**	**PCR:** **VSH:**
EXAME FÍSICO			
127. Peso ☐☐☐	**128. Altura** ☐☐☐	**129. IMC** ☐☐☐	**130.1 PAS** ☐☐☐ **130.2 PAD** ☐☐☐

HAQ – *Health Assessment Questionnaire*

Atividade	Sem dificuldade 0	Pouca dificuldade 1	Muita dificuldade 2	Não consegue 3	Maior valor
1. Vestir-se, inclusive amarrar os cordões dos sapatos e abotoar as roupas					
2. Lavar sua cabeça e seus cabelos					
3. Levantar-se de maneira ereta de uma cadeira de encosto reto e sem braços					
4. Deitar-se e levantar-se da cama					
5. Cortar pedaços de carne					
6. Levar à boca um copo ou xícara cheio de café, leite ou água					
7. Abrir um saco (caixa) de leite comum					
8. Caminhar em lugares planos					
9. Subir 5 degraus					
10. Lavar e secar seu corpo após o banho					
11. Tomar banho de chuveiro					
12. Sentar-se e levantar-se de um vaso sanitário					
13. Levantar os braços e pegar um objeto de aproximadamente 2,5 quilos que está posicionado pouco acima da cabeça					
14. Curvar-se para pegar roupas no chão					
15. Segurar-se em pé no ônibus ou metrô					
16. Abrir potes ou vidros de conservas que tenham sido previamente abertos					
17. Abrir e fechar torneiras					
18. Fazer compras nas redondezas onde mora					
19. Entrar e sair de um ônibus					
20. Realizar tarefas tais como usar vassoura para varrer e ou rodo para a água					
SOMATÓRIO					
SOMATÓRIO DIVIDIDO POR 8 (RESULTADO DO HAQ)					

ESCALA VISUAL ANALÓGICA

Faça uma marca na linha abaixo (10 cm) referente ao grau de limitação apresentada pelo indivíduo.

1. Na semana passada, quanto os seus problemas com o Fenômeno de Raynaud (dedos que alternam de cor entre roxo, pálido e vermelho pelo frio) interferiram nas suas atividades? Faça uma marca na linha para indicar a gravidade desse problema.

Não interfere _____ Limitações muito graves

2. Na semana passada, quanto os seus problemas com as feridas nos dedos interferiram nas suas atividades? Faça uma marca na linha para indicar a gravidade desse problema.

Não interfere _____ Limitações muito graves

3. Na semana passada, quanto os seus problemas gastrointestinais interferiram nas suas atividades? Faça uma marca na linha para indicar a gravidade desse problema.

Não interfere _____ Limitações muito graves

4. Na semana passada, quanto os seus problemas com os pulmões interferiram nas suas atividades? Faça uma marca na linha para indicar a gravidade desse problema.

Não interfere _____ Limitações muito graves

5. Na semana passada, quanto o conjunto de seus problemas causados pela esclerodermia interferiram nas suas atividades? Faça uma marca na linha para indicar a gravidade desse problema.

Não interfere _____ Limitações muito graves

ESCORE

1. Converter cada valor de EVA em subscores de 0-3
2. Somar cada valor do subescore das EVA's (0-3), com o maior valor de cada um dos 8 domínios
3. Dividir o valor da somatória por 13.

APÊNDICE C – Artigo de revisão

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Review Article

The Role of PPAR Gamma in Systemic Sclerosis

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Fibrosis is recognized as an important feature of many chronic diseases, such as systemic sclerosis (SSc), an autoimmune disease of unknown etiology, characterized by immune dysregulation and vascular injury, followed by progressive fibrosis affecting the skin and multiple internal organs. SSc has a poor prognosis because no therapy has been shown to reverse or arrest the progression of fibrosis, representing a major unmet medical need. Recently, antifibrotic effects of PPARγ ligands have been studied *in vitro* and *in vivo* and some theories have emerged leading to new insights. Aberrant PPARγ function seems to be implicated in pathological fibrosis in the skin and lungs. This antifibrotic effect is mainly related to the inhibition of TGF-β/Smad signal transduction but other pathways can be involved. This review focused on recent studies that identified PPARγ as an important novel pathway with critical roles in regulating connective tissue homeostasis, with emphasis on skin and lung fibrosis and its role on systemic sclerosis.

1. Introduction

Fibrosis is defined as an inappropriate repair by connective tissue characterized by excessive deposition of collagen and other extracellular matrix (ECM) components, promoting disruption of tissue homeostasis. It is recognized as an important feature of many chronic diseases, including myocardial infarction, glomerulosclerosis, idiopathic pulmonary fibrosis, liver cirrhosis, and systemic sclerosis (SSc) [1].

Fibroblasts are major effector cells in the development of fibrosis and an inappropriate fibroblast activation is the fundamental pathogenic alteration underlying pathologic fibrosis. A subgroup of resident fibroblasts, in response to transforming growth factor-β (TGF-β) stimulation, transdifferentiate into myofibroblasts expressing high levels of α-smooth muscle actin (α-SMA) with a significant functional role in pathologic fibrosis. The myofibroblasts show accelerated synthesis of extracellular matrix proteins, are resistant to apoptosis, and have contractile properties. Furthermore, bone-marrow-derived mesenchymal progenitors such as fibrocytes and monocytes might traffic to damaged tissue and undergo *in situ* differentiation into activated fibroblasts and myofibroblasts. Nonfibroblastic cell lineages (such as epithelial or endothelial cells or adipocytes) can also differentiate into fibroblasts and myofibroblasts through a process called epithelial-mesenchymal transition (EMT) [2–4].

Regulation of these cellular transitions, collagen gene expression, and ECM accumulation is tightly controlled. Various chemokines/cytokines can induce cell migration and proliferation, as well as stimulation of cell-cell adhesion and collagen production, which is associated with the pathogenesis of fibrosis. TGF-β is considered the main regulator of physiologic fibrogenesis and pathological fibrosis, and it has emerged as an important therapeutic target in fibrotic diseases [5, 6].

Intracellular TGF-β signaling is primarily mediated via the canonical Smad pathway. Binding of TGF-β to type 2 TGF-β receptor recruits type 1 TGF-β receptors (TGF-βRI), forming a heterotetrameric structure that phosphorylates Smad2 and Smad3, which then binds to Smad4. The resulting Smad complex then translocates to the nucleus and binds to the Smad binding elements (SBE) in the gene promoter in order to regulate the transcription of target genes [7]. Smads regulate transcription of target genes by interacting with other transcription factors and by recruiting transcriptional coactivators or corepressors, such as CREB (cAMP response element binding protein) binding protein (CBP)/p300 [8].

Although the Smad pathway is the central intracellular mediator of signals from the TGF-β receptors, recent evidence indicates that alternative non-Smad pathways exist. This also mediates TGF-β responses, involving protein kinases (MAP kinases p38 and JNK, focal adhesion kinase FAK, and TGF-β activated kinase TAK1), lipid kinases such as PI3 kinase and its downstream target Akt, the calcium-dependent phosphatase calcineurin, and the nonreceptor tyrosine kinase c-Abl [9, 10].

Besides TGF-β, multiple cytokines, growth factors, and chemokines regulate collagen production, ECM accumulation, and mesenchymal cell function and are also expressed abnormally in fibrotic diseases. These mediators, such as connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), interleukin (IL)-4, IL-6, IL-13, and IL-8, interact with TGF-β and directly contribute to the pathogenesis of fibrosis and might also represent potential targets for antifibrotic therapy [11].

Although the diagnosis and pathophysiology of most fibrosing diseases have been better characterized over the past few years, there remains no effective therapy for this group of diseases. Systemic sclerosis is an autoimmune disease of unknown etiology, characterized by immune dysregulation and vascular injury, followed by progressive fibrosis affecting the skin and multiple internal organs, mainly the lung. The disease has a poor prognosis because no therapy has been shown to reverse or arrest the progression of fibrosis, representing a major unmet medical need.

Therapies initially targeted to inflammation proved to be ineffective. Thus, studies have focused on modulation of profibrotic molecules, targeting myofibroblast differentiation, recruitment, and activity as a potential antifibrotic treatment. Hence, the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) appears to participate in controlling fibrogenesis by inhibiting the TGF-β pathway. Aberrant PPARγ function seems to be implicated in pathological fibrosis in the skin, lung, liver, heart, kidney, and pancreas. This review focused on recent studies that identified PPARγ as an important novel pathway with critical roles in regulating connective tissue homeostasis, with emphasis on skin and lung fibrosis and its role in systemic sclerosis.

2. Role of PPARγ in Fibrosis Signaling

PPARγ is a ligand-dependent nuclear receptor that belongs to the nuclear hormone receptor superfamily and regulates the expression of target genes. Some studies demonstrated the pivotal role of PPARγ in glucose homeostasis, lipid metabolism, and cell growth regulation and, posteriorly, in inflammation, innate immunity, and regulation of connective tissue biology [12]. It is now recognized that PPARγ modulates connective tissue synthesis and degradation, mesenchymal cell activation, transdifferentiation, and survival [13].

PPARγ is activated by natural and pharmacological agents. Endogenous ligands include 15-deoxy–Δ12,14-prostaglandin J2 (15d-PGJ2), lysophosphatidic acid, and nitrolinoleic acid. PPARγ can also be activated by synthetic ligands including the thiazolidinediones (TZD) as well as oleanic acid derivatives known as triterpenoids (2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid (CDDO)) [14]. The TZDs are highly potent PPARγ agonists. They consist of rosiglitazone (RGZ), pioglitazone (PGZ), and troglitazone (TGZ) and were originally approved for the treatment of type 2 diabetes but their commercialization has been questioned in many countries mainly due to cardiovascular safety profile and increased risk of cancer and bone fractures [15–17].

In unstimulated cells, the PPARγ receptors are located in the cytoplasm as heterodimers complexed to their repressors. After ligation with its agonist, PPARγ heterodimerizes with retinoid X receptor (RXR) and coactivators such as p300 are recruited; this complex is translocated to the nucleus where it recognizes specific DNA sequence elements termed as peroxisome proliferator response element (PPRE) in promoters of target genes. PPARs regulate numerous genes through ligand-dependent transcriptional activation and repression [18, 19]. In the absence of ligands, the PPAR/RXR complex is bound to transcriptional corepressors and histone deacetylases, which prevents its binding to PPRE [13].

PPARγ express two isoforms: PPARγ1, present in macrophages, colonic epithelial cells, endothelial cells, and vascular smooth muscle cells, and PPARγ2, mainly expressed in adipose tissue associated with the regulation of adipogenesis. The expression level of PPARγ in a given cell or tissue determines the intensity and duration of the cellular response to endogenous or synthetic PPARγ ligands [6, 19].

Recent studies have established that PPARγ is a negative regulator of profibrotic signal-induced collagen synthesis and blunts fibrogenesis in a wide variety of organs. The antifibrotic effects of PPARγ ligands were studied *in vitro* and *in vivo* and some theories have emerged leading to new insights. Indeed, it is possible that they act through a variety of distinct mechanisms according to different cell types or type of agonist (natural or synthetic) [13, 20–27].

An inverse relationship between fibrosis and PPARγ expression/function was reported in multiple human fibrosing disorders as well as in animal models of fibrosis. Under physiologic conditions, PPARγ shows a low level of constitutive activation, driven by natural ligands controlling fibrotic responses. Prolonged or recurrent fibrogenic stimulation decreases the expression of PPARγ, inhibiting cellular responsiveness to natural endogenous PPARγ ligands. In multiple organ-specific human fibrotic diseases, fibrosis is preceded by reduced tissue PPARγ levels, suggesting a causal role for reduced PPARγ expression or activity in the development or progression of fibrosis [13]. It is not clear

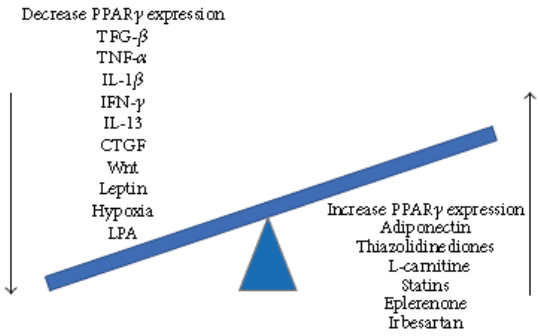

FIGURE 1: Effects of different molecules on PPARγ expression.

in these conditions whether fibrosis is the cause of reduced PPARγ or whether reduced PPARγ causes fibrosis [6].

Some cytokines and chemokines are recognized as regulators of PPARγ expression. Cytokines implicated in fibrosis generally suppress PPARγ expression in mesenchymal effector cells. As an example, TGF-β seems to reduce PPARγ expression in fibroblasts and hepatic stellate cells, although it stimulates PPARγ expression in monocytes and macrophages [13]. Other inhibitors of PPARγ expression include CTGF, IL-13, Wnt, leptin, lysosphosphatidic acid (LPA), and hypoxia [28, 29].

On the contrary, adiponectin, which is regulated itself by PPARγ, enhances the expression of PPARγ in the liver and adipose tissue [30, 31]. Some molecules (L-carnitine, eplerenone, statins, and irbesartan) were studied as potential antifibrotic agents because of their effect on increasing PPARγ expression [32–35] (Figure 1).

The molecular pathways underlying the antifibrotic effects of PPARγ are not completely defined. One of the proposed mechanisms is the antagonistic relationship between PPARγ and TGF-β signaling in fibrosis. As previously discussed, TGF-β promotes myofibroblasts differentiation from fibroblasts. In contrast, PPARγ ligands induce adipogenic differentiation of skin fibroblasts [21]. TGF-β negatively regulates both the expression and function of PPARγ, thereby desensitizing fibroblasts to PPARγ ligands. On the other hand, PPARγ ligands can directly disrupt TGF-β signal transduction and suppress TGF-β production [13, 21].

Activation of PPARγ by either naturally occurring or synthetic ligands inhibits the induction of profibrotic responses induced by TGF-β in fibroblasts. While the effects of PPARγ ligands (15d-PGJ2 and troglitazone) on collagen expression were only modest in unstimulated skin fibroblasts, these ligands significantly prevented collagen synthesis and expression in TGF-β-stimulated fibroblasts [21, 25–27]. PPARγ agonists (troglitazone, 15d-PGJ2, and CDDO) also prevented α-SMA expression induced by TGF-β in skin fibroblasts [25, 27]. In hepatic stellate cells, skin fibroblasts, and aortic muscle cells, PPARγ ligands suppressed CTGF expression induced by TGF-β1 [36, 37].

In normal fibroblasts, PPARγ ligands can inhibit profibrotic signaling triggered by TGF-β and can interfere with downstream signal transduction. Blockage of the canonical Smad signaling pathway was demonstrated by some authors [26, 27, 38]. In hepatic stellate cells, PPARγ ligands prevented Smad3 phosphorylation [38]. In contrast, in the TGF-β-mediated fibroblast activation, PPARγ agonists did not prevent Smad2/3 phosphorylation or nuclear accumulation, but, instead, prevented recruitment of the coactivator p300 to the transcriptional complex [39]. In cultures of explanted normal fibroblasts, the PPARγ agonist CDDO prevented fibrogenic responses induced by TGF-β. Such effects occurred via disruption of Smad-dependent transcription, but without preventing Smad2/3 activation, and were also associated with inhibition of Akt activation [27].

In contrast, in dermal fibroblasts, rosiglitazone treatment did not attenuate expression of phosphorylated Smad2, suggesting that PPARγ ligands can abrogate TGF-β-induced responses independent of Smad activation [21, 25, 40]. For example, rosiglitazone reduced the induction of Egr-1, an early immediate transcription factor of TGF-β signaling [41]. Studies also implicate upregulation of the tumor suppressor phosphatase and tensin homolog (PTEN) as responsible for the inhibition of profibrotic effector functions by PPARγ. *In vitro* studies showed that PTEN inhibits fibroblast-myofibroblast differentiation and expression of α-SMA and collagen in human and mouse lung fibroblasts [42]. Accordingly, 15d-PGJ2 inhibited transcription of the TGF-β1 gene via PTEN upregulation in mouse fibroblasts [43].

In mesangial cells, PPARγ ligands (15d-PGJ2, troglitazone, and ciglitazone) stimulated the expression of hepatocyte growth factor (HGF), an endogenous antifibrotic agent. HGF induces the Smad transcriptional corepressor TG-interacting factor (TGIF) thus mediating autocrine suppression of TGF-β-induced fibrogenic responses [44, 45].

Contrary to the mentioned findings, some studies suggested that antifibrotic effects of PPARγ ligands could not be related to PPARγ activation [46–49]. Ferguson et al. demonstrated that CDDO inhibited α-SMA expression by a PPARγ-independent mechanism, promoting dysregulation of acetylation of the TGF-β gene transcription coactivator CBP/p300 [48]. Similarly, Kulkarni et al. showed that PPARγ ligands inhibited TGFβ-induced Akt phosphorylation and this effect was not restored by PPARγ antagonist [49]. Figure 2 illustrates some effects of PPARγ in TGFβ signaling pathway.

These data argue that PPARγ agonists have a role in limiting fibrosis, in addition to their already known anti-inflammatory and immunomodulatory effects. Tables 1 and 2 summarize *in vitro* and *in vivo* studies of antifibrotic effects of PPARγ agonists. This antifibrotic effect is mainly related to the inhibition of TGF-β/Smad signal transduction but other pathways may be involved. This knowledge has stimulated the development of further studies examining PPARγ role in fibrotic diseases and the potential therapeutic use of their ligands.

3. PPARγ and Lung Fibrosis

Lung fibrosis occurs in a wide variety of illnesses, including systemic disorders, as systemic sclerosis, as well as primary

Table 1: *In vitro* studies of antifibrotic effects of PPARγ agonists.

Cell type	PPARg Ligand	Effects	References
Healthy human lung fibroblast	15d-PGJ2, CGZ and RGZ	↓ TGF-β-induced myofibroblast differentiation ↓ TGF-β-induced type I collagen protein production	[40]
Normal lung fibroblasts and fibroblasts isolated from patients with IIP human fetal lung fibroblast (IMR-90) cells	CGZ and TGZ	↓ Proliferation of human lung fibroblasts ↓ Proliferative responses of undifferentiated Fibroblasts and myofibroblasts to PDGF Inhibited TGF-β1-induced myofibroblast differentiation	[47]
MRC-5 cells derived from human lung fibroblasts	PGZ	↓ TGFβ-mediated increase in procollagen I and CTGF expression	[60]
	RGZ	↓ Lung fibroblast migration and proliferation ↓ Myofibroblast transdifferentiation	[56]
Normal human lung fibroblast cell	15 d-PGJ2, RGZ and CDDO	↓ TGF-β–stimulated differentiation of fibroblasts to myofibroblasts ↓ TGF-β–induced fibronectin expression	[48]
A549 human alveolar cell line	RGZ and CGZ	↓ Profibrotic changes (elevation of N-cadherin, CTGF and collagen I) in alveolar epithelial cells	[59]
Primary lung human fibroblasts Lung fibroblasts from IIP patients	CDDO and 15d-PGJ2	↓ TGFβ-induced phosphorylation of Akt ↓ myofibroblast differentiation	[49]
SSc lung fibroblasts	RGZ	↑ MMP-1 expression ↓ Collagen type I expression in white patients ↓ CTGF and α-SMA expression	[53]
Primary cultures of human dermal fibroblasts	15d-PGJ2 and TGZ	↑ PPARγ nuclear levels in skin fibroblasts ↓ type I collagen synthesis and expression by TGFβ-stimulated fibroblasts ↓ α-SMA expression by TGFβ-stimulated fibroblasts	[25]
	TGZ	↓ TGFβ1, type I collagen and fibronectin expression and secretion	[67]
Human foreskin fibroblasts	15d-PGJ2 and TGZ	↓ Collagen synthesis and of COL1A2 promoter activity induced by TGF-β ↓ Smad3-dependent transcriptional responses without blocking Smad activation ↓ TGFβ-induced interaction of p300 with Smad3 ↓ Recruitment of p300 to the DNA-bound transcriptional complex	[39]
Healthy and scleroderma fibroblasts	RGZ	↓ α-SMA, type I collagen and CTGF protein expression in dcSSc fibroblasts ↑ PPARγ expression	[79]
	Ajulemic acid	↓ Supernatant levels of procollagen type I propeptide and TGFb	[69]
Human scleroderma fibroblasts	RGZ	Reduced CXCL10 secretion induced by IFNγ e TNFα	[86]
	PGZ and RGZ	↓ Cell proliferation and cell viability Increased apoptosis	[80]
	CDDO	↓ Cellular and secreted type I collagen levels ↓ COL1A1 and COL1A2 mRNA expression	[27]
Explanted normal human skin Fibroblasts	CDDO	↓ COL1A2 and aSMA expression induced by TGFb	[27]
Organotypic human skin raft model (epidermal keratinocytes and dermal fibroblasts)	CDDO	↓ COL1A1, COL1A2, and α-SMA expression in fibroblasts	[27]
Human A540 epithelial cells	CDDO	↓ TGF-β-induced epithelial–mesenchymal transition	[27]

CGZ = ciglitazone, RGZ = rosiglitazone, TGF-β = transforming growth factor-β, TGZ = troglitazone, IIP = idiopathic interstitial pneumonia, PDGF = platelet-derived growth factor, PGZ = pioglitazone, CTGF = connective tissue growth factor, CDDO = 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid, SSc = systemic sclerosis, MMP-1 = matrix metalloproteinase-1, dSSc = diffuse systemic sclerosis.

Table 2: *In vivo* studies of antifibrotic effects of PPARγ agonists.

Animal model	PPARγ ligand	Effects	Reference
Bleomycin-induced model of lung fibrosis	15d-PGJ2 and RGZ	↓ Histological evidence of lung fibrosis	[61]
	TGZ	↓ Hydroxyproline and collagen deposition in lung tissue Ameliorated histopathological changes	[47]
	PGZ	↓ Hydroxyproline content in lung tissue Ameliorated histopathological changes	[60]
	RGZ	Prevented onset of fibrotic radiological changes	[63]
	RGZ	↓ Hydroxyproline content in lung tissue ↓ Lung TGF-β1 concentration Ameliorated histopathological changes	[62]
Bleomycin-induced model of skin fibrosis	RGZ	Attenuated severity of dermal fibrosis and local collagen deposition ↓ Tissue accumulation of myofibroblasts ↓ Levels of TGF-β levels in lesional skin	[41]
	Ajulemic acid	Prevented development of skin fibrosis ↓ Skin thickness dermal ↓ Hydroxyproline content ↓ Myofibroblasts number	[69]
	CDDO	↓ Collagen deposition and dermal thickness ↓ α-SMA and TGFβ1 expression	[27]
Constitutively active TGFβ receptor type I mouse model (AdTGFbRI)	Ajulemic acid	Prevented development of skin fibrosis ↓ Skin thickness dermal ↓ Hydroxyproline content ↓ Myofibroblasts number	[69]

RGZ = rosiglitazone, TGZ = troglitazone, PGZ = pioglitazone, CDDO = 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid, TGF-β = transforming growth factor-β, α-SMA = α-smooth muscle actin.

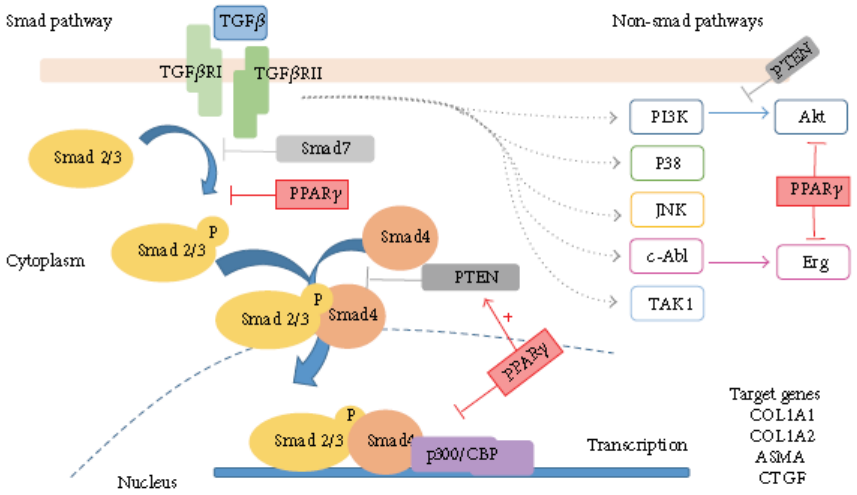

Figure 2: Smad and non-Smad signaling TGF-β pathways and potential effects of PPARγ ligands. Binding of TGF-β to type 2 TGF-β receptor (TGF-βRII) recruits type 1 TGF-β receptors (TGF-βRI), forming a heterotetrameric structure that phosphorylates Smad2 and Smad3, which then binds to Smad4. Smad complex then translocates to the nucleus, where it interacts with various transcription factors to regulate the transcription of target genes (COL1A1, COL1A2, ASMA, CTGF). After TGF-β binding, TGF-βRII recruits a TGF-βRI and activates it by phosphorylation. Nonclassic pathways are also illustrated. PPARγ ligands can block TGF-β signaling by blocking Smad and non-Smad pathways.

lung disease, such as idiopathic interstitial pneumonia (IIP). Fibrotic remodeling of lung tissue is also an important feature of other lung diseases, including sarcoidosis, asthma, and chronic obstructive pulmonary disease. In general, it is characterized by inflammatory cell infiltration and alveolar epithelial cell injury with failure of alveolar reepithelialization, followed by recruitment and persistence of fibroblasts that differentiate into myofibroblasts. The excessive collagen and extracellular matrix production results in distortion of the lung architecture and consequently decreased gas exchange and reduced pulmonary compliance [50, 51].

Many types of lung cells express PPARγ, including fibroblasts, T lymphocytes, ciliated airway epithelial cells, alveolar type II pneumocytes, alveolar macrophages, and airway smooth muscle cells [52]. Reduced PPARγ expression was demonstrated in lung fibroblasts from patients with SSc [22, 53] and in alveolar macrophages of patients with sarcoidosis [54] and pulmonary alveolar proteinosis [55], suggesting that insufficient PPARγ activity may contribute to ongoing dysregulated inflammation and fibrosis.

PPARγ ligands have negative regulatory effects on human lung fibroblasts, by inhibiting proliferation and migration of healthy or IIP fibroblasts and by inhibiting proliferative responses of undifferentiated fibroblasts and myofibroblasts to mitogenic growth factors, as PDGF [47, 56]. Furthermore, PPARγ agonists inhibited the human lung fibroblast transdifferentiation mediated by TGF-β to the myofibroblast phenotype [40, 47, 48, 56] and significantly reduced expression of fibronectin [48] and type I collagen TGF-β-stimulated [40, 47].

TGF-β is a potent stimulus for induction of pulmonary fibrosis *in vivo* [57]. Wei et al. demonstrated that normal lung fibroblasts stimulated with TGF-β showed a decrease in PPARγ expression [22]. In another experiment, primary lung fibroblasts showed a small and not significant increase, followed by an expressive downregulation of PPARγ expression after exposure to TGF-β, beginning after an hour and persisting for at least 48 hours. This effect was reduced in Smad3-deficient lung fibroblasts, suggesting that TGF-β1 modulates PPARγ expression, in part, via Smad3 signaling. Additionally, the inhibition of transcriptional ability of PPARγ by TGF-β1 was overcome by overexpression of PPARγ [24].

Other mechanisms are also proposed to explain PPARγ agonists action. Activation of ERK-MAPK pathway by TGF-β plays an important role in fibrosis by regulating myofibroblast transdifferentiation, cell proliferation, and survival, as well as ECM synthesis [58]. In lung fibroblasts, RGZ showed an antifibrotic effect by decreasing ERK phosphorylation induced by PDGF and TGF-β1 [56].

Recent studies provide evidence that alveolar epithelial cells (AEC) can undergo a TGF-β1-induced epithelial-mesenchymal transition (EMT), acquiring a fibroblast-like phenotype and possibly contributing to lung fibrosis. Phenotypic markers associated with EMT include the diminished expression of E-cadherin, a cell anchoring protein expressed specifically by epithelial cells, and an elevated expression of N-cadherin, normally present at relatively high levels in fibroblasts. Tan et al. demonstrated that RGZ and CGZ inhibited the elevation of markers of profibrotic phenotype (N-cadherin, CTGF, and collagen I) in TGF-β1-stimulated A549 cells, a model of AEC type II [59].

Studies with animal models found that the PPARγ agonists troglitazone [47], pioglitazone [60], and rosiglitazone [61, 62] were able to inhibit lung fibrosis bleomycin induced. This inhibition was observed either before or even after bleomycin administration. The initial period of postinflammatory fibrosis could correspond to the period in which patients are likely to present symptoms [47, 60]. More recently, using microcomputed tomography to evaluate radiological changes in the murine model of lung fibrosis, two authors demonstrated that the treatment of bleomycin-instilled mice with RGZ prevented the development of [63] or improved [64] typical features of lung fibrosis, like ground glass opacity and consolidation.

In condusion, there are some evidences that PPARγ agonists have antifibrotic effects on human lung fibroblasts, as demonstrated by the attenuation of bleomycin-induced lung injury and downregulation of TGF-β1-mediated collagen deposition in fibrotic lung tissues (Figure 3).

4. PPARγ and Skin Fibrosis

Wound repair is a very complex and dynamic process, involving the interactions of multiple cell types and growth factors, cytokines, and soluble mediators. Normal cutaneous tissue repair involves an initial inflammatory phase, characterized by migration of inflammatory cells to the injured site, followed by a fibroproliferative phase, with synthesis and deposition of granulation tissue and neovascularization. Finally, the resolution phase is characterized by replacement of damaged and granulation tissue by newly synthesized fibrous matrix protein collagens. In response to tissue injury, myofibroblasts repopulate the wound and synthesize and remodel new ECM. Dysregulation of this process could result in chronic wounds or fibrosis [65, 66].

It is suggested that PPARγ may in part be responsible for initiating endogenous mechanisms of wound repair and the activation of PPARγ by its natural ligands controls fibrotic responses. Normal dermal fibroblasts constitutively express low levels of PPARγ, distributed in both nucleus and cytoplasm [21, 25, 67]. Kapoor et al. demonstrated that PPARγ is upregulated during the resolution phase of normal wound healing [68]. Besides, PGJ2 physiologically increases and there is an upregulation of PPARγ expression, leading to blocking of fibroblast activation and collagen neosynthesis [6, 68]. Migration of dermal fibroblasts plays a critical role in both normal wound healing and pathological fibrogenesis. Treatment with rosiglitazone abrogated stimulation of fibroblast migration and wound closure elicited by TGF-β [41].

In TGF-β-stimulated dermal fibroblasts, there was a significant time-dependent decrease in PPARγ expression and a similar inhibition of matrix metalloproteinase- (MMP-) 1 and Smad3. At the same time, there was an increase in the expression of fibrosis-related genes such as ASMA, SERPINE1, CTGF, and COMP1 [22]. In addition, it was demonstrated *in vivo* a decline in cutaneous PPARγ expression in a mouse model of bleomycin-induced skin fibrosis [41].

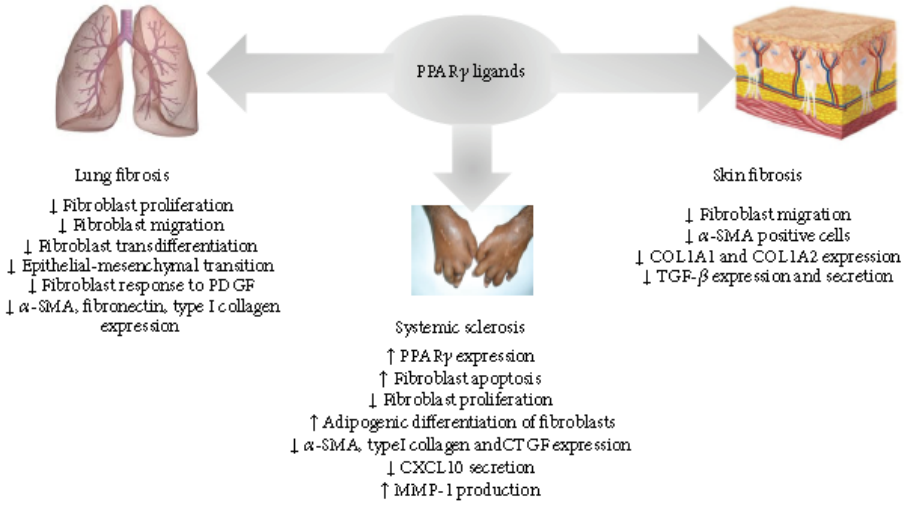

FIGURE 3: Effects of PPARγ ligands in fibrotic diseases.

Skin fibrosis associated with progressive loss of PPARγ expression seems to be prevented or reduced by the administration of PPARγ ligands. In the model of bleomycin-induced skin fibrosis, treatment with PPARγ ligands (RGZ, CDDO) prevented the development of skin fibrosis and also reduced established fibrosis [21, 27]. Gonzalez et al., using ajulemic acid (AjA), a nonpsychoactive synthetic analogue of tetrahydrocannabinol that can bind to PPARγ, also demonstrated prevention of experimental bleomycin-induced dermal fibrosis and interruption of further progression of established fibrosis, but did not alter preexisting ECM accumulation [69].

In human dermal fibroblasts troglitazone reduced TGF-β1 secretion [67] and administration of rosiglitazone substantially prevented the upregulation of TGF-β1 [21]. PPARγ agonists also attenuated the upregulation of the fibrogenic genes COL1A1 and COL1A2 and reduced the number of α-SMA-positive fibroblastic cells [21]. An interesting finding was that the overexpression of PPARγ resulted in complete suppression of COL1A2 gene transcription stimulated by TGF-β, even in the absence of an activation ligand, and caused a sensitization of fibroblasts to the ligands. These observations suggest that the relatively low level of PPARγ expression in normal fibroblasts may be a limiting factor for negative regulation of TGF-β responses [25].

Ghosh et al. found that basal type I collagen gene expression was markedly elevated in mouse embryonic fibroblasts (MEFs) lacking PPARγ. The agonist 15d-PGJ2 failed to suppress the elevated levels of collagen in MEFs. At the same way, activity of the COL1A2 promoter was markedly elevated in PPARγ null MEFs and reconstitution of these cells with ectopic PPARγ resulted in downregulation of COL1A2 promoter activity. PPARγ null MEFs displayed elevated expression of type I TGF-β receptor (TβRI) and produced more TGF-β1. Furthermore, PPARγ null MEFs showed Smad2/3 phosphorylation, with nuclear accumulation, even in the absence of stimulation by exogenous TGF-β. These results indicate that absence of PPARγ in MEFs is associated with constitutive upregulation of collagen gene expression and Smad activation, at least in part, due to autocrine TGF-β stimulation [70].

In animal models, PPARγ null skin fibroblasts showed an enhanced responsiveness to tissue injury, as shown by increased rate of dermal wound closure, concomitant with increased collagen deposition, greater expression of α-SMA, CTGF, and proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. They also showed elevated phosphorylation of Smad3, Akt, and ERK. Conversely, loss of PPARγ expression by itself was not sufficient to promote skin fibrosis, since PPARγ-deficient skin did not show significant alterations in skin thickness or matrix accumulation [71].

In line with these findings, Kapoor et al., using bleomycin-induced skin fibrosis in PPARγ knockout mice, showed enhanced susceptibility to skin fibrosis as demonstrated by enhanced dermal thickness, higher scores for collagen content, and greater expression of α-SMA. PPARγ-deficient mice also showed elevated Smad3 phosphorylation, indicating a potentiation of the profibrotic TGFβ1/Smad signaling pathway in the absence of PPARγ. TGF-β1-stimulated dermal fibroblasts isolated from PPARγ-KO mice had an increase in expression of α-SMA and type I collagen [72]. Taken together, these findings suggest that PPARγ normally suppresses fibrogenesis *in vivo* and that loss of PPARγ expression in skin results in elevated profibrotic signaling [72, 73].

These data indicate that PPARγ plays an important role in suppressing the skin fibrogenic response by antagonizing TGF-β signaling in physiological conditions and highlight the potential ability of PPARγ agonists to inhibit abnormal synthesis and tissue accumulation of collagen in fibrotic diseases (Figure 3).

5. PPARγ and Systemic Sclerosis

Systemic sclerosis is a clinically heterogeneous disease, known as the most severe connective tissue disorder, and associated with a high mortality risk. Patients with SSc may exhibit proliferative small artery and obliterative microvascular disease. There is also inflammation and fibrosis affecting the skin, oesophagus, respiratory tract, and other target organs. Loss of cutaneous elasticity and accompanying tightness followed by thickening and hardening of the skin (sclerosis) is almost always present and it has an important impact on quality of life. Skin involvement is a marker of disease activity and presents correlation with disease prognosis [74]. Pulmonary involvement is also common in patients with SSc and most often comprises fibrosis or interstitial lung disease and pulmonary vascular disease leading to pulmonary arterial hypertension (PAH). Currently, pulmonary manifestations are the leading cause of disease-related morbidity and mortality in patients with SSc [75].

The pathological events in SSc are complex and include impaired communication between endothelial cells, epithelial cells, and fibroblasts; lymphocyte activation; autoantibody production; inflammation; connective tissue fibrosis. These events result in an accumulation of constituents of the ECM, which replaces the normal tissue architecture, thus culminating in organ failure [76]. Scleroderma fibroblasts display an activated phenotype characterized by overproduction of collagen, secretion of profibrotic cytokines and chemokines, and expression of cell-surface integrin adhesion molecules and receptors for TGF-β, PDGF, and CCL2. Furthermore, SSc fibroblasts show increased expression of α-SMA and resistance to apoptosis [77, 78].

Reduced expression of PPARγ mRNA and protein was demonstrated in SSc skin biopsies, as well as in explanted skin fibroblasts [22, 69, 70, 79]. Although the cause underlying the PPARγ deficit in SSc and other fibrosing conditions is not yet known, multiple factors implicated in the pathogenesis of fibrosis, such as TGF-β, CTGF, and IL-13, potently inhibit PPARγ expression and function [3].

PPARγ expression shows an inverse relationship with enhanced TGF-β signaling in SSc lesional tissue. Microarray-based expression profiling of SSc skin biopsies showed an inverse correlation between PPARγ mRNA and levels of plasminogen activator inhibitor-1 (PAI-1), a TGF-β-regulated gene and marker of TGF-β activity [22].

Although fibroblasts from lesional SSc skin show reduced PPARγ expression, treatment with PPARγ ligands was able to increase the levels of the endogenous PPARγ ligand 15d-PGJ2 and the PPARγ expression [69, 79]. Furthermore, rosiglitazone attenuated the activated phenotype of scleroderma fibroblasts, by suppressing α-SMA, type I collagen, and CTGF protein expression and by reducing the ability of these fibroblasts to contract collagen matrix [79]. Other nonthiazolidinic PPARγ ligands, AjA and CDDO, also reduced collagen neosynthesis by scleroderma fibroblasts *in vitro*, an action that was reversed completely by cotreatment with a selective PPARγ antagonist [27, 69].

The expression of PPARγ is also reduced in lung fibroblasts from SSc patients [22, 53]. Treatment with RGZ resulted in significantly increased levels of PPARγ in SSc but not in normal lung fibroblasts. In addition, RGZ increased the production of MMP-1 and inhibited collagen type I, CTGF, and α-SMA expression [53]. Besides, RGZ or PGZ significantly reduced cell proliferation and viability and increased apoptosis in SSc fibroblasts, whereas they did not present a significant influence on healthy fibroblasts [80].

As mentioned above, the role of myofibroblasts as the principal mesenchymal cell responsible for the formation of fibrotic tissue is already well established in SSc and other fibrotic diseases. However, the origin of myofibroblasts is not completely understood. Recently, it was suggested that myofibroblasts in fibrotic skin could originate from adiponectin-positive intradermal progenitors via adipocyte-myofibroblast transition [81]. In line with this, development of dermal fibrosis is accompanied by progressive atrophy of the subcutaneous adipose layer and fibrous tissue replacement. An interesting finding is that PPARγ ligands induced adipogenic differentiation of mature dermal fibroblasts as well as preadipocytes, and this process was reversed by TGF-β [21].

SSc patients showed reduced serum levels and skin expression of adiponectin. An inverse correlation between serum adiponectin levels and skin fibrosis was also observed in these patients [23, 82, 83]. Adiponectin is a direct transcriptional target of PPARγ, whose levels directly reflect PPARγ activity, and it could mediate the antifibrotic effects of PPARγ [84, 85]. It was demonstrated that this adipokine suppressed the expression of type I collagen and α-SMA in normal and scleroderma fibroblasts and abrogated the stimulation of these responses elicited by TGF-β [85]. Thus, adiponectin levels might be a potential biomarker of the level of PPARγ expression and progression of fibrosis.

Rosiglitazone attenuated the CXCL10/IP-10 secretion in explanted SSc fibroblasts, suggesting other potential effects of PPARγ ligands in SSc apart from antifibrotic action [86]. CXCL10 has been implicated in SSc pathogenesis since increased serum levels and epidermis expression were demonstrated in SSc patients [87] in addition to an association with more severe clinical phenotype [88].

These studies demonstrated that PPARγ expression and activity are reduced in SSc. This impaired PPARγ expression resulting from its suppression by TGF-β and related cytokines might contribute to unregulated fibroblast activation and persistent fibrogenesis and represent an important advance in understanding the pathophysiology of SSc. Therefore, more studies are needed to evaluate the therapeutic potential of PPARγ ligands in SSc (Figure 3).

6. Conclusion

Fibrosis is a major medical problem, which can lead to progressive dysfunction of many organs and eventually the death of patients. Many aspects of its molecular mechanisms are still unclear. Currently, no effective antifibrotic treatment is available. There are many studies suggesting a key physiologic function of PPARγ signaling as an endogenous mechanism to prevent excessive fibrogenesis following injury. PPARγ is a negative regulator of profibrotic signal-induced collagen

synthesis and reduces fibrogenesis in a wide variety of organs in experimental animal models of fibrosis. Activation of cellular PPARγ receptors using either synthetic or natural PPARγ ligands blocks the induction of profibrotic responses. Experimental studies in systemic sclerosis demonstrated an impaired PPARγ expression and function, supporting a potential pathogenic role of PPARγ in this disease. Thus the use of synthetic agonists to induce the activation of PPARγ signaling or to enhance defective PPARγ tissue expression might be investigated as novel therapeutical approaches to the treatment of fibrosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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