

## *In This Issue:*

**Consensus Statement:**  
Core Variables in the Assessment of the  
Patient with Systemic Sclerosis

*Gabriele Valentini, MD*  
*Marco Matucci Cerinic, MD, PhD*  
*Stefano Bombardieri, MD*

**Eighth International Workshop**  
on Scleroderma Research Bridges  
Basic and Clinical Research

**49 Workshop Abstracts**

## Mission Statement

*Scleroderma Care and Research* is an independent, quarterly journal committed to elevating the standards of care in scleroderma and presenting new and useful information from ongoing clinical trials. It is the official journal of the Scleroderma Clinical Trials Consortium. The journal is distributed to rheumatologists in the United States and additional physicians internationally.

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*Scleroderma Care and Research*

DataMedica

P.O. Box 1688

Westhampton Beach, NY 11978

Tel (631) 288-7733 Fax (631) 288-7744

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## Editor's Memo

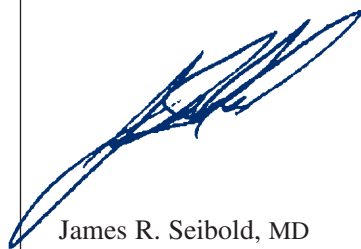
This issue of *Scleroderma Care and Research* marks the end of our second year of publication. We of the scleroderma research community have been gratified by the positive feedback from the readership and are committed to upgrading the journal.

Plans for the future include clinical vignettes, suggested management algorithms, and enhanced flow of information from the research community. Philip J. Clements, MD, will assume the position of Editor-in-Chief. Dr Clements is a superlative researcher and clinician but is also an extremely effective communicator. He will enjoy your support and feedback.

The gap between the scleroderma specialist and the primary care rheumatologist is ever smaller, and the therapeutic options for this challenging condition are continually expanding.

The world has become interconnected and is slowly becoming more interdependent. The contents of this issue are illustrative examples. Development of a core set of variables was stimulated by our esteemed colleagues in Italy but rapidly became an international endeavor. The biannual scleroderma research conference has been international from the outset, with alternating venues between North America and Europe. The growth of this important meeting has been extraordinary, and the breadth and depth of scleroderma research has benefited immensely.

We continue to thank our collaborators in the scleroderma-based patient groups and from the private sector for their commitment to partnership in the fight to solve scleroderma.



James R. Seibold, MD  
Editor-in-Chief

# Consensus Statement: Core Variables in the Assessment of the Patient with Systemic Sclerosis

**Gabriele Valentini, MD**, Professor of Rheumatology, Second University of Naples, Naples, Italy  
**Marco Matucci Cerinic, MD, PhD**, Professor of Rheumatology, University of Florence, Florence, Italy  
**Stefano Bombardieri, MD**, Professor of Rheumatology, University of Pisa, Pisa, Italy

Effective study of systemic sclerosis is hampered by the clinical heterogeneity of the disorder but also by the lack of uniform standards for assessment. A standardized data set permitting comparability among patients enrolled in different centers would be instrumental toward the goal of advancing knowledge in diverse areas of research, including genetic, etiologic, pathogenetic, pathophysiologic, and clinical therapeutics. It is equally true that these uniformly applied data gathering standards would be applicable to clinical practice and would lead to elevated standards of care and improved patient outcome.

Preliminary criteria for the classification of systemic sclerosis were defined by a committee of the American College of Rheumatology (ACR) in 1980.<sup>2</sup> The ACR subcommittee derived their conclusions from information obtained in 797 patients, of whom 264 had definite systemic sclerosis, 35 had probable or early stage systemic sclerosis, 85 had overlap syndromes, 172 had systemic lupus erythematosus, 120 had polymyositis/dermatomyositis, and 121 had Raynaud phenomenon. In 97% of systemic sclerosis patients (definite, probable, and overlap syndromes) and 96% of comparison cases, the diagnosis had been made within 2 years from entry into study. According to ACR criteria, the presence of either skin thickening proximal to MCP (the major criterion) or any two among sclerodactyly, digital pitting scars/loss of substance at finger pad, and bibasilar lung fibrosis (minor criteria) served to classify a patient as having systemic sclerosis with 97% sensitivity and 98% specificity. All experienced clinicians recognize that there are many disorders, for instance, eosinophilic fasciitis and scleredema adultorum, in which the major criterion is satisfied in the absence of the diagnosis of systemic sclerosis. It is also accepted that many patients, for instance, those with systemic sclerosis sine scleroderma or undifferentiated connective tissue disease, have conditions clearly within the scleroderma spectrum of disease but in whom criteria for classification are not fulfilled. The very high specificity and sensitivity of the ACR criteria are based on patient selection issues and are validated in comparison only with systemic lupus erythematosus, polymyositis/dermatomyositis, and Raynaud phenomenon.

At a minimum, in order to facilitate comparability with other researchers, the clinical investigator is commonly required to assess the fulfilment of criteria for the classification of the relevant disease.<sup>1</sup> The ACR criteria were not intended to assist the clinician in the diagnosis of the single patient but are more intended to assure uniformity in medical reportage. They are usually satisfied

**Table 1—Epidemiological and Clinical Features of the SSc Series Investigated**

Sex	F/M
Age	Median (range)
Disease duration	Median (range)
ACR criteria	N
Subset	ISSc/dSSc
Autoantibody profile	ANA positive ACA positive Anti-Scl 70 positive (anti-RNA polymerase I-III positive)
Altered inflammation indices	N
Activity	Active/Inactive

Organ	Involvement	Severity
General	Yes/No	Median (range)
Peripheral vascular	Yes/No	Median (range)
Skin	Yes/No	Median (range)
Joint/tendon	Yes/No	Median (range)
Muscle	Yes/No	Median (range)
GI tract	Yes/No	Median (range)
Lung	Yes/No	Median (range)
Heart	Yes/No	Median (range)
Kidney	Yes/No	Median (range)

G Valentini et al. *Clin Exp Rheumatol*. 2003

only in patients with definite disease.

Recent evidence, derived from a prospective European study devoted to define criteria to assess disease activity in systemic sclerosis patients, has clearly pointed out that the fulfilment of ACR classification criteria for systemic sclerosis does not facilitate comparison among patients from different sources. Careful analysis of the clinical charts of 290 systemic sclerosis patients enrolled at 19 tertiary European centers revealed significant differences in subset distribution, autoantibody profile, disease duration, and prevalence of internal organ involvement among different single center series.<sup>3</sup> This evidence may help to explain why conflicting results have been reported in some relevant research topics<sup>4,5</sup> and underscores the pressing need for scleroderma investigators to agree on a “core” set of variables to be used to describe individual patients in clinical investigation.

**Table 2—Core Set Variables to be Assessed to Define Each Organ System Involvement**

Skin	Modified Rodnan skin score
Vessels	Raynaud; digital ulcers (active /healed)
Joints	FTP (finger tip distance)
Tendons	Tendon friction rubs at hands, wrists, elbows, knees, ankles
Muscles	Proximal weakness; serum CPK
GI tract	Dysphagia, heart burn, early satiety, bloating, vomiting, diarrhea, constipation, malabsorption, intestinal distention, barium esophageal and intestinal X-ray
Lung	Dyspnea, lung fibrosis, (chest X-ray), restrictive defect (by FVC, DLCO), pulmonary hypertension (echo color Doppler)
Heart	Dyspnea, palpitation, chest pain, dizziness, presyncope/syncope, edema, venous congestion, cardiac block, arrhythmias (ECG), pericardial disease, EF, E/A (echo color Doppler)
Kidney	Arterial pressure, serum creatinine, urinalysis

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**Table 3—Revised Preliminary SSc Severity Scale**

Organ system	0 (normal)	1 (mild)	4 (end stage)
1. General	Wt loss <5% Hb 12.3+ g/dL	Wt loss 5.0-9.9% Hb 11.0-12.2 g/dL	Wt loss 20+ % Hb <8.3 g/dL
2. Peripheral	RP not requiring vasodilators	RP requiring vasodilators	Digital gangrene
3. Skin	TSS 0	TSS 1-14	TSS 40+
4. Joint/tendon	FTP 0-0.09 cm	FTP 1.0-1.9 cm	FTP 5.0+ cm
5. Muscle	Normal proximal muscle strength	Proximal weakness mild	Ambulation aids required
6. GI tract	Normal esophagram	Distal esophageal hyperistalsis	Hyperalimentation required
7. Lung	FVC-DLCO 80+ %; No fibrosis; sPAP <35 mmHg	FVC-DLCO 70-79% Fibrosis sPAP 35-49 mmHg	Oxygen required
8. Heart	EKG normal; LVEF 50+ %	Conduction defect; LVEF 45-49%	CHF
9. Kidney	No Hx SRC creatinine <1.3 mg/dL	Hx SRC creatinine <1.6 mg/dL	Hx SRC dialysis required

TA Medsger et al. *Clin Exp Rheumatol*. 2003

In order to define such a core set, an international conference was organized in Portonovo, Italy, on February 7-9, 2002. On the basis of their areas of expertise, the participants were divided into

**Table 4—European SSc Activity Index**

Item	Score
mRTSS >14	1.0
Scleredema	0.5
Delta-Skin	2.0
Digital necrosis	0.5
Delta-Vasc	0.5
Arthritis	0.5
DCLO <80%	0.5
Delta-HL	2.0
ERS >30 mm/1 st h	1.5
Hypocomplementemia	1.0
Total maximum disease activity index	10.0

G Valentini et al. *Ann Rheum Dis*. 2005 (in press)

nine subcommittees. Each subcommittee provided a list of clinical, laboratory, and instrumental tests useful to investigate the relevant disease aspect. Core variables were chosen in large measure to reflect cost effectiveness and general availability in diverse clinical settings. These are not intended to serve as measures of outcome. The conclusions were discussed and accepted in a final plenary session.<sup>6</sup>

It was proposed that whatever clinical investigation study in the future would be accompanied by a “**Table 1**” listing the main epidemiological and clinical features of the systemic sclerosis patients being investigated. A consensus was reached on the core clinical, laboratory, and instrumental investigations to be carried out to assess the involvement of the main disease features (**Table 2**). The need to assess the severity of each organ/system involvement by a revised Medsger scale (**Table 3**) was stressed.<sup>7</sup> Finally, as an interim measure, it was decided to rely on preliminary European activity criteria for systemic sclerosis (**Table 4**).<sup>8</sup>

Providing all clinical investigational studies with the previously defined **Table 1** variables and investigating each patient by predefined parameters is clearly expected to improve the comparability among different systemic sclerosis series. It should be stressed, however, that the conclusions reached at Portonovo have been derived by a Delphi technique (that is, the consensus among experts in the field) and will require extensive study for validation. Prospective studies on systemic sclerosis patients from different centers are needed to unequivocally demonstrate the improvement in scientific communication arising from the use of these tools.

The need to carefully assess the systemic sclerosis patient in a routinized fashion is not confined to clinical investigation, but also involves clinical practice and therapeutic trials. Guidelines to plan the timing and the frequency of follow-up in the single systemic sclerosis patient according to the subset and the disease stage have been recently proposed.<sup>9</sup> The clinical researcher planning or performing clinical trials should enroll homogeneous populations, for instance, patients with early diffuse disease,<sup>10</sup> and must rely on validated measures, which should be sensitive to change.<sup>11</sup> These

core variables are not intended to supplant the need for closer study of specific clinical problems in the individual patient.

The authors gratefully acknowledge the participants in the Ancona Conference: A Akesson (S), R Becvar (Cz), W Bencivelli (I), P Bruhlmann (CH), P Clements (USA), L Czirikak (H), S D'Angelo (I), A Della Rossa (I), CP Denton (UK), O Distler (CH), A Drosos (Gr), M Emdin (I), C Ferri (I), G Fiori (I), A Gabrielli (I), L Ghattas (Gr), R Giacomelli (I), B Kahaleh (USA), T Krieg (D), MD Mayes (USA), N McHugh (UK), TA Medsger (USA), PA Merkel (USA), O Meyer (F), H Nielsen (Dk), G Riemekasten (D), R Scorza (I), JR Seibold (USA), AJ Silman (UK), R Silver (USA), V Steen (USA), P Vlachoyannopoulos (Gr), D Veale (Irl), FHJ van den Hoogen (NI)

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# **Eighth International Workshop on Scleroderma Research Bridges Basic and Clinical Research**

The Eighth International Workshop on Scleroderma Research was held in conjunction with the Scleroderma Clinical Trials Consortium from August 1 to 4, 2004, in Cambridge, England. More than 200 attendees from throughout the world attended, including substantial numbers from the United States, Europe, the Middle East, and the Far East. This workshop, since its establishment in 1990, has focused on basic disease mechanisms in the pathogenesis of scleroderma. For the last several workshops, the Scleroderma Clinical Trials Consortium has held its meetings jointly and the program has bridged basic and clinical research.

Basic science sessions were held on cell signaling, immunology, extracellular matrix, animal models of disease, vascular remodeling, endothelial cell function, and regulation of fibrosis. In addition, talks focused on cutting-edge technologies and approaches to the study of cells and proteins. Outstanding keynote talks were delivered by Richard Flavell, Benoit de Crombrughe, and Dean Sheppard, and these set a standard for the high level of basic science. A summary of these invited presentations will be published elsewhere.

In addition to the invited lectures, a record number of abstracts were received. These covered areas ranging from

clinical medicine and clinical trials related to scleroderma, including clinical trial methodology, to basic laboratory investigations of microfibrillar proteins. These abstracts are reproduced here for the interest of our readers, as the subject matter may not be available elsewhere unless abstracts have been coincidentally submitted to the American College of Rheumatology or other groups. The enormous interest in the abstracts at the workshop has led in some cases to planned collaborative ventures as well as discussion about potential clinical trials. The initiation of this kind of interaction was one of the goals of the first international workshop, held in Chicago in 1990 and cochaired by Drs Carol Black of the Royal Free Hospital and Joseph Korn of Boston University, who have remained as organizers and chairs of the workshop.

The Ninth International Workshop will be held in 2006. Ideas for workshop topics or speakers and volunteers to participate in workshop organization are welcome. Please contact Kate Brennan in the United States (e-mail [kbrennan@arthritis.bu.edu](mailto:kbrennan@arthritis.bu.edu)) or Millie Williams in the United Kingdom ([m.williams@rfc.ucl.ac.uk](mailto:m.williams@rfc.ucl.ac.uk)) or Drs Black or Korn.

## **Carol A. Black, MD**

Royal Free Hospital  
London, England

## **Joseph H. Korn, MD**

Boston University School of Medicine  
Boston, Massachusetts

# Abstracts From the Eighth International Workshop on Scleroderma Research

## 1. Gravitational stress in systemic sclerosis and Raynaud's phenomenon. Rationale and results

Isasi ME, Isasi ES

Gravitational Therapy Center, Cardiology Center of Maciel Hospital, Uruguayan Scleroderma Society and Related Syndromes, Montevideo, Uruguay

**Background:** Raynaud's phenomenon (RPh) is present in almost all patients with systemic sclerosis (SS). Recurrent painful attacks, frequent digital ischemic lesions and critical ischemia that can cause deep tissue injury and digital loss are a major clinical problem in patients with SS, occurring in about one third of patients per year. In previous studies we demonstrated that: a) the endothelium critically situated at the blood-tissue interface, is an important target of gravitational stress (GS), b) GS constitutes a mechanical stimulus over the vessel wall that enhances prostacyclin and nitric oxide (PGI<sub>2</sub> and NO) synthesis, causes sustained vascular dilation, induces endothelial cell (EC) desquamation of impaired cells and improves the functional capacity of patients with coronary artery disease and obstructive peripheral arteriopathy, with or without diabetes mellitus. The fact that EC damage and impaired endothelium relaxing-derived factors (PGI<sub>2</sub>, NO) have been identified to be underlying causes of digital ulceration and critical ischemia in patients with SS and RPh led us to the present study.

**Materials and Methods:** We analyzed the peripheral circulation and clinical evolution of 46 patients (6 male, 40 female, mean age 48±14 yrs) with SS and RPh with critical ischemia and 10 normal volunteers (NV) at baseline and after GS (with a protocol of ROR +GZ). Eleven of the enrolled patients had prior partial or total digital amputation of 23 fingers and 37 finger necrosis occurring in 20 patients at the entrance of the study. Digital photoplethysmographic studies (DPHG) and quantification of EC in venous samples at rest and after GS were performed at the beginning of the study. Blood samples were processed with MGG for optic microscopic observation and the number of ECs was assessed counting 100 white cells. The Ethical Committee of the Center approved the study and all patients gave their informed consent. GS procedure: After training, all subjects run on the couch of a human centrifugation machine and were exposed to accelerative and decelerative profiles (0-6 "g") of rapid onset at

peak acceleration and a rapid ramp back to control. Gravitational therapy procedure: All patients were exposed to protocol of ROR +GZ hypergravity, for one hour, three times a week, during six weeks (protocol of +GZ hypergravity).

**Results:** 1) An increase in pulse amplitude ( $P<.001$ ) was registered after GS in both groups. 2) There was a significant difference in endothelial cell desquamation between both groups. EC in SS increased from  $4.80 \pm 3.18$  to  $9.13 \pm 3.81$  Ecs/100 WC ( $P<.001$ ). In NV no significant EC desquamation was observed. 3) Clinical evolution: since the beginning patients achieved pain relief, reduced the Raynaud's Attacks, had beneficial effects in healing their ulcers and avoiding the digital amputation of 35 fingers. The follow-up of these patients was  $37 \pm 14$  months and all received between 10 to 20 sessions of gravitational therapy by year prior to winter as a maintenance treatment. Ulcers or new digital necrosis did not recur during the follow-up of these patients.

**Conclusions:** Gravitational stress improves flow-mediated dilation and induces endothelial desquamation of impaired cells in patients with systemic sclerosis and Raynaud's phenomenon. GS as a therapy had notorious beneficial effects in painful Raynaud's attacks, healing digital ischemic lesions and avoiding the digital amputation.

## 2. AGP-2 Overexpression increases type I collagen: New insights into the molecular basis for dermal fibrosis in scleroderma

Raphael Lemaire, Joseph Korn, Robert Lafyatis

Boston University School of Medicine, Boston, Massachusetts

**Background:** Prominent clinical features of scleroderma include skin fibrosis resulting from excess accumulation of matrix proteins, especially collagen. Animal models for scleroderma such as the *tsk* mouse, as well as genetic studies, have shown an association between fibrosis and mutations within fibrillin-1 a major structural protein of connective tissue microfibrils.

One hypothesis proposed for the effect of mutant fibrillin-1 such as *tsk*-fibrillin-1 is that fibrosis is due to altered TGF- $\beta$  activity. Motifs in fibrillin-1, referred to as eight cysteine repeat motifs, are homologous to regions within latent TGF- $\beta$  binding proteins shown to bind latent TGF- $\beta$ . Duplication of 8-cysteine repeats in

tsk- fibrillin-1 might alter interactions with TGF- $\beta$  and subsequently to increased signaling for expression of fibrotic genes, including collagen. Although theoretically appealing, this hypothesis has not been shown to account for fibrosis in tsk animals.

An alternative novel hypothesis is that tsk-fibrillin alters extracellular matrix assembly. Indeed, the observation that mutant fibrillin-1 in tsk mice affects the structure and morphology of fibrillin-1 fibers suggests that altered fibrillin-1 structure might mediate the effect of tsk-fibrillin on matrix. According to such a hypothesis, fibrillin-1 might interact directly with type I collagen, or alternatively other proteins that associate with fibrillin-1 might mediate the effects of altered fibrillin-1 structure on type I collagen deposition. Fibrillin-1-associated proteins might bind more or less avidly, or not at all, to a disrupted or conformationally altered fibrillin-1, providing faulty signaling for type I collagen deposition.

Since no direct binding has been described between fibrillin-1 and collagen, we propose that fibrillin-1-associated proteins interface between fibrillin-1 and collagen. In that context, we have previously shown that microfibril-associated glycoprotein-2 (MAGP-2) is increased in fibrotic skin of tsk mouse and scleroderma (Lemaire et al., *A&R*, 2004, 50:915-926). MAGP-2 is an extracellular matrix protein known to bind to fibrillin-containing microfibrils as well as to  $\alpha\text{v}\beta 3$  integrin through an RGD motif. However, its biological function remains unknown.

**Objective:** We investigated whether MAGP-2 could regulate type I collagen and thus account for increased collagen in scleroderma skin.

**Methods:** We created tetracycline-regulated mouse embryonic fibroblasts (MEF) that conditionally overexpress MAGP-2. Cell cultures were analyzed by Northern blot, Western blot, immunofluorescence and pulse-chase labeling to look at the effect of MAGP-2 on the expression, secretion and matrix deposition of type I collagen.

**Results:** MEF cells overexpressing MAGP-2 showed a 3-fold increase in intracellular levels of type I procollagen that was associated with increased levels of type I collagen secreted into the culture medium and deposited into the extracellular matrix. Accordingly, immunofluorescence studies showed that MAGP-2 and type I collagen matrices co-localize. Strikingly, MAGP-2 overexpression had no effect on type I procollagen mRNA, but markedly increased the half-life of type I procollagen protein. Although exogenous TGF- $\beta$  greatly increased deposition of MAGP-2 matrix, the MAGP-2-induced increase in type I collagen was independent of TGF- $\beta$  signaling. In contrast, MAGP-2 signals to type I collagen before being cross-linked into the extracellular matrix. MEF cells overexpressing MAGP-2 with a mutated RGD also showed increased type I collagen suggesting that the RGD of MAGP-2 does not signal to type I collagen.

**Conclusion:** This study strongly suggests that MAGP-2 contributes to dermal fibrosis in scleroderma. It also identifies for the first time a biological function for MAGP-2 as it stabilizes the type I procollagen protein.

### 3. Monitoring activity of systemic sclerosis—potential markers and their clinical correlations

*Becvar R, Pesáková V, Stánová A, Hulejová H, Rysová M, Institute of Rheumatology, Stork J, Clinic of Dermatovenereology, Jáchymová M, Pourová L, Clinic of Medicine 2, General Teaching Hospital, Zatloukalová A, Zatloukal P, Clinic of Pneumology, Prague, Czech Republic*

The aims of the study were: (a) to establish a group of with a confirmed diagnosis of SSc, (b) to determine concentrations of selected activity marker, (c) to make a comprehensive control examination after one year.

A total of 49 patients were examined (36 with limited, 9 with diffuse, and 4 with other forms). All underwent routine laboratory and organ examination. Plasma or serum levels of N-terminal propeptide of procollagen type III (NPIIP), interleukin-6 (IL-6), soluble receptor for interleukin-2 (sIL-2r), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), von Willebrand factor antigen (vWFAg), big endothelin-1 (BET-1) and urinary excretion of pyridinoline (PYR) and deoxypyridinoline (D-PYR) were assayed. Functional disability was assessed by a functional questionnaire (FQ).

The mean levels of sICAM-1, sVCAM-1, vWFAg, sIL-2r, BET-1 and PYR were insignificantly increased, the other markers were normal. The values after one year did not differ from the entry ones. At entry NPIIP concentrations correlated with the finger to palm distance and D-PYR also with the FQ. IL-6 levels correlated with the leukocyte count and sIL-2r with the FQ. After one year there was the same correlation for NPIIP and D-PYR. IL-6 correlated with the FQ and BET-1 concentrations correlated with DLCO values.

Our data confirmed the correlations of collagen metabolism markers with skin involvement and FQ as it was reported previously. The correlation of ET-1 with DLCO is remarkable.

This study was supported by a research grant of the Czech Ministry of Health #000 000 23728.

### 4. Functional genomics-based search for novel antifibrotic proteins

*Christine Power, David Abraham, Georg Feger, Maria Karmirantzou, Yan Lavrovsky, Serono Pharmaceutical Research Institute, Geneva, Switzerland; Royal Free Hospital, London, UK*

Modern methods of functional genomics starting from human genome mining to identify novel cDNAs, encoding secreted proteins, cloning of corresponding cDNAs, purification and assay of pharmacological activities of human secreted proteins have been employed to search for novel pharmaceuticals to treat and prevent fibrotic diseases. Several novel and known proteins have been shown to be active in in vitro and in vivo models of fibrosis. One of the proteins identified, osteoprotegerin, had a protective effect in models of fibrotic diseases. Osteoprotegerin is a secreted protein that has been shown to modulate osteoclast maturation and the protein has been extensively studied in relevance to different aspects of bone physiology and pathology. However, the role of

osteoprotegerin in fibroblast biology has not been extensively studied. A microarray comparison of mRNA expression in normal and diseased fibroblasts from scleroderma patients revealed that osteoprotegerin mRNA levels were significantly lower in diseased fibroblasts compared to that in normal fibroblasts. This observation was further confirmed at the level of osteoprotegerin protein expression.

We have shown that human recombinant osteoprotegerin produced in HEK 293 and CHO cells has antifibrotic effects in vitro and in vivo, thus demonstrating that recombinant proteins may have the potential to be developed as antifibrotic drugs.

## 5. Healthy children have a significantly increased skin score assessed with the modified Rodnan skin score

*Ivan Foeldvari, Angela Wierk. Paediatric Rheumatology Clinic at AK-Eilbek, Hamburg, Germany*

Modified Rodnan skin score (MRSS) is used as a primary outcome measure in most of the therapeutic trials in systemic sclerosis (SSc) in adults. If we want to use this outcome measure in trials in juvenile patients with SSc, we need to evaluate this assessment method in children without sclerodermatous skin changes to establish values for the “normal pediatric population.”

To determine the “healthy pediatric population,” patients of the outpatient clinic with mechanical pain or with juvenile idiopathic arthritis (JIA) at the age of 16 years or under were assessed from January 1 till March 31, 2004. Patients with any sign of connective tissue disease or skin disorders, like psoriasis or ectopic dermatitis, were excluded. The MRSS was determined in standardized locations and with the standardized pinching method.

Two hundred seventeen patients, including 100 females, were assessed. The mean age of the patients was 10.5 years (2.9-16), the mean body mass index (BMI) was 18.3 (9.3-35.7), and the mean MRSS was 13.92 (range 4-25). The MRSS score did not show a correlation to Tanner stage summarizing the data for males and females. The mean scores for Tanner 1, 2, 3, and 4 were 13.1, 15.5, 14.1, and 13.5. There was a tendency for lower MRSS scores in the males comparing Tanner 1 with 12.4 MRSS and Tanner 4 with 10.6 MRSS. There was no correlation between age and MRSS. But there was a linear correlation between MRSS and BMI independently from age and Tanner stage.

The mean MRSS in healthy children is 13.92 and already in an abnormal range looking at adult studies for patients with SSc. The MRSS score in children correlates with the BMI, so if MRSS is used in a pediatric trial, the score has to be corrected for the BMI, according to this pilot study.

## 6. Beta-thromboglobulin and platelet factor-4 are elevated in bronchoalveolar lavage fluid in early scleroderma lung disease

*Otylia Kowal-Bielecka<sup>1</sup>, Krzysztof Kowal<sup>2</sup>, Joanna Rojecka<sup>1</sup>, Andrzej Lewszuk<sup>3</sup>, Anna Bodzenta-Lukaszyk<sup>2</sup>, Jerzy Walecki<sup>3</sup>, Stanislaw Sierakowski<sup>1</sup>*

*1Department of Rheumatology and Internal Diseases,*

*2Department of Allergology and Internal Diseases, 3Department of Radiology, Medical University of Bialystok, Poland*

**Objective:** Inflammation and progressive fibrosis of lower respiratory tract are main features of scleroderma interstitial lung disease (SLD). Blood platelets are a source of mediators and growth factors that were shown to play an important role in the processes of inflammation and fibrosis. Therefore, we investigated markers of platelet activation:  $\beta$ -thromboglobulin (BTG) and platelet factor-4 (PF-4) in bronchoalveolar lavage fluid (BALF) from patients with and without SLD

**Patients and methods:** BTG and PF-4 were measured by means of ELISA in BALF of 37 patients with systemic sclerosis (SSc). Control group consisted of 10 healthy subjects. BALF was collected during routine bronchoscopy from the right middle lobe. SLD was diagnosed on the basis of high-resolution computed tomography (HRCT) of the lungs.

**Results:** BTG was detected in 11/37 (29.7%) of patients with SSc (106.87 $\pm$ 69.79 IU/mL) and PF-4 was found in 8/37 (21.6%) of patients with SSc (35.21 $\pm$ 17.35 IU/mL). In all BALFs with detectable platelet activation markers, the BTG:PF-4 ratio was greater than 2:1, indicating in vivo release. Both markers were found exclusively in patients with SLD. Neither BTG nor PF-4 were detectable in any of the subjects from the control group or the group of patients without SLD. Moreover, SLD patients with detectable platelet activation markers had significantly shorter disease duration than SLD patients and undetectable BTG/PF-4.

**Conclusions:** The results of our study indicate that activation of blood platelets takes place within the lungs of patients with SLD and may contribute to the development of lung fibrosis.

## 7. Increased transforming growth factor $\beta$ receptor type I: Type II ratio contributes to elevated collagen protein synthesis by upregulation of CTGF expression in a Smad-independent manner

*Jaspreet Pannu, Edwin A. Smith and Maria Trojanowska*  
*Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina*

Alterations in the TGF $\beta$  signaling pathway have been implicated in the ‘activated’ phenotype of scleroderma fibroblasts. Our recent studies demonstrated that SSc fibroblasts express higher endogenous ratio of TGF $\beta$ R1:TGF $\beta$ R2 (as compared to normal controls). Furthermore we showed that forced expression (using adenoviral vectors) of TGF $\beta$ R1 increased basal collagen protein levels in a dose dependant manner, while similar expression of TGF $\beta$ R2 had no effect (Pannu et al., A&R, 2004). The maximal stimulation of collagen protein synthesis in response to AdTGF $\beta$ R1 was observed in the dose range of MOI 25-50 corresponding to the elevated TGF $\beta$ R1 levels in SSc fibroblasts, while higher doses had no effect. The goal of this study was to gain further insight into the mechanism involved in increased collagen gene expression associated with elevated TGF $\beta$ R1 expression.

Using Northern blot analysis, we observed maximal stimulation of COL1A2 mRNA (2.22 fold) 48 hours post transduction, at a dose of MOI 25 that corresponds to a twofold increase of

TGF $\beta$ R1 over endogenous levels. Importantly, CTGF mRNA was also up-regulated (1.42 fold) under this condition. To determine if the upregulation of CTGF and collagen by elevated TGF $\beta$ R1 levels is mediated by Smads, we determined Smad2 phosphorylation levels in fibroblasts expressing elevated TGF $\beta$ R1 levels. In contrast to robust response observed with TGF $\beta$  stimulation (2.5 ng/mL) for 30 mins (used as positive control), dermal fibroblasts expressing increased TGF $\beta$ R1 did not exhibit increased Smad2 phosphorylation suggesting that TGF $\beta$ R1 effect is Smad-independent. Ras/MEK/ERK pathway activation, in addition to Smad activation, has been implicated in induction of CTGF by TGF $\beta$ . We examined the activation status of MEK1/2 pathway in response to elevated TGF $\beta$ R1 levels. We found higher levels of phospho-ERK1/2 in fibroblasts expressing elevated TGF $\beta$ R1 while total ERK1/2 levels were unchanged. Treatment of cells with a specific MEK1/2 inhibitor UO126 (10  $\mu$ M) abolished both the phosphorylation of ERK1/2 and the concomitant stimulation of collagen type I protein.

To determine whether MEK1/2 pathway is involved in the "activation" of SSc fibroblasts, we compared matched pairs of normal and scleroderma fibroblasts. We observed elevated ERK1/2 phosphorylation in SSc fibroblasts that exhibited elevated endogenous TGF $\beta$ R1. We conclude that the increased TGF $\beta$ R1:TGF $\beta$ R2 ratio contributes to CTGF upregulation in SSc fibroblasts in an Smad-independent manner.

## 8. Increased amino terminal propeptid of type III procollagen levels are unfavorable predictors of survival in systemic sclerosis

*Jaspreet Pannu, Edwin A. Smith and Maria Trojanowska*

*Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina*

**Objective:** Investigation of the impact on survival of inflammatory parameters (C-reactive protein, ESR, markers of immune activation (serum soluble IL-2 receptor, soluble CD30), and collagen C aminopeptide terminal levels (PIIINP) in systemic sclerosis (SSc).

**Design:** In a prospective follow up study, clinical and laboratory data of 80 patients with SSc were evaluated. Kaplan-Meier survival curves and Cox proportional hazards model were used.

**Subjects:** Eighty cases with SSc were evaluated. Female/male ratio was 8/72. The mean ( $\pm$ SD) age was 49.3 ( $\pm$ 12.3) years, 16 patients died during our mean follow up of 58.1 months (min: 5 max: 79 months).

**Results:** In the univariate analysis, the presence of a C-reactive protein level above 20 mg/L was an unfavourable prognostic sign ( $P < .001$ ). Increased level of PIIINP level also caused an unfavourable outcome of disease ( $P < .001$ ). Conversely, increased ESR, soluble IL-2 receptor, soluble CD30 levels, presence of anemia, did not influence the prognosis. Male gender ( $P < .005$ ), diffuse cutaneous SSc, clinically significant lung involvement ( $P < .001$ ), kidney ( $P < .0001$ ), cardiac ( $P < .05$ ) manifestations including pericarditis ( $P < .02$ ) were unfavorable prognostic signs by univariate Kaplan-Meier method. Multivariate analysis by Cox

proportional hazards model showed that the increased level of PIIINP (RR: 6.98), and presence of diffuse cutaneous SSc (RR: 5.14) were independent unfavorable prognostic signs.

**Conclusions:** An increased collagen metabolism unfavourably influences the outcome of SSc. This parameter may also be a potential candidate as disease activity marker.

## 9. Circulating levels of the endothelial markers are dysregulated in systemic sclerosis

*S Guiducci<sup>1</sup>, M Cinelli<sup>1</sup>, A Del Rosso<sup>1</sup>, AF Milia<sup>1</sup>, A Gabrielli<sup>3</sup>, R Giacomelli<sup>4</sup>, S Generini, M Del Rosso<sup>2</sup>, M Matucci-Cerinic<sup>1</sup>*

**Background:** NO, produced by eNOS and ACE are mostly released by endothelial cells together with adhesion molecules sP-selectin and sPECAM-1. The interaction of adhesion molecules with the endothelium and extracellular matrix is critical in the circulation and recirculation of white blood cells. This includes migration to site of tissue damage, infection and inflammation as well as homing to lymphoid tissue. In SSc, endothelial dysfunction is a key event in the disease pathogenesis. Endothelial damage may be investigated through the study of endothelial derived products.

**Aims:** To investigate the endothelial derived markers and the correlation with the main clinical parameters [Diffuse lung capacity for carbon monoxide (DLCO); forced vital capacity (FVC); autoantibodies ACA and DTPA clearance].

**Patients and methods:** circulating serum levels of endothelial markers were examined by ELISA in 40 SSc patients, [34 limited SSc (lSSc) and 6 diffuse SSc (dSSc)] and 20 age and sex-matched healthy controls.

**Results:** In SSc, basal levels of sP-selectin are significantly lower than controls [87.5 $\pm$ 28.4 vs 149.1 $\pm$ 11.2 ng/mL;  $P < .001$ ] and do not differ in dSSc vs lSSc. (74.2 $\pm$ 26.7 vs 89.5 $\pm$ 28.4 ng/mL;  $P > .05$ ). SSc levels of sP-selectin correlate with DLCO test ( $r = 0.346$ ,  $P < .05$ ). In SSc, basal levels of sPECAM-1 are significantly higher than controls (56.1 $\pm$ 10.8 vs 43.36 $\pm$ 3.79 ng/mL;  $P < .001$ ) and this increase is higher in lSSc vs dSSc (57.96 $\pm$ 10.51 vs 49.05 $\pm$ 3.25 ng/mL;  $P < .01$ ). sPECAM-1 correlate with DLCO ( $r = 0.42$ ,  $P < .01$ ) and FVC ( $r = 0.51$ ,  $P < .01$ ). sPECAM-1 are significantly higher in ACA+ patients vs ACA- (59.82 $\pm$ 11.99 vs 52.62 $\pm$ 4.88  $P < .05$ ), and lower in patients with pulmonary fibrosis (52.67 $\pm$ 5.23 vs 60.32 $\pm$ 12.34  $P < .05$ ). NO levels are significantly lower than controls [21.34 $\pm$ 11.32 vs 31.03 $\pm$ 9.39 mM;  $P < .01$ ] and do not differ in dSSc vs lSSc (17.33 $\pm$ 11.13 vs 22.32 $\pm$ 11.71 mM;  $P > .05$ ). NO levels are significantly higher in patients with systemic hypertension (32.05 $\pm$ 7.44 vs 18.95 $\pm$ 11.05  $P < .01$ ). ACE levels are significantly lower than controls (435.7 $\pm$ 160.0 vs 593.9 $\pm$ 58.96 ng/mL;  $P < .001$ ), not different in dSSc vs lSSc (421.3 $\pm$ 108.4 vs 441.5 $\pm$ 177.0 ng/mL;  $P > .05$ ), and correlate with DTPA clearance ( $r = 0.359$ ,  $P < .05$ ).

**Conclusion:** Low levels of NO, ACE and sP-selectin suggest an endothelial dysfunction in SSc. Low ACE may be due to pulmonary involvement (correlation ACE and DTPA clearance). Markers of endothelial cell dysfunction might help the clinician to follow up disease activity and organ involvement in SSc.

## 10. Opposing effects of PKCa and PKCe on collagen expression are mediated via MEK/ERK and caveolin-1 signaling

*Elena Tourkina, Pal Gooz, Richard Silver, Stanley Hoffman.*

*Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina*

Scleroderma is a complex autoimmune disease characterized by small-vessel vasculopathy and fibrosis of the skin, lungs, and other organs. The major cause of morbidity and mortality in scleroderma is pulmonary fibrosis. Signaling cascades that regulate collagen expression in normal and fibrotic lung tissue were studied using primary cultures of normal (NLF) and scleroderma (SLF) human lung fibroblasts. Results obtained with caveolin-1 siRNA were particularly striking. A 70% decrease in caveolin-1 in NLF caused a fivefold increase in MEK/ERK activation and in collagen expression. The combined data reveal a branched signaling pathway. In the pathway's central portion MEK activates ERK, leading to increased collagen expression. Two branches converge on MEK/ERK. In one, increased PKCe expression leads to MEK and ERK activation. In the second, increased PKCa expression induces caveolin-1 expression which in turn inhibits MEK/ERK activation and collagen expression. Experiments using SLF showed that these signaling cascades are altered during lung fibrosis. Consistent with their overexpression of collagen, SLF contained much more activated MEK and ERK and much less caveolin-1 than NLF. Moreover, because MEK and ERK are already hyperactivated in SLF, two treatments (overexpression of constitutively active PKCe, inhibition of caveolin-1 expression) that strongly increase MEK/ERK activation and collagen expression in NLF do not affect MEK/ERK activation and collagen expression in SLF. Finally, we confirmed the biological relevance of these signaling cascades in vivo, utilizing the bleomycin model of murine lung fibrosis. In summary, these results demonstrate that a branched signaling pathway involving MEK, ERK, PKCe, PKCa, and caveolin-1 regulates collagen expression in normal lung tissue and that this signaling pathway is altered in SLF and in models of lung fibrosis.

## 11. DNA microarray studies of chemokines and chemokine receptors in murine sclerodermatous graft versus host disease, a model for human scleroderma

*David L Zhou, Caiyun Wu, Guofen Chen, David Askew,*

*Anita C Gilliam Department of Dermatology, Case University and University Hospitals of Cleveland, Cleveland, Ohio*

Chronic graft versus host disease (GVHD) is a major complication of allogeneic bone marrow transplantation that can have autoimmune features resembling scleroderma (sclerodermatous GVHD, Scl GVHD). The molecular mechanisms governing skin fibrosis in Scl GVHD are not known. Our hypothesis is that chemokines that attract monocyte/macrophages and T cells are critical in the early pathogenesis of fibrosis, and may be targets for interventions in scleroderma. We used DNA microarrays to characterize chemokines and chemokine receptor gene expression in skin during early stages of fibrosing disease in irradiated BALB/c

(H-2<sup>d</sup>) mice transplanted with B10.D2 (H-2<sup>d</sup>) bone marrow and spleen cells. These mice develop skin thickening, while control mice transplanted with BALB/c (H-2<sup>d</sup>) bone marrow and spleen cells (syngeneic BMT control) do not develop skin thickening. We found consistent differences in gene expression in skin of between mice with Scl GVHD and controls. Messenger RNA for chemokines CCL11 (eotaxin), CCL19 (MIP-3 β), CCL22 (MDC), CXCL5 (ENA-78), CXCL10 (IP-10), CXCL11 (I-TAC), CXCL14 (BRAK), CXCL16 were up regulated in Scl GVHD mice but not in the syngeneic BMT mice. CCL17 (TARC), CXCL9 (MIG), CXCL13 (BLC, BCA-1) were up regulated in both groups. Chemokines CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CCL9 (MIP-1γ), CCL12 (MCP-5) were up regulated in Scl GVHD mice, and down regulated in the syngeneic BMT mice. CXCL1 (GROβ), CXCL2 (GROα), CXCL15 (Lungkine) were down regulated in both groups. Chemokine receptor mRNAs CCR1, CCR5, CXCR6 were increased, while CCR2, CCR9, CXCR4 were decreased. Therefore, chemokines and their receptors for both T cells and macrophages appear to be important in early fibrosing disease, consistent with human scleroderma and with our previously published results that T cells and macrophages are both found in early cutaneous Scl GVHD. This chemokine environment and time course of chemokine upregulation in skin of animals developing Scl GVHD will help us to better understand pathogenesis of fibrosis and to devise more effective strategies for intervention at the various stages in the development of early scleroderma and GVHD.

Chemokines/receptors	Scl GVHD	Syngeneic group
CXCL13,CXCL9,CCL17,	Up	Up
CCL5, CCL12, CCL2,CCL7,CCL9, CCR1,CCR5,CXCR6	Up	Down
	Down	Up
CXCL1,CXCL15,CXCL2 CCR2,CCR9,CXCR4	Down	Down
CCL11,CCL22,CXCL14,CXCL16, CXCL10,CXCL11,CXCL5, CCL19	Up	No change

## 12. T cell-antigen presenting cell interactions and the innate immune system may both be important in the development of murine sclerodermatous GVHD, a model for scleroderma

*David Askew, David L. Zhou, Caiyun Wu, Guofen Cheng, and*

*Anita C. Gilliam Department of Dermatology, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, Ohio*

We are modeling human scleroderma with murine sclerodermatous graft versus host disease (Scl GVHD), in which transplantation of bone marrow and spleen cells of B10.D2 (H-2<sup>d</sup>) into lethally irradiated BALB/c (H-2<sup>d</sup>) across minor histocompatibility loci (MiHC) produces skin thickening and lung fibrosis rather than classic cytotoxic GVHD. By day 7 post-bone marrow transplant (BMT), we find an increase of activated CD4<sup>+</sup> T cells pro-

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ducing IFN $\gamma$  and IL-2 in the spleens of mice undergoing Scl GVHD compared to syngeneic BMT controls (BALB/c  $\rightarrow$  BALB/c). At this time, we find that both CD11c<sup>+</sup> dendritic cells (DCs) and CD11b<sup>+</sup> macrophages (M $\phi$ s) upregulate expression of MHC-I, MHC-II, CD80, CD86, and PDL1, molecules important in interacting with T cells. While IFN $\gamma$ -producing CD4<sup>+</sup> T cells remain in the spleen 21 days post-BMT, the expression of costimulatory molecules on DCs and M $\phi$ s decrease to normal levels. We see IFN $\gamma$ -producing CD4<sup>+</sup> T cells infiltrating skin of mice undergoing Scl GVHD by 14 days post-BMT. Suggesting T cell-APC interactions occurring first in the spleen and then in the skin. To understand the role of T cell-APC interactions in Scl GVHD we examined mixed leukocyte reactions using BALB/c APCs and B10.D2 T cells. We found that CD11c<sup>+</sup> DCs are more efficient in stimulating CD4<sup>+</sup> T cells than are CD11b<sup>+</sup> M $\phi$ s. While CD11c<sup>+</sup> DCs stimulate CD4<sup>+</sup> T cells to produce IFN $\gamma$  and IL-2, CD11b<sup>+</sup> M $\phi$ s fail to stimulate cytokine production by CD4<sup>+</sup> T cells. Toll-like receptor (TLR)-agonists added to DC/T cell cultures fail to enhance T cell proliferation, but they do enhance production of IFN $\gamma$  and IL-2 produced by CD4<sup>+</sup> T cells. Of interest is that TLR agonists added to M $\phi$ /T cell cultures enhance T cell proliferation and production of IL-4 by CD4<sup>+</sup> T cells. Because Scl GVHD and scleroderma are considered a mixed Th1/Th2 phenotype, these results suggest that CD11c<sup>+</sup> DCs, CD11b<sup>+</sup> M $\phi$ s, and TLR agonists may all be required for the development of the cytokine profile associated with this fibrosing disease. The prominence of macrophages in cutaneous immune cell infiltrates and our observation that TLR ligands could play a role in early disease suggest that in addition to classical T cell-APC interactions, the innate immune system (macrophages and TLRs) may also be important. These findings have clinical relevance in the hypothesis that bacterial or viral antigens could trigger autoimmune disease via molecular mimicry.

### 13. Intravenous prostaglandin E1 reduces skin fibrosis in early Systemic Sclerosis

*Lidia Rudnicka, Justyna Sicinska, Elzbieta Szymanska*

*Department of Dermatology, CSK MSWiA, Warsaw, Poland*

Prostaglandin E1 (PGE1) has been shown to reduce the severity of Raynaud's phenomenon and improve acral trophic skin lesions associated with scleroderma. The aim of the study was to evaluate the effect of intravenous PGE1 on the extend of skin indurations in patients with systemic sclerosis. Sixteen patients with systemic sclerosis (14 females and 2 males; 10 Topo I - positive and 4 ACA positive) received intravenous PGE1-alpha-cyclodextrin (alprostadiol) at the dose of 20 $\mu$ g, 40 $\mu$ g and 60 $\mu$ g on 3 consecutive days at 5 week intervals. Modified Rodnan skin score was assessed by 3 independent investigators every 3 cycles of infusion for evaluation of the extend of skin involvement. In all patients basic laboratory tests, blood pressure and electrocardiogram was monitored. In 1 patient PGE1 therapy was discontinued due to significant hypotonia. In one other patient PGE1 was discontinued due to lack of any efficacy after 4 cycles of infusions. In 12 patients significant improvement in peripheral circulation

after PGE1 treatment was observed. The severity and frequency of Raynaud's phenomenon was reduced and acral trophic skin lesions healed. In all these patients improvement in peripheral circulation was associated by reduction in skin fibrosis. The average skin score decreased from 18 to 10 after 9 cycles of infusions. In one 33-year-old male patient with early TopoI+ scleroderma complete resolution of skin indurations of fingers and dorsum of hands was observed (reduction of Rodnan score from 10 to 0) after 9 cycles of intravenous treatment. In this patient coexisting morphea lesions on the trunk subsided with no additional treatment, leaving minimal hyperpigmentation. In a 57-year-old female with a 6 year history of ACA+ scleroderma Rodnan score subsided from 12 to zero after 9 cycles. In other patients, with longer-lasting scleroderma (over 8 years) progression of skin fibrosis was stopped, or incomplete improvement was observed. The efficacy of PGE1 treatment depended on the length of disease history and was partially related to the extent of skin fibrosis. It remains unclear whether resolution of skin fibrosis after PGE1 might be due to its direct antifibrotic activity, to immunosuppressive activity and reduction of immune-dependent fibrosis or due to interference with an early vascular stage of the pathogenic pathway in scleroderma.

### 14. Is thermography a sufficient tool to detect active disease in juvenile localised scleroderma?

*Visentin<sup>1</sup> M, Howell<sup>2</sup> KJ, Lavorato A<sup>3</sup>, Jones CD<sup>4</sup>, Martini G<sup>1</sup>, Smith RE<sup>3</sup>, Denton CP<sup>2</sup>, Zulian F<sup>1</sup>, Black CM<sup>2</sup>, Harper JT<sup>5</sup> and Woo P<sup>6</sup>*

*1 Department of Paediatrics, University of Padua, Italy.*

*2 Departments of Rheumatology and 3 Medical Physics, Royal Free Hospital, London, UK.*

*4 School of Computing, University of Glamorgan, Pontypridd, UK.*

*5 Departments of Dermatology and 6 Rheumatology, Great Ormond Street Hospital, London, UK.*

**Background** Infrared thermography (IRT) has been shown to be of value in the detection of active localised scleroderma (LS) lesions in children. Plaques with a temperature  $>0.5^{\circ}\text{C}$  warmer than adjacent or contralateral uninvolved skin can be considered "thermography +ve." On this basis, a positive thermogram detects clinically active LS lesions with a sensitivity of 92% and a specificity of 68% (Martini et al, 2002). Skin surface temperature is influenced not just by dermal blood flow, but also by the morphological skin changes that can occur in LS. Increased heat transfer through the skin in older, clinically quiescent lesions associated with fat and muscle atrophy has been thought to be the reason for the "false-positive" thermograms.

**Method** We have evaluated a new protocol for the assessment of LS in children. This combines IRT with laser Doppler flowmetry (LDF, a measure of microvascular skin erythrocyte flow), 20 MHz Doppler ultrasound (US, a technique for imaging skin structure), and digital photography (to record the extent of the clinically visible lesion). The digital images are then superimposed on the infrared thermograms to assist the physician in relat-

ing the anatomy to the extent of skin involvement. We reassessed 15 clinically quiescent LS patients who were found to have “false-positive” thermograms on previous assessment by IRT (11F, 4M). Up to 3 involved skin sites from each patient were selected for assessment by IRT, LDF and US. Equivalent measurements were also made from contralateral or adjacent uninvolved skin. Each involved site was reassessed by two experienced clinicians to confirm that the area had remained clinically quiescent. Only data from such quiescent sites was included in our analysis. We calculated temperature data from the infrared thermograms at each skin site, and also LDF flux data from each site. All involved LS sites were then grouped into those that remained “thermography +ve” and those that were reclassified “thermography -ve.”

**Results** The difference in LDF red cell flux between involved LS skin and contralateral/adjacent uninvolved skin was expressed as a percentage of the flux in the uninvolved skin. The mean ( $\pm$ standard deviation) flow difference for the “thermography +ve” group was 131 $\pm$ 153% vs 11 $\pm$ 51% for the “thermography -ve” group ( $P < .004$ , t-test).

**Conclusions** Microvascular skin blood is increased in thermographically warm LS plaques that are considered to be clinically quiescent. This may suggest residual disease activity and could have implications for the length of medical treatment. Further work is in progress to refine our imaging techniques in order to define whether the increase in blood flow is related to inflammation or to structural changes.

## 15. The effect of a novel TGF- $\beta$ inhibitor on TGF- $\beta$ /Smad signaling in normal dermal fibroblasts

*Yasuji Mori, Wataru Ishida, Swati Bhattacharyya, John Varga*  
*University of Illinois at Chicago, Chicago, Illinois*

**Abstract:** Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a pleiotropic cytokine implicated in the pathogenesis of fibrosis. Targeting extra- and intracellular TGF- $\beta$  signaling represents a potential approach to the treatment of scleroderma and related fibrotic disorders. The aim of present study was to investigate how SB-431542, a recently identified small molecule inhibitor of the ALK5, TGF- $\beta$  receptor, affects TGF- $\beta$  signaling in normal skin fibroblasts.

We examined the effect of SB-431542 on intra-cellular shuttling of activated Smads. Preincubation of normal fibroblasts with the inhibitor (10  $\mu$ M) caused >90% inhibition of TGF- $\beta$  triggered cytoplasmic-nuclear transport of endogenous Smad2/3 and Smad4. SB-431542 induced complete blockade of Smad2 and Smad3 phosphorylation upon TGF- $\beta$  treatment. We next examined the activation of TGF- $\beta$  signaling and target gene expression in the presence of SB-431542. SB-431542 inhibited the autoinduction of TGF- $\beta$ 1 and of CTGF and extracellular matrix mRNA expression. SB431542 repressed TGF- $\beta$  induced SBE4-luc, -772COL1A2/CAT and AR3-luc activities. SB431542 partially prevented the stimulation of type I collagen and PAI-1 protein expression at 24 hours. TGF- $\beta$  induced marked stimulation of alpha smooth muscle actin expression (a marker for myofibroblasts), and SB-431542 completely abrogated this response. In

summary, SB-431542 is a potent novel inhibitor of TGF- $\beta$  signaling through interference with cellular Smad activation. This molecule is thus a powerful tool to understand the detailed mechanism of normal and aberrant TGF- $\beta$  signaling and its target genes. Furthermore, SB-431542 may help in identifying novel approaches for the treatment of fibrosis.

## 16. A positive signaling loop involving ROS/ERK/ Ha-RAS maintains the activation of SSC fibroblasts and is triggered by serum stimulatory antibodies against $\alpha\beta$ PDGF receptors.

*Svegliati S., Spadoni T., Bevilacqua F., Paroncini P., Luchetti M., Di Carlo A., Santillo M.\*, Avvedimento E\*., Gabrielli A*

*Istituto di Clinica Medica Generale, Ematologia ed Immunologia Clinica, Università Politecnica delle Marche, Ancona, Italy*

*\*Dipartimento di Biologia e Patologia Molecolare e Cellulare, Università Federico II, Napoli, Italy*

**Background:** Systemic sclerosis (SSc) phenotype is characterized by accumulation of components of extracellular matrix, such as collagen, and excessive oxidative stress in monocytes, endothelial cells and fibroblasts. The molecular mechanisms that trigger and maintain ROS generation in SSc fibroblasts are, however, unknown. Therefore we decided to test whether Ha- and Ki-Ras were involved in ROS generation and whether they have an impact on cell function.

**Materials and Methods:** For the ROS measurement fibroblasts were loaded with 2'-7'-dichlorofluorescein diacetate (DCHF-DA 10  $\mu$ M) for 15 minutes at 37  $^{\circ}$ C and the fluorescent intensity was measured by a CytoFluor plate reader (excitation 485, emission 530). For Ha-Ras protein expression whole cells lysates were immunoprecipitated with Pan-Ras antibody and protein A-agarose and then subjected to SDS-PAGE and immunoblotted with the specific antibody. Effectene Transfection reagents (Quiagen) were used for fibroblast transient transfection. Total serum IgG were purified with Immunopure (A/G) purification kit (Pierce).

**Results:** We have found that the selective increase of the mass and the activity of Ha-Ras induce, via ERK1-2, an abnormal release of ROS. High ROS levels in SSc fibroblasts stabilize Ha-Ras by stimulating ERK 1-2. This positive self-perpetuating loop linking ROS/ERK 1-2/ Ha-Ras is persistently active and since ROS are crucial for collagen gene expression in SSc fibroblasts, it explains the persistent activation of these cells in vitro, and likely in vivo, even in the absence of growth factors. Furthermore, we have found that serum of SSc patients contain stimulatory antibodies to the  $\alpha\beta$  PDGF receptor chains. The autoantibodies trigger PDGF receptor activation, stabilize Ha Ras, induce ROS and ERK1/2 signaling.

**Conclusions:** We conclude that anti PDGF receptor antibodies initiate the Ras-ERK1/2-ROS cascade. ROS, by stabilizing Ha-Ras protein, maintain and amplify focal ERK1/2 signaling and are responsible for fibroblast activation in vitro.

## 17. Prolactin is produced by activated lymphocytes and stimulates further immune activation in patients with Systemic Sclerosis

Malgorzata Olszewska, Joanna Czuwara-Ladykowska, Lidia Rudnicka

Department of Dermatology CSK MSWiA, Warsaw, Poland,  
Department of Dermatology, Warsaw Medical School, Warsaw, Poland

Increased serum levels of prolactin have been reported to correlate with disease activity in systemic sclerosis. The aim of this study was to investigate the source of excessive prolactin in patients with SSc, its effects on autoimmune phenomena in SSc and possible usefulness of bromocriptine, a prolactin inhibitor, in treatment of patients with systemic sclerosis. The study group consisted of 52 patients with diffuse SSc (44 women and 8 men) and 52 age and sex matched healthy controls. The following methods were used: ELISA, indirect immunofluorescence, capillaroscopy and patient's self assessment sheet for Raynaud's severity score. Our results show that: T lymphocytes of patients with systemic sclerosis produce significantly increased amounts of prolactin ( $24.5 \pm 11.0$  vs  $13.4 \pm 5.0$  absorb. units), prolactin has immunostimulatory activity, as shown by increase in IL-2 receptor concentration in supernatants of lymphocytes from healthy donors (basal level  $2253 \pm 1276$  and PRL induced  $8769 \pm 3426$  ( $P < .01$ ) and patients ( $7234 \pm 3491$  and  $9345 \pm 3820$ ), serum levels of prolactin significantly decreased in patients receiving oral cyclophosphamide (50 mg/day) for 3 months from  $96.84 \pm 89.06$   $\mu\text{g/L}$  to  $36.84 \pm 30.79$   $\mu\text{g/L}$  ( $P < .05$ ). In conclusion, the results of this study show that hyperprolactinemia in patients with SSc at least partially results from and induces immune activation.

## 18. Development of skin fibrosis and microvessel injury in mice with reduced levels of Fli1 (Fli1+/-)

Margaret Markiewicz<sup>1</sup>, Masahide Kubo<sup>1</sup>, Rachele Childress<sup>1</sup>, Dennis K. Watson<sup>2</sup> and Maria Trojanowska<sup>1</sup>

<sup>1</sup>Division of Rheumatology and Immunology, <sup>2</sup>Department of Pathology, Medical University of South Carolina, Charleston, South Carolina

Systemic sclerosis (SSc) is an autoimmune disease characterized by microvascular injury and elevated deposition of extracellular matrix (ECM) proteins. We have recently observed that a transcriptional regulator of ECM genes, Fli1, is consistently reduced in SSc skin and may directly contribute to activation of SSc fibroblasts and uncontrolled matrix deposition in SSc skin *in vivo* (Kubo et al, AJP, 2003). The goal of this study was to determine the consequences of the reduced Fli1 gene dosage in the mouse model. The targeted deletion of Fli1 gene is homozygous lethal at 11.5 dpc (Spyropoulos et al, MCB, 2000). The early lethality is reported to be associated with vascular abnormalities. Heterozygous Fli1<sup>+/-</sup> mice (backcrossed to C57/B1/6) are viable and were used for this study. In the initial experiment mice at 3 months of age (male/WT, female/WT, male Fli1<sup>+/-</sup>, female Fli1<sup>+/-</sup>) were analyzed. Hematoxylin-eosin staining of skin sections revealed increased dermal thickening in Fli1<sup>+/-</sup> male and

female mice as compared to wild-type littermates. 8 mm punch biopsies from the lower back were used to obtain soluble collagen by acetic extraction method. Analysis of extracted collagen by SDS-acrylamide electrophoresis showed markedly increased (twofold)  $\alpha 1(I)$  and  $\alpha 2(I)$  collagen levels in Fli1<sup>+/-</sup> male and female mice as compared to wild-type littermates. To further delineate the role of Fli1 in regulation of ECM related genes, skin fibroblasts were isolated from wild-type and Fli1<sup>+/-</sup> female mice. Fibroblasts from Fli1<sup>+/-</sup> mice produced elevated levels of collagen type I protein as determined by Western blot. Furthermore, northern blot analysis revealed increased expression of COL1A1 (1.8 fold), COL1A2 (1.4 fold), and CTGF (2.2 fold) mRNAs in Fli1<sup>+/-</sup> fibroblasts as compared to fibroblasts obtained from wild type littermates. Interestingly, preliminary data indicate that upregulation of CTGF gene expression was not observed in Fli1<sup>+/-</sup> fibroblasts obtained from ovariectomized females, suggesting hormonal involvement in development of skin fibrosis in Fli1<sup>+/-</sup> mice. To determine whether reduced levels of Fli1 affect vascular cells, the presence of apoptotic cells was examined using TUNEL technique and immunostaining with active caspase-3 antibody. Very few apoptotic cells were identified in the skin sections of wild-type mice, whereas apoptotic cells (endothelial cells and pericytes) were detected in a proportion of blood vessels in Fli1<sup>+/-</sup> mice. Together, these data demonstrate that mice with reduced expression of Fli1 gene develop scleroderma-like features, including increased collagen synthesis and microvascular injury. Furthermore, similar to scleroderma fibroblasts isolated from the skin of Fli1<sup>+/-</sup> female mice exhibit activated phenotype. Because of the current lack of the mouse models that recapitulate both vascular and stromal characteristics of SSc, Fli1<sup>+/-</sup> mice should be very useful for studying pathogenesis of this disease.

## 19. Simultaneous occurrence of malignancies and connective tissue diseases

Cecilia Varjú, Szilvia Üveges, and László Czirják

Hungarian Brothers of St. John of God and University of Pécs Faculty of Medicine, Department of Immunology and Rheumatology, Pécs, Hungary

**Objective:** To analyze the simultaneous onset of malignancies and connective tissue diseases in a large number of patients with systemic sclerosis (SSc), dermatomyositis (DM/PM) and systemic lupus erythematosus (SLE).

**Methods:** Retrospective analysis of our clinical database was performed. Those cases were taken as tumor associated CTD where the time elapsed between the two diseases was less than 2 years.

**Results:** In DM six (25 %) of the 30 patients had malignant tumours. There were one lung and one vaginal cancer, one non-Hodgkin lymphoma, and three gastrointestinal adenocarcinomas. None of the DM patients with tumors tested positive for rheumatoid factor or anti-Jo-1 antibody. Two malignant tumors were detected out of the 35 PM patients. In SSc malignant tumors developed in 13 (6.5 %) out of the 200 patients (three haematological malignancies, three gastrointestinal cancers, four breast

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cancers, one lung, one ovarian cancer and one malignant melanoma). Among the 13 patients with malignant tumors six (46 %) were positive for antitopoisomerase antibody, whereas among the 187 tumor free patients 51 (36 %) had this particular autoantibody (NS). In diffuse cutaneous SSc (dcSSc) 4 of 60 patients (6.7 %) developed malignant tumors, in limited cutaneous SSc (lcSSc) 9 out of 141 (6.4 %). In the group of the 207 SLE patients only three malignant tumors (1.4 %) were detected.

Conclusions: Malignant tumors were more likely to occur in early DM and in both subsets of SSc indicating that in early lcSSc there also be present some risk for a simultaneous development of malignancy. In SSc the occurrence of malignant tumours was only slightly increased in patients with antitopoisomerase I antibody positivity compared to the rest of the cases. In DM and PM the elevated anti-Jo-1 antibody in the sera is definitely not associated with the presence of early development of malignant tumors. In early PM patients the risk for development of a tumor is low. In early lupus paraneoplastic syndrome seems to be also rare.

### **20. Mice expressing a conditional fibroblast-specific, constitutively active TGF- $\beta$ type I receptor ( $T\beta RI^{CA}$ ) recapitulate histological and biochemical features of the human disease systemic sclerosis**

*Sonali Sonnylal<sup>1</sup>, Christopher Denton<sup>2</sup>, Bing Zeng<sup>1</sup> and*

*Benoit de Crombrughe<sup>1</sup>: 1UT M. D. Anderson Cancer Center, Dept. of Molecular Genetics, Houston, Texas; 2Royal Free and University College Medical School, London, England*

Scleroderma or systemic sclerosis (SSc) is a disorder of the connective tissues affecting various organ systems. The disease is complex and is characterized by excessive accumulation of collagen and other extracellular matrix components in systemic organs. Increased signaling by TGF- $\beta$  has been implicated in the disease process. To establish a mouse model of Scleroderma, we have disrupted the TGF- $\beta$  signaling pathway in fibroblasts of transgenic mice. This was achieved after completion of embryonic development using the Cre-Lox system in order to circumvent the embryo lethality caused by this disruption. DNA for a constitutively active TGF- $\beta$ RI ( $T\beta RI^{CA}$ ) DNA was inserted 3' to a transcription stop cassette flanked by *Lox-P* sites and targeted to the ubiquitously expressed ROSA 26 locus in mice. These mice were crossed with transgenic mice encoding Cre-recombinase fused to a mutant ligand-binding domain of the estrogen receptor under control of a fibroblast-specific *Colla2* promoter/enhancer. Expression of  $T\beta RI^{CA}$  was turned on in offspring two weeks after birth by administering tamoxifen.

The phenotype of tamoxifen-treated *Colla2-CreER*,  $T\beta RI^{CA}$  mice were compared with age-matched controls treated with oil. Histological examination of skin biopsies showed pronounced and

generalized fibrosis, increase in total collagen content, loss of subcutaneous fat and a decrease in the number of hair follicles. In addition to the skin, thickening of small blood vessel walls was also observed in lung and kidney. In vitro analysis with primary skin fibroblasts showed elevated expression of downstream TGF- $\beta$  targets including CTGF, Timp-1, Collagen type I, fibronectin, osteopontin, and Smad 7. Transgenic fibroblasts from tamoxifen injected mouse also showed elevated basal expression of TGF- $\beta$  regulated promoters, PAI-1 and TP-3. The results strongly suggest that constitutive activation of TGF- $\beta$  signaling in fibroblastic cells of mice causes downstream activation of TGF- $\beta$  responsive genes resulting in a fibrotic phenotype with clinical features similar to those found in patients with SSc. These mice should be excellent models to test therapies aimed at correcting TGF- $\beta$  signaling in scleroderma.

## 21. Digital botox injections for non-healing scleroderma associated ulcerations

Marc R. Pritzker, MD; Paul Lim, MD; Adam Lokeh, MD, Allen Van Beek, MD

Minneapolis Heart Institute Foundation and the Divisions of Cardiology and Plastic Surgery, University of Minnesota Medical School

Patients with CREST variant scleroderma may develop refractory digital ulcerations, thereby becoming candidates for digital sympathectomy. Botox has been shown to block sympathetic nerve induced vascular smooth muscle contraction as well as norepinephrine release at the neuromuscular junction. Seven patients with persistent digital ulcerations refractory to treatment with prostacyclin, plavix, aspirin, folate, B12, B6, pletal, and sildenafil alone or in combination underwent palmar injection of Botox. Botox was injected immediately adjacent to the common and superficial digital arteries, and the palmar arch. Only one hand was treated in each patient. Ulcer healing was accomplished in all patients within two months (range 27 to 55 days). Digital temperature rose in the injected hand by an average of 2.1 degrees. All patients experienced relief of rest pain. There were no systemic complications. Two patients experienced mild grip weakness. All patients had a greater than 75% reduction in the frequency of vasospastic episodes. We suggest that peri-arterial injection of Botox produces a local, pharmacologic sympathectomy, which may allow for the healing

## 22. Effect of endothelin-1 to CD40 expression on skin fibroblasts

Jae-Bum Jun, Young In Na, Jae-Won Choi

Division of Rheumatology, The Hospital for Rheumatic Diseases, Hanyang University, Seoul, Korea

**Background:** Systemic sclerosis (SSc) is a chronic inflammatory disease characterized by fibrosis and resultant multiple organ damage. CD40/CD40L system has been known to be involved in the pathogenesis of various fibrotic diseases. We and others have shown that CD40 expression is elevated in SSc skin fibroblasts. Interferon gamma (IFN- $\gamma$ ) is the well-known powerful

stimulator of CD40 expression. However, IFN- $\gamma$  expression in involved skin of SSc is not elevated.

**Objectives:** The objective of this study is to investigate the effect of endothelin-1 (ET-1) to CD40 expression on skin fibroblasts as a potential upregulator.

**Methods:** We performed FACS analysis for CD40 expression on cultured normal skin fibroblasts (#CCL-110 from ATCC) after stimulation with ET-1, TNF- $\alpha$ , and IFN- $\gamma$  as a positive control.

**Results:** IFN- $\gamma$  upregulated CD40 expression, but various concentration of ET-1 did not elevate its expression. Moreover, pretreatment with ET-1 or simultaneous ET-1 did not stimulate IFN- $\gamma$ -induced CD40 expression.

**Conclusion:** ET-1 does not seem to have a role of upregulation of CD40 expression on SSc skin fibroblasts. We need to study this issue with adult normal and SSc skin fibroblasts. Furthermore, other potential stimulators will be needed to be studied.

## 23. c-Myb protooncogene plays a key role in type I collagen gene regulation in chicken UCD200

Jae-Bum Jun, Young In Na, Jae-Won Choi

Division of Rheumatology, The Hospital for Rheumatic Diseases, Hanyang University, Seoul, Korea

c-Myb is a transcription factor that exerts a pivotal role in the differentiation and proliferation of hematopoietic cells (1). Recently, we have demonstrated that *c-myb* gene is involved in the regulation of type I collagen gene expression also in fibroblasts. In fact, *c-myb*, is able to transactivate mouse, rat (2) and human type I collagen gene promoters (3). Moreover, mouse fibroblasts derived from *c-myb*<sup>-/-</sup> embryos do not express type I collagen gene (3) and type I collagen is not expressed in the *whole-mount c-myb*<sup>-/-</sup> embryos. In conclusion, the studies we have conducted so far have demonstrated that *c-myb* gene exerts an important physiologic role in fibroblast regulating type I collagen gene expression and since it is over-expressed in scleroderma (SSc) fibroblasts cultured in serum-deprived medium (4) and in SSc skin, *c-myb* may have a key role in maintaining the augmented production of collagen in scleroderma.

In the present work we show that *c-myb* expression also plays a key role in the regulation of type I collagen gene expression in fibroblasts from UCD200 chickens, which represents one of the animal models of scleroderma. As in fibroblasts from SSc patients, *c-myb* gene displayed an increased expression in embryonic UCD200 chicken fibroblasts when compared to embryonic *wt*-chicken fibroblasts. Since chicken *c-myb* exhibits an extensive homology to human *c-myb*, the same siRNAs anti-*c-myb* were used to treat embryonic UCD200 chicken fibroblasts and human SSc skin fibroblasts, obtaining a significant reduction of type I collagen gene expression in both cells. In conclusion, *c-myb* gene is upregulated in UCD200 chicken fibroblasts also, and its expression plays a key role in maintaining type I collagen expression either in UCD200 chicken fibroblasts and SSc human skin fibroblasts.

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## 24. South Australian Scleroderma Register—

### Ten year review

*P.J. Roberts-Thomson, J.G. Walker, S.R. Cox, M.D. Smith, M.J. Ahern and other participating SA Rheumatologists.*

*Department of Immunology, Allergy & Arthritis, Flinders Medical Centre, Bedford Park, SA*

The population-based South Australian Scleroderma Register was established in 1993 to determine the prevalence, incidence, mortality, and disease characteristics of scleroderma in SA (state population of  $1.5 \times 10^6$ ). We have continued to systematically identify new patients over the last 10 years and now report our conclusions concerning scleroderma.

Mean prevalence of  $21.4$  per  $10^5$  (95% C1 20.2-22.6).

Mean cumulative incidence of  $1.50$  per  $10^5$  (95% C1 1.32-1.73) with a trend to increase over the period 1993-2002 but not at a significant level ( $P=.13$ ).

Cumulative survival has improved. Diffuse disease and male gender have significantly reduced survival as compared with limited disease and female gender.

Proportion with diffuse disease (~20%) has remained steady. small but significant ( $P<.001$ ) predisposition in patients with European birth place.

Family history of scleroderma in 1.6% with  $\Lambda 1$  (familial risk) of 14.3 (95% C1 5.9-34.5). However a family history of systemic autoimmunity (especially rheumatoid arthritis) was more common (6.0%).

Occurs most commonly in women involved with home duties ( $P=0.01$ , in comparison with ABS data).

No identified geographic predisposition.

No spatiotemporal association either at time of initial symptom or at 10 years prior to onset.



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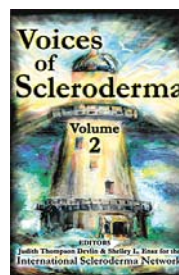
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## 25. Six minute walk test (6MWT) in interstitial lung disease in systemic sclerosis (SSc): Preliminary results from the BUILD2 Trial

James R. Seibold<sup>1</sup>, Carol M Black, Christopher P. Denton<sup>2</sup>, Daniel E. Furst<sup>3</sup>, Loic Guillevin<sup>4</sup>, Vivien M. Hsu<sup>1</sup>, Pascal Charef, Harbajan Chadha-Boreham, Sebastien Roux<sup>5</sup>, Joseph H. Korn<sup>6</sup> for the BUILD2 Study Group and the Scleroderma Clinical Trials Consortium.

1 UMDNJ New Brunswick, New Jersey; 2 Royal Free Hospital, London, UK; 3 UCLA, Los Angeles, California; 4 Hopital Cochin, Paris, France; 5 Actelion Pharmaceuticals, Allschwil, Switzerland; 6 Boston University, Boston, Massachusetts

The six minute walk test (6MWT) is an established measure of exercise capacity in studies of pulmonary hypertension and serves as a surrogate for WHO classification, hemodynamics and survival. The 6MWT has also been employed as a measure of outcome in investigations of interstitial lung disease (ILD). There has been no validated experience in SSc. BUILD2 is a multicenter randomized placebo controlled comparison with bosentan in the treatment of ILD secondary to SSc. The scientific rationale is based on the prominent profibrotic and proinflammatory effects of endothelin. This trial is in progress and seeks 132 subjects with SSc and exertional dyspnea. Subjects with disease <3 yrs duration must have significant ILD on HRCT, 6MWT  $\pm$ 150 m or <500 m and DLCO <80%. Subjects >3 yrs SSc duration must have worsening pulmonary function, increasing dyspnea, new areas of ILD on HRCT or alveolitis by bronchoalveolar lavage. These characteristics serve to identify a population of "active" ILD at risk of clinical and physiologic worsening. Pulmonary hypertension (estimated PASP >50 mm Hg by echo) is an exclusion. 6MWT data are available on the initial 35 subjects including 10 with limited and 25 with diffuse SSc. Subjects are 51.4 yrs old (SD 10.2 yrs) with disease duration of 6.7 yrs (SD 7.3 yrs, range 2-38 years). Screening 6MWT test was 403 m  $\pm$  89 and repeat test prior to randomization was 394 m  $\pm$  93. The mean difference between studies was 9 m  $\pm$  30. Inter-test correlation was 0.94 by Pearson. 6MWT were highly correlated with end of test patient ratings of dyspnea (Borg Dyspnea Index) ( $R = 0.83$ ,  $P < .0001$ , Pearson) but not with % predicted DLCO ( $R = -0.19$ ) or % predicted FVC ( $R = 0.07$ ) (both comparisons NS). These data show that patients with SSc can perform 6MWT. 6MWT test has high within-patient consistency and correlates closely with clinical ratings of dyspnea. Evaluation of the 6MWT as a measure of outcome will require longitudinal data.

## 26. Ultrasound measurement of skin thickness correlates with skin hardness and clinical skin score

Eugene Y Kissin, Aileen M Schiller, Robert W Simms, Joseph H Korn, Peter A Merkel.

Boston University School of Medicine, Boston, Massachusetts

**Purpose:** To determine whether dermal thickness, as measured by ultrasound, correlates with dermal hardness and clinical skin score in patients with systemic sclerosis.

**Methods:** Participants of this study included 20 women and

4 men with systemic sclerosis (14 with diffuse; 10 with limited skin disease, age 50 $\pm$ 10 years, weight 68 $\pm$ 20 kg), and 9 controls (all women, age 40 $\pm$ 11 years, weight 81 $\pm$ 15 kg). Skin thickness was measured by portable, high-resolution ultrasonography (Titan 10Mhz linear array transducer), skin hardness was measured by digital durometry (Rex Gauge Type OO), and skin involvement was measured semiquantitatively by 8 site (scored 0-3 at each) modified Rodnan skin scoring. Sites of skin assessment included the dorsum of the third phalanx, dorsum of the mid hand, dorsum of the mid forearm, and dorsum of the mid upper arm. Associations between ultrasound, durometry and clinical measurements were made by Pearson correlations.

**Results:** Correlation between ultrasound-measured skin thickness and durometer-measured skin hardness was high in the hands, forearms, and upper arms ( $r=0.70$ ,  $0.65$  and  $0.67$ ,  $P < .01$ ) but low in the fingers ( $r=0.10$ ,  $P=.51$ ). Correlations between ultrasound and skin score, and durometer and skin score were also high ( $r=0.64-0.78$ ,  $P < .01$ ) for all regions but the fingers ( $r=0.24$ ,  $0.25$ ,  $P=.08$ ,  $0.09$ ). Average skin thickness and skin hardness increased with increasing skin scores for all regions studied. There was a weak inverse correlation between disease duration and both total skin ultrasound measured skin thickness ( $r=-0.38$ ,  $P=.07$ ) and total clinical skin score ( $r=-0.40$ ,  $P=.05$ ) but not between disease duration and total durometer measured skin hardness ( $r=-0.25$ ,  $P=.25$ ).

**Conclusions:** In subjects with systemic sclerosis, skin thickness as measured by ultrasound is highly correlated to skin hardness and clinical skin score. The weaker associations in the fingers are likely due to the proximity of dermis to the underlying bone, making skin hardness and clinical skin score difficult to assess. Our data also suggest that disease duration correlates with skin thinning but not skin softening. Prior work has shown both durometry and ultrasound to have higher reliability than clinical skin score. Ultrasound and durometry are simple to learn and perform, and allow skin thickness and hardness to be measured on a continuous scale at the bedside. These tools provide complementary information about the degree of skin involvement by systemic sclerosis and could be used to monitor response to treatment in clinical trials.

## 27. Validity, reliability, and feasibility of durometer measurements of scleroderma skin disease in a multicenter treatment trial

Peter A Merkel<sup>1</sup>, Nancy Silliman<sup>2</sup>, Christopher P Denton<sup>3</sup>, Philip J Clements<sup>4</sup>, Paul Emery<sup>5</sup>, Daniel E Furst<sup>4</sup>, Vivien M Hsu<sup>6</sup>, Richard P Polisson<sup>2</sup>, James B Streisand<sup>2</sup>, James R Seibold<sup>6</sup>, Carol M Black<sup>3</sup>, and Joseph H Korn<sup>1</sup> for the CAT-192 Research Group and The Scleroderma Clinical Trials Consortium

1Boston University, Boston, MA; 2Genzyme Corporation, Cambridge, MA; 3Royal Free Hospital, London, United Kingdom; 4UCLA, Los Angeles, CA; 5University of Leeds, Leeds, United Kingdom; 6UMDNJ, New Brunswick, NJ

**Purpose:** Determine the validity, reliability, and feasibility of

durometer (DUR) measurements of skin hardness as an outcome measure for scleroderma (SSc) when compared to skin scoring and patient self-assessment tools.

**Methods:** The DUR was tested during a multicenter treatment trial for scleroderma. Skin *hardness* was measured using handheld digital DURs (Rex Gauge Type OO) with a continuous scale; 3 readings were taken at each of 6 skin sites (forearms, thighs, legs). Skin *thickness* was measured by 17-site (scored 0-3 at each) modified Rodnan skin scoring (MRSS). Investigators were trained in both DUR and MRSS techniques. Other outcome data collected included the Scleroderma Health Assessment Questionnaire (SHAQ). In a reliability exercise in advance of the trial, 9 investigators examined the same 5 patients with SSc by MRSS and DUR.

**Results:** Forty-three patients (33 women) with early diffuse cutaneous SSc were studied at 11 international centers: mean age 49 years (range 24-76); median duration of disease 6.4 months (0.3-23); median baseline MRSS = 22 (11-38). Investigators found the DUR simple and quick to use. The reliability of DUR measurements was extremely high, with interobserver intraclass correlations (ICC) = 0.82 (forearms), 0.82 (thighs), 0.91 (legs), 0.92 (6 sites) each result the same or higher than the corresponding ICCs for MRSS: 0.82 (forearms), 0.54 (thighs), 0.85 (legs), 0.84 (6 sites). DUR scores highly correlated with 17-site MRSS throughout the trial:  $r=0.69$  (baseline),  $0.70$  (month 3),  $0.70$  (month 6),  $P<.001$ . Correlations were similarly high at each of the 6 individual DUR sites or using a combined MRSS-6 measure as well as throughout the spectrum of total skin scores. DUR scores within a given skin score ranged widely, indicating durometry may provide a greater dynamic range than MRSS. *Change* in DUR scores was highly correlated with both improving and worsening *change* in MRSS:  $r=0.70$ ,  $P<.0001$  ( $N=32$ ). DUR scores also correlated with patient self-assessments of skin disease ( $r=0.69$ ,  $P<.0001$ ) and HAQ disability scores ( $r=0.34$ ,  $P=.03$ ).

**Conclusions:** DUR measurements of skin hardness in SSc are reliable, simple, accurate, and demonstrate good sensitivity to change compared to traditional skin scoring. DUR scores also reflect patient self-assessments. Durometry may offer an increased range of values for skin assessment in SSc compared to semi-quantitative MRSS. DUR measurements are valid, objective, and scalable, and should be considered for use as a complementary outcome measure to skin scoring in clinical trials of SSc.

## 28. Multiple potential levels of cross-talk between monocyte chemoattractant protein-3 (MCP-3) and TGF $\beta$ 1 in fibrosis

Voon H Ong, Maresa Carulli, Gisela E Lindahl, Xu Shiwen, David J Abraham, Carol M Black, Christopher P Denton  
Royal Free Hospital, London, United Kingdom

**Purpose:** The  $\beta$  chemokine MCP-3 is highly overexpressed in early diffuse cutaneous scleroderma (SSc) and the type 1 tight skin mouse model. We have examined potential cross-talk between MCP-3 and TGF $\beta$ 1 in driving fibrosis.

**Methods:** We have investigated the protein expression in

fibroblasts cultured from a transgenic mouse strain (TBR11 $\Delta$ k-fib) with a constitutive TGF $\beta$  activated phenotype and we have previously shown that the fibroblasts demonstrate biochemical properties similar to scleroderma fibroblasts. Transcriptional activation of MCP-3 was examined using a 1kb promoter fragment subcloned into pSEAP2-Basic reporter vector and transiently transfected into neonatal fibroblasts. Later the effect of TGF $\beta$ 1 on MCP-3 promoter activity in transgenic or littermate wildtype fibroblasts was assessed. Interplay between downstream signaling pathways regulated by MCP-3 and TGF $\beta$ 1 was examined using pharmacological inhibitors to modulate activation of a transiently transfected minimal pro $\alpha$ 2(I)collagen (*COL1A2*) promoter. A similar strategy was used to characterise TGF $\beta$ 1 dependent activation of the MCP-3 promoter. All experiments were performed in triplicate and cotransfected control reporter plasmids were used to correct transfection efficiency.

**Results:** Western blot confirmed MCP-3 is inducible by TGF $\beta$  in cultured wildtype fibroblasts. Time-course analysis revealed significant activation (mean  $\pm$  SEM % basal expression) of the MCP-3 promoter by recombinant TGF $\beta$  (4 ng/mL) from 24 hours, maximum at 48 hours in wildtype fibroblasts ( $232 \pm 79\%$ ). Although there was basal promoter activation by TBR11 $\Delta$ k-fib transgenic fibroblasts ( $157 \pm 57\%$ ), there was no further upregulation by TGF $\beta$ . This TGF $\beta$ -induced activation (Mean  $\pm$  SEM % basal) at 30 hours ( $336 \pm 28\%$ ,  $P=.02$ ) was inhibited by U0126, a direct inhibitor of p42/44 MAPK activity ( $69.2 \pm 15.1$ ,  $P=.04$ ). A series of independent transient transfection studies showed activation of the minimal *COL1A2* promoter in wildtype fibroblasts by recombinant MCP-3 ( $136 \pm 16\%$ ) and this was inhibited by SB203580 ( $44.8 \pm 7.7\%$ ,  $P=.02$ ), a p38 MAPK inhibitor. This was comparable to inhibition of TGF $\beta$ -dependent activation by SB203580 ( $65.4 \pm 4.1\%$ ,  $P=.03$ ). Time-course profile of activation of p38 MAPK by MCP-3 showed a sustained response in transgenic fibroblasts and an early response from 20 to 120 minutes in wildtype fibroblasts. In contrast, there was a delayed and sustained phosphorylation of p38 MAPK by TGF $\beta$  in wildtype fibroblasts with activation from 2 hour to 24 hours.

**Conclusions:** These results confirm that upregulation of MCP-3 by TGF $\beta$ 1 is mediated primarily via the p42/44 MAPK pathway. Conversely, p38 MAPK appears more important in regulating collagen expression by these two cytokines.

## 29. Clinical and pathological significance of monocyte chemoattractant protein-1 ligand-receptor axis upregulation in scleroderma

M. T Carulli, V. Ong, M. Ponticos, X. Shiwen, D.J. Abraham, C.M. Black, C.P. Denton  
Centre for Rheumatology, Royal Free Hospital, London, UK

**Background:** Fibroblast derived monocyte chemoattractant protein-1 (MCP-1 or CCL2) is a candidate mediator linking inflammatory and fibrotic processes in scleroderma (SSc). The present study addresses the hypothesis that MCP-1 is a key mediator of intercellular cross-talk in the pathogenesis of early stage SSc and that fibroblasts are a target for activation for this cytokine.

**Methods:** Serum samples and skin biopsies were examined from 64 patients with SSc and 12 healthy controls. Of our cohort of patients 30 had early (within 2 years from onset) diffuse cutaneous SSc (dcSSc), 14 late dcSSc and 20 limited cutaneous SSc (lcSSc). Levels of MCP-1 in serum were measured by commercial ELISA. Expression of MCP-1 and its major receptor CCR2 in snap-frozen skin biopsies was determined by immunohistochemistry. Co-localisation studies were performed to identify the cell types expressing MCP-1. Expression of chemokine receptors (CCR2, CXCR2 and CCR5) on 9 SSc and 9 normal fibroblasts cell lines was determined by flow cytometry.

**Results:** Immunohistochemistry confirmed strong expression of MCP-1 and CCR2 in skin from patients with early stage dcSSc, but expression in late-stage dcSSc and lcSSc was minimal. In particular MCP-1 co-localised with endothelial cells, myofibroblasts, macrophages and T lymphocytes. Furthermore only SSc fibroblasts cell lines from early stage disease showed expression of CCR2 (5 of 9 lines) and CXCR2 (6 of 9). No fibroblast lines expressed CCR5. MCP-1 serum levels (mean±sem) were significantly elevated in SSc patients (380±16 pg/mL) compared to controls (132±26 pg/mL,  $P = .002$ ) and were highest in the early dcSSc subset (557±61 pg/mL) compared to late-stage disease (307±41 pg/mL,  $P = .01$ ) or lcSSc (276±28 pg/mL,  $P = .0007$ ). Analysis of clinical and serological characteristics of patients with early dcSSc showed association of elevated serum levels of MCP-1 defined as >3SD above mean serum levels of healthy controls (or >401 pg/mL), with hallmark antibodies of diffuse disease.

**Conclusions:** Colocalisation of MCP-1 ligand with surface markers of cell types known to be activated in SSc is consistent with a key role in pathogenesis. Furthermore selective upregulation of CCR2 on SSc fibroblasts supports the hypothesis that MCP-1 may be a key activator in early-stage disease. MCP-1/CCR2 ligand-receptor axis antagonism may be a potential future therapeutic strategy for early stage disease.

### 30. Co-stimulation with ET-1 and thrombin significantly enhances matrix remodeling in lung fibroblasts: Implications for scleroderma lung fibrosis

*X Shiwen<sup>1</sup>, A Leask<sup>1</sup>, E Renzoni<sup>2</sup>, CP Denton<sup>1</sup>, M Eastwood<sup>3</sup>, R Stratton<sup>1</sup>, A Holmes<sup>1</sup>, R duBois<sup>2</sup>, DJ Abraham<sup>1</sup>, CM Black<sup>1</sup>*  
*1Centre for Rheumatology, Royal Free and University College Medical School, London. 2Interstitial Lung Disease Unit, Imperial College School of Medicine, London. 3Centre for Tissue Engineering Research, University of Westminster, London*

**Purpose:** Endothelin-1 (ET-1) is a potent endothelial cell-derived vasoconstrictor. In addition, ET-1 promotes matrix remodeling during tissue repair. Thrombin (TH) also appears to play an important role in influencing tissue repair and fibrosis outside the vasculature. We and others have shown that activation of protein kinase C epsilon (PKCε) is involved in ET-1 and TH signal transduction. ET-1 and TH are both increased in bronchoalveolar lavage fluid in pulmonary fibrosis associated with scleroderma (FASSc). In this study, we investigate the effects of co-stimulation

with ET-1 and TH in lung fibroblasts and the signalling pathways involved.

**Methods:** Pulmonary fibroblasts (PF; n=5) were obtained from control and FASSc lung tissue. PKCε wild type (WT) and knock out (KO) mice fibroblasts were cultured from animal skin. Protein was extracted from lung fibroblast samples treated and untreated with ET-1 (100 nM) and TH (2-4U). Proteins including α-smooth muscle actin (SMA), connective tissue growth factor (CTGF) and collagen were examined by immunocytofluorescence staining and/or Western blot analysis. The ability of ET-1 and TH to induce matrix remodeling in both floating and fixed 3D collagen contraction models was examined.

**Results:** FASSc lung fibroblasts showed enhanced ability to contract a 3-D collagen matrix. Although ET-1 and TH individually induced growth factor production (including CTGF) and alpha-SMA expression, together ET-1 and TH synergized to enhance these activities. Furthermore, human lung fibroblast treated with ET-1 and TH promoted gel contraction (control; 182.46±13.62 vs ET-1; 87.19±26.13 vs TH; 91.47±19.05 vs ET-1/TH; 62.36±16.54 ( $P < .05$ )). Similar results were obtained using WT mouse fibroblasts ( $P < .01$ ), whereas the effect was significantly diminished in PKCε KO mouse fibroblasts ( $P > .05$ ).

**Conclusions:** FASSc lung fibroblasts show increased ability to contract a collagen matrix. ET-1 and TH synergized to enhance matrix remodeling and expression of the pro-contractile protein α-SMA by lung fibroblasts. The enhanced gel contraction may be due, at least in part, to signaling through PKCε. Thus, selective modulation of kinase cascades would seem to have utility in targeting specific aspects of the fibrotic phenotype.

### 31. Anti-TGFβ1 therapy for diffuse cutaneous systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT192

*Christopher P Denton<sup>1</sup>, Peter A Merkel<sup>2</sup>, Daniel E Furst<sup>3</sup>, Dinesh Khanna<sup>3</sup>, Paul Emery<sup>4</sup>, Vivien M Hsu<sup>5</sup>, Nancy Silliman<sup>6</sup>, James Streisand<sup>6</sup>, John Powell<sup>7</sup>, Joseph H Korn<sup>2</sup>, Carol M Black<sup>1</sup>, James R Seibold<sup>5</sup> with CAT192 Study Group and SCTC*

*1Royal Free Hospital, London, United Kingdom; 2BUSM, Boston, MA; 3UCLA, Los Angeles, CA; 4Leeds University, Leeds, United Kingdom; 5UMDNJ, New Brunswick, NJ; 6Genzyme Corporation, Boston, MA; 7CAT, Cambridge, United Kingdom*

**Purpose:** TGFβ, overactivity is implicated in systemic sclerosis (SSc) pathogenesis. We report a phase I/II trial of CAT192, a human recombinant IgG4 antibody that neutralizes TGFβ1, in early stage diffuse cutaneous (dc)SSc.

**Methods:** Forty-five subjects within 18 months of SSc onset were enrolled from 11 centers in the USA and Europe and randomly allocated to one of four treatment arms: 10 mg/kg, 5 mg/kg, 0.5 mg/kg or placebo infused on day 0 and weeks 6, 12, and 18. Primary outcomes were safety and pharmacokinetics (PK). Secondary outcomes included modified Rodnan skin score

(mRSS), durometer measures of skin hardness, SSc health assessment questionnaire, assessment of organ-based disease, and biomarkers.

**Results:** The primary end-points were achieved as treatment-related morbidity was undetectable and PK parameters were established. There were 4 deaths, 1 in the 0.5 mg/kg and 3 in the 5 mg/kg group, none attributable to treatment. Serious adverse events occurred in 13 subjects, including 2 receiving placebo. A total of 275 adverse events occurred in 42 subjects. Biologically active serum levels (>10 µg/mL) of CAT192 were confirmed in treatment groups with a half-life (mean±se) of 24.0±2.1 days. No secondary outcome showed significant change among treatment groups. The table summarizes mRSS data throughout the trial. Improvement in mRSS associated significantly with disease duration ( $P = .0008$ ). TGFβ1 and β2 mRNA levels were increased in affected (twofold) and clinically unaffected (1.6 fold) SSc skin ( $P = .011$  and  $P = .002$  respectively) compared with control biopsies. Serum level of N-terminal procollagen peptide (PINP, µg/L) was greater in SSc (8.5±3.5) than controls (5.1±2.4; n=100,  $P < .0001$ ). Although change in PINP correlated with change in skin score ( $r=0.37$ ,  $P = .027$ ), there was no treatment effect for any biomarker. Levels of soluble IL2 receptor (ng/L) were elevated in SSc (1.8±0.9) versus controls (0.8±0.3;  $P < .0001$ ) and did not change with treatment.

**Conclusion:** Systemic administration of CAT192 in patients with dcSSc is safe and well tolerated. The study was not powered to determine efficacy. Therefore although mRSS improved more in the 5 or 10 mg/kg subgroups, this may reflect longer disease duration at baseline. Additionally, the feasibility of multicenter trials in early dcSSc is confirmed.

	CAT192 dose (mg/kg)			
Median (range)	Placebo	0.5	5	10
Disease duration: Months	5.9 (1, 23)	4.6 (1, 14)	7.2 (1, 17)	9.4 (2, 16)
Baseline mRSS: Units	25 (11, 38)	21 (14, 27)	23 (14, 30)	24(13,30)
Change in mRSS: Units	-1 (-12, 9)	3 (-9, 14)	-3 (-10, 12)	-4 (-17, 6)
Percentage of baseline	-4.5 (-60, 31)	14.3 (-64, 70)	-13.0 (-67, 52)	-21.1 (-57, 31)

**Disclosure:** The authors have received research funding, been paid consultants, or are employees of Genzyme Corp (Mass, USA) or CAT (Cambridge UK).

### 32. Racial disparities in patients with pulmonary hypertension associated with systemic sclerosis

Marcy B. Bolster, MD, Paul J. Nietert Ph. D., Stephanie Shaftman, MSc. MS, Holly Mitchell, MD, Richard M. Silver, MD, Medical University of South Carolina, Charleston, South Carolina

**Purpose:** We evaluated racial disparities in the presentation,

detection, and management of pulmonary hypertension (PAH) in a cohort of patients with systemic sclerosis (SSc).

**Methods:** Encounters of patients with SSc seen at the Medical University of South Carolina are recorded in a computerized database. PAH was defined as having a peak right ventricular systolic pressure (PRVSP) >30 mm Hg on echocardiogram (ECHO), or a pulmonary artery pressure (PAP) >25 mm Hg on right heart catheterization (RHC). Disparities between Caucasian (Cauc) and African-American (AA) patients with SSc were assessed by examining encounters from 11/97 through 1/04.

**Results:** There were 788 encounters from 328 patients enrolled in the database, including 245 Cauc, and 83 AA patients. There were no differences in the sub-types of SSc between ethnic groups. Of the 245 Cauc patients, 105 (42.9%) had diffuse cutaneous (dcSSc) and 105 (42.9%) had limited cutaneous (lcSSc) disease. There were 14 (5.7%) SSc sine scleroderma, and 21 (8.6%) uncertain (lcSSc disease associated with positive Scl-70 antibody, tendon friction rubs, and/or nailfold capillaroscopy with “active” changes). Of the 83 AA patients, 58 (69.9%) had dcSSc and 12 (14.5%) had lcSSc disease. There were 3 (3.6%) SSc sine scleroderma and 10 (12.1%) uncertain patients. An ECHO had been performed in 177 (72.2%) of the Cauc patients and in 59 (71.1%) of the AA patients. A RHC was performed in 18 (7.4%) of the Cauc patients and in 5 (6%) of the AA patients. PAH was present in 82 (33.5%) of the Cauc patients and in 34 (41%) of the AA patients. AA patients presented with PAH at a younger age (47.3 years vs 59.3 years for Cauc,  $P < .0001$ ) and had a shorter disease duration from non-Raynaud’s onset of symptoms until diagnosis with PAH (5.8 years vs 8.5 years in Cauc,  $P = .057$ ). On physical examination, there was no difference in the presence of an increased P2 (19.5% of the Cauc PAH patients vs 17.7% of the AA PAH patients). A disparity was noted in the presence of lower extremity edema (48.8% Cauc vs 20.6% AA patients,  $P = .005$ ); however, there was no difference in the number of patients having ECHO evidence of right ventricular dilatation (17.5% of Cauc vs 21.2% of AA patients). There were no significant differences in the pulmonary function test results in Cauc and AA patients with PAH, in terms of forced vital capacity (FVC) (mean 77.3% predicted in Cauc vs 72.3% predicted in AA), diffusion capacity (DLCO) (62.6% predicted in Cauc vs 54.6% predicted in AA), or FVC/DLCO ratio >1.6 (25.4% Cauc vs 34.6% AA). There was no significant difference in the number of patients with PAH having an FVC <70% (an estimation of the presence of underlying interstitial lung disease) (36.2% of Cauc and 48.2% of AA with PAH). There were no disparities between Cauc and AA patients in terms of treatment of PAH, including home oxygen (24.4% Cauc vs 17.7% AA patients with PAH), Coumadin (15.9% Cauc patients vs 14.7% AA patients), calcium channel blockers (61% Cauc vs 76.5% AA patients), intravenous prostacyclin (Flolan) (4.9% Cauc patients vs 5.9% AA patients), and bosentan (Tracleer) (12.2% Cauc patients vs 8.8% AA patients).

**Conclusions:** In this cohort, the proportion of AA patients with SSc that have PAH appears to be quite high (41%). The AA patients presented at a significantly younger age and with a short-

er disease duration from onset of symptoms to time of diagnosis of PAH. Although, the Cauc patients had a higher incidence of lower extremity edema, they did not have an increased incidence of right ventricular dilatation (a sign of right heart failure); thus this physical finding disparity is of unknown significance. There were neither ethnic disparities found in the studies that were obtained to detect PAH, nor in the treatment of PAH.

### 33. Diminished TGF- $\beta$ 2 production leads to increased expression of a profibrotic procollagen- $\alpha$ 2 type I mRNA variant in embryonic fibroblasts of UCD-200 chickens, a model for systemic sclerosis

*Martina Prelog, Georg Wick, and Roswitha Sgonc*

*Institute of Pathophysiology, University of Medicine, Innsbruck, Austria*

**Objective:** In a previous *ex vivo* study we have identified an  $\alpha$ 2(I)-mRNA variant, that is increased during early, acute scleroderma-like disease in University of California at Davis line 200 (UCD-200) chickens. This  $\alpha$ 2(I)-mRNA transcript is represented by a 115bp band in RNase protection assays (RPA), whereas the expected band is 180bp in size. The aim of the present study was to investigate the influence of cytokines on the expression of these two  $\alpha$ 2(I)-mRNA variants.

**Methods:** Chicken embryonic fibroblasts (CEF) of UCD-200 and normal White Leghorns (NWL) were grown in monolayer or 3-dimensional collagen gels. Procollagen-mRNA expression was analyzed by RPA, proliferation by  $^3\text{H}$ -thymidine incorporation. TGF- $\beta$ 1 and TGF- $\beta$ 2 in culture supernatants was measured by ELISA.

**Results:** In collagen gels, UCD-200-CEF expressed 7.2 times more of the smaller profibrotic  $\alpha$ 2(I)-mRNA variant than NWL-CEF, the latter lacking the 115bp band in - the less physiologic - monolayers completely. TGF- $\beta$ 1 stimulated the proliferation of UCD-200-CEF, but not NWL-CEF. The 115/180bp ratio was increased by TGF- $\beta$ 1 in both, NWL- and UCD-200-CEF. TGF- $\beta$ 2 and TGF- $\beta$ 3 reduced the expression of the profibrotic  $\alpha$ 2(I)-mRNA in UCD-200-CEF to the same levels seen in healthy control NWL-CEF. Moreover, TGF- $\beta$ 2 also reduced the 180bp transcript in UCD-200 and NWL-CEF, whereas TGF- $\beta$ 3 reduced the 180bp band only in NWL, but not in UCD-200-CEF. Interestingly, analysis of cell culture supernatants revealed that NWL-CEF produced 4.1 times more TGF- $\beta$ 2 than UCD-CEF.

**Conclusions:** We could show that TGF- $\beta$ 2 reduces the expression of a profibrotic  $\alpha$ 2(I)-mRNA variant in UCD-200-CEF. The constitutive overproduction of this  $\alpha$ 2(I)-mRNA variant and diminished TGF- $\beta$ 2 synthesis found in untreated UCD-200-CEF suggest that TGF- $\beta$ 2 is an antifibrotic cytokine and might be a key player during fibrosis onset. These results also may shed some light on the contradictory reports on TGF- $\beta$ 2 in human SSC.

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### 34. Sitaxsentan improves 6MW and hemodynamics in patients with pulmonary arterial hypertension (PAH) related to connective tissue disease (CTD)

*Vallerie V McLaughlin, University of Michigan, Ann Arbor, MI; Nicholas Hill, Tufts University, Boston, MA; Victor Tapson, Duke University, Durham, NC; Adaani Frost, Baylor College of Medicine, Houston, TX; David Langleben, The Sir Mortimer B. Davis - Jewish General Hospital, Montreal, PQ, Canada; Ronald Oudiz, UCLA School of Medicine, Torrance, CA; Shelley Shapiro, University of Southern California, Los Angeles, CA; Ivan Robbins, Vanderbilt University Medical Center, Nashville, TN; Robyn Barst, Columbia University, New York, NY; STRIDE-1 Study Group*

**Purpose:** PAH related to CTD is progressive and difficult to manage. In a randomized, controlled clinical trial, IV epoprostenol improved 6MW and hemodynamics in patients with PAH related to CTD. The multicenter, randomized, double-blind, placebo (PBO) controlled bosentan BREATHE-1 16 week PAH trial reported a trend towards a 6MW treatment effect in the CTD subgroup (47 of the 213 patients in BREATHE-1 had PAH related to CTD). However, this was due to deterioration in the PBO group rather than an improvement in the bosentan treatment group. Sitaxsentan (SITAX) is a selective (6500:1) once daily oral endothelin A receptor antagonist in clinical development for the treatment of PAH.

**Method:** The Sitaxsentan to Relieve Impaired Exercise trial (STRIDE-1) was a multicenter, randomized, double-blind, placebo-controlled, 12 week trial evaluating SITAX 100 mg, 300 mg, and PBO in 178 patients with PAH. A post hoc analysis was performed to evaluate the effect of SITAX in the intent-to-treat CTD subgroup (42 of the 178 patients had PAH related to CTD). Due to similar treatment effects in total ITT population, the SITAX 100 mg and 300 mg groups were pooled.

**Results:** At baseline (BL) all CTD patients were NYHA class II or III. Mean BL 6MW = distance was 356 meters; CI = 2.6 L/min/m<sup>2</sup>; RAPm = 8 mm Hg; PVR = 732 dyne.sec.cm<sup>-5</sup>; and PAPm 45 mm Hg. 6MW treatment effect was 58m ( $P = .0274$ ), due both to an increase in 6MW in the SITAX group from BL (+20 m;  $P = .0327$ ) and a decrease in 6MW in the PBO group from BL (-38m). Eight of 33 (24%) SITAX patients improved by one NYHA functional class on SITAX compared with 1/9 (11%) PBO patients. Hemodynamic treatment effects were observed in CI (+0.55 L/min/m<sup>2</sup>;  $P = .0007$ ); RAPm (-4.14 mm Hg;  $P = .0023$ ); PVR (-320 dyne.sec.cm<sup>-5</sup>;  $P = .0042$ ) and mPAP (-7.66 mm Hg;  $P = .0556$ ). SITAX was well tolerated. No patients experienced LFT abnormalities and no patients discontinued due to adverse events.

**Conclusion:** SITAX improves 6MW, NYHA functional class and hemodynamics in PAH related to CTD.

### 35. Myofibroblast phenotype and reduced expression of collagenase (MMP-1) in human fibroblasts overexpressing intracellular IL-1 receptor antagonist (icIL-1ra): Implications in the pathogenesis of fibrosis in systemic sclerosis.

Siva Kanangat, PhD, Karen Hasty, PhD, Gloria Higgins, MD, PhD, and Arnold Postlethwaite MD

Systemic sclerosis (SSc, Scleroderma) is an autoimmune disease with excessive deposition of matrix components (especially Type I collagen) in the extracellular tissue spaces. Fibroblasts explants grown from involved skin of patients with SSc express higher levels of intracellular IL-1 $\alpha$  and intracellular (ic) IL-1 receptor antagonist (icIL-1ra). We examined the relationship between the expression of matrix metalloproteinase (MMP-1)/collagenase, a potent enzyme involved in the degradation of collagen and icIL-1ra. In this report, we show enhanced expression of icIL-1ra, and reduced levels of MMP-1 in fibroblasts explanted from involved skin of patients with SSc. Therefore we postulate that icIL-1ra is involved in the downregulation of MMP-1 expression. To test this, we stably transfected normal human foreskin fibroblasts with a plasmid encoding icIL-1ra Type I. The transfected cells overexpressing icIL-1ra had very low basal levels of MMP-1 expression compared to control fibroblasts. When transfected cells overexpressing icIL-1ra were stimulated with IL-1 $\beta$ , TNF- $\alpha$ , or PMA, the levels of MMP-1 were significantly lower compared to the control cells transfected with plasmid alone. Pretreatment of transfected cells overexpressing icIL-1ra with antisense oligonucleotide directed against the sense mRNA of icIL-1ra restored MMP-1 expression upon stimulation with IL-1 $\beta$ . Therefore, enhanced expression of Type I icIL-1ra isoform is related to decreased expression of MMP-1 in fibroblasts from SSc patients and suggest that it may contribute to with subsequent accumulation of Type I collagen in the extracellular matrix.

**Purpose:** The aim of the present investigation was to determine the relationship between overexpression of icIL-1ra and MMP-1 production and thereby to better our understanding of the pathophysiology of systemic sclerosis (scleroderma).

**Materials and Methods:** Fibroblasts were derived from explant cultures of skin biopsies obtained from involved and uninvolved skin of scleroderma patients. For icIL-1ra overexpression studies, fibroblasts were derived from the foreskin of normal human beings. Fibroblasts in both cases were between 5-6 passages. Normal human foreskin fibroblasts were stably transfected with a plasmid encoding icIL-1ra (HF-icIL-1ra). Fibroblasts transfected with the plasmid alone (HF-Vector) served as control. Overexpression of icIL-1ra in transfected cells was assessed by RT-PCR and by ELISA. To determine effect of icIL-1ra on MMP-1 expression, HF-vector and HF-icIL-1ra were exposed to varying concentrations of IL-1 $\beta$ , TNF- $\alpha$  or PMA. Fibroblasts were harvested after 8-12 hours of stimulation for mRNA analysis of MMP-1 by Semiquantitative RT-PCR and ribonuclease protection assay (RPA) and 48 hours after stimulation for ELISA for MMP-1 protein. TIMP-1 and MMP-1 protein expression in stimulated and unstimulated SSc fibroblasts were examined by Western blot

and ELISA. The specificity of icIL-1ra was assessed by blocking icIL-1ra expression using antisense oligonucleotide directed against icIL-1ra mRNA. Paired Student's *t* test was used for analysis of significance.

### 36. Human dermal endothelial cell apoptosis induced by scleroderma serum is mediated by non-caspase-3 mechanisms

Sohail Ahmed, MD, Boston University School of Medicine, Boston, Massachusetts

**Purpose:** As our previous studies have demonstrated increased caspase-3 gene expression in human dermal endothelial cells (HDEC) undergoing apoptosis in response to scleroderma (SSc) serum, this study explored the effects of caspase-3 inhibition on SSc serum-induced HDEC apoptosis.

**Methods:** HDEC were grown to subconfluence and incubated for 18 hours with sera from 3 different controls, 3 patients with limited SSc and centromere antibodies (ACA), and 3 patients with diffuse SSc and Scl70 antibodies (SCL70). As cardiolipin antibodies are known to induce endothelial cell apoptosis, similar experiments were performed with sera containing cardiolipin antibodies from 3 patients with systemic lupus erythematosus (SLE). Control sera plus camptothecin (5  $\mu$ M) served as a positive control for caspase-3-mediated apoptosis. Apoptosis was determined by flow cytometry for annexin V staining of HDEC. Caspase-3 activity was determined by the protease's cleavage of Z-DEVD. Caspase-3 inhibition was achieved by the addition of 100 nM Ac-DEVD-CHO.

**Results:** ACA sera, SCL70 sera, and SLE sera resulted in levels of apoptosis (86%, 89%, and 85%, respectively) similar to that induced by control sera containing camptothecin (90%). Control sera by itself demonstrated 6% HDEC apoptosis. Low levels of cell necrosis were demonstrated in HDEC exposed to any of the autoimmune sera (0%-3%) while none was seen with exposure to control sera. Relative to caspase-3 activity resulting from HDEC exposure to camptothecin, the autoimmune sera demonstrated 72% caspase-3 activity compared to 56% induced by control sera ( $P < .02$ ). As expected, the addition of the caspase-3 inhibitor Ac-DEVD-CHO resulted in 91% reduction in camptothecin-induced HDEC apoptosis. However, the addition of Ac-DEVD-CHO resulted in only 27% reduction in autoimmune sera-induced HDEC apoptosis. Caspase-3 activity induced by autoimmune sera was decreased to control levels by the addition of Ac-DEVD-CHO which, as expected, inhibited camptothecin-induced protease activity to control levels.

**Conclusion:** Additional caspases or noncaspase proteases are primarily responsible for SSc serum-induced HDEC apoptosis. The increased activity of the caspase-3 protease in HDEC exposed to autoimmune sera may suggest a role for this protease in the generation of SLE autoantigens such as U1-70kd RNP and SSc autoantigens such as topoisomerase I, both of which are known substrates for caspase-3.

### 37. Hematopoietic stem cell transplantation for severe systemic sclerosis—phase I and II study results and update on the ASTIS trial

A Tyndall, D Farge, J van Laar on behalf of the EBMT/EULAR Scleroderma Study Group.

**Objective:** To assess the benefits and risks of the use of high dose cyclophosphamide therapy followed by hematopoietic stem cell transplantation (HSCT) in patients with systemic sclerosis (SSc).

**Methods:** All SSc patients who underwent HSCT in various pilot studies performed in Europe and registered in the EBMT/EULAR database since 1996 with at least 6 months follow up were evaluated up to 3 years after HSCT. Transplant regimens followed published EBMT/EULAR guidelines. Based on serial skin scores, and major organ function tests, disease outcome was categorized as complete, partial or non-response (CR, PR, NR, respectively). In addition the probability of disease progression and survival after HSCT (Kaplan-Meier) were calculated. Results (median, range) were compared with the Wilcoxon's test.

**Results:** Fifty-seven SSc patients (median age 40 yrs, range 9-68; 10 male, 47 female) were included with a median follow-up of 20 months (range 0.3-81.1). reduction of skin scores of more than 25% of the starting score were considered significant, and this occurred in 26/37 (70.2%) patients at 6 months, in 20/30 (66.7%) patients at 12 months, in 15/19 (78.9%) patients at 24 months and in 6/10 (60%) patients at 36 months after HSCT ( $P < .005$ ). No significant changes were observed in pulmonary or renal function overall, though some individuals experienced clinically relevant improvement. Disease outcome was categorized as PR in 32/50 (64%), CR in 14/50 (28%) and NR in 4/50 (8%). Relapses occurred in 16/46 (35%) patients with an initial PR or CR within 10 (2.2 - 48.7) months after HSCT. Disease progression was documented in 19/50 (38%) patients. Thirteen of 57 (22.7%) patients died during follow-up, 5/13 (8.7%) treatment related and 8/13 (14%) due to disease progression. At 5 years, the probability of disease progression was 48% (95% CI, 28-68) and the projected survival was 72 % (95% CI, 59-75).

**Conclusion:** This EBMT/EULAR database analysis shows durable responses in two thirds of patients up to 3 yrs after HSCT. TRM was lower than the initial reports probably due to better patient selection. The ongoing Autologous Stem cell Transplantation International Scleroderma (ASTIS) trial was launched in 2001 to investigate whether HSCT showed benefit over conventional chemotherapy in terms of risk/benefits. With 32 patients randomized (median follow-up 17 months, range 1-28) to either HSCT or pulse-therapy cyclophosphamide no treatment-related mortality has yet been observed in either arm. Based on encouraging results from the interim safety analysis on the first 20 patients, the protocol has been amended to include patients with early diffuse scleroderma.

### 38. The role of cell-cell and cell-matrix contacts in connective tissue remodeling

Boris Hinz, Laboratory of Cell Biophysics, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland

Fibroblast/myofibroblast modulation represents a crucial step in producing the connective tissue deformations typical of fibrocontractive diseases. In addition to synthesizing extracellular matrix (ECM) components, myofibroblasts develop tensile force through the neo-formation of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA)-containing cytoplasmic stress fibers. In order to coordinate tension distribution during connective tissue remodeling, stress fiber associated cell-matrix and cell-cell contacts appear essential. Myofibroblasts develop complex adhesion structures with the ECM that are called “fibronexus” *in vivo* and “supermature” focal adhesions (FA) *in vitro*. They contribute to the transmission of force and to the detection of stress level in the ECM. Increased matrix stress results in FA maturation, which is a prerequisite for the incorporation of  $\alpha$ SMA into stress fibers. In a mechanical feedback loop, this incorporation leads to FA supermaturation and increased myofibroblast adhesion. Treatment of myofibroblasts with  $\alpha$ SMA fusion peptide (SMA-FP) that specifically inhibits  $\alpha$ SMA-mediated contractile activity leads to disassembly of supermature FAs and reduced cell adhesion. Tissue myofibroblasts develop intercellular adherens junctions (AJs) that are absent from normal fibroblasts *in vivo* and exhibit significant molecular and functional differences *in vitro*. Cadherin expression changes from N-cadherin in early wound fibroblasts to OB-cadherin in contractile wounds, populated with  $\alpha$ SMA-positive myofibroblasts. Expression of a specific cadherin pattern appears to cause the spontaneous segregation of suspended fibroblasts and myofibroblasts in culture. AJs of plated myofibroblasts are reinforced by  $\alpha$ SMA-mediated contractile activity, resulting in high a mechanical resistance as demonstrated by subjecting cell pairs to hydrodynamic forces in a flow chamber. Application of SMA-FP to myofibroblasts causes reorganization of large stripe-like AJs into belt-like contacts and reduces their mechanical resistance. In turn, anti-OB-cadherin but not anti-N-cadherin peptides decrease the contraction of myofibroblast-populated collagen gels, suggesting that AJs are instrumental for myofibroblast contractile activity. We conclude that  $\alpha$ SMA-mediated contractile activity is important in both, regulating mechanical communication between myofibroblasts and with the ECM.

### 39. Autoantibody frequencies in British Caucasians and black South Africans with systemic sclerosis

A Rands<sup>1</sup>, M Tikly<sup>2</sup>, N McHugh<sup>1</sup> <sup>1</sup>Bath University, Bath, England, and <sup>2</sup>University of the Witwatersrand, Johannesburg, South Africa.

Clinical features and autoantibody frequencies vary in patients with systemic sclerosis (SSc) of different ethnic background. We undertook a cross-sectional study to compare autoantibody frequencies in 2 distinct ethnic groups of patients with SSc, ie, British Caucasians (BC) and black South Africans (SA).

**Patients and Methods:** Sera from 229 BC and 45 SA

patients with SSc were screened for anti-cytoplasmic, antinuclear and antinucleolar antibody reactivities by IF. Positive sera were further examined by <sup>35</sup>S-immunoprecipitation and, where necessary, by Ouchterlony double immunodiffusion to identify autoantibody specificities.

**Results:** Autoantibody frequencies are shown in the Table:

Auto-antibody	BC (%)	SA (%)	<i>P</i> <sub>corr</sub> value	Auto-antibody	BC (%)	SA (%)	<i>P</i> <sub>corr</sub> value
ACA	89 (38.9)	4 (8.9)	<.001	ATA	37 (16.2)	7 (15.6)	NS
Anti-U3RNP	2 (0.9)	8 (17.8)	<.01	ARA I	38 (16.6)	2 (4.4)	NS
Sum ARA II	34 (14.9)	1 (2.2)	<.05	ARA III	27 (11.8)	2 (4.4)	NS
Anti-Ro	16 (7)	10 (22.2)	<.05	Anti-La	13 (5.7)	4 (8.9)	NS
Anti-Pm-Scl	10 (4.4)	1 (2.2)	NS	Anti-Jo-I	10 (4.4)	0	NS
Anti-U1RNP	16 (7)	8 (17.8)	<.05	Unidentifiable antibody	18 (7.9)	10 (22.2)	<.01

Only 26.5% of SA patients were included in the 3 ‘major serological groups’ compared to 67% of BC patients (*P* <.0001). In BC patients, ACA was significantly associated with the limited cutaneous SSc (*P* <.0001), while ARA III and ATA were associated with diffuse cutaneous SSc (*P* <.0005 and <.0001, respectively). In SA patients, disease subset was not significantly associated with any of the autoantibody groups.

**Conclusion:** Distinct differences in autoantibody frequencies between the 2 ethnic groups are evident, even though the SA cohort was small. These differences might be related to the differences in clinical features between the 2 groups. A larger cohort of SA patients needs to be studied to confirm these results and to test for associations with clinical subsets and specific clinical complications in SA patients.

#### 40. Intravenous epoprostenol for severe digital ischemia in scleroderma

*Robert W Simms, Harrison Farber, Eugene Y Kissin, Peter A Merkel, Joseph H Korn*

*Boston University School of Medicine, Boston, MA*

**Purpose:** Severe digital ischemia and gangrene is an uncommon complication of Raynaud’s phenomenon in systemic sclerosis or other autoimmune disease. Available therapies include calcium channel blockers, alpha blocking agents, topical nitrates, phosphodiesterase inhibitors, sympathetic nerve blockade and anticoagulation. Some patients experience progressive digital ischemia leading to gangrene despite these therapies in combination. Intravenous epoprostenol has been shown to be effective in treatment of pulmonary hypertension associated with scleroderma and anecdotal evidence has suggested simultaneous improvement in Raynaud’s phenomenon. We herein report our experience with

iv epoprostenol for treatment of severe digital ischemia in association with scleroderma.

**Methods:** Five patients with severe digital ischemia (defined as persistent digital temperature loss and cyanosis with pain for greater than 24 hrs with threatened digital infarction.) received a total of 7 epoprostenol infusions administered over a mean of 5 days with a mean dose of 8 ng/kg/min. Four of five patients had limited scleroderma and one had systemic lupus erythematosus. Two had prior digital amputations before epoprostenol therapy. All patients were female, with a mean age of 52 and an average disease duration of was 5 years. Previous therapies administered prior to iv epoprostenol included nifedipine, prazosin, pentoxifylline, sildenafil, topical nitroglycerin, anticoagulation, and sympathetic blockade.

**Results:** Of the 7 infusions, complete reversal of digital ischemia was seen on 2 occasions and improvement or stabilization occurred in the remaining 5 infusions, although subsequent digital amputation occurred in 2 patients (both had early gangrene at the time of the infusion). Following improvement or stabilization, iv epoprostenol was tapered and then discontinued with resumption of oral vasodilator therapy.

**Conclusions:** Intravenous epoprostenol appears to be an effective therapy for severe digital ischemia associated with scleroderma unresponsive to conventional therapies including oral vasodilators and sympathetic blockade. When administered early in the course of severe digital ischemia, it may act to reverse severe digital vasospasm before permanent tissue injury and gangrene develop.

#### 41. The collagen type Iα2 enhancer regulates expression in vascular smooth muscle cells via a novel regulatory element

*Markella Ponticos<sup>1</sup>, David Abraham<sup>1</sup>, Carol Black<sup>1</sup> and George Bou-Gharios<sup>2</sup>*

*1 Department of Medicine, Royal Free & University College Medical School, Royal Free Campus, London, UK. 2 Renal Medicine, Imperial College London, Hammersmith Campus, London, UK.*

**Background:** The collagen type I α 2 (col1α2) far upstream enhancer (FUE) is crucial for the temporal and tissue-specific transcriptional regulation of collagen type I in embryonic development. The enhancer is also thought to be involved in controlling collagen type I expression in tissue remodeling and repair following injury and in fibrosis. Using transgenesis, this enhancer has been shown to contain elements that direct transcription of reporter genes to type I collagen-producing cells in the dermis, tendon, intra-membranous bones and internal organs. Our purpose is to further refine and delineate the tissue specific elements within col1α2 FUE.

**Methods:** We have used transgenic mice experiments, transient transfection assays, site directed mutagenesis, and electrophoretic mobility shift assays (EMSA).

**Results:** Analysis in transgenic mice revealed that a 400bp deletion from the 5’ end of the minimal 3.5kb FUE region result-

ed in loss of collagen expression in vascular smooth muscle cells (vSMCs). A 170bp sequence located around the deletion site was refined further and shown to direct high level of reporter gene expression to vSMCs using transient transfection assays with A7R5 vSMCs, and both primary human and mouse vSMCs, but not in fibroblasts. Investigation of the 170bp element revealed E2-box sequences and putative binding sites for the transcription factors Nkx2.5, AP1, and GATA. Using site directed mutagenesis, EMSAs and by over-expression of specific transcription factor cDNAs, it was possible to identify two transcription factor families that bind to and regulate the fragment in vSMCs but not other cell types. Nkx-homeodomain family members activated transcription whereas the zinc finger homeodomain transcription factor (deltaEF1) repressed transcriptional activity.

**Conclusion:** Two homeodomain transcription factors interact with a novel element in the col1 $\alpha$ 2 FUE and regulate collagen type I expression in vSMCs.

## 42. Over-expression of endoglin in cutaneous scleroderma fibroblasts

A. Holmes, A. Leask, DJ Abraham, X. Shi-Wen, Y. Chen, CM Black

Centre for Rheumatology and Connective Tissue Diseases, Royal Free and University College Medical School, University College, London, UK

**Objective** Transforming growth factor  $\beta$  (TGF $\beta$ ) induces extracellular matrix synthesis and fibroblast differentiation, thus TGF $\beta$  has long been proposed to mediate fibrosis. Indeed, anti-TGF $\beta$  strategies seem effective at blocking the onset of fibrosis. However, clinically fibrosis is often a chronic disorder and the role of TGF $\beta$  signaling in persistent fibrosis is not fully understood. Systemic sclerosis (SSc) is a chronic fibrotic disorder with unknown etiology. As an initial approach to understanding the basis of the SSc phenotype, we have sought to identify whether the expression of TGF $\beta$  receptors is dysregulated in lesional SSc fibroblasts relative to their normal counterparts.

**Methods** We have used FACS and over expression analysis to assess the potential role of TGF $\beta$  receptors in fibrosis. Flow cytometry: The expression levels of TGF $\beta$  receptor type I (TGF $\beta$ RI), TGF $\beta$  receptor type RII (TGF $\beta$ RII), Endoglin (CD105), and total TGF $\beta$  receptor binding were assessed by flow cytometry using FACScalibur (Becton Dickinson). Transfection and reporter assays: NIH3T3 fibroblasts were cultured in 6 well plates and transfected with CTGF or collagen 1 $\alpha$ 2 promoter reporter constructs in the presence or absence of endoglin or SMAD3 and SMAD4 and TGF- $\beta$ .

**Results** SSc fibroblasts showed elevated expression of the endothelial cell-specific TGF- $\beta$  accessory receptor, endoglin but not TGF $\beta$  type I and II receptors. Transfection of endoglin in fibroblasts suppressed both CTGF and collagen reporter gene activity by TGF $\beta$  and this repression was alleviated by co-transfection with the down stream signalling genes SMAD3 and SMAD4. Further SSc fibroblasts though basally expressing high levels of CTGF and collagen type I appeared to refractory to stim-

ulation with exogenous TGF $\beta$  compared to normal fibroblasts.

**Conclusion** SSc is characterized by overproduction of matrix and matrix associated genes. These results show SSc fibroblasts to be refractory to TGF $\beta$  stimulation and exhibit elevated expression of the TGF $\beta$  accessory receptor endoglin. Thus the refractory nature exhibited by SSc fibroblasts to TGF $\beta$  treatment may be due to the over expression of endoglin as a negative feedback mechanism in an attempt to block further induction of profibrotic genes by TGF $\beta$ .

## 43. Expression of STRO-1 by pericytes identifies the microvasculature as a potential source of stromal stem cells

Vineeth S Rajkumar<sup>1</sup>, Katalin Csiszar<sup>2</sup>, David Abraham<sup>1</sup>, Carol Black<sup>1</sup>

<sup>1</sup>University College London, London, United Kingdom

<sup>2</sup>University of Hawaii, Honolulu, HI

**Purpose:** The mechanisms by which an initial microvascular insult develops into a fibrotic lesion in diffuse cutaneous systemic sclerosis (dcSSc) are unclear. Microvascular pericytes have been proposed as mesenchymal stem cells and can be phenotypically linked to both myofibroblasts and bone marrow derived stem cells (BMSCs) by their mutual expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA). We hypothesized that a transition of pericytes to myofibroblasts contributes to the development of a fibrotic lesion from vascular injury.

**Methods:** We used a double immunofluorescence labeling approach in combination with confocal microscopy to determine whether pericytes and myofibroblasts share phenotypic markers in early dcSSc (n=8), late stage dcSSc (n=5) and control skin (n=6) samples. Additionally, we investigated whether STRO-1, a marker recognizing multipotent BMSCs in the microvascular compartment.

**Results:** Using  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), the ED-A splice variant of fibronectin (ED-A FN) and Thy-1 to identify myofibroblasts, we demonstrated the presence of myofibroblasts in half of the early dcSSc samples analysed. Neither myofibroblasts nor ED-A FN were detected in control skin or late stage dcSSc skin. The presence of myofibroblasts correlated significantly ( $P<.05$ ) with the expression of ED-A FN but not collagen synthesis in early dcSSc skin. Pericytes expressing  $\alpha$ SMA were also found to adopt a myofibroblast phenotype in early dcSSc skin by expressing ED-A FN and Thy-1. Using antibodies against PCNA, we also found evidence of pericyte proliferation in a proportion of dcSSc microvessels. Furthermore, pericytes were also shown to express the multipotent stem cell marker STRO-1.

**Conclusions:** These observations clearly demonstrate that the major matrix component in the early dcSSc lesion is ED-A FN synthesised by both pericytes and myofibroblasts and not collagen. Pericytes and myofibroblasts were found to share a common phenotype in dcSSc skin and the expression of PCNA by ED-A FN synthesising pericytes suggests that pericyte proliferation may contribute to dcSSc pathology, possibly by generating a pool of ED-A FN synthesising myofibroblasts. Furthermore the expres-

sion of STRO-1, a marker for multipotent BMSCs by pericytes, supports the hypothesis that pericytes may represent an *in situ* stem cell with the ability to transdifferentiate into other mesenchymal cell types.

#### 44. Correlation between echocardiographic estimate of pulmonary artery pressure and right heart catheterization (RHC) in Scleroderma

**Davidoff R<sup>3</sup>, Merkel PA, Korn JH<sup>1</sup>.** *Sections of Rheumatology<sup>1</sup>, Pulmonary<sup>2</sup> and Cardiology<sup>3</sup>, Boston University Medical Center, Boston, Massachusetts*

Pulmonary arterial hypertension (PAH) is a common complication of limited scleroderma and patients typically present with progressive dyspnea. Accurate assessment of PAH traditionally requires invasive RHC with direct measurement of pulmonary artery systolic pressure (PAS), and calculation of pulmonary vascular resistance (PVR). Echocardiography with color Doppler (ECHO) currently provides the optimal non-invasive estimate of PAS obtained by RHC, but requires tricuspid regurgitation, and the degree to which it correlates with RHC has varied in the literature.

**Methods:** All scleroderma patients (n=27) with PAS estimated to be greater than 35 mmHg by ECHO and who were symptomatic underwent RHC. All ECHOs were performed and read at BMC by an experience echocardiographer blinded to hemodynamic data. RHC was performed within 4 weeks of the ECHO by the same pulmonologist (HF). Patients with left ventricular dysfunction defined as those with a pulmonary capillary wedge pressure 15 mm Hg or greater were excluded.

**Results:** The following graph shows the correlation between ECHO estimation of PAS and PAS obtained at RHC (correlation coefficient 0.768,  $P = .0001$ ). For PAS estimates by ECHO of 50 mm Hg or less, the correlation was higher: 0.904 ( $P = .0021$ ).

**Conclusion:** ECHO provides a reliable estimate of PAS in symptomatic patients with scleroderma and PHT, and is a useful screening tool for PHT. RHC is still required to definitively assess left ventricular function, gauge PHT severity and guide therapy.

#### 45. Endothelin-1 mediated regulation of intercellular adhesion molecule-1 (ICAM-1) in human dermal fibroblasts

**Charlotte E. Waters<sup>1,3</sup>, Xu Shi-Wen<sup>2</sup>, David Abraham<sup>2</sup>, Jeremy D. Pearson<sup>1</sup>**

*1. Centre for Cardiovascular Biology and Medicine, Kings College London, London, UK*

*2. Centre for Rheumatology, Royal Free & University College Medical School, London, UK*

*3. Corresponding and presenting author.*

Endothelin-1 (ET-1) is implicated in the pathogenesis of fibrotic and inflammatory diseases including scleroderma. In addition to modulating vascular tone and extracellular matrix turnover, ET-1 up-regulates cell surface adhesion molecules, including ICAM-1. The signaling pathways involved under normal conditions have not been fully investigated, but are of interest

because basal expression of ICAM-1 increases in cells from fibrotic lesions in scleroderma, and sensitivity to ET 1 is significantly reduced whereas sensitivity to interleukin-1 mediated upregulation of ICAM-1 is enhanced. In normal human dermal fibroblasts (HDF) ET-1 induced ICAM-1 mRNA and surface protein expression in a dose- and time-dependent manner. The maximally effective concentration of ET-1 was 100 nM, and increased ICAM-1 protein was detected within 4h. Under these conditions, antagonists against ET-1 receptors ETA (JKC-301, 10  $\mu$ M) and ETB (BQ788, 10  $\mu$ M) inhibited ICAM-1 protein expression by 47 $\pm$ 13% and 46 $\pm$ 11% respectively, whilst the dual antagonist Bosentan (10 $\mu$ M) abolished the ET-1 response, indicating that ET-1 signaling occurs via both receptor subtypes. Inhibition of PI-3 kinase (wortmannin) or p38 MAPK (SB203580) had no effect, whereas inhibition of PKC by 500nM Bis-1 reduced ICAM-1 expression by 45 $\pm$ 11%. 100 nM Bis-1 failed to inhibit the ET-1 response, suggesting activation of a novel PKC isoform. An ICAM-1 promoter construct driving luciferase transiently transfected into mouse wild type fibroblasts showed robust induction by ET-1, which was absent in transfected PKC $\epsilon$  knockout fibroblasts. The MEK inhibitor UO126 (10 $\mu$ M) reduced ET-1-induced ICAM-1 expression in HDF by 35 $\pm$ 9%. Involvement of MEK was corroborated by western blot, detecting time-dependent ET-1-induced phosphorylation of p42/p44 MAP kinase and its inhibition by UO126. Inhibition of NF $\kappa$ B with PG490 (100 nM) decreased ET-1-mediated ICAM-1 expression by 78 $\pm$ 11%. Activation of NF $\kappa$ B by ET-1 was confirmed by EMSA using a consensus NF $\kappa$ B binding site oligonucleotide. Combining PG490 and UO126 abolished the ability of ET-1 to increase ICAM-1. Incubating cells with PG490 and either receptor antagonist (JKC-301 or BQ788) also completely blocked ET-1-induced ICAM-1 expression. Bis-1 had no added effect in the presence of either PG490 or UO126. We conclude that in normal HDF ET-1 upregulates ICAM-1 by signalling pathways in which receptor-mediated PKC $\epsilon$  activation leads to activation of MEK, with a parallel receptor-mediated activation of NF $\kappa$ B, both of which regulate ICAM-1 transcription. In future experiments we aim to determine the additional transcription factors activated downstream from MAP kinase, and will test whether the balance of these signaling pathways is altered in HDF from scleroderma lesions.

This work is funded by the Wellcome Trust.

#### 46. The role of anti-endothelial cell antibodies in the pathogenesis of systemic sclerosis

**Sarah E Riley, Jeremy D Pearson.**

*Centre for Cardiovascular Biology and Medicine, King's College London, Guy's Campus, London, UK*

Systemic sclerosis (SSc) or scleroderma is an autoimmune connective tissue disease. The pathogenesis of SSc is unclear. Microvascular dysfunction is an early and prominent component along with endothelial-fibroblast interactions promoting disease progression. Autoantibodies are found in the majority of SSc patients, which include anti-endothelial cell antibodies (AECA). This study is designed to investigate the role of AECA in the

pathogenesis of SSc, and characterize the intracellular signaling cascades activated when AECA bind to endothelial cells. Cell based enzyme linked immunosorbent assays (ELISAs) were used to screen SSc patient samples to detect antibodies (IgG) that bind to human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMEC). ELISAs were also used to detect any consequent increase in leukocyte adhesion molecules, including E-selectin, ICAM-1 and VCAM following pretreatment with SSc samples. These ELISAs were optimised using interleukin-1 beta (IL-1b), before using SSc patient serum samples. Certain SSc samples, but not normal serum or IgG, caused an increase in leukocyte adhesion molecules and showed a high level of AECA binding. Lipopolysaccharide contamination was ruled out by showing that responses were insensitive to polymyxin B. Reverse transcription polymerase chain reaction (RT-PCR) was also conducted, using HUVEC stimulated over a time course with IL-1b. A peak of mRNA expression was seen at 4-6 hours with E-selectin, ICAM-1, VCAM and MCP-1, when normalised against 28S RNA. This work is being continued to determine whether AECA cause upregulation of similar mRNAs. cDNA arrays using IL-1b stimulated HUVEC have been carried out, allowing a huge range of genes to be investigated, those of interest have been confirmed using real time RT-PCR. This technique will also be used with AECA stimulated HUVEC. Western blots have been used to detect p42/p44 MAP kinase activation, using IL-1b and AECA stimulated HUVEC and HMEC. These show activation at 15 and 30 minutes after stimulation.

This work is funded by the Arthritis Research Campaign.

## 47. Altered pattern of gene expression in endothelial cells in scleroderma

Howat, S.L., †Abraham D., Pearson, J.D.

Cardiovascular Division, King's College London, Guy's Campus, London, and †Rheumatology Research Unit, Royal Free and UC Medical School, London.

**Introduction:** The aim of this work was to use an *in vitro* approach to identify the altered pattern of gene expression in endothelial cells induced by co-culture with SSc lesional fibroblasts, which may reflect the phenotypic changes in endothelial cells in SSc. Paracrine factors secreted from activated endothelial cells have been implicated in the consequential fibroblast dysfunction and excessive deposition of extracellular matrix in lesional tissues (eg, Denton et al., 1996). Activated lesional fibroblasts, which maintain their variant phenotype in culture for several passages, in turn, act upon the endothelial cells causing a 'cross-talk' which perpetuates the SSc phenotype of both endothelial cells and fibroblasts.

**Materials and Methods:** Human dermal microvascular endothelial cells (HMEC-1., Ades et al., 1992) were grown to confluence and incubated in co-culture with dermal fibroblasts (SSc or normal) for up to 96 hours. Dermal fibroblasts were obtained from biopsies of lesional areas of the skin of individuals with scleroderma or normal dermal fibroblasts. Fibroblasts, used at passage 2, were grown to confluence on co-culture inserts. Then fibrob-

lasts with their conditioned media were transferred into co-culture with HMEC-1. HMEC-1 treated with TGF beta (2 ng/mL) over the same time period were used as a positive control. Total RNA was extracted from HMEC-1 using RNeasy total RNA isolation kit (Qiagen) and mRNA levels of selected genes were subsequently measured in the endothelial cells, by real time PCR using a LightCycler™ (Roche). Atlas™ Nylon cDNA Expression Arrays; human broad range 1.2 (Clontech) were also used to identify a general pattern of gene expression and to identify any other genes which may be affected by SSc fibroblasts

**Results:** Results obtained by real time PCR fell into five categories (1) messages up regulated only by co-culture with fibroblasts from patients with SSc (LFA-1, MMP-1, MMP-11, ezrin); (2) messages up regulated by co-culture with fibroblasts from patients with SSc and also by treatment with TGF beta (ET-2 and ICAM-1); (3) messages which were up regulated by co-culture with SSc fibroblasts and normal fibroblasts (Caspase 10); (4) messages up regulated only by treatment with TGF beta (PAI-1 and CTGF); (5) messages which were unchanged after any treatment (thymosin beta 4). We find that consistently a third of the cDNAs on the array were expressed in HMEC-1 of which 5%-10% of the cDNAs were differentially expressed in diseased relative to control. The expression of individual genes varied according to the duration of co-culture and the culture conditions.

**Discussion:** Upregulation of ICAM-1 and LFA-1 in HMEC-1 co-cultured with SSc lesional fibroblasts demonstrates an activation of the endothelial cells by SSc fibroblasts. Molecules required for leukocyte trafficking are found to be up regulated in lesional tissue. Excessive deposition of extracellular matrix (ECM) is also characteristic of SSc pathology caused by a dysregulation of turnover of ECM molecules. Upregulation of metalloproteinases such as MMP-1 and MMP-11 reflect such dysregulation. This work demonstrates that the pattern of HMEC-1 gene expression is affected by co-culture with fibroblasts from patients with SSc.

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## 48. Enhancement of TGF-β gene expression in fibroblasts by IL-4 and IL-13

Cristina M. Stoica<sup>1</sup>, Tracy L. McGaha<sup>2</sup>, Constantin A. Bona<sup>1</sup>

1. Department of Microbiology, Mount Sinai School of Medicine, New York

2. Department of Molecular Genetics and Immunology, The Rockefeller University, New York

Recently, we demonstrated that IL-4 stimulated the transcription of TGF-β1 gene in fibroblasts, which plays a critical role in connective tissue metabolism. We found that *in vitro* stimulation of

fibroblasts with IL-4 enhanced the transcription of TGF- $\beta$ 1 gene and disruption of the IL-4 gene prevented skin sclerosis in TSK mice. Based on these results, we hypothesised that there is an epistatic interaction between IL-4 and TGF- $\beta$ 1 genes. The major aim of this study was to investigate the mechanisms by which IL-4 and IL-13 control TGF- $\beta$ 1 expression in fibroblasts. TGF- $\beta$ 1 promoter analysis showed the presence of several IL-4 responsive DNA binding motifs. Studying the promoter activity in fibroblasts transfected with deletion TGF- $\beta$ 1/CAT constructs containing various DNA binding motifs for transcription factors, we found that only the construct containing STAT6 site was crucial for IL-4 induction of TGF- $\beta$ 1 promoter. Mutation of this site abrogated IL-4's effect on promoter activity. Additionally, we found that ectopic expression of wild type (wt) or constitutively active STAT6 (VT) enhanced this biological activity, while introduction of a dominant negative STAT6 (dn) reduced the TGF- $\beta$ 1 promoter activity at basal level. The fibroblasts transfected with STAT6 wt and STAT6 VT increased the transcription of TGF- $\beta$ 1 gene that paralleled with an increased synthesis of TGF- $\beta$ 1. Three constructs containing all 6 Sp1 binding motifs ([-2-+20], [-113-+55], [-231-+20]) showed minimal promoter activity which was not enhanced by stimulation with cytokines, indicating that Sp-1 does not play an essential role in IL-4 mediated stimulation of TGF- $\beta$ 1 promoter activity. Conversely, the -406-+55 construct containing the STAT6 binding site showed significant basal activity which was enhanced by the addition of IL-4 and IL-13. Our results clearly show that IL-4 and IL-13 have a direct effect on TGF- $\beta$ 1 promoter activity and strongly suggest that they principally exert their effect through a STAT6 dependent mechanism.

#### **49. Cartilage oligomeric matrix protein is overexpressed by scleroderma dermal fibroblasts**

*Giuseppina Farina, Joseph H. Korn, Russell L. Widom*

*Arthritis Center, Boston University School of Medicine, Boston, Massachusetts*

Cartilage Oligomeric Matrix Protein (COMP) belongs to the thrombospondin gene family and is an extracellular glycoprotein found predominantly in cartilage, tendon, ligament, and bone. Mutations in the COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia. COMP influences the organization of collagen fibrils by interacting with collagens I, II, and IX. Gene expression profiling of cultured fibroblasts (fb) suggested that COMP mRNA levels were elevated in scleroderma. We therefore examined COMP expression in SSc and normal fb cultures and skin biopsies. Our results confirm that COMP mRNA levels are elevated in SSc versus normal fb. Immunohistochemistry of SSc skin showed striking COMP deposition in the papillary dermis and to a lesser extent, throughout the dermis. Due to its involvement in the development of fibrosis, TGF $\beta$  was examined for a possible role in regulating COMP expression. In cultured fb, TGF $\beta$ -1 treatment greatly increased COMP expression at both the mRNA and protein level. SSc and normal fb demonstrated from 5- to 20-fold induction after 24h of TGF $\beta$ -1 stimulation, while foreskin fb showed an almost 70-fold increase. COMP protein levels generally correlated with mRNA expression. Cultured SSc fb demonstrated greater staining for COMP compared to normal controls. Taken together, these data demonstrate that COMP is overexpressed in SSc skin and fb possibly due to TGF $\beta$  stimulation. By interactions with other matrix proteins, COMP may play a role in pathogenic matrix deposition.

# **NATIONAL REGISTRY FOR CHILDHOOD ONSET SCLERODERMA**

**LOCALIZED AND SYSTEMIC  
FORMS OF SCLERODERMA**

**CHILDREN AND ADULTS  
(WITH ONSET BEFORE AGE 16)**

**University of Pittsburgh  
Thomas A. Medsger, Jr., MD  
Principal Investigator**

**Children's Hospital of Pittsburgh  
Thaschawee Arkachaisri, MD, FACR  
Co-Investigator**

**one blood sample required (sent via mail kit)**

**no visits to Pittsburgh necessary**

**yearly questionnaire (HAQ or CHAQ) to be completed by patient**

**IRB approved, HIPAA compliant yearly one page data collection  
form to be completed by patient's attending physician**

The goals of the Registry are to perform serum autoantibody profiles and to identify associations of specific autoantibodies with clinical and laboratory manifestations and prognosis.

We hope to stimulate future research on childhood onset scleroderma by having a large compilation of data and specimens available. Investigators may apply for access to de-identified clinical data, serum, peripheral blood mononuclear cells, and DNA from Registry subjects; and may use the Registry as a vehicle to make their projects known to this subject population.

We have thus far enrolled 18 patients with systemic sclerosis and 61 with localized scleroderma. We expect to have 75 systemic and 200 localized patients in the Registry by the end of 2004.

**For further information please contact Jennifer Jablon, the Study Coordinator,  
at 412-383-8674 or [HYPERLINK "mailto:jablonj@msx.dept-med.pitt.edu"](mailto:jablonj@msx.dept-med.pitt.edu)  
[jablonj@msx.dept-med.pitt.edu](mailto:jablonj@msx.dept-med.pitt.edu)**

Please ask your interested patients to call the Registry at 1-800-603-8960.

## New National Institutes of Health Study Entitled:

# Pathogenic Studies in Families With Twins or Siblings Discordant for Systemic Rheumatic Disorders

A new unit of the National Institute of Environmental Health Sciences, called the Environmental Autoimmunity Group (EAG), has been established in Bethesda, Maryland, at the National Institutes of Health (NIH) in the US Department of Health and Human Services to conduct pioneering research in understanding the genetic and environmental risk factors that may result in autoimmune diseases.

The EAG is currently enrolling families in which an adult or child meets criteria for systemic sclerosis (scleroderma), rheumatoid arthritis/juvenile rheumatoid arthritis, systemic lupus erythematosus, or Myositis and in which a twin or sibling of the same gender, who is within 4 years of age, does not have any one of these four illnesses or another autoimmune disease. Subjects may enroll at the NIH Clinical Center in Bethesda, Maryland, or in their local doctors' offices. Patients remain under the care of their personal physicians while participating in the study. There is no charge for study-related evaluations and medical tests at the NIH. Compensation is available to both physicians and subjects for enrollment.

For information about the NIH Twin-Sibs study, please call the persons below, or visit the Web site: <http://dir.niehs.nih.gov/direag/>

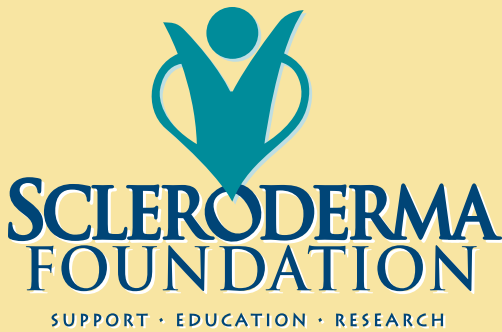
**Call Drs. Frederick Miller, Lisa Rider or Mark Gourley  
at (301) 451-6280 or toll-free at 1-888-271-3207**

### Overview of the Study

- The goal of the study is to understand the genetic and environmental factors that may result in systemic rheumatic diseases.
- The study will perform evaluations to assess why one twin or sibling developed disease and why the other brother or sister did not.
- Subjects may enroll at the NIH Clinical Center in Bethesda, Maryland or their local doctors' offices.
- A letter from a referring physician is required.
- Twins or siblings as well as their biological parents will be enrolled.
- 400 pairs of twins or siblings, in which one has disease and one does not, will be enrolled.
- Medical records, questionnaires and blood and urine samples will be collected at enrollment and at the end of the study after 5 years.
- For each subject, annual questionnaire follow-ups will be collected by mail.
- Subjects who develop new autoimmune diseases during the study will be reevaluated.

### Subject Eligibility

- Families are eligible when an adult or child member meets criteria for:
  - Systemic sclerosis (SSc, scleroderma)
  - Rheumatoid arthritis (RA) or
  - Juvenile Rheumatoid Arthritis (JRA) or
  - Systemic lupus erythematosus (SLE) or
  - idiopathic inflammatory myopathy (IIM, meaning any form of adult or juvenile dermatomyositis, polymyositis or inclusion body myositis)
- **And** when a twin or brother or sister of the same gender, and within 4 years of age, does not have rheumatic or autoimmune disease.
- The diagnosis of SSc, RA, SLE or IIM has to be within 4 years of enrollment.
- Affected and unaffected brothers or sisters must be of the same gender (both male or both female) and be offspring of the same parents.
- Normal healthy volunteers, who do not have a blood relative with a rheumatic or autoimmune disease, and who are matched to enrolled patients, are also eligible to enroll in the study.



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