

Scleroderma

Care and Research

Volume 4, Number 1
Autumn 2006

Journal of the
Scleroderma Clinical
Trials Consortium



Joseph H. Korn, MD
1947-2005

In This Issue:

*The 9th International Workshop on
Scleroderma Research*

Mission Statement

Scleroderma Care and Research is an independent, biannual 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 is circulated to the community of physicians caring for patients with scleroderma.

Editor's Memo

This issue of *Scleroderma Care and Research* contains the abstracts that were presented at the Ninth International Scleroderma Workshop held in Boston in 2006. Joseph H. Korn, MD, and Carol M. Black, MD, were the prime movers in developing content and maintaining the quality of the biannual International Workshops. Unfortunately Joe passed away shortly before this Workshop was to take place. It is fitting, therefore, that we dedicate this issue of *Scleroderma Care and Research* to his memory.

The International Scleroderma Workshop was the brainchild of Joe Korn and Carol Black, who years ago decided that a workshop focused on very basic research (much of which was not directly linked to scleroderma) would be good for investigators interested in scleroderma. Many of the invited speakers were from outside the field of scleroderma. They were asked to present work that was, in the broadest of senses, "interesting" to investigators in scleroderma, but it often was seemingly quite tangential. The plan was for scleroderma investigators to see work that would "tweak" them into thinking about new and entirely different approaches to investigating the disease.

Joe and Carol held their first Workshop in Chicago in 1990, with funding from an interested patron. It was a small, select group of interested folks. From there, the workshop alternately bounced between the United States and the United Kingdom on a biannual basis, with the Ninth Workshop being held in Boston in 2006. The audience has increased accordingly, with more than 200 investigators attending this year.

Although Carol's career (she is Professor and Chief of Rheumatology at Royal Free Hospital in London) has always been based in both basic and clinical research, Joe started out as a basic researcher. Over the years he blossomed. As Professor of Medicine, Chief of Rheumatology, and Head of the Scleroderma Unit at Boston University School of Medicine, he learned how to take care of patients with scleroderma. He became the rare division head who was comfortable at the bench taking care of fibroblasts as well as in the clinic taking care of patients with scleroderma. As a result, he encouraged and supported both basic and clinical research, both at Boston and in the world arena.

This dual role that Joe and Carol assumed in their careers (basic research coupled with clinical care) spilled over into the content of the International Workshop. Although it began as a forum for basic research, the Workshop began to add segments that addressed clinical research. In 2002 the Scleroderma Clinical Trials Consortium helped develop a half-day segment relating to new aspects of clinical research as it pertains to systemic sclerosis. Over the last few Workshops, I am happy to say that the conference has continued to include segments dealing with new aspects of clinical research alongside the basic research that has been the hallmark of this conference.

The entire scleroderma community misses Joe. Importantly, the Workshop is continuing, even in his absence. As it continues, it should remain a reminder of the major contributions he made to this field.

Philip J. Clements, MD
Editor-in-Chief

Abstracts Presented– Ninth International Workshop on Scleroderma Research

1. The contribution of connective tissue growth factor (CTGF) to fibroblast biology

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CCN2 is induced by TGFbeta in fibroblasts and is overexpressed in connective tissue disease such as scleroderma. CCN2 has been proposed to be a downstream mediator of TGFbeta action in fibroblasts; however, the role of CCN2 in regulating this process unclear. Here, we show using embryonic fibroblasts isolated from *Ccn2*^{-/-} mice that CCN2 is required for the induction of 345/942 TGFbeta-responsive mRNAs. Although TGFbeta properly induced a generic Smad3-responsive promoter in *Ccn2*^{-/-} fibroblasts, TGFbeta-induced focal adhesion kinase (FAK) and Akt activation was reduced in *Ccn2*^{-/-} fibroblasts. Akt1 overexpression in *Ccn2*^{-/-} fibroblasts rescued the TGFbeta induced transcription of CCN2-dependent mRNA. Induction of TGFbeta-induced fibroblast adhesion to fibronectin and type I collagen was significantly diminished in *Ccn2*^{-/-} fibroblasts. *Ccn2*^{-/-} fibroblasts showed reduction in basal expression of approximately 500 genes and diminished cell migration and matrix contraction, which depended on MEK/ERK. Thus CCN2 is necessary for the activation of a matrix synthesis and remodeling program, and therefore is likely to represent, compared to TGFbeta, a selective target for drug intervention in scleroderma.

2. Development of a minimal standard for the evaluation of patients with juvenile systemic sclerosis

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Introduction: Juvenile systemic sclerosis (jSSc) is a rare disease. Currently just retrospective data exist regarding organ involvement, and evolution of the disease. No standard assessment

of the organ involvement has been developed.

Objectives: The goal of the project is to develop a minimal standard to assess organ involvement in jSSc and to develop and follow a jSSc inception cohort. Using the standard assessments, prospective data will be collected to describe organ involvement and changes in organ involvement over time.

Methods: A group of interested pediatric rheumatologists have held two face-to-face meetings and several rounds of E-mail discussions to develop a consensus regarding the data set and study methodology.

Results: For each organ system involved in jSSc, a consensus was reached regarding aspects of physical examination and diagnostic tests, and intervals between consecutive evaluations that would be appropriate for patient care. A minimal scheme for patient evaluation and follow-up was determined, with a requirement that all diagnostic tests be available in virtually all pediatric rheumatology centers. Additional tests were suggested as option where available. Emphasis was placed on collection of quantitative data whenever possible. An international, multicenter, prospective study of the disease manifestations and evolution of jSSc has been planned. The data set for the study will be presented.

Conclusion: A minimal standard of initial and follow-up evaluation has been proposed for jSSc. Based on this standard, an inception cohort will be developed and data on these patients will be prospectively collected in an international study group. The results of this study will not only advance our understanding of jSSc, but also help guide other physicians in their care of children with systemic sclerosis.

3. Controlled trial of tadalafil in Raynaud phenomenon (RP) secondary to systemic sclerosis (SSc)

Elena Schiopu, MD¹, Vivien M. Hsu, MD², Ann J. Impens, PhD¹, Jennifer A. Guerriere-Rothman, MD², Deborah A. McCloskey, BSN², Julianne E. Wilson, RN², James R. Seibold, MD¹

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Objective: Type V cGMP phosphodiesterase inhibitors (PDE-5) are reported as useful in the treatment of RP and for the ischem-

ically threatened digit in SSc. Controlled trials are lacking.

Methods: 39 patients with SSc and RP were recruited for a randomized, double-blinded, placebo-controlled, cross-over study of tadalafil at 20 mg daily. Quality of female sexual function was a co-primary outcome hence all patients were women. The mean age was 52.9 ± 10.6 years. Of the 39 subjects, 29 (74.4%) had limited and 10 (25.6%) had diffuse scleroderma. The mean duration of RP was 11.8 ± 10 years. Eligible subjects recorded daily diaries of RP episodes and Raynaud Condition Score (RCS) for 2 weeks. The average number of RP attacks per week was 20.7 ± 12.3. Subjects that had at least 6 RP attacks per week were randomized to 20 mg tadalafil or placebo daily for 4 weeks followed by a 2 week wash-out and then 4 weeks of crossover therapy. The safety and tolerability were assessed by monitoring adverse effects (AE), vital signs, clinical laboratories and physical examination findings. Efficacy was assessed utilizing a daily paper diary including RCS. Duration and frequency of RP attacks were secondary efficacy outcomes.

Results: There were no severe AEs. Common AEs included headache (32.5%), myalgia (22.5%), fluid retention (10%), vasomotor changes (7.5%), fatigue (5%), sleep disturbances (5%) and palpitations (5%). Five subjects reported no AEs. Measures of efficacy are reported as mean change from baseline and revealed:

	RCS (cm)	RP Frequency (per day)	RP Duration (min)
Baseline	3.76	2.93	53.42
Tadalafil	-1.33	-0.85	-12.81
Placebo	-1.23	-0.83	-6.42

All differences were not significant: RCS (t (38) = -0.36, *P* = .71), RP Frequency (t (38) = -0.08, *P* = .93) and RP Duration (t (38) = -1.15, *P* = .25). There were too few digital ulcers to permit analysis. Several validated questionnaires of quality of female sexual function showed no effects (data not presented).

Conclusions: Tadalafil is a long acting PDE-5 inhibitor amenable to once daily dosing. It appears to be well tolerated in women with SSc and RP. The present data do not support its use as a therapy for RP secondary to SSc although studies in pulmonary hypertension-SSc are in progress. Placebo effect remains a prominent issue in RP clinical trial design.

4. Influences of clinical features of systemic sclerosis (SSc) on the Michigan Hand Questionnaire (MHQ)

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Rationale: Domains of SSc hand impairment include digital ulcers, skin thickening, tendon/joint involvement and atrophy. The MHQ is a 37-item hand-specific outcomes questionnaire with 6 subscales: overall hand function, activities of daily living (ADL),

pain, work performance, aesthetics, and patient satisfaction with hand function. MHQ is highly validated in rheumatoid arthritis (RA). We have shown reliability and validity in SSc. We examine the influence of SSc hand features on the MHQ.

Methods and Patients: Ninety-four ambulatory SSc patients, 47 with limited and 47 with diffuse disease, completed MHQ along with SF36 and SHAQ. Eighty-one (86%) were women. Mean age was 51 y ± 12 and disease duration 7y ± 7. SSc patients were subgrouped by disease features into 1) tendon involvement (palpable tendon friction rubs/impaired fist closure) 2) digital tip ulcerations 3) upper extremity skin score (fingers, hands, forearms; 0-3 per area, range 0-18), and 4) disease classification.

Results:

Spearman's Correlations for the MHQ, SHAQ, and Domains of Scleroderma Hand Function

	Skin Score			Digital Ulcers			Tendon Involvement		
	Combined	Limited	Diffuse	Combined	Limited	Diffuse	Combined	Limited	Diffuse
MHQ Combined	-.35§	-.15 ^{NS}	-.36¶	-.08 ^{NS}	-.11 ^{NS}	.03 ^{NS}	-.52*	-.40±	-.57*
Overall Hand Function	-.34±	-.21 ^{NS}	-.31¶	.01 ^{NS}	.02 ^{NS}	.03 ^{NS}	-.51*	-.46±	-.48±
Activities of Daily Living	-.34±	-.01 ^{NS}	-.26 ^{NS}	-.05 ^{NS}	-.02 ^{NS}	-.06 ^{NS}	-.47*	-.30¶	-.44±
Pain +	.21 ^{NS}	.11 ^{NS}	.19 ^{NS}	.19 ^{NS}	.23 ^{NS}	.15 ^{NS}	.36§	.30 ^{NS}	.42±
Aesthetics	-.25¶	-.16 ^{NS}	-.30 ^{NS}	-.12 ^{NS}	-.14 ^{NS}	.07 ^{NS}	-.35§	-.34¶	-.38¶
Work Performance	-.27¶	-.15 ^{NS}	-.39¶	-.02 ^{NS}	-.08 ^{NS}	.06 ^{NS}	-.34±	-.29 ^{NS}	-.41±
Satisfaction Hand Function	-.34±	-.08 ^{NS}	-.36¶	-.04 ^{NS}	.00 ^{NS}	-.02 ^{NS}	-.50*	-.35¶	-.57*
SHAQ	.25¶	.10 ^{NS}	.04 ^{NS}	.03 ^{NS}	-.05 ^{NS}	.07 ^{NS}	.46*	.56§	.11 ^{NS}

Lower scores on MHQ reflect higher impairment except for MHQ Pain where higher scores reflect higher pain levels

* *P* < .0001; § *P* ≤ .001; ± *P* < .01; ¶ *P* < .05; NS = Not Significant

Conclusions: Digital ulcers and skin thickening had minimal impact whereas tendon involvement dominated patient ratings of hand function. No significant correlations were found between number of digital ulcers and MHQ, MHQ subscales, SHAQ or SF36 for all patients and diffuse SSc patients. Tendon involvement correlated with several MHQ scales in the total patient sample and both SSc groups. These observations are consistent with clinical studies of digital ulcers wherein neither SHAQ nor other hand functional tools have been shown to be sensitive to change. MHQ is a valid and reliable measure of hand function in SSc although sensitivity to change has not been assessed. Occupational therapy focused on fist closure and tendonitis would be predicted to influence hand function whereas future studies of digital ulcers and skin involvement will require more specific instruments.

5. Validation of the Michigan Hand Outcomes Questionnaire (MHQ) in systemic sclerosis (SSc)

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Rationale: Hand involvement is a universal feature of systemic sclerosis (SSc) and hand impairment is a key determinant of functional status in SSc. Validated outcome measures that are practical for SSc are lacking. The MHQ is a 37-item hand-specific outcomes questionnaire with 6 subscales: overall hand function, activities of daily living (ADL), pain, work performance, aesthetics, and patient satisfaction with hand function. The MHQ has been validated in rheumatoid arthritis (RA) and has face and content validity for application in SSc.

Methods and Patients: Ambulatory SSc patients completed the MHQ along with SF-36 and SHAQ. Thirty-two subjects underwent repeated testing (2-week intervals) to assess test-retest reliability. MHQ responses were compared with detailed clinical measurements of SSc.

Results: Ninety-four SSc patients were studied, including 47 with limited and 47 with diffuse SSc. Eighty-one (86%) were women. Mean age was 51 y ± 12 and disease duration 7y ± 7. Test-retest reliability for MHQ was .84 (Spearman correlation), which ranged from .61 for aesthetic to .86 for ADL subscales. Internal consistency including overall MHQ and each subscales all scored > .80, except for a .62 correlation with aesthetic scale. Both limited and diffuse groups had similar internal consistency scores.

Spearman's Correlations between the MHQ, SHAQ, and subscales of SF-36

	SHAQ	SF-36 Physical+Pain	SF-36 Physical	SF-36 Pain	SF-36 Mental Health	SF-36 General Health
MHQ Combined	-.65*	.66*	.43*	.64*	.26¶	.38§
Overall Hand Function	-.56*	.55*	.39§	.56*	.17 ^{NS}	.33¶
Activities of Daily Living	-.70*	.68*	.51*	.60*	.19 ^{NS}	.41*
Pain +	.54*	-.58*	-.31±	-.65*	-.19 ^{NS}	-.27¶
Aesthetics	-.29±	.30±	.17 ^{NS}	.30±	.25¶	.17 ^{NS}
Work Performance	-.55*	.69*	.44*	.60*	.36§	.36*
Satisfaction Hand Function	-.59*	.55*	.37*	.54*	.25¶	.39§

Lower scores on MHQ reflect higher impairment except for MHQ Pain, where higher scores reflect higher pain levels. The negative sign in the pain scale indicates reverse scoring: patients with a higher pain score (more pain) have worse performance. * $P < .0001$; § $P < .001$; ± $P < .01$; ¶ $P < .05$; NS = Not Significant

Conclusions: The MHQ has test-retest reliability and good internal consistency in a large sample of SSc patients. MHQ has construct validity for SSc because its function and pain domains correlated with similar domains in SHAQ and the SF-36. The non-

significant low correlation between the MHQ and the SF-36 mental scale is sensible, while the low correlation between the MHQ aesthetic domain and other functional domains adds to the construct validity of the aesthetic domain, which is often overlooked. The MHQ is a promising outcomes tool for SSc and its responsiveness will be tested in clinical trials.

6. Caveolin-1 regulates collagen expression and myofibroblast differentiation and inhibits bleomycin-induced lung fibrosis

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Abnormal deposition of extracellular matrix proteins, mainly collagen, in many lung diseases, including scleroderma, is the hallmark of lung fibrosis. Our previous studies suggested that a PKC/MEK/ERK/caveolin-1 signaling cascade regulates collagen accumulation in normal lung fibroblasts (NLF). We found that caveolin-1 depletion leads to increased collagen levels in NLF, but not in scleroderma lung fibroblasts (SLF), in which caveolin-1 is already expressed at low levels. To further investigate the role of caveolin-1 on collagen regulation we overexpressed full-length caveolin-1 using an adenoviral construct and increased its function using the caveolin-1 scaffolding domain peptide (CSD). Both treatments gave the expected decrease in MEK/ERK activation and collagen levels in NLF and SLF. In addition to regulating their collagen expression caveolin-1 also regulates the differentiation of fibroblasts to myofibroblasts. Caveolin-1 depletion leads to a-smooth muscle actin (ASMA) upregulation in NLF, but not in SLF, while increasing caveolin-1 expression decreases ASMA levels in SLF, but not in NLF. Although caveolin-1 perturbations had no effect on the level of expression of PKC alpha and epsilon, immunohistochemical analyses revealed alterations in the localization of PKC isoforms after CSD treatment in both NLF and SLF, suggesting that caveolin-1 may regulate signaling molecule function by their translocation. In vivo studies have shown that systemic CSD delivery inhibits bleomycin-induced lung fibrosis in mice. Thus caveolin-1 plays an important role in the regulation of collagen synthesis and myofibroblast differentiation, and thereby inhibits bleomycin-induced lung fibrosis.

7. Adenosine modulates dermal fibrogenesis

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Background: Adenosine, acting at its receptors, is a potent modulator of inflammation and tissue repair. We have recently report-

IN PAH, TAKE AIM AT ET-1 THROUGH ET_A SELECTIVITY

Circulating levels of ET-1, the most potent subtype of ET, have been associated with disease severity in PAH.¹ The deleterious effects of elevated ET-1 include cellular proliferation, vasoconstriction, and vascular remodeling.²⁻⁴

In pulmonary arterial hypertension (PAH), endothelin (ET-1) exerts its cardiovascular effects through 2 receptors: ET_A and ET_B. When ET-1 activates the ET_A receptor on the vascular smooth muscle, it leads to vasoconstriction and vascular remodeling.^{4,5} Endothelial ET_B receptors mediate the release of vasodilating nitric oxide (NO) and prostacyclin (PGI₂), while inhibiting and clearing ET-1 from circulation.^{5,6} Blockade of ET_B receptors may significantly impair the balance of endothelium-derived vasodilating substances.^{4,7}

Endothelial dysfunction has been shown to improve with selective ET_A blockade.⁸ Hence, preemptive targeting of ET-1 through selective ET_A receptor antagonism can slow the progression of PAH, and may even provide better overall outcomes.^{2-4,8}

TARGETED ET-1 TREATMENTS MAY PROVIDE BETTER OUTCOMES

Imbalances in the key endothelial cell-derived mediators NO, PGI₂, and specifically ET-1 are thought to be central to the pathogenesis of PAH.⁹ NO and PGI₂ are potent vasodilators with antiproliferative activity.¹⁰ ET-1 is a potent vasoconstrictor with proliferative activity.⁵ Chronically elevated levels of ET-1 are associated with pulmonary vascular resistance, excessive scar formation and cardiac remodeling, cellular proliferation, and cardiac hypertrophy.^{1,11-13}

A reduction of excess ET-1 levels may result in positive outcomes for patients with PAH. It has been shown that in patients with congestive heart failure, elevated ET-1 plasma

Figure 1: ET_A receptor pathway

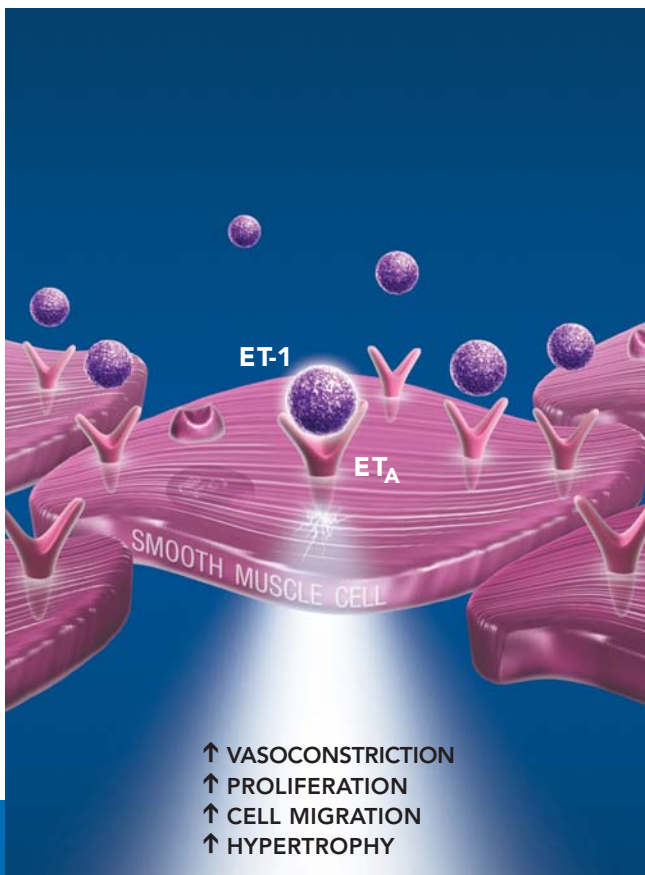
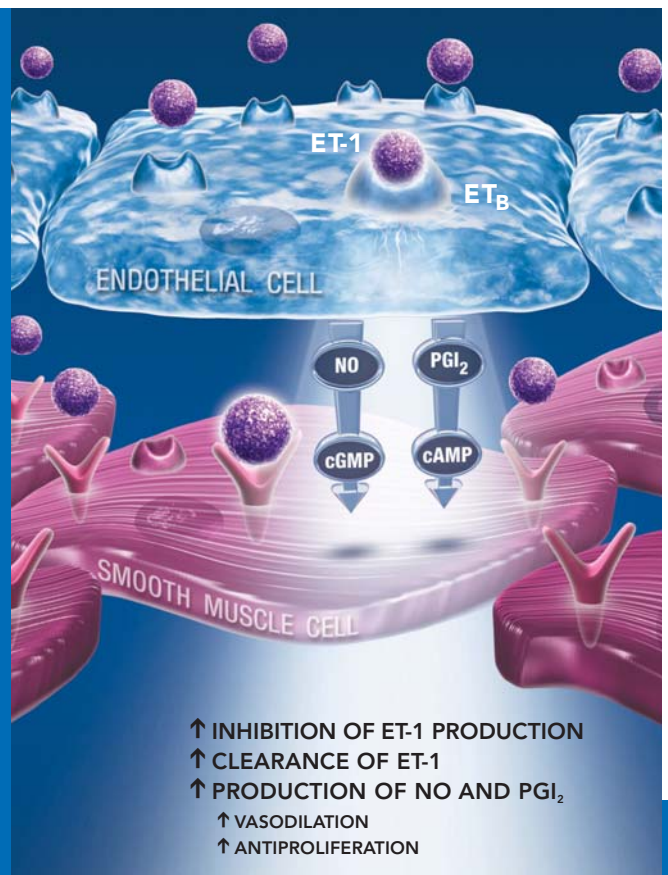


Figure 2: ET_B receptor pathway





levels are at least partly associated with impaired ET_B receptor-mediated clearance.¹³ Furthermore, the long-term administration of a selective ET_B receptor antagonist was found to have unfavorable effects on vascular remodeling.⁴ This is in sharp contrast to the benefits of selective ET_A antagonism.¹⁴

THE DIFFERENCE LIES IN ET_A SELECTIVITY

Vasoconstriction, cellular proliferation, and vascular remodeling are the hallmarks of PAH.¹² Studies have demonstrated that selective ET_A antagonists play a pivotal role in the regulation of ET-1 levels in PAH and have been beneficial for vascular remodeling.^{4,7,13}

ET-1 AND RECEPTOR-MEDIATED ACTIVITIES

Highly selective ET_A blockade maintains ET-1 clearance, NO and PGI₂ levels, and reduces or maintains circulating ET-1 levels, resulting in vasodilation, increased blood flow, and repair of remodeled vasculature compared to less selective agents.^{5-7,14} (See Figures 1,2)

HOW SELECTIVE TO ET_A SHOULD TREATMENT BE?

The more selective, the better. One should always be aware of the varying degrees of selectivity, as they equate to differences in blockade of the ET_A and ET_B receptors and resulting levels of ET-1.^{8,15,16} Figure 3 illustrates the difference between a less selective agent and highly selective agents. These in vitro selectivity ratios demonstrate the stark differences in ET_A selectivity. Figure 4 depicts how agents with low selectivity of the ET_A receptor (<2400) increase ET-1 levels whereas highly selective ET_A receptor (>2400) antagonists have been shown to

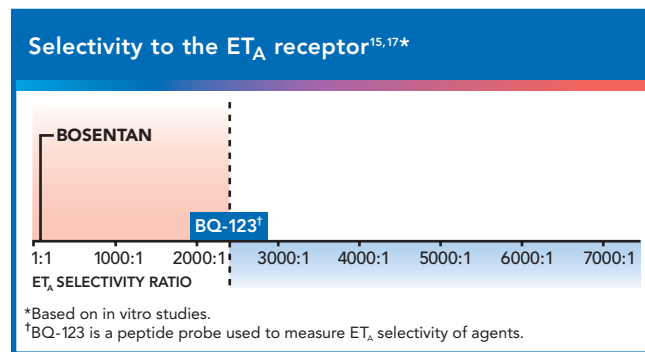


Figure 3

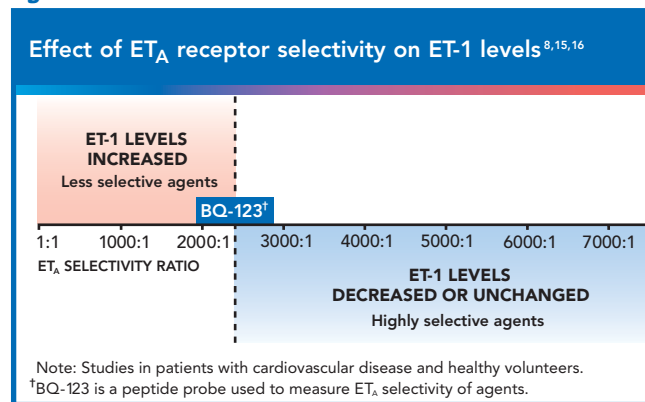


Figure 4

decrease ET-1 levels or leave them unchanged.^{6,8,15} The benefits of ET_A selectivity are being recognized.

TOWARD BETTER OUTCOMES IN PAH

Currently, there are no highly selective ET_A antagonists available for the treatment of PAH. In vivo studies have shown that highly selective ET_A antagonism may lead to better overall outcomes.^{7,8,12}

References: 1. Rubens C, Ewert R, Halank M, et al. Big endothelin-1 and endothelin-1 plasma levels are correlated with the severity of primary pulmonary hypertension. *Chest*. 2001;120:1562-1569. 2. Lüscher TF, Yang Z, Tschudi M, et al. Interaction between endothelin-1 and endothelin-derived relaxing factor in human arteries and veins. *Circ Res*. 1990;66:1088-1094. 3. Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*. 1988;332:411-415. 4. Murakoshi N, Miyauchi T, Kakinuma Y, et al. Vascular endothelin-B receptor system in vivo plays a favorable inhibitory role in vascular remodeling after injury revealed by endothelin-B receptor-knockout mice. *Circulation*. 2002;106:1991-1998. 5. Peacock AJ, Rubin LJ, eds. *Pulmonary Circulation: Diseases and Their Treatment*. 2nd ed. London: Arnold; 2004. 6. Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ET_B receptors in rats. *Biochem Biophys Res Commun*. 1994;199:1461-1465. 7. Verhaar MC, Strachan FE, Newby DE, et al. Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation*. 1998;97:752-756. 8. Halcox JPJ, Nour KRA, Zalos G, Quyyumi AA. Coronary vasodilation and improvement in endothelial dysfunction with endothelin A receptor blockade. *Circ Res*. 2001;89:969-976. 9. Giaid A, Yanagisawa M, Langleben D, et al. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med*. 1993;328:1732-1739. 10. Hankins SR, Horn EM. Current management of patients with pulmonary hypertension and right ventricular insufficiency. *Curr Cardiol Rep*. 2000;2:244-251. 11. Spieker LE, Noll G, Ruschitzka FT, Lüscher TF. Endothelin receptor antagonists in congestive heart failure: a new therapeutic principle for the future? *J Am Coll Cardiol*. 2001;37:1493-1505. 12. Jeffery TK, Wanstall JC. Pulmonary vascular remodeling: a target for therapeutic intervention in pulmonary hypertension. *Pharmacol Ther*. 2001;92:1-20. 13. Lüscher TF, Barton M. Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circulation*. 2000;102:2434-2440. 14. Chen SJ, Chen YF, Opgenorth TJ, et al. The orally active nonpeptide endothelin A-receptor antagonist A-127722 prevents and reverses hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling in Sprague-Dawley rats. *J Cardiovasc Pharmacol*. 1997;29:713-725. 15. Ihara M, Noguchi K, Saeki T, et al. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci*. 1992;50:247-255. 16. Williamson DJ, Wallman LL, Jones R, et al. Hemodynamic effects of bosentan, an endothelin receptor antagonist, in patients with pulmonary hypertension. *Circulation*. 2000;102:411-418. 17. Clozel M, Breu V, Gray GA, et al. Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther*. 1994;270:228-235.

ed that blockade or deletion of adenosine A_{2A} receptors in mice protects against bleomycin-induced dermal fibrosis, a murine model of scleroderma. Adenosine may be formed intracellularly from adenine nucleotides or extracellularly through their sequential dephosphorylation by nucleoside triphosphate diphosphohydrolase (CD39) and ecto-5' nucleotidase (CD73). Adenosine deaminase (ADA) converts adenosine to its inactive metabolite inosine, and its deficiency leads to marked increases in extracellular adenosine levels. To further characterize the contribution of endogenous adenosine to skin fibrosis, we determined whether changes in nucleoside levels, due to either injury (as in the bleomycin-induced model of dermal fibrosis) or ADA deficiency, regulate dermal fibrosis.

Methods: Male ADA deficient mice (ADAKO) were supplemented with PEG-ADA enzyme therapy for the first 3 weeks after birth. After the last enzyme injection, animals were maintained without PEG-ADA for 2 weeks and then sacrificed. Male CD39/CD73 deficient mice (CD39/73KO) were injected with the fibrosing agent bleomycin (0.1U sc qod) for 21 days. After sacrifice, dermal morphometric measurements were assessed on freshly excised skin and 6mm skin punch biopsies. Dermal collagen content was measured by hydroxyproline determination and skin adenosine levels were assessed by HPLC.

Results: ADAKO mice showed significant increases in dermal thickness (0.63±0.01 vs. 0.49±0.01 mm), skin-fold thickness (1.53±0.03 vs. 1.09±0.03 mm), breaking tension (275±11 vs. 201±6 g), and hydroxyproline content (10.2±0.4 vs. 6.5±0.2 ìg/mg tissue) (n=5, *P* < .01 for each) compared with their wild type (WT) littermates even in the absence of the exogenous fibrosant bleomycin. Adenosine levels in supernates of cultured skin from ADAKO mice were significantly higher than WT littermate controls (2.3±0.1-fold increase, n=5, *P* < .05). Conversely, CD39/CD73KO mice showed lower dermal thickness (0.29±0.05 vs. 0.36±0.10 mm), skin-fold thickness (0.79±0.16 vs. 0.97±0.37 mm), tensile strength (198.2±7.3 vs. 248.7±7.0 g) and hydroxyproline content (21.7±1.2 vs. 26.5±1.1 ìg/mg tissue) (n=6, *P* < .01 for each) compared to control mice after bleomycin treatment.

Conclusion: Adenosine is a critical regulator of dermal fibrosis whether increased as the result of a metabolic abnormality (ADA deficiency) or as the result of a toxic insult (bleomycin). Using models of both increased and decreased tissue adenosine, our results suggest that modulation of extracellular adenosine production may represent a useful therapeutic means to regulate dermal fibrogenesis in conditions such as scleroderma.

8. Gravitational stress effects in the digestive tract of patients with systemic sclerosis. Clinical and endoscopic findings. Preliminary communication

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Systemic Sclerosis (SS) or scleroderma is an autoimmune connective tissue disease characterized by vasculopathy, inflammation

and fibrosis that involves skin and internal organs. Most of the clinical manifestations in SS are secondary to vascular damage. Microvascular dysfunction, ECS damage and leakage are the components of the early inflammatory stage and fibrosis is the final stage of pathological process. Clinically, Raynaud's Phenomenon is practically a constant in SS and it precedes in years or decades "skin fibrosis", the hallmark of Scleroderma. Raynaud's Phenomenon in SS patients is not limited to the fingers: the microvascular dysfunction is a widespread phenomenon and the digestive tract doesn't escape it. In SS, in a patient with Raynaud's Phenomenon and pathological capillaroscopy, the most frequent digestive involvement is that of the esophagus, and this can be detected before the appearance of the sclerosis of the dermis.

Materials and Methods: Clinical and endoscopic findings of the digestive tract were analyzed in 27 outpatients with SS (22 F and 5 M, age 48±13 years) before and after Gravitational Therapy (Protocol GS (+GZ)). SS diagnosis was carried out according to American College of Rheumatology criteria for Scleroderma. The study was approved by the Ethical Committee of the Center and the patients' participation committee; an informed consent was obtained from each patient. All patients were evaluated by:

1) *Clinical assessment of gastrointestinal involvement* including: oral aperture measurement, hyposalivation, sclerosis of the facial skin, lip retraction, heartburn, reflux, dysphagia, dysphonia, chest pain, night asphyxia episodes, postprandial abdominal pain, constipation or diarrhea, anal sclerosis, rectal incontinence, anorexia and weight loss.

2) *Endoscopic esophagus-gastro-duodenal studies and the arytoids' characteristics* were documented. Endoscopic data of the esophagus were categorized. In 10 patients barium esophagograms, and in 3 patients esophageal manometry were performed.

3) *Digital photoplethysmography* with serial records, documented wave pulse profiles and their evolution. *Pharmacological considerations:* in this investigation all the patients suspended nonsteroidal anti-inflammatory drugs (NSAIDs), calcium channel blockers' vasodilators or antiplatelets, and / or drugs that influenced the fibrosis metabolism like corticoides, penicillamine, colchicine, etc. Patients who previously received antacids or proton-pump inhibitors (a total of 15 patients) maintained the same medication during this whole investigation.

Gravitational Therapy Procedure: All patients were placed on the couch of a human centrifuge in supine decubitus. After a week of training, all patients were exposed to different acceleration and deceleration profiles from 1 to 6 "g", from head to feet (+GZ) as previously described, during one hour, three times per week, over two months.

Results: Clinical findings before gravitational therapy: Gastrointestinal disorders involved many regions of the gut from the mouth to the anus. 1) Limitation of oral and anal opening by skin sclerosis was observed: at oral level in 13 pts. (48%) and anal level in 2 pts. (7%). 2) esophagus dysfunction in 18 pts. (67%). Heartburn in 18 pts. (67%); dysphagia in 16 pts (59%), dysphonia in 10 pts (37%); chest pain in 4 pts (15%); night asphyxia episodes in 4 pts. (15%); postprandial abdominal pain in 6 pts. (22%); constipation in 5 pts (19%); anal sclerosis in 2 pts (7%); rectal pro-

lapse in 1 patient. (4%); anorexia in 9 pts. (33%) and weight loss in 12 pts. (44%).

Endoscopic findings before Gravitational Therapy: 1) normal esophagus in 6 pts (22%). Despite endoscopy not being the preferred technique to evaluate motility, this method was used to observe: 2) mild esophagitis with esophageal motility conservation in 4 pts (15%); 3) esophageal hypomotility in 4 pts (15%); 4) distal esophagus lineal erosions in 3 pts (11%); 5) esophagus linear ulcers in 8 pts (30%); 6) hiatal hernia in 8 pts (30%); 7) Barrett's esophagus in 4 pts (15%); 8) cardias fibrosis in 1 pt (4%); 9) chronic gastritis in 5 pts (22%). 10) arythenoides edema in 10 pts. (37%). No patient in the study displayed esophagus stenosis for chronic reflux.

Photoplethysmographic findings before Gravitational Therapy: Raynaud's Phenomenon was present in 26 pts (96%), absence of pulse wave in 6 pts (22%) and pulse wave of vasospastic diathesis in 20 pts (74%).

Clinical findings after Gravitational Therapy: 1) Increase of the mouth opening in 13 pts (100%); 2) improvement of esophagus dysfunction by disappearance of the heartburn in 17 pts (94%), disappearance of the dysphagia in 12 pts (75%); correction of the dysphonia in 9 pts (90%), improvement of the chest pain in 4 pts (100%); disappearance of the episodes of night asphyxia in 4 pts (100%); improvement of the postprandial abdominal pain in 5 pts (83%); correction of constipation in 5 pts (100%); improvement of the anal sclerosis in 2 pts (100%); improvement of the anorexia in 8 pts (89%). improvement of weight loss in 10 pts (83%).

Endoscopic findings after gravitational therapy: Improvement of mild esophagitis with esophageal motility conservation in 4 pts (100%). Improvement of esophageal hypomotility in 3 pts (75%). Improvement of distal esophagus lineal erosions in 3 pts (100%). Healing of esophagus lineal ulcers in 7 pts (87%). Notorious improvement of the arythenoides edema in 10 pts (100%). No changes were observed in hiatal hernia, Barrett's esophagus, cardias fibrosis and chronic gastritis.

Photoplethysmographic findings after gravitational therapy: Clinically, Raynaud's phenomenon improved in 100% of the patients. Photoplethysmographic studies documented a significant improvement of digital perfusion ($P < .001$) with an increase of the pulse wave amplitude and changes of pulse wave profiles. Reappearance of the pulse wave was observed in 6 pts (100%).

Discussion and Conclusions: The mechanism of the microvascular dysfunction and damage in SS remains unknown. Vasodilation related to flow is severely impaired in patients with SS and Raynaud's phenomenon. Vasodilation independent from endothelium is also abnormal in patients with SS and Raynaud's phenomenon, demonstrating a functional and structural alteration of the smooth vascular muscle.

Endothelial dysfunction can play a critical role in the development of fibrosis in Systemic Sclerosis. Moreover, endothelial cell dysfunction and damage to the striated muscle and smooth muscle that make up the digestive tract, can generate leakage, edema and injury by ischemia, leading to muscle fiber necrosis, and a later stage, to scarring. Edema and fibrosis result in hypomotility, specially of the distal esophagus, which in turn alters esophageal emptying, reflux and esophagitis. Gravitational Therapy in

patients with SS induces a diversity of functional and structural responses linked to spatial and temporal effects of the gravitational stress on the vascular endothelium, the control of the capillary filtration and the tissue drainage. In previous studies we have demonstrated that the frictional vector of the hemodynamic forces exerted by the gravitational stress, constitutes a mechanical stimulus over the vessel walls, inducing vasodilation that is dependent on the flow and on the synthesis and release of prostacyclin and nitric oxide (Isasi & Isasi). The final result of the synthesis of PGI₂ is the increase of intracellular cAMP. In turn, cAMP inhibits the "cellular proliferation inducing Ras pathway". This inhibiting effect is observed particularly on fibroblasts.

Conclusion: Exposure to gravitational stress shows an overall benefit on the digestive tract of patients with SS, which is brought about by different mechanisms: a) improving the tissue perfusion, b) regulating the capillary filtration, c) facilitating the tissue drainage, d) inhibiting the fibrosis.

9. Alveolar epithelial cell injury triggers lung fibrosis in a novel mouse model of scleroderma

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Purpose: Sustained activation of transforming growth factor (TGF β) pathways has been implicated in the pathogenesis of systemic sclerosis (SSc). Transgenic mice with fibroblast-specific perturbation of TGF β signalling (T β RII Δ k-fib) develop cutaneous fibrosis, and 25% develop lung fibrosis from 16 weeks; the sporadic nature of lung involvement mimics human SSc. We postulate that greater susceptibility to alveolar epithelial cell (AEC) injury may determine pulmonary disease in the T β RII Δ k-fib model.

Methods: Littermate wild-type (WT) and transgenic (TG) animals, aged 6 weeks, were injured with intratracheal saline (0.9%, pH 5.7) or bleomycin (0.03 Units), and sacrificed at day 3, 7, 10, 14, and 21, together with matched untreated controls. Lungs were processed for ultrastructural analysis by electron microscopy, histology, and RNA and protein analysis of lung homogenates.

Results: There was ultrastructural evidence of baseline epithelial abnormality in untreated TG lungs, with AEC crenellation. Similar changes occurred after saline or bleomycin injury in WT mice. Moreover, TG mice after bleomycin (TG-B) showed severe damage to the AEC layer at 3 days. Increased lung cellularity, parenchymal connective tissue, and non cross-linked collagen in TG-B compared with WT-B animals was demonstrated from 7 days with H&E, Masson's trichrome and picrosirius red stains respectively. In addition, immunostaining revealed that, compared with WT-B, TG-B had increased parenchymal staining for TGF β 1 and PAI-1 in a peribronchial distribution, α -SMA persistence from day 21, and reduced staining for the type II AEC marker TTF-1, consistent with impaired AEC II hyperplasia. TG mice treated

with saline (TG-S) and WT-B had phenotypic similarities, implying greater susceptibility to fibrosis in response to minor injury in the TG model. These findings support the notion that TG animals exhibit an exaggerated fibroproliferative response to AEC injury, compounded by a reduced capacity for epithelial repair. Northern blotting revealed that at day 21 post injury, collagen mRNA expression was significantly elevated ($P < .0001$) in TG compared with WT animals when treated with saline or bleomycin. Colorimetric analysis showed that from day 10 post injury, TG-B had a mean newly synthesised collagen content of 45 ± 0.64 mg/g wet weight, compared with 37 ± 0.43 mg/g in WT-B ($P = .002$), whereas TG-S had 34 ± 0.44 mg/g compared with 10 ± 0.03 mg/g in WT-S ($P = .0002$). Thus, RNA and protein analysis confirm increased matrix deposition in TG animals after injury. Despite exaggerated fibrosis, lung neutrophil infiltration, quantified by myeloperoxidase ELISA, was attenuated in TG-B compared with WT-B animals (168 ± 4 ng/mL -vs- 448 ± 13 ng/mL, $P = .008$).

Conclusions: Sporadic pulmonary fibrosis in the context of systemic perturbation of TGF β bioactivity and generalised skin thickening make the T β RII Δ k-fib strain a powerful model for the fibrotic complications of SSc. Our results support a model in which altered AEC structure and exaggerated response to AEC injury, rather than inflammation, trigger lung fibrosis in this mouse model of SSc.

10. LA-B35 Influences the response to bosentan in Italian scleroderma patients with pulmonary hypertension: a new era of pharmacogenetics?

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Background: In a previous report, we have described an increased risk for developing pulmonary arterial hypertension (PAH) in Italian SSc patients carrying the HLA-B35 allele.¹ Recently, we have provided a possible explanation for this association. Indeed, different kinds of cells expressing the HLA-B35 antigens show increased apoptotic rates when exposed in vitro to thapsigargin or taxol² and human ECV304 cells transfected with the HLA-B35 allele under stimulation with physiological concentrations of IL-1 β produce increased amounts of endothelin-1 (ET-1) as compared to cells transfected with other HLA class I antigens or untransfected cells.³ Bosentan, a nonselective endothelin receptor antagonist (ERA) has been shown to improve the patient's exercise tolerance, World Health Organisation (WHO) functional class and survival, in SSc-PAH.⁴ Nonetheless, not all the patients equally respond to treatment and the reason for this discrepancy are presently unknown. We conducted the present retrospective study to verify whether the HLA-B35 allele might influence the response to bosentan in patients with SSc-PAH.

Material and Methods: Twenty-six SSc patients aged 62 ± 13.6 years (range 37-82), with a right ventricular systolic pressure

(RVSP) ≥ 45 mmHg and treated with bosentan (62.5 mg bid for 1 month and then 125 mg bid thereafter) for at least 6 months were considered. Patients with a decrease in the WHO functional class were considered as responders, otherwise as nonresponders; the percent change in the RVSP (Δ RVSP) with respect to baseline was also calculated. The presence of the HLA-B35 allele and WHO functional class responses were compared by the Fischer's exact test. To analyse the contributing factor to the Δ RVSP a linear regression model was used with the HLA-B35 allele, the patient's disease subset and disease duration, the observational time and the baseline RVSP as covariates. Student's t test was also used when appropriate.

Results: Patients were mostly females ($n=25$, 96%), with the limited cutaneous form (lcSSc) of the disease ($n=19$, 73.1%) and a mean disease duration of 12.7 ± 5.1 years (range 3-23); all the patients but two were in WHO functional class III at the beginning of therapy. Six patients (23.1%) carried the HLA-B35 antigen. Bosentan was given for a mean of 21.3 ± 9.7 months (range 6-42); 3 patients died (11.5%) in this lapse of time and 2 patients discontinued the drug due to untoward reactions. Overall, an improvement in the WHO functional class was observed in 13 patients (50%), a deterioration in 4 (15.3%), while no changes were observed in 8 cases (24.7%). In no patient carrying the HLA-B35 allele an improvement in the WHO functional class was observed, while 13 out of 20 "naïve" patients improved ($P = .015$). In patients with a WHO functional class improvement a significant decrease in the mean RVSP was observed as compared to unresponsive patients (Δ RVSP: $-27.8 \pm 15\%$ vs $3.2 \pm 14\%$). Patients carrying the HLA-B35 allele showed no RVSP responses at the end of the observational period, with a significant increase in the ϕ RVSP as compared to HLA 'naïve' patients ($4.6 \pm 14.9\%$ vs $-17.4 \pm 20.5\%$; $P = .023$). Linear regression analysis showed that the HLA-B35 allele was the only contributive factor, among those considered, to the Δ RVSP in SSc patients treated with bosentan ($P = .022$, $B = 24.5$, 95% CI for $B = 3.91-45.08$).

Conclusions: Bosentan is effective in treating SSc-PAH in most, but not all the patients. The HLA B-35 allele negatively influences the functional and the hemodynamic responses in Italian SSc-PAH subjects. Our results suggest the existence of a pharmacogenetics of responses to ERAs.

1. Scorza R, Caronni M, Bazzi S, et al. Post-menopause is the main risk factor for developing isolated pulmonary hypertension in systemic sclerosis. *Ann NY Acad Sci.* 2002;966:238-46.

2. Slazar G, Colombo G, Lenna S, et al. HLA-B35 influences the apoptosis rate in human peripheral blood mononucleated cells and HLA-transfected cells. *Human Immunol.* 2006, in press.

3. Santaniello A, Slazar G, Lenna S, et al. HLA-B35 Upregulates the Production of Endothelin-1 in HLA-Transfected Cells: A Possible Pathogenetic Role in Pulmonary Hypertension. *Tissue Antigens.* 2006, in press.

4. Williams MH, Das C, Handler CE, et al. Systemic sclerosis associated pulmonary hypertension: improved survival in the current era. *Heart.* 2006 Jul;92(7):926-32.

Important: Correction of Information about Remodulin[®] (treprostinil sodium) Injection

Dear Health Care Provider:

This letter provides important information about Remodulin relating to the treatment of pulmonary arterial hypertension. We are notifying you that United Therapeutics Corporation recently received a Warning Letter from the Food and Drug Administration (FDA) concerning the promotion of Remodulin[®] (treprostinil sodium) Injection. The Warning Letter concluded that United Therapeutics disseminated an advertisement and a promotional booklet that contained **unsubstantiated comparative claims, unsubstantiated effectiveness claims, omitted material facts, and minimized risks** relating to the use of Remodulin.

This letter provides accurate information about Remodulin and corrects certain information from our promotional materials.

Specifically, the FDA letter stated that these promotional materials contained misleading comparative claims about the benefits of Remodulin administration versus Flolan (epoprostenol sodium) because the booklet did not also disclose other comparative information that Flolan has a proven effect on walking distance and survival in the indicated patient population, while Remodulin has not demonstrated these benefits.

FDA also stated that these promotional materials contained unsubstantiated effectiveness claims by implying that Remodulin had a dose-response effect on walk distance, a statistically significant effect on walk distance, and that the effect on walk distance exceeded 10 meters. FDA concluded that these claims were not supported by substantial evidence.

These promotional materials also contained statements minimizing the risks of the infusion site reactions and pain associated with the subcutaneous administration of Remodulin. The FDA considered these statements as misleading because they did not include the incidence rates for severe reactions and pain from our clinical trials. In clinical trials, severe infusion site reactions occurred in 38% of subjects and severe pain occurred in 39% of subjects treated with Remodulin.

The FDA approved Remodulin as a continuous subcutaneous or intravenous infusion (for those not able to tolerate a subcutaneous infusion) for the treatment of pulmonary arterial hypertension in patients with NYHA Class II-IV symptoms to diminish symptoms associated with exercise. Remodulin is also indicated to diminish the rate of clinical deterioration in patients requiring transition from Flolan[®]; the risks and benefits of each drug should be carefully considered prior to transition.

The Clinical Effects section of the Remodulin PI states:

“The effect of Remodulin on 6-minute walk, the primary end point of the studies, was small and did not achieve conventional levels of statistical significance. For the combined populations, the median change from baseline on Remodulin was 10 meters and the median change from baseline on placebo was 0 meters from a baseline of approximately 345 meters. Although it was not the primary endpoint of the study, the Borg dyspnea score was significantly improved by Remodulin during the 6-minute walk, and Remodulin also had a significant effect, compared with placebo, on an assessment that combined walking distance with the Borg dyspnea score.”

Important Safety Information

In clinical trials, the most common side effects reported with subcutaneous Remodulin therapy included infusion site pain (85%) and infusion site reaction (83%). Subcutaneous infusion site pain required the use of narcotics in 32% of Remodulin treated patients and led to the discontinuation of treatment in 7% of Remodulin treated patients. Other adverse events included headache (27%), diarrhea (25%), nausea (22%), rash (14%), jaw pain (13%), vasodilatation (11%), dizziness (9%), edema (9%), pruritus (8%) and hypotension (4%). In controlled studies of Remodulin administered subcutaneously, there were no reports of infection related to the drug delivery system. There were 187 infusion system complications reported in 28% of patients (23% Remodulin, 33% placebo); 173 (93%) were pump related and 14 (7%) related to the infusion set. There are no controlled clinical studies with Remodulin administered intravenously. Among patients (n=38) treated for twelve weeks with intravenous Remodulin in an open-label study, two patients experienced either line infections or sepsis. Other events potentially related to intravenous dosing of Remodulin include arm swelling, paresthesias, hematoma and pain. Remodulin is a potent pulmonary and systemic vasodilator and should be used only by clinicians experienced in the diagnosis and treatment of pulmonary arterial hypertension. Abrupt withdrawal or sudden large reductions in dosage of Remodulin may result in worsening of PAH symptoms and should be avoided. Reduction in blood pressure caused by Remodulin may be exacerbated by drugs that by themselves alter blood pressure, such as diuretics, antihypertensive agents, or vasodilators. Since Remodulin inhibits platelet aggregation, there is also a potential for increased risk of bleeding, particularly among patients maintained on anticoagulants. Remodulin should be used with caution in patients with hepatic or renal impairment. Remodulin has not been studied in conjunction with Flolan[®] or Tracleer[®] (bosentan).

If you have any questions regarding this important corrective information, please contact United Therapeutics Corporation at 919-485-8350. Please refer to the full prescribing information for Remodulin.

REMODULIN® (treprostinil sodium) Injection

BRIEF SUMMARY

The following is a brief summary of the full prescribing information on Remodulin (treprostinil sodium) Injection. Please review the full prescribing information prior to prescribing Remodulin.

INDICATIONS AND USAGE

Remodulin® is indicated as a continuous subcutaneous infusion or intravenous infusion (for those not able to tolerate a subcutaneous infusion) for the treatment of pulmonary arterial hypertension in patients with NYHA Class II-IV symptoms to diminish symptoms associated with exercise.

Remodulin is indicated to diminish the rate of clinical deterioration in patients requiring transition from Flolan®; the risks and benefits of each drug should be carefully considered prior to transition.

DESCRIPTION

Remodulin® (treprostinil sodium) Injection is a sterile sodium salt supplied in 20 mL vials in four strengths, containing 1 mg/mL, 2.5 mg/mL, 5 mg/mL or 10 mg/mL of treprostinil. Each mL also contains 5.3 mg sodium chloride (except for the 10 mg/mL strength which contains 4.0 mg sodium chloride), 3.0 mg metacresol, 6.3 mg sodium citrate, and water for injection.

CONTRAINDICATIONS

Remodulin is contraindicated in patients with known hypersensitivity to the drug or to structurally related compounds.

WARNINGS

Remodulin is indicated for subcutaneous or intravenous use only.

PRECAUTIONS

General

Remodulin should be used only by clinicians experienced in the diagnosis and treatment of PAH. Remodulin is a potent pulmonary and systemic vasodilator. Initiation of Remodulin must be performed in a setting with adequate personnel and equipment for physiological monitoring and emergency care. Therapy with Remodulin may be used for prolonged periods, and the patient's ability to administer Remodulin and care for an infusion system should be carefully considered. Dose should be increased for lack of improvement in, or worsening of, symptoms and it should be decreased for excessive pharmacologic effects or for unacceptable infusion site symptoms. Abrupt withdrawal or sudden large reductions in dosage of Remodulin may result in worsening of PAH symptoms and should be avoided.

Information for Patients

Patients receiving Remodulin should be given the following information: Remodulin is infused continuously through a subcutaneous or surgically placed indwelling central venous catheter, via an infusion pump. Therapy with Remodulin will be needed for prolonged periods, possibly years, and the patient's ability to accept and care for a catheter and to use an infusion pump should be carefully considered. In order to reduce the risk of infection, aseptic technique must be used in the preparation and administration of Remodulin. Additionally, patients should be aware that subsequent disease management may require the initiation of an alternative intravenous prostacyclin therapy, Flolan® (epoprostenol sodium).

Drug Interactions

Reduction in blood pressure caused by Remodulin may be exacerbated by drugs that by themselves alter blood pressure, such as diuretics, antihypertensive agents, or vasodilators. Since Remodulin inhibits platelet aggregation, there is also a potential for increased risk of bleeding, particularly among patients maintained on anticoagulants. During clinical trials, Remodulin was used concurrently with anticoagulants, diuretics, cardiac glycosides, calcium channel blockers, analgesics, antipyretics, nonsteroidal anti-inflammatories, opioids, corticosteroids, and other medications. Remodulin has not been studied in conjunction with Flolan or Tracleer® (bosentan).

Effect of Other Drugs on Remodulin

In vivo studies: Acetaminophen - Analgesic doses of acetaminophen, 1000 mg every 6 hours for seven doses, did not affect the pharmacokinetics of Remodulin, at a subcutaneous infusion rate of 15 ng/kg/min.

Effect of Remodulin on Other Drugs

In vitro studies: Remodulin did not significantly affect the plasma protein binding of normally observed concentrations of digoxin or warfarin. In vivo studies: Warfarin - Remodulin does not affect the pharmacokinetics or pharmacodynamics of warfarin. The pharmacokinetics of R- and S- warfarin and the INR in healthy subjects given a single 25 mg dose of warfarin were unaffected by continuous subcutaneous Remodulin at an infusion rate of 10 ng/kg/min.

Hepatic and Renal Impairment

Caution should be used in patients with hepatic or renal impairment.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies have not been performed to evaluate the carcinogenic potential of treprostinil. In vitro and in vivo genetic toxicology studies did not demonstrate any mutagenic or clastogenic effects of treprostinil. Treprostinil sodium did not affect fertility or mating performance of male or female rats given continuous subcutaneous infusions at rates of up to 450 ng treprostinil/kg/min (about 59 times the recommended starting human rate of infusion (1.25 ng/kg/min) and about 8 times the average rate (9.3 ng/kg/min) achieved in clinical trials, on a ng/m² basis). In this study, males were dosed from 10 weeks prior to mating and through the 2-week mating period. Females were dosed from 2 weeks prior to mating until gestational day 6.

Pregnancy

Pregnancy Category B - In pregnant rats, continuous subcutaneous infusions of treprostinil sodium during organogenesis and late gestational development, at rates as high as 900 ng treprostinil/kg/min (about 117 times the starting human rate of infusion, on a ng/m² basis) and about 16 times the average rate achieved in clinical trials, resulted in no evidence of harm to the fetus. In pregnant rabbits, effects of continuous subcutaneous infusions of treprostinil during organogenesis were limited to an increased incidence of fetal skeletal variations (bilateral full rib or right rudimentary rib on lumbar 1) associated with maternal toxicity (reduction in body weight and food consumption) at an infusion rate of 150 ng treprostinil/kg/min (about 41 times the starting human rate of infusion, on a ng/m² basis, and 5 times the average rate used in clinical trials). In rats, continuous subcutaneous infusion of treprostinil from implantation to the end of lactation, at rates of up to 450 ng treprostinil/kg/min, did not affect the growth and development of offspring. Because animal reproduction studies are not always predictive of human response, Remodulin should be used during pregnancy only if clearly needed.

Labor and delivery

No treprostinil sodium treatment-related effects on labor and delivery were seen in animal studies. The effect of treprostinil sodium on labor and delivery in humans is unknown.

Nursing mothers

It is not known whether treprostinil is excreted in human milk or absorbed systemically after ingestion. Because many drugs are excreted in human milk, caution should be exercised when Remodulin is administered to nursing women.

Pediatric use

Safety and effectiveness in pediatric patients have not been established. Clinical studies of Remodulin did not include sufficient numbers of patients aged ≤16 years to determine whether they respond differently from older patients. In general, dose selection should be cautious.

Geriatric use

Clinical studies of Remodulin did not include sufficient numbers of patients aged 65 and over to determine whether they respond differently from younger patients. In general, dose selection for an elderly patient should be cautious, reflecting the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy.

ADVERSE REACTIONS

Patients receiving Remodulin as a subcutaneous infusion reported a wide range of adverse events, many potentially related to the underlying disease (dyspnea,

fatigue, chest pain, right ventricular heart failure, and pallor). During clinical trials with subcutaneous infusion of Remodulin, infusion site pain and reaction were the most common adverse events among those treated with Remodulin. Infusion site reaction was defined as any local adverse event other than pain or bleeding/bruising at the infusion site and included symptoms such as erythema, induration or rash. Infusion site reactions were sometimes severe and could lead to discontinuation of treatment. In addition, generalized rashes, sometimes macular or papular in nature, and cellulitis have been infrequently reported in postmarketing experience.

Percentages of subjects reporting subcutaneous infusion site adverse events:

	Reaction		Pain	
	Placebo	Remodulin	Placebo	Remodulin
Severe	1	36	2	39
Requiring narcotics*	NA**	NA**	1	32
Leading to discontinuation	0	3	0	7

* based on prescriptions for narcotics, not actual use

** medications used to treat infusion site pain were not distinguished from those used to treat site reactions

Other adverse events included diarrhea, jaw pain, edema, vasodilatation and nausea, and these are generally considered to be related to the pharmacologic effects of Remodulin, whether administered subcutaneously or intravenously.

Adverse Events During Chronic Dosing:

The following table lists adverse events that occurred at a rate of at least 3% and were more frequent in patients treated with subcutaneous Remodulin than with placebo in controlled trials in PAH.

Adverse Events in Controlled 12-Week Studies of Patients with PAH, Occurring with at Least 3% Incidence and More Common on Subcutaneous Remodulin than on Placebo.

Adverse Event	Remodulin (N=236) Percent of Patients	Placebo (N=233) Percent of Patients
Infusion Site Pain	85	27
Infusion Site Reaction	83	27
Headache	27	23
Diarrhea	25	16
Nausea	22	18
Rash	14	11
Jaw Pain	13	5
Vasodilatation	11	5
Dizziness	9	8
Edema	9	3
Pruritus	8	6
Hypotension	4	2

Reported adverse events (at least 3%) are included except those too general to be informative, and those not plausibly attributable to the use of the drug, because they were associated with the condition being treated or are very common in the treated population.

Adverse Events Attributable to the Drug Delivery System

In controlled studies of Remodulin administered subcutaneously, there were no reports of infection related to the drug delivery system. There were 187 infusion system complications reported in 28% of patients (23% Remodulin, 33% placebo); 173 (93%) were pump related and 14 (7%) related to the infusion set. Eight of these patients (4 Remodulin, 4 Placebo) reported non-serious adverse events resulting from infusion system complications. Adverse events resulting from problems with the delivery systems were typically related to either symptoms of excess Remodulin (e.g., nausea) or return of PAH symptoms (e.g., dyspnea). These events were generally resolved by correcting the delivery system pump or infusion set problem such as replacing the syringe or battery, reprogramming the pump, or straightening a cramped infusion line. Adverse events resulting from problems with the delivery system did not lead to clinical instability or rapid deterioration; there are no controlled clinical studies with Remodulin administered intravenously. Among the subjects (n=38) treated for 12-weeks in an open-label study, 2 patients had either line infections or sepsis. Other events potentially related to the mode of infusion include arm swelling, paresthesias, hematoma and pain.

OVERDOSAGE

Signs and symptoms of overdose with Remodulin during clinical trials are extension of its dose-limiting pharmacologic effects and include flushing, headache, hypotension, nausea, vomiting, and diarrhea. Most events were self-limiting and resolved with reduction or withholding of Remodulin.

In controlled clinical trials, seven patients received some level of overdose and in open-label follow-on treatment seven additional patients received an overdose; these occurrences resulted from accidental bolus administration of Remodulin, errors in pump programmed rate of administration, and prescription of an incorrect dose. In only two cases did excess delivery of Remodulin produce an event of substantial hemodynamic concern (hypotension, near-syncope). One pediatric patient was accidentally administered 7.5 mg of Remodulin via a central venous catheter. Symptoms included flushing, headache, nausea, vomiting, hypotension and seizure-like activity with loss of consciousness lasting several minutes. The patient subsequently recovered.

DOSAGE AND ADMINISTRATION

Remodulin® is supplied in 20 mL vials in concentrations of 1 mg/mL, 2.5 mg/mL, 5 mg/mL and 10 mg/mL. Remodulin can be administered as supplied or diluted for intravenous infusion with Sterile Water for Injection or 0.9% Sodium Chloride Injection prior to administration.

Initial Dose for Patients New to Prostacyclin Infusion Therapy

Remodulin is administered by continuous infusion. Remodulin is preferably infused subcutaneously, but can be administered by a central intravenous line if the subcutaneous route is not tolerated, because of severe site pain or reaction. The infusion rate is initiated at 1.25 ng/kg/min. If this initial dose cannot be tolerated because of systemic effects, the infusion rate should be reduced to 0.625 ng/kg/min.

Dosage Adjustments

The goal of chronic dosage adjustments is to establish a dose at which PAH symptoms are improved, while minimizing excessive pharmacologic effects of Remodulin (headache, nausea, emesis, restlessness, anxiety and infusion site pain or reaction). The infusion rate should be increased in increments of no more than 1.25 ng/kg/min per week for the first four weeks and then no more than 2.5 ng/kg/min per week for the remaining duration of infusion, depending on clinical response. There is little experience with doses >40 ng/kg/min. Abrupt cessation of infusion should be avoided (see PRECAUTIONS).

Administration

Subcutaneous Infusion

Remodulin is administered subcutaneously by continuous infusion, via a self-inserted subcutaneous catheter, using an infusion pump designed for subcutaneous drug delivery. To avoid potential interruptions in drug delivery, the patient must have immediate access to a backup infusion pump and subcutaneous infusion sets. The ambulatory infusion pump used to administer Remodulin should: (1) be small and lightweight, (2) be adjustable to approxi-

mately 0.002 mL/hr, (3) have occlusion/no delivery, low battery, programming error and motor malfunction alarms, (4) have delivery accuracy of ±6% or better and (5) be positive pressure driven. The reservoir should be made of polyvinyl chloride, polypropylene or glass.

For subcutaneous infusion, Remodulin is delivered without further dilution at a calculated Subcutaneous Infusion Rate (mL/hr) based on a patient's Dose (ng/kg/min), Weight (kg), and the Vial Strength (mg/mL) of Remodulin being used. During use, a single reservoir (syringe) of undiluted Remodulin can be administered up to 72 hours at 37°C. The Subcutaneous Infusion rate is calculated using the following formula:

$$\text{Subcutaneous Infusion Rate (mL/hr)} = \frac{\text{Dose (ng/kg/min)} \times \text{Weight (kg)} \times 0.00006^*}{\text{Remodulin Vial Strength (mg/mL)}}$$

* Conversion factor of 0.00006 = 60 min/hour x 0.000001 mg/ng

Intravenous Infusion

Remodulin must be diluted with either Sterile Water for Injection or 0.9% Sodium Chloride Injection and is administered intravenously by continuous infusion, via a surgically placed indwelling central venous catheter, using an infusion pump designed for intravenous drug delivery. To avoid potential interruptions in drug delivery, the patient must have immediate access to a backup infusion pump and infusion sets. The ambulatory infusion pump used to administer Remodulin should: (1) be small and lightweight, (2) have occlusion/no delivery, low battery, programming error and motor malfunction alarms, (3) have delivery accuracy of ±6% or better of the hourly dose, and (4) be positive pressure driven. The reservoir should be made of polyvinyl chloride, polypropylene or glass. Diluted Remodulin has been shown to be stable at ambient temperature for up to 48 hours at concentrations as low as 0.004 mg/mL (4,000 ng/mL). When using an appropriate infusion pump and reservoir, a predetermined intravenous infusion rate should first be selected to allow for a desired infusion period length of up to 48 hours between system changeovers. Typical intravenous infusion system reservoirs have volumes of 50 or 100 mL. With this selected Intravenous Infusion Rate (mL/hr) and the patient's Dose (ng/kg/min) and Weight (kg), the Diluted Intravenous Remodulin Concentration (mg/mL) can be calculated using the following formula:

The Amount of Remodulin Injection needed to make the required Diluted Intravenous Remodulin Concentration for the given reservoir size can then be calculated using the following formula:

$$\text{Step 1 Diluted Intravenous Remodulin Concentration (mg/mL)} = \frac{\text{Dose (ng/kg/min)} \times \text{Weight (kg)} \times 0.00006}{\text{Intravenous Infusion Rate (mL/hr)}}$$
$$\text{Step 2 Amount of Remodulin Injection (mL)} = \frac{\text{Diluted Intravenous Remodulin Concentration (mg/mL)} \times \text{Total Volume of Diluted Remodulin Solution in Reservoir (mL)}}{\text{Remodulin Vial Strength (mg/mL)}}$$

The calculated amount of Remodulin Injection is then added to the reservoir along with the sufficient volume of diluent (Sterile Water for Injection or 0.9% Sodium Chloride Injection) to achieve the desired total volume in the reservoir.

In patients requiring transition from Flolan:

Transition from Flolan to Remodulin is accomplished by initiating the infusion of Remodulin and increasing it, while simultaneously reducing the dose of intravenous Flolan. The transition to Remodulin should take place in a hospital with constant observation of response (e.g., walk distance and signs and symptoms of disease progression). During the transition, Remodulin is initiated at a recommended dose of 10% of the current Flolan dose, and then escalated as the Flolan dose is decreased (see table below for recommended dose titrations).

Patients are individually titrated to a dose that allows transition from Flolan therapy to Remodulin while balancing prostacyclin-limiting adverse events. Increases in the patient's symptoms of PAH should be first treated with increases in the dose of Remodulin. Side effects normally associated with prostacyclin and prostacyclin analogs are to be first treated by decreasing the dose of Flolan.

Recommended Transition Dose Changes

Step	Flolan Dose	Remodulin Dose
1	Unchanged	10% Starting Flolan Dose
2	80% Starting Flolan Dose	30% Starting Flolan Dose
3	60% Starting Flolan Dose	50% Starting Flolan Dose
4	40% Starting Flolan Dose	70% Starting Flolan Dose
5	20% Starting Flolan Dose	90% Starting Flolan Dose
6	5% Starting Flolan Dose	110% Starting Flolan Dose
7	0	110% Starting Flolan Dose + additional 5-10% increments as needed

HOW SUPPLIED

Remodulin® is supplied in 20 mL multi-use vials at concentrations of 1 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL treprostinil, as sterile solutions in water for injection, individually packaged in a carton. Unopened vials of Remodulin are stable until the date indicated when stored at 15 to 25°C (59 to 77°F). Store at 25°C (77°F), with excursions permitted to 15-30°C (59-86°F) [see USP Controlled Room Temperature].

During use, a single reservoir (syringe) of undiluted Remodulin can be administered up to 72 hours at 37°C. Diluted Remodulin Solution can be administered up to 48 hours at 37°C when diluted to concentrations as low as 0.004 mg/mL in Sterile Water for Injection or 0.9% Sodium Chloride Injection. A single vial of Remodulin should be used for no more than 30 days after the initial introduction into the vial.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit. If either particulate matter or discoloration is noted, Remodulin should not be administered.

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11. Increased expression of secreted frizzled-related protein 4 in systemic sclerosis and the tight skin mouse: potential role in skin fibrosis

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The tight skin (Tsk) mouse, an animal model for skin fibrosis in human Systemic Sclerosis (SSc), possesses a large in-frame duplication within fibrillin-1 gene, a major structural protein of connective tissue microfibrils. Tsk mice develop skin fibrosis with features that parallel skin disease in SSc patients, such as altered elastogenesis of the hypodermis and increased collagen expression. Our objective is to understand better the molecular mechanisms mediating the effect of Tsk fibrillin on skin fibrosis and how these mechanisms might be important in human SSc. Gene expression profiling and Northern blot analysis were used to identify differentially regulated molecules in skin of Tsk/+ and control wild-type, one month old mice. These results pointed to several potential candidates and signaling pathways causing altered matrices in Tsk mice. Among the most highly upregulated genes in Tsk skin, we identified several molecules of the Wnt signaling pathway, including Wingless molecules (Wnt2, Wnt9a), Wnt1 Inducible Signaling pathway Protein 2 (WISP2), Dapper homolog 2 Antagonist of b-catenin (DACT1, DACT2) and Secreted Frizzled Related Proteins (SFRP2 and SFRP4). Wnts proteins are a family of secreted cytokines that play important signaling role in a wide range of developmental processes, by binding to Frizzled receptors. The function of SFRP4 in the Tsk pathology was more specifically investigated. SFRP4 is a family member of soluble Wnt antagonists that bind directly to Wnt or Frizzled and inhibit signaling along this pathway. Consistent with mRNA data, we found increased SFRP4 protein level in Tsk skin by Western blot. Microarray and quantitative Real-Time PCR analyses showed that SFRP4 mRNA is upregulated in lesional SSc skin compared to normal individuals' skin (5-fold). These data were confirmed detecting increased expression of SFRP4 protein by immunohistochemistry. We are currently developing conditional mouse embryonic fibroblasts overexpressing SFRP4, which will permit us to study in vitro the role of SFRP4 in skin fibrosis. Our findings show here a dysregulation of Wnt signaling pathway, especially SFRP4, in Tsk and SSc skin. A recent study has demonstrated that recombinant SFRP4 reduces the number of myofibroblasts and modulates the progression of renal fibrosis, induced by unilateral ureteral obstruction. Together with these data, our results suggest a novel function of SFRP4, as a putative regulator of the cytokine cascade that leads to skin fibrosis in Tsk mice and in patients with SSc, by controlling Wnt activation.

12. CD4+ CD8+ double positive (DP) T cells with very high IL-4 production potential are present in lesional skin of systemic sclerosis (SSc) patients

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Background: Fibrotic skin changes in systemic sclerosis (SSc) are preceded by an inflammatory infiltrate rich in T cells poised to high IL-4 production. Since no direct comparison with T cells in normal skin has been performed, we aimed at functionally characterize T cells infiltrating the skin of early active SSc patients and normal skin.

Methods: Skin biopsies were from 5 individuals suffering of early active diffuse SSc and 4 healthy individuals. Biopsy fragments were cultured in IL-2-containing medium to generate T cell lines and clones which expression of coreceptors, TcR usage, cytokine production, helper and cytolytic activity was characterized. Immunofluorescence analysis of skin biopsies and peripheral blood was performed to confirm the presence of specific subsets in vivo.

Results: T cell lines were generated from all normal and SSc skins. A distinct subset expressing both CD4 and CD8 $\alpha\beta$ coreceptors at high levels (double positive, DP) was present in T cell lines from normal and, at higher frequency, from SSc skin ($P = .006$). DP T cells actively transcribed both accessory molecules, were endowed with clonally distributed cytolytic and helper activity and expressed TcR clonotypes distinct from CD4+ and CD8+ single positive (SP) T cells. In SSc, DP compared to CD4+ SP T cells produced very high levels of IL-4. Furthermore, DP T cells were directly identified in SSc skin, thus arguing for the existence of DP T cells as a distinct subset in vivo.

Conclusions: T cells with the unusual CD4+CD8+ DP phenotype are present in the skin. Their very high IL-4 production in early active SSc may contribute to enhance extracellular matrix deposition by fibroblasts. Further, the higher frequency of DP T cells in SSc may reflect an improper negative selection process during thymic maturation.

Work supported in part by the Swiss National Science Foundation, grant No 3100A1-100478/1 (CC), and the Association des Sclérodermiques de France.

13. Fli1 represses fibrillar collagen genes in mouse skin

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Biosynthesis of fibrillar collagen in the skin is precisely regulated to maintain proper tissue homeostasis. In Systemic Sclerosis (SSc) disruption of this homeostasis leads to elevated deposition of extracellular matrix (ECM) proteins, mainly collagen. We have

previously shown that Fli1 is involved in collagen repression in dermal fibroblast (Czuwara-Ladykowska et al., 2001). The levels of Fli1 protein is consistently reduced in lesional fibroblast in patients with scleroderma (Kubo et al., 2003). The goal of this study was to determine the role of Fli1 gene in collagen biosynthesis *in vivo* using two mouse models, heterozygous Fli1 mice (Fli1^{+/-}) and mice with homozygous deletion of C-terminal activation domain of Fli1 gene (Fli1^{rec/rec}). To determine the effect of Fli1 gene dosage on collagen gene expression in the skin *in vivo* we used Fli1^{+/-} and Fli1^{rec/rec} mice. The levels of procollagen type I, II, III and V mRNAs were measured by quantitative real-time PCR in the skin taken from the dorsa of the female and male mice. Absence of the C-terminal domain (Fli1^{rec/rec}) had a dramatic stimulatory effect on collagen mRNA levels, ranging from 3.6- to 16.4-fold increase in female mice and 3.2- to 6.2-fold in males. These data indicate that C-terminal domain of Fli1 plays an important role in repressing fibrillar collagen gene expression in mouse skin. The mRNA levels of various collagen chains were also modestly increased in males and females Fli1^{+/-} mice (1.1-2.5 fold increase). To assess newly synthesized collagen in Fli1^{rec/rec}, Fli1^{+/-} and control mice, acetic acid extraction method was employed. Punches (8 mm) from the dorsa of each mouse were used and analyzed by SDS-PAGE. The pattern of constituent collagen bands was similar in all samples, suggesting no qualitative differences in collagen fibril composition. However, significant quantitative differences were observed in procollagen $\alpha 1(I)$ and $\alpha 2(I)$ chains between male and female mice. In addition differences were observed in the amounts of the acetic acid extractable collagen. The increases were more pronounced in female mice (3.1-4.3 fold increase over control) than in the male mice (1.9-2.5 fold increase). To verify these observations, collagen type I was analyzed by Western blot using skin protein extracts. The amounts of type I collagen were elevated in male and female mice skin extracts in comparison to wild type mice. Fibroblasts cultured from the skin of Fli1^{rec/rec} mice maintained elevated synthesis of collagen mRNA and protein indicating that Fli1-mediated effects on matrix synthesis are the intrinsic property of these cells. To determine whether Fli1 directly targets collagen genes, we utilized chromatin immunoprecipitation (ChIP) assay. Mouse embryonic fibroblasts (MEFs) from wild type and Fli1 null mice were used. We observed that Fli1 occupies pro-Col1 $\alpha 1$ promoter in living cells. Similar results were obtained in human adult dermal fibroblasts. Together, our data show that Fli1 is a key regulator of the collagen homeostasis in the skin. Furthermore, this study supports the view that the absence of Fli1 plays a pathological role in scleroderma skin fibrosis.

14. Validation of the six-minute walk test in patients with scleroderma

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Purpose: Systemic sclerosis (SSc) associated lung disease is the most common cause of scleroderma-related deaths. The six-minute walk test (6MWT) is a validated measure in therapeutic studies of some pulmonary diseases including pulmonary hypertension (PAH). However, unlike patients with idiopathic PAH, SSc patients may have disease-related features which have an impact on walking performance and their perception of dyspnea. This prospective study evaluates the 6MWT in patients with SSc, both with regard to the patient's subjective assessment of breathing difficulty and the effects of physical limitation on performance.

Methods: Patients with SSc who were felt to be at risk for developing pulmonary vascular disease completed the 6MWT as part of an established exercise echocardiogram study. In addition to the 6MWT, patients completed pulmonary function tests, an exercise echocardiogram, the University of California San Diego shortness of breath questionnaire (UCSD), and the Scleroderma Health Assessment Questionnaire (SHAQ).

Results: Fifty patients completed 61 6MWTs. Study patients were predominantly female. Their age ranged from 34 to 77 years (mean 54) and the disease duration ranged from 1 to 28 years (mean 8.8). The 6-minute walk distance (6MWD) did not correlate with age, weight or disease duration. Low diffusion capacity (DLCO) was strongly correlated to performance on 6MWT (OR 5.4, CI 1.33-21.98, $P = .013$) in these patients. Other pulmonary function tests including TLC, FVC, and FVC/FEV₁ ratio did not correlate to 6MWT. All 3 dyspnea indices correlated to 6MWT, and this relationship was strongest for the UCSD score (OR 5.2, CI 1.71-15.83, $P < .003$). The SHAQ visual analog scales (VAS) were used to evaluate the relationship of non-pulmonary symptoms of SSc and performance on 6MWT. The respiratory VAS correlated to the distance walked ($P = .002$), but the pain and Raynaud's VAS did not. Health assessment questionnaires (HAQ) performed as part of the SHAQ allow calculation of a disability index (DI). The DI correlated with distance walked, but on logistic regression the arising component of the HAQ was the most significant predictor of poor 6MWT performance.

Conclusions: In SSc patients at increased risk for PAH, 6MWT correlates with DLCO, but not with other pulmonary function test abnormalities. Performance on the 6MWT is not influenced by demographic features, and it validly reflects subjective perception of dyspnea as measured on the UCSD and the respiratory VAS. Physical function as represented by the HAQ-DI correlates to poor performance on the 6MWT, with the "arising" component as the major contributing factor. The 6MWT provides a valid measure of exercise capacity in this subset of SSc patients. It adequately reflects symptoms without excessive interference from other disease factors, and should be a useful test to monitor patients at high risk or with PAH. These findings should hold true for SSc patients with pulmonary fibrosis, but the correlation with specific fibrotic lung disease parameters needs to be studied.



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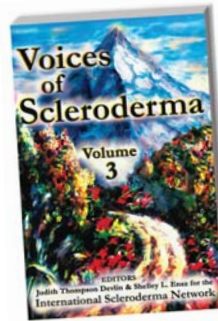
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15. MCP-1 (CCL2) knockout mice exhibit severe fibrosis in murine sclerodermatous graft versus host disease, a model for human scleroderma

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Murine sclerodermatous GVHD recapitulates many features of human scleroderma in which increased TGFβ, drive excessive fibroblast collagen synthesis. Lethally irradiated female BALB/c (H-2^d) mice transplanted with male B10.D2 (H-2^d) bone marrow and spleen cells are used to generate Scl GVHD, in which skin thickening and lung fibrosis are seen by 21 days post bone marrow transplantation (BMT). In this model, monocyte/macrophages, the main producer of TGFβ, and T cells are found in the dermis in early stages of the disease, suggesting that tissue-selective trafficking mediators may play a crucial role in cutaneous fibrosis. We have previously shown that MCP-1(CCL2) and RANTES (CCL5) mRNAs are upregulated in Scl GVHD mice compared to syngeneic BMT control mice, similar to human scleroderma. To evaluate the role of MCP-1 and macrophage influx in skin fibrosis in vivo, we used irradiated MCP-1 KO mice on the BALB/c background and wild-type littermates as recipients of B10.D2 cells. Surprisingly, those MCP-1 KO mice showed more

fibrosis in skin and lungs than wild-type Scl GVHD mice despite reduced CD45, CD3, CD11b cells in skin on day 7 compared with wild type mice with Scl GVHD. There was also a significant difference in the cutaneous gene expression profile by genechip analysis between MCP-1 KO and wild-type BALB/C mice with Scl GVHD. Therefore, we hypothesize that in addition to its function as a chemokine for immune cell; MCP-1 plays an important regulatory role in murine Scl GVHD and possibly in human scleroderma.

16. TGFβ, Receptor type I dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways: relevance for scleroderma fibrosis

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Alterations in the TGFβ signaling pathway have been implicated in scleroderma (SSc) fibrosis. We have earlier reported that SSc fibroblasts express higher endogenous ratio of TGF,RI:TGF,RII

(as compared to control fibroblasts). Furthermore, we demonstrated that forced expression (using adenoviral vectors) of TGF β ,RI (RI) in control fibroblasts at the expression levels corresponding to those in SSc fibroblasts resulted in an upregulation of the basal collagen type I gene expression (Pannu et al., A&R, 2004) suggesting that this system may be used as a model of fibrosis. Using this model, we carried out Affymetrix genechip analysis and have demonstrated an upregulation of matrix gene program (that closely resemble the Scleroderma phenotype) in dermal fibroblasts overexpressing TGF β ,RI (Pannu et al., A&R, in press). The present study was aimed at determining the molecular mechanisms underlying the profibrotic program in this model of fibrosis. We demonstrated that the TGF β ,RI-dependent up regulation of collagen and CCN2 (CTGF) genes did not involve Smad2/3 activation but was mediated by ALK1/Smad1 and ERK1/2 pathways. The following findings support this conclusion: i) Smad2 and 3 were not phosphorylated in response to TGF β ,RI, ii) a TGF β ,RI mutant defective in Smad2/3 activation, ALK5(3A) potently stimulated collagen production, iii) Smad1 was persistently phosphorylated in response to elevated TGF β ,RI, iv) blockade of Smad1 via siRNA abrogated collagen and CCN2 up regulation in this model, v) elevated TGF β ,RI led to a prolonged activation of ERK1/2, vi) pharmacologic inhibitor of ERK1/2 inhibited profibrotic effects of elevated TGF β -RI. To understand the mechanistic basis of this observation, we have utilized CCN2/CTGF promoter constructs containing site-specific mutations. We have demonstrated that the effects of RI overexpression on the CTGF promoter are mediated through a GC rich motif located between the TATA box and the transcription start site (previously characterized as the Sp1 response element) and did not require functional Smad response element. This GC rich element was shown previously to mediate upregulation of CCN2 promoter in SSc fibroblasts. Furthermore, this motif in the CCN2 promoter mediated Smad1-dependent increase of promoter activity in this model. In conclusion, this study defines a novel ALK1/Smad1 and ERK1/2 dependent, Smad3-independent mode of TGF β , signaling that may operate during chronic stages of fibrosis in SSc.

17. Proteomic analysis of scleroderma lesional skin showing an epidermal wound healing phenotype

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Scleroderma is a severe connective tissue disorder of unknown etiology characterized by a persistent fibrotic response in the skin and internal organs. Changes in the dermis in scleroderma have received much attention where persistent over production of extracellular matrix proteins by dermal fibroblasts explain in part the pathological changes seen in the disease. In order to obtain new insight into the changes in scleroderma we performed a proteomic analysis of whole skin biopsy material taken from the fibrotic skin lesions of patients with recent onset scleroderma, drawing comparison with the array of proteins expressed in healthy control

material. Proteins of altered abundance in the disease were formally identified by mass spectroscopy, and include proteins involved in fibroblast differentiation and extracellular matrix production. In addition we were surprised to find that proteins specific for the epidermal layer and involved in epidermal keratinocyte differentiation were of altered abundance in the disease. We go on to show that in scleroderma maturation of epidermal keratinocytes is delayed and abnormal and resembles a wound healing phenotype. The epidermis is endowed with fibrosis enhancing factors including endothelin-1 and transforming growth factor beta and we show altered expression of these factors in the epidermis in scleroderma. We speculate that changes in the epidermal layer might contribute to the generalized fibrotic tendency in scleroderma.

18. Autoantibodies to CD22 in patients with systemic sclerosis

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CD22 is a B cell-specific Siglec protein expressed on the cell surface as B cells mature to express IgD. CD22 has six tyrosine residues within the cytoplasmic domain, some of which are phosphorylated following B cell antigen receptor ligation. Furthermore, CD22 ligation also induces tyrosine phosphorylation of CD22. Phosphorylated CD22 recruits SHP-1 tyrosine phosphatase, and thus acts as a negative regulator of B cell activation. Therefore, CD22 engagement can modulate B cell function. Herein we examined the presence of autoantibodies to CD22 in the sera from patients with systemic sclerosis (SSc) by ELISA using recombinant protein of CD22 extracellular domain. Of 55 SSc patients, 15 (27%) were positive for anti-CD22 antibodies. While functional analyses of these autoantibodies are currently being undertaken, these results suggest that autoantibodies produced in SSc patients can influence B cell functions in vivo.

19. Effect of mycophenolate mofetil on pulmonary function in scleroderma-associated interstitial lung disease

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Purpose: To determine the effectiveness of mycophenolate mofetil (MMF) in scleroderma-associated interstitial lung disease (SSc-ILD).

Methods: We retrospectively identified patients who received MMF for treatment of SSc-ILD. Patients were included if they met ACR criteria for systemic sclerosis, had an abnormal high-resolution chest CT, received more than 1 gram/day of MMF for

at least 6 months, and had pulmonary function measured before and during treatment. Diffusion capacity (DLCO), vital capacity (VC), and modified Rodnan skin scores at treatment onset were compared with those on treatment using paired t-tests. Standardized selection rules were used to identify DLCO and VC measurements made ~12 months prior to treatment, and these values were also compared to DLCO and VC at treatment onset using paired t-tests.

Results: Thirteen patients with systemic sclerosis (9 diffuse, 4 limited) were treated with a median dose of 2 g/day of MMF. Patients were diagnosed with systemic sclerosis and SSc-ILD a median of 6 and 2 years, respectively, prior to treatment with MMF. Pulmonary function abnormalities at treatment onset were mild-to-moderate (VC 2.7 ± 0.6 L or $70 \pm 15\%$ predicted; DLCO 16 ± 6 mL/mmHg/min or $51 \pm 13\%$ of the predicted value). During median follow-up of 21 months, patients treated with MMF experienced an improvement in VC (mean difference +129 mL [CI +52 to +205] and +4% of the predicted value [CI +2 to +7]) but no change in DLCO (mean difference 0.1 mL/mmHg/min [CI -1.1 to +1.3] and +1 % of the predicted value [CI -3 to +5]). In contrast, during a median 14-month period prior to treatment onset, patients experienced a decrease in VC (mean difference -228 mL [CI -384 to -72] and -5% of the predicted value [CI -9 to -1]) and trend towards decrease in DLCO (mean difference -1.5 mL/mmHg/min [CI -3.3 to +0.4] and -4 % of the predicted value [-9 to +1]). Modified Rodnan skin scores did not change during treatment with MMF (mean difference -1 [CI -5 to +3]).

Conclusion: These retrospective data suggest MMF improves VC and may prevent deterioration in DLCO in patients with SSc-ILD.

20. Monitoring of gene expression by microarrays: bosentan attenuates the pro-fibrotic gene expression profile of lung fibroblasts in scleroderma

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Purpose: A body of indirect evidence implicates endothelin-1 (ET-1) in pathogenesis of scleroderma (SSc). Bosentan is a dual specificity endothelin receptor antagonist that has beneficial effects on vasculopathy in SSc. ET-1 has previously been shown to have profibrotic activity, although recent clinical studies of bosentan in fibrotic lung disease have been inconclusive. In the present study we have explored the potential attenuation of fibrotic gene expression by bosentan using explanted fibroblasts from scleroderma associated pulmonary fibrosis (SSc-PF) and from nonfibrotic control lung samples.

Methods: The effect of an 18 hour treatment of bosentan on the expression of genes in normal and FASSc lung fibroblasts (N=5) was assessed by U133A Affymetrix gene chips analyzed by D-Chip software, real-time polymerase chain reaction (RT-PCR) and Western blot analysis. In addition, the effect of bosentan on the phenotype of SSc-PF lung fibroblasts was assessed by functional assays.

Results: Hierarchical cluster analysis of Affymetrix gene arrays revealed that FASSc lung fibroblasts differentially overexpressed over 350 genes compared to control normal lung fibroblasts, of which approximately 25% were dependent upon endogenous ET-1 signaling. Cluster analysis revealed that mRNAs involved with metabolism, cytoskeleton, adhesion, transcription, extracellular matrix (ECM) and cell proliferation were sensitive to bosentan. RT-PCR and Western analysis confirmed that members of all cohorts were upregulated in SSc-PF lung fibroblasts and sensitive to endothelin antagonist treatment. Functional analysis revealed that the overexpression of ECM and ECM-associated genes, including type I collagen, fibronectin and connective tissue growth factor, by SSc-PF lung fibroblasts was blocked by bosentan ($P < .05$). Similarly, the elevated adhesive, migratory and contractile phenotype of SSc-PF lung fibroblasts was blocked by bosentan.

Conclusion: These data suggest that elevated endogenous ET-1 signaling contributes to the pro-fibrotic phenotype observed in SSc-PF lung fibroblasts. Our findings provide support for continued exploration of the potential clinical benefit of endothelin receptor antagonists in SSc and other fibrotic diseases.

21. The transcription factor Egr-1 is a novel mediator of TGF- β responses and plays key roles in fibrosis in vitro and in vivo

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Purpose: We showed previously that the zinc finger transcription factor Egr-1 was rapidly induced by TGF- β in fibroblasts, and played a key role in stimulation of collagen gene expression by TGF- β . Because the significance of Egr-1 in fibrosis is unknown, we investigated its expression, regulation, mechanism of action and biological role in fibrotic responses in vitro and in vivo.

Methods: Normal and scleroderma skin fibroblasts, and fibroblasts from Egr-1 null mice, were used. Gene expression was examined with Northern and Western analysis and transient transfection assays. Skin and lung fibrosis in wildtype and Egr-1 null mice was induced in vivo with bleomycin. Lesional tissue was examined by histology, immunohistochemistry and PCR. Tissue collagen was quantitated with SIRCOL assays. Ex vivo cytokine production by bone marrow macrophages was assessed by ELISA.

Results: In normal fibroblasts, Egr-1 (protein and mRNA) was rapidly induced by TGF- β . Transient transfection assays with Egr promoter-luc constructs demonstrated that stimulation was mediated at the transcriptional level. Stimulation was blocked by inhibitors of ERK1/2, but not the ALK5, indicating that this response was MAPK-dependent and ALK5/Smad-independent. Egr-1 overexpression in normal fibroblasts caused increased collagen synthesis, and Egr-1 directly transactivated COL1A2 via a consensus DNA element (EBS) located within the TGF- β response region of the COL1A2 promoter. Stimulation of collagen gene expression by TGF- β was markedly impaired in Egr-1-null

fibroblasts. In BALB/c mice, bleomycin induced striking early skin inflammation, and subsequent fibrosis associated with elevation in lesional Egr-1. In contrast, in Egr-1-null mice both acute inflammation and cutaneous fibrosis were markedly reduced. Fibrosis in the lung was also attenuated. Furthermore, collagen mRNA expression, collagen accumulation and organization were impaired, and alpha smooth muscle expression and fibrogenic cytokine levels were reduced. Egr-1-null macrophages showed impaired TGF- β -dependent cytokine production *ex vivo*.

Summary: Egr-1 is a novel TGF- β -inducible gene that plays key roles in mediating TGF- β -dependent profibrotic activation of fibroblasts. Tissue fibrosis in a mouse model is associated with marked increase in Egr-1 expression in lesional tissue, and loss of Egr-1 in these mice is associated with fibrosis resistance.

Conclusion: This is the first evidence implicating Egr-1 as an important component of TGF- β -driven Smad-independent fibrotic responses relevant to the pathogenesis of scleroderma. Therapeutic targeting of Egr-1 therefore represents a novel approach to controlling fibrosis.

22. The role of Gata3 as a regulator of ECM homeostasis in SSc

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Systemic sclerosis (SSc) is a connective tissue disease that is distinguished by the increased production and deposition of extracellular matrix (ECM), especially collagen. In the past, cultured fibroblasts from SSc patients were used to study the mechanism of the disease, but a recent cDNA microarray study revealed that with passaging SSc fibroblasts lose many of their characteristics (Gardner *et al.*, in press). The data suggested that analyzing the SSc biopsy as a whole with powerful methods, like cDNA microarray analysis, may provide many more leads on which pathways are altered in SSc fibroblasts. Interestingly, array data indicated that expression levels of a transcription factor, Gata3, are significantly reduced in SSc skin. To determine the distribution of Gata3 in skin cell types, we performed immunohistochemical analysis of 7 normal and 15 SSc dermal sections. In healthy skin, immunoreactivity was detected in epithelial cells in the epidermis and hair follicles, endothelial cells lining blood vessels and fibroblasts. In contrast, in the majority of SSc dermal sections, Gata3 expression was seen in epithelial cells in the epidermis and hair follicles (9/15), but was drastically downregulated in dermal fibroblasts (12/15) and endothelial cells (14/15). These data reveal for the first time that Gata3, a T-cell specific transcription factor, is expressed in dermal fibroblasts and differentially expressed in SSc skin. To determine a potential role of Gata3 in fibroblast function, a cDNA microarray analysis was performed with Gata3 suppressed by siRNA. 503 genes were upregulated

and 540 genes were downregulated, indicating that Gata3 functions as both a repressor and activator in fibroblasts. Most importantly, this experiment showed that when Gata3 is knocked down in fibroblasts, cellular matrix proteins including thrombospondin1 (TSP1), tenascin C (TN-C), procollagen-lysine 2-oxoglutarate 5-dioxygenase (PLOD2) and lysyl oxidase-like 2 (LOXL2) are upregulated. This was a significant finding because these matrix proteins are markedly upregulated in fibrosis. In addition, many other characteristics of the SSc biopsy were recapitulated in fibroblasts with Gata3 suppressed. For example, the Wnt pathway was one of the major pathways upregulated in the biopsy microarray analysis and it was also upregulated in the Gata3 knockdown array. To further investigate the role of Gata3 as a transcriptional repressor in fibroblasts, we are focusing on the TN-C promoter, which contains 7 putative GATA sites. Using a series of 5' deletions of the TN-C promoter linked to the chloramphenicol acetyltransferase reporter gene, we cotransfected Gata3 and analyzed promoter activity. The deletion constructs, -2100, -1300, -516, and -248, which contain 5, 3, 1, and 0 putative GATA sites, respectively, were cotransfected with 0.5 mg of Gata3. Both distal portions, -2100 and -1300, showed about a 50% decrease in promoter activity in the presence of Gata3, while the -516 promoter activity was suppressed about 90%. Gata3 had no significant effect on the activity of the -248 promoter. These data suggest that Gata3 may be a functional repressor of the TN-C promoter and that the putative GATA site within the -516 promoter may be a key negative GATA response element.

Conclusion: Based on these findings, we hypothesize that Gata3 may play a role in maintaining a quiescent state of fibroblasts by regulating programs responsible for ECM production. Loss of Gata3 may directly contribute to SSc fibrosis.

23. Fibrillin-1 mutants in Marfan syndrome and tight-skin mouse antagonistically affect expression of CCN3 and MAGP-2: novel molecular insights into extracellular matrix regulation by fibrillin-1 in connective tissue

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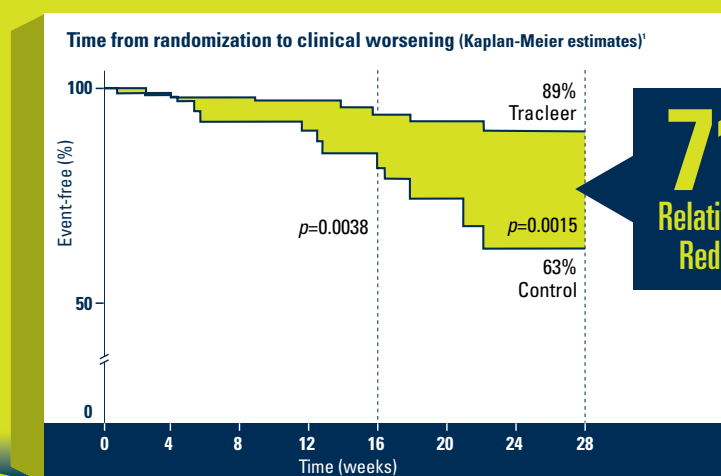
Purpose: Fibrillins are major structural components of connective tissue microfibrils and play important functions in extracellular matrix homeostasis. Mutations in fibrillins are associated with various connective tissue dysfunctions that range from fibrosis in the skin of Tight-skin (Tsk) mouse (gain-of-function mutation) to reduction of structural integrity in the skin and the aorta in Marfan syndrome (loss-of-function mutation). The molecular basis for extracellular matrix dysregulation by these mutant fibrillins is unclear. We investigated here altered gene expression and matrix deposition associated with fibrillin-1 mutations in both the Tsk mouse, a model of skin fibrosis in scleroderma, and in Marfan syndrome.

Methods: Gene expression in Tsk and control mouse skin was analyzed by Affymetrix microarray. Further analysis was performed using northern blot, western blot, immunohistochemistry, and immunofluorescence. Gene expression in Marfan syndrome

In pulmonary arterial hypertension (PAH) WHO Class III or IV secondary to scleroderma, identify and treat early.



Tracleer: The Only Oral Therapy Proven to Reduce the Risk of Clinical Worsening



Clinical worsening is defined in bosentan clinical trials as the combined endpoint of:

- Death
- Hospitalization for treatment related to PAH
- Discontinuation of therapy due to worsening PAH
- Initiation of epoprostenol therapy

BREATHE-1 All patients (n=144 in the Tracleer group and n=69 in the control group) participated in the first 16 weeks. A subset of this population (n=35 in the Tracleer group and n=13 in the control group) continued for up to 28 weeks.

- Treatment effect was notable because both the Tracleer groups and the control groups could have received background therapy, which excluded IV epoprostenol but may have included vasodilators (calcium channel blockers or ACE inhibitors), digoxin, anticoagulants, and/or diuretics²

Liver and pregnancy warnings

Requires attention to two significant concerns: Potential for serious liver injury (including very rare cases of unexplained hepatic cirrhosis after prolonged treatment)—Liver monitoring of all patients is essential prior to initiation of treatment and monthly thereafter. **High potential for major birth defects**—Pregnancy must be excluded and prevented by two forms of birth control; monthly pregnancy tests should be obtained.

Prescribed to over 30,000 patients³



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THE DUAL ENDOTHELIN RECEPTOR ANTAGONIST

A Cornerstone of Oral Therapy



Please see brief summary of prescribing information and full reference list on following page.



62.5 mg and 125 mg film-coated tablets

Brief Summary: Please see package insert for full prescribing information.

Use of TRACLEER® requires attention to two significant concerns: 1) potential for serious liver injury, and 2) potential damage to a fetus.

WARNING: Potential liver injury. TRACLEER® causes at least 3-fold (upper limit of normal: ULN) elevation of liver aminotransferases (ALT and AST) in about 11% of patients, accompanied by elevated bilirubin in a small number of cases. Because these changes are a marker for potential serious liver injury, serum aminotransferase levels must be measured prior to initiation of treatment and then monthly (see WARNINGS: Potential Liver Injury and DOSAGE AND ADMINISTRATION). In the post-marketing period, in the setting of close monitoring, rare cases of unexplained hepatic cirrhosis were reported after prolonged (> 12 months) therapy with TRACLEER® in patients with multiple co-morbidities and drug therapies. There have also been rare reports of liver failure. The contribution of TRACLEER® in these cases could not be excluded.

In at least one case the initial presentation (after > 20 months of treatment) included pronounced elevations in aminotransferases and bilirubin levels accompanied by non-specific symptoms, all of which resolved slowly over time after discontinuation of TRACLEER®. This case reinforces the importance of strict adherence to the monthly monitoring schedule for the duration of treatment and the treatment algorithm, which includes stopping TRACLEER® with a rise of aminotransferases accompanied by signs or symptoms of liver dysfunction. (see DOSAGE AND ADMINISTRATION).

Elevations in aminotransferases require close attention (see DOSAGE AND ADMINISTRATION). TRACLEER® should generally be avoided in patients with elevated aminotransferases (> 3 x ULN) at baseline because monitoring liver injury may be more difficult. If liver aminotransferase elevations are accompanied by clinical symptoms of liver injury (such as nausea, vomiting, fever, abdominal pain, jaundice, or unusual lethargy or fatigue) or increases in bilirubin $\geq 2 \times$ ULN, treatment should be stopped. There is no experience with the re-introduction of TRACLEER® in these circumstances.

CONTRAINDICATION: Pregnancy. TRACLEER® (bosentan) is very likely to produce major birth defects if used by pregnant women, as this effect has been seen consistently when it is administered to animals (see CONTRAINDICATIONS). Therefore, pregnancy must be excluded before the start of treatment with TRACLEER® and prevented thereafter by the use of a reliable method of contraception. Hormonal contraceptives, including oral, injectable, transdermal, and implantable contraceptives should not be used as the sole means of contraception because these may not be effective in patients receiving TRACLEER® (see Precautions: Drug Interactions). Therefore, effective contraception through additional forms of contraception must be practiced. Monthly pregnancy tests should be obtained.

Because of potential liver injury and in an effort to make the chance of fetal exposure to TRACLEER® (bosentan) as small as possible, TRACLEER® may be prescribed only through TRACLEER® Access Program by calling 1 866 228 3546. Adverse events can also be reported directly via this number.

INDICATIONS AND USAGE: TRACLEER® is indicated for the treatment of pulmonary arterial hypertension (WHO Group I) in patients with WHO Class III or IV symptoms, to improve exercise ability and decrease the rate of clinical worsening.

CONTRAINDICATIONS: TRACLEER® is contraindicated in pregnancy, with concomitant use of cyclosporine A, with administration of glyburide, and in patients who are hypersensitive to bosentan or any component of the medication.

Pregnancy Category X. TRACLEER® is expected to cause fetal harm if administered to pregnant women. The similarity of malformations induced by bosentan and those observed in endothelin-1 knockout mice and in animals treated with other endothelin receptor antagonists indicates that teratogenicity is a class effect of these drugs. There are no data on the use of TRACLEER® in pregnant women. TRACLEER® should be started only in patients known not to be pregnant. For female patients of childbearing potential, a prescription for TRACLEER® should not be issued by the prescriber unless the patient assures the prescriber that she is not sexually active or provides negative results from a urine or serum pregnancy test performed during the first 5 days of a normal menstrual period and at least 11 days after the last unprotected act of sexual intercourse. Follow-up urine or serum pregnancy tests should be obtained monthly in women of childbearing potential taking TRACLEER®. The patient must be advised that if there is any delay in onset of menses or any other reason to suspect pregnancy, she must notify the physician immediately for pregnancy testing. If the pregnancy test is positive, the physician and patient must discuss the risk to the pregnancy and to the fetus.

WARNINGS: Potential Liver Injury: Elevations in ALT or AST by more than 3x ULN were observed in 11% of bosentan-treated patients (N = 658) compared to 2% of placebo-treated patients (N = 280). The combination of hepatocellular injury (increases in aminotransferases of > 3 x ULN) and increases in total bilirubin ($\geq 3 \times$ ULN) is a marker for potential serious liver injury. Elevations of AST and/or ALT associated with bosentan are dose-dependent, occur both early and late in treatment, usually progress slowly, are typically asymptomatic, and to date have been reversible after treatment interruption or cessation. These aminotransferase elevations may reverse spontaneously while continuing treatment with TRACLEER®. Liver aminotransferase levels must be measured prior to initiation of treatment and then monthly. If elevated aminotransferase levels are seen, changes in monitoring and treatment must be initiated. If liver aminotransferase elevations are accompanied by clinical symptoms of liver injury (such as nausea, vomiting, fever, abdominal pain, jaundice, or unusual lethargy or fatigue) or increases in bilirubin $\geq 2 \times$ ULN, treatment should be stopped. There is no experience with the re-introduction of TRACLEER® in these circumstances. **Pre-existing Liver Impairment:** TRACLEER® should generally be avoided in patients with moderate or severe liver impairment. In addition, TRACLEER® should generally be avoided in patients with elevated aminotransferases (> 3 x ULN) because monitoring liver injury in these patients may be more difficult.

PRECAUTIONS: Hematologic Changes: Treatment with TRACLEER® caused a dose-related decrease in hemoglobin and hematocrit. The overall mean decrease in hemoglobin concentration for bosentan-treated patients was 0.9 g/dl change to end of treatment. Most of this decrease of hemoglobin concentration was detected during the first few weeks of bosentan treatment and hemoglobin levels stabilized by 4–12 weeks of bosentan treatment. In placebo-controlled studies of all uses of bosentan, marked decreases in hemoglobin (> 15% decrease from baseline resulting in values of < 11 g/dl) were observed in 6% of bosentan-treated patients and 3% of placebo-treated patients. In patients with pulmonary arterial hypertension treated with doses of 125 and 250 mg b.i.d., marked decreases in hemoglobin occurred in 3% compared to 1% in placebo-treated patients. A decrease in hemoglobin concentration by at least 1 g/dl was observed in 57% of bosentan-treated patients as compared to 29% of placebo-treated patients. In 80% of cases, the decrease occurred during the first 6 weeks of bosentan treatment. During the course of treatment the hemoglobin concentration remained within normal limits in 68% of bosentan-treated patients compared to 76% of placebo patients. The explanation for the change in hemoglobin is not known, but it does not appear to be hemorrhage or hemolysis. It is recommended that hemoglobin concentrations be checked after 1 and 3 months, and every 3 months thereafter. If a marked decrease in hemoglobin concentration occurs, further evaluation should be undertaken to determine the cause and need for specific treatment. **Fluid retention:** In a placebo-controlled trial of patients with severe chronic heart failure, there was an increased incidence of hospitalization for CHF associated with weight gain and increased leg edema during the first 4–8 weeks of treatment with TRACLEER®. In addition, there have been numerous post-marketing reports of fluid retention in patients with pulmonary hypertension, occurring within weeks after starting TRACLEER®. Patients required intervention with a diuretic, fluid management, or hospitalization for decompensating heart failure.

Information for Patients: Patients are advised to consult the TRACLEER® Medication Guide on the safe use of TRACLEER®. The physician should discuss with the patient the importance of monthly monitoring of serum aminotransferases and urine or serum pregnancy testing and of avoidance of pregnancy. The physician should discuss options for effective contraception and measures to prevent pregnancy with their female patients. Input from a gynecologist or similar expert on adequate contraception should be sought as needed.

Drug Interactions: Bosentan is metabolized by CYP2C9 and CYP3A4. Inhibition of these isoenzymes will likely increase the plasma concentration of bosentan. Bosentan is an inducer of CYP3A4 and CYP2C9. Consequently, plasma concentrations of drugs metabolized by these two isoenzymes will be decreased when TRACLEER® is co-administered. **Contraceptives:** Co-administration of bosentan and the oral hormonal contraceptive Ortho-Novum® produced decreases of norethindrone and ethinyl estradiol levels by as much as 56% and 66%, respectively, in individual subjects. Therefore, hormonal contraceptives, including oral, injectable, transdermal, and implantable forms, may not be reliable when TRACLEER® is co-administered. Women should practice additional methods of contraception and not rely on hormonal contraception alone when taking TRACLEER®. **Cyclosporine A:** During the first day of concomitant administration, trough concentrations of bosentan were increased by about 30-fold. Steady-state bosentan plasma concentrations were 3–4-fold higher than in the absence of cyclosporine A (see CONTRAINDICATIONS). Tacrolimus: Co-administration of tacrolimus and bosentan has not been studied in man. Co-administration of tacrolimus and bosentan resulted in markedly increased plasma concentrations of bosentan in animals. Caution should be exercised if tacrolimus and bosentan are used together. **Glyburide:** An increased risk of elevated liver aminotransferases was observed in patients receiving concomitant therapy with glyburide (see CONTRAINDICATIONS). Alternative hypoglycemic agents should be considered. Bosentan is also expected to reduce plasma concentrations of other oral hypoglycemic agents that are predominantly metabolized by CYP2C9 or CYP3A4. The possibility of worsened glucose control in patients using these agents should be considered. **Ketoconazole:** Co-administration of bosentan 125 mg b.i.d. and ketoconazole, a potent CYP3A4 inhibitor, increased the plasma concentrations of bosentan by approximately 2-fold. No dose adjustment of bosentan is necessary, but increased effects of bosentan should be considered. **Simvastatin and Other Statins:** Co-administration of bosentan decreased the plasma concentrations of simvastatin (a CYP3A4 substrate), and its active β -hydroxy acid metabolite, by approximately 50%. The plasma concentrations of bosentan were not affected. Bosentan is also expected to reduce plasma concentrations of other statins that have significant metabolism by CYP3A4, eg, lovastatin and atorvastatin. The possibility of reduced statin efficacy

should be considered. Patients using CYP3A4 metabolized statins should have cholesterol levels monitored after TRACLEER® is initiated to see whether the statin dose needs adjustment. **Warfarin:** Co-administration of bosentan 500 mg b.i.d. for 6 days decreased the plasma concentrations of both S-warfarin (a CYP2C9 substrate) and R-warfarin (a CYP3A4 substrate) by 29 and 38%, respectively. Clinical experience with concomitant administration of bosentan and warfarin in patients with pulmonary arterial hypertension did not show clinically relevant changes in INR or warfarin dose, and the need to change the warfarin dose during the trials due to changes in INR or due to adverse events was similar among bosentan- and placebo-treated patients. **Digoxin, Nimodipine and Losartan:** Bosentan has been shown to have no pharmacokinetic interactions with digoxin and nimodipine, and losartan has no effect on plasma levels of bosentan.

Sildenafil: In healthy subjects, co-administration of multiple doses of 125 mg b.i.d. bosentan and 80 mg t.i.d. sildenafil resulted in a reduction of sildenafil plasma concentrations by 63% and increased bosentan plasma concentrations by 50%. A dose adjustment of neither drug is necessary. This recommendation holds true when sildenafil is used for the treatment of pulmonary arterial hypertension or erectile dysfunction.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Two years of dietary administration of bosentan to mice produced an increased incidence of hepatocellular adenomas and carcinomas in males at doses about 8 times the maximum recommended human dose (MRHD) of 125 mg b.i.d., on a mg/m² basis. In the same study, doses greater than about 32 times the MRHD were associated with an increased incidence of colon adenomas in both males and females. In rats, dietary administration of bosentan for two years was associated with an increased incidence of brain astrocytomas in males at doses about 16 times the MRHD. **Impairment of Fertility/Testicular Function:** Many endothelin receptor antagonists have profound effects on the histology and function of the testes in animals. These drugs have been shown to induce atrophy of the seminiferous tubules of the testes and to reduce sperm counts and male fertility in rats when administered for longer than 10 weeks. Where studied, testicular tubular atrophy and decreases in male fertility observed with endothelin receptor antagonists appear irreversible. In fertility studies in which male and female rats were treated with bosentan at oral doses of up to 50 times the MRHD on a mg/m² basis, no effects on sperm count, sperm motility, mating performance or fertility were observed. An increased incidence of testicular tubular atrophy was observed in rats given bosentan orally at doses as low as about 4 times the MRHD for two years but not at doses as high as about 50 times the MRHD for 6 months. An increased incidence of tubular atrophy was not observed in mice treated for 2 years at doses up to about 75 times the MRHD or in dogs treated up to 12 months at doses up to about 50 times the MRHD. There are no data on the effects of bosentan or other endothelin receptor antagonists on testicular function in man.

Pregnancy, Teratogenic Effects: Category X

SPECIAL POPULATIONS: Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, breastfeeding while taking TRACLEER® is not recommended. **Pediatric Use:** Safety and efficacy in pediatric patients have not been established. Use in Elderly Patients: Clinical experience with TRACLEER® in subjects aged 65 or older has not included a sufficient number of such subjects to identify a difference in response between elderly and younger patients.

ADVERSE REACTIONS: Safety data on bosentan were obtained from 12 clinical studies (8 placebo-controlled and 4 open-label) in 777 patients with pulmonary arterial hypertension, and other diseases. Treatment discontinuations due to adverse events other than those related to pulmonary hypertension during the clinical trials in patients with pulmonary arterial hypertension were more frequent on bosentan (5%; 8/165 patients) than on placebo (3%; 2/80 patients). In this database the only cause of discontinuations > 1%, and occurring more often on bosentan was abnormal liver function. In placebo-controlled studies of bosentan in pulmonary arterial hypertension and for other diseases (primarily chronic heart failure), a total of 677 patients were treated with bosentan at daily doses ranging from 100 mg to 2000 mg and 288 patients were treated with placebo. The duration of treatment ranged from 4 weeks to 6 months. For the adverse drug reactions that occurred in 3% of bosentan-treated patients, the only ones that occurred more frequently on bosentan than on placebo (2% difference) were headache (16% vs. 13%), flushing (1% vs. 2%), abnormal hepatic function (6% vs. 2%), leg edema (5% vs. 1%), and anemia (3% vs. 1%). Additional adverse reactions that occurred in > 3% of bosentan-treated pulmonary arterial hypertension patients were: nasopharyngitis (11% vs. 8%), hypotension (7% vs. 4%), palpitations (5% vs. 1%), dyspepsia (4% vs. 0%), edema (4% vs. 3%), fatigue (4% vs. 1%), and pruritus (4% vs. 0%). Post-marketing experience: hypersensitivity, rash, angiodema.

Special Considerations: Patients with Congestive Heart Failure (CHF): Based on the results of a pair of studies with 1613 subjects, bosentan is not effective in the treatment of CHF with left ventricular dysfunction.

OVERDOSAGE: Bosentan has been given as a single dose of up to 2400 mg in normal volunteers, or up to 2000 mg/day for 2 months in patients, without any major clinical consequences. The most common side effect was headache of mild to moderate intensity. In the cyclosporine A interaction study, in which doses of 500 and 1000 mg b.i.d. of bosentan were given concomitantly with cyclosporine A, trough plasma concentrations of bosentan increased 30-fold, resulting in severe headache, nausea, and vomiting, but no serious adverse events. Mild decreases in blood pressure and increases in heart rate were observed. There is no specific experience of overdosage with bosentan beyond the doses described above. Massive overdosage may result in pronounced hypotension requiring active cardiovascular support.

DOSAGE AND ADMINISTRATION: TRACLEER® treatment should be initiated at a dose of 62.5 mg b.i.d. for 4 weeks and then increased to the maintenance dose of 125 mg b.i.d. Doses above 125 mg b.i.d. did not appear to confer additional benefit sufficient to offset the increased risk of liver injury. Tablets should be administered morning and evening with or without food.

Dosage Adjustment and Monitoring in Patients Developing Aminotransferase Abnormalities

ALT/AST levels	Treatment and monitoring recommendations
> 3 and = 5 x ULN	Confirm by another aminotransferase test; if confirmed, reduce the daily dose or interrupt treatment, and monitor aminotransferase levels at least every 2 weeks. If the aminotransferase levels return to pre-treatment values, continue or re-introduce the treatment as appropriate (see below).
> 5 and = 8 x ULN	Confirm by another aminotransferase test; if confirmed, stop treatment and monitor aminotransferase levels at least every 2 weeks. Once the aminotransferase levels return to pre-treatment values, consider re-introduction of the treatment (see below).
> 8 x ULN	Treatment should be stopped and re-introduction of TRACLEER® should not be considered. There is no experience with re-introduction of TRACLEER® in these circumstances.

If TRACLEER® is re-introduced it should be at the starting dose; aminotransferase levels should be checked within 3 days and thereafter according to the recommendations above. If liver aminotransferase elevations are accompanied by clinical symptoms of liver injury (such as nausea, vomiting, fever, abdominal pain, jaundice, or unusual lethargy or fatigue) or increases in bilirubin $\geq 2 \times$ ULN, treatment should be stopped. There is no experience with the re-introduction of TRACLEER® in these circumstances. Use in Women of Child-bearing Potential: See CONTRAINDICATIONS and Drug Interactions. **Dosage Adjustment in Renally Impaired Patients:** The effect of renal impairment on the pharmacokinetics of bosentan is small and does not require dosage adjustment. **Dosage Adjustment in Geriatric Patients:** Clinical studies of TRACLEER® did not include sufficient numbers of subjects aged 65 and older to determine whether they respond differently from younger subjects. In general, caution should be exercised in dose selection for elderly patients given the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy in this age group. **Dosage Adjustment in Hepatically Impaired Patients:** The influence of liver impairment on the pharmacokinetics of TRACLEER® has not been evaluated. Because there is *in vivo* and *in vitro* evidence that the main route of excretion of TRACLEER® is biliary, liver impairment would be expected to increase exposure to bosentan. There are no specific data to guide dosing in hepatically impaired patients; caution should be exercised in patients with mildly impaired liver function. TRACLEER® should generally be avoided in patients with moderate or severe liver impairment. **Dosage Adjustment in Children:** Safety and efficacy in pediatric patients has not been established. **Dosage Adjustment in Patients with Low Body Weight:** In patients with a body weight below 40 kg but who are over 12 years of age the recommended initial and maintenance dose is 62.5 mg b.i.d. **Discontinuation of Treatment:** There is limited experience with abrupt discontinuation of TRACLEER®. No evidence for acute rebound has been observed. Nevertheless, to avoid the potential for clinical deterioration, gradual dose reduction (62.5 mg b.i.d. for 3 to 7 days) should be considered.

HOW SUPPLIED: 62.5 mg film-coated, round, biconvex, orange-white tablets, embossed with identification marking "62.5" NDC 66215-101-06: Bottle containing 60 tablets. 125 mg film-coated, oval, biconvex, orange-white tablets, embossed with identification marking "125" NDC 66215-102-06: Bottle containing 60 tablets.

Rx only.

STORAGE: Store at 20°C – 25°C (68°F – 77°F). Excursions are permitted between 15°C and 30°C (59°F and 86°F). [See USP Controlled Room Temperature].

References for previous pages: 1. Rubin LJ, Badesch DB, Barst RJ, et al. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med.* 2002;346:896–903. 2. Tracleer (bosentan) full prescribing information. Actelion Pharmaceuticals US, Inc. 2005. 3. Data on file, Actelion Pharmaceuticals.

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was analyzed in an in vitro model using doxycycline (dox)-regulated mouse embryonic fibroblasts (MEF) conditionally overexpressing a C-terminal truncated Marfan-like fibrillin-1 mutant that prevents microfibril formation. Genes found dramatically altered by both Tsk and Marfan-like fibrillin-1 mutants (mainly CCN3 and MAGP-2) were further investigated using dox-regulated MEF cell conditionally overexpressing these genes.

Results: Among the genes with the most highly increased expression in Tsk mouse skin were CCN3 (23-fold increase), a member of the CCN gene family that includes CTGF, and microfibril-associated-glycoprotein-2 (MAGP-2, 10-fold increase), a matrix protein that binds to fibrillin-1. In contrast, overexpression of the deficient Marfan-like fibrillin-1 mutant decreased both CCN3 and MAGP-2. Similar to the effect of Tsk fibrillin-1 expression, overexpression of CCN3 increased MAGP-2, while MAGP-2 overexpression did not alter CCN3. Together, these data suggest that fibrillin-1 matrix may control MAGP-2 matrix via CCN3. Finally, unlike Tsk fibrillin-1 that increases type I collagen matrix, Marfan-like fibrillin-1 mutant decreased type I collagen in vitro.

Conclusion: We show here that fibrillin-1 regulates MAGP-2 expression and that CCN3 might mediate this effect. Since MAGP-2 stimulates type I collagen (Lemaire et al., A&R, 2005, 2005, 52:1812-1823) and elastogenesis (Lemaire et al, A&R, 2005, 52, S366), these results suggest that the fibrillin-1 / CCN3 / MAGP-2 pathway might also contribute to the altered regulation of extracellular matrix in human connective tissue disorders, such as scleroderma and Marfan syndrome.

24. Peroxisome proliferator-activated receptor- γ (PPAR- γ) inhibits collagen gene expression: implications for fibrosis

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Purpose: By stimulating collagen synthesis and myofibroblast differentiation, TGF- β has a pivotal role in the pathogenesis of fibrosis. PPAR- γ is a broadly expressed nuclear receptor that functions as a ligand-activated transcription factor induced by prostaglandins, lipids, and antidiabetic drugs. In addition to its role in regulation of energy metabolism, PPAR- γ is increasingly implicated in inflammation and tissue repair. We previously showed that PPAR- γ blocked stimulation of collagen gene expression in skin fibroblasts. In the present study, we investigated the molecular mechanisms underlying PPAR- γ suppression of profibrotic TGF- β responses.

Methods: Primary cultures of normal and scleroderma skin fibroblasts, and fibroblasts from PPAR- γ null mouse embryos, were used. COL1A2 transcriptional activity was studied by transient transfection assays. Protein expression and phosphorylation were determined by Western analysis. Nuclear protein DNA binding activities were examined by gel shift and DNA affinity precipitation assays. Subcellular Smad distribution was examined by confocal microscopy.

Results: The levels of collagen and COL1A2 promoter activity

were strikingly elevated in the absence of TGF- β in mouse embryonic fibroblasts lacking PPAR- γ . Incubation of normal skin fibroblasts with PPAR- γ ligands (15d-PGJ₂ and troglitazone) blocked stimulation of collagen synthesis elicited by TGF- β , or by ectopic expression of its downstream signal mediator Smad3. However, TGF- β -dependent Smad activation and inducible sequence-specific DNA binding activities were not abrogated. TGF- β enhanced the direct interaction between Smad3 and the ubiquitous coactivator p300 in the COL1A2 transcriptional complex. Significantly, this response was completely abrogated by PPAR- γ ligands. Furthermore, ectopic p300 was able to rescue TGF- β stimulation of COL1A2 promoter activity in the presence of PPAR- γ , suggesting that interference with the p300-dependent Smad transcriptional complex assembly accounted for inhibition of Smad-dependent TGF- β responses by PPAR- γ . Levels of cellular PPAR- γ were reduced in three out of five SSc skin fibroblast lines compared to matched normal controls.

Conclusion: PPAR- γ inhibited profibrotic responses induced by TGF- β , and in fibroblasts appeared to function as an endogenous natural repressor of collagen production. Ligand activation of PPAR- γ resulted in disruption of inducible Smad3-coactivator interactions, and blocked Smad-mediated transcriptional responses. The findings establish a mechanism of action for the antifibrotic effect of PPAR- γ and suggest that pharmacological PPAR- γ activation may be a novel approach to controlling fibrosis in scleroderma.

Acknowledgments: Supported by grants from the Scleroderma Foundation and NIH.

25. Regulation of the coactivator and histone acetyltransferase (HAT) p300

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Purpose: p300 is a ubiquitous transcriptional coactivator with intrinsic HAT activity. Previously we have shown that inducible interaction of p300 with TGF- β -activated Smad2/3 played a fundamental role in profibrotic TGF- β signaling. In normal fibroblasts, elevation of the level of p300 markedly enhanced the stimulation of collagen gene expression induced by TGF- β , whereas p300 suppression blocked the response, suggesting that the relative abundance of cellular p300 governed the intensity of TGF- β responses. Further, we and others showed that both the expression of p300, and its interaction with Smad3 were significantly elevated in scleroderma lesional fibroblasts. Because the regulation of p300 expression is not well understood, we investigated the regulation of p300 in fibroblasts.

Methods: Primary cultures of human foreskin and lung fibroblasts, and mouse embryonic fibroblasts (MEFs) were used. mRNA steady-state levels and transcript stability were examined by RT-PCR. Protein levels and acetylation state were determined by Western analysis. p300 gene promoter activity was studied in transient transfection assays. HAT activity was determined using a kit.

Results: Incubation of normal skin and lung fibroblasts with

TGF- β resulted in substantial time- and dose-dependent increase in p300 mRNA and protein accumulation, and was accompanied by enhanced HAT activity and histone acetylation. Whereas stability of p300 mRNA transcripts was unaffected by TGF- β , the activity of the p300 gene promoter was markedly induced in transiently transfected fibroblasts. SB431542, a selective inhibitor of the ALK5 TGF- β receptor, did not prevent stimulation of p300 expression, whereas the ERK1/2 inhibitor PD98059 fully blocked the response. Increased cellular p300 accumulation in TGF- β -treated fibroblasts could account for the increase in HAT activity and H4 acetylation in these cells.

Conclusion: These results indicate for the first time that expression of p300, an integral component of the TGF- β intracellular signal transduction pathways, can be modulated by cytokines. Stimulation of p300 by TGF- β involved ALK5-independent transcriptional activation of the p300 gene. Because the relative levels of p300 govern cellular responsiveness to TGF- β , enhanced p300 expression and/or function could have a key role in the fibrotic phenotype of scleroderma fibroblasts. Furthermore, selective targeting of p300, or its interaction with Smads, may represent novel therapeutic approaches to fibrosis.

Acknowledgments: Supported by grants from the Scleroderma Foundation and NIAMS-NIH.

26. Host conditioning and the “cytokine storm” are not required for generation of sclerodermatous graft-versus-host disease

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Our laboratory has modeled 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^d) into lethally irradiated BALB/c (H-2^d) across minor histocompatibility loci (MiHC) produces skin thickening and lung fibrosis rather than classic cytotoxic GVHD. The focus of these studies is to examine the early immune events in Scl GVHD that may provide insight to changes that occur in early scleroderma, during which immune-based therapies may be used. Cytotoxic GVHD is thought to be initiated by the conditioning phase that generates the “cytokine storm” in which recipient antigen presenting cells (APCs) are activated by cytokines released by damaged gut and tissue. The role that host conditioning and the “cytokine storm” has on the induction of Scl GVHD is unknown. The Scl GVHD model (B10.D2 \rightarrow Balb/c) depends on irradiation (conditioning) to ablate the host bone marrow. Rag 2 knockout mice have been used successfully to generate Scl GVHD (1). We used Rag 1 knockout mice on Balb/c background to avoid the effects of irradiation and determine the role of conditioning. When CD4⁺ T cells from B10.D2 mice were injected into irradiated or non-irradiated Rag 1 mice, Scl GVHD was generated in both recipient groups. When C57BL/6 CD4⁺ T cells were injected into Rag1 knockout mice, irradiated mice developed cytotoxic GVHD as expected. However, when non-irradiated mice were the recipient, Scl

GVHD developed. Suggesting that important APC-T cell interactions that lead to one or the other form of GVHD. Also,, the cytokine environment is important because TNF α appears to be critical cutaneous cytokine for cytotoxic GVHD, but is absent in Scl GVHD.

1. Ruzek MC, Jha S, Ledbetter S, Richards SM, Garman RD. A modified model of graft versus-host-induced systemic sclerosis (scleroderma) exhibits all major aspects of the human disease. *Arthritis Rheum.* 2004;50:1319-1331.

27. Establishment of immortal SSc fibroblast clonal cell lines by introduction of the hTERT gene

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The lack of an adequate experimental model has hindered our ability to understand scleroderma (SSc) pathogenesis. Current scleroderma research is based on the study of cultured fibroblasts from SSc skin biopsies. Gene array analysis has shown that cultured fibroblasts do not fully maintain the fibrotic phenotype observed in SSc biopsies (*Gardner et. al, in press*). This may be a result of the heterogeneity of biopsy fibroblast populations. With passaging a certain fibroblast phenotype may overtake the culture population so that the *in vivo* phenotype is lost. The goal of this study was to use hTERT immortalization to determine if passaging leads to a change in the phenotypic culture population. hTERT immortalization was chosen because it is the least disruptive to the cellular genome and gene expression profiling. Fibroblasts from two pairs of closely matched normal and SSc biopsies were used in this study. Fibroblasts from one pair were immortalized in passage five and the second pair was immortalized in passage zero by infection with an hTERT lentivirus and selection with zeocin. hTERT infected clones were isolated and cultured for several months. The presence of telomerase was confirmed using RT-PCR and telomeric repeat amplification protocol (TRAP). We used QPCR to measure the mRNA levels of 9 genes shown by microarray to differ in biopsy and culture including collagen I (COL1A1), thrombospondin 1 (TSP1), lysyl hydroxylase 2 (PLOD2), connective tissue growth factor (CCN2), collagen XI, fibrillin 2, desmin, calponin 1, and tenascin C. Protein levels of CCN2 and COL1A1 were confirmed using western blotting. Clonal phenotypes were compared to whole cell biopsy populations to compare phenotype stability. Ten normal and 8 SSc clones from the first pair of biopsy samples were immortalized in passage zero. A normal clone with low levels of COL1a1 expression was selected and used to normalize all mRNA and protein measurements. Among the normal clones five produced low levels of COL1A1 mRNA (1-1.9) and four clones produced intermediate levels (3.2-5.8). Of the SSc clones, three produced low levels of COL1A1 (1-1.8), one produced intermediated levels (5.5), and four produced high levels (7.3-11.1). TSP1 mRNA levels in normal clones ranged from 0.9-2.3 and from 1-12.7 in SSc clones with a strong correlation to COL1A1 levels. Likewise, CCN2 levels ranged from 0.9-2.7 in normal clones and from 0.9-4.7 in SSc clones with a high correlation to COL1A1. Several genes includ-

ing PLOD2 showed only minimal variation. In contrast to these observations, normal and SSc clones immortalized in passage five showed only slight variation in the levels of COL1A1, TSP1, and CCN2 expression.

These data show that some of the SSc biopsy characteristics including expression of COL1A1, TSP1, and CCN2 are lost in culture due to passaging. Other genes that were significantly changed in the SSc biopsies, including PLOD2, are not maintained in culture. These genes may require the presence of other cell types or the *in vivo* matrix environment that cannot be recapitulated in cell culture. The scleroderma cell lines immortalized in passage zero may present a new and useful model to investigate scleroderma pathogenesis.

28. The role of Fli1 in the collagen processing and fibrillogenesis

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Fli1, a member of Ets family of transcriptional factors, has been implicated in collagen gene regulation. We have previously observed markedly reduced levels of Fli1 protein in lesional fibroblasts of patients with scleroderma, suggesting that an absence of Fli1 may contribute to the process of cutaneous fibrosis. The aberrant ultrastructure of collagen has been observed in involved skin of scleroderma, however the basis for the abnormalities of the collagen processing and/or fibrillogenesis remains to be clarified. The goal of this study was to investigate the role of Fli1 in the collagen processing and fibrillogenesis using *in vitro* cultivated foreskin fibroblasts and *in vivo* mouse model.

In *in vitro* studies, we compared by quantitative realtime RT-PCR the effects of TGFβ1 and siRNA-induced gene silencing of Fli1 on mRNA levels of the following eleven genes which participate in the collagen processing and fibrillogenesis; (i) Procollagen assembly-related genes; prolyl-4-hydroxylase (P4H), heat shock protein 47 (HSP47), protein disulfide isomerase (PDI), (ii) Procollagen cleavage-related genes; a disintegrin and metalloproteinase with thrombospondin motifs 2 (ADAMTS2), bone morphogenetic protein 1 (BMP1), procollagen C terminal peptidase enhancer protein (PCPE), (iii) Pyridinoline cross-link-related genes; procollagen-lysine 2-oxyglutarate 5-dioxygenase 2 (PLOD2), lysyl oxidase-like 2 (LOXL2), (iv) small leucine-rich proteoglycans (sLRPs); decorin, fibromodulin, lumican. The treatment with TGF-β1 significantly increased mRNA levels of P4H, HSP47, PDI, ADAMTS2, BMP1 and PLOD2, and significantly decreased mRNA levels of sLRPs. TGFβ1 stimulation did not affect the mRNA levels of PCPE and LOXL2. Among the 9 TGFβ1-responsive genes, the effect of TGFβ1 was reproduced by Fli1 siRNA on 5 genes including P4H, PLOD2, decorin, fibromodulin, and lumican. On the other hand, Fli1 siRNA showed no effect on the mRNA levels of other 6 genes. These results of quantitative RT-PCR were also confirmed at the protein level by immunoblotting.

For the *in vivo* mouse model, we used Fli1^{rec/rec} mice, which have homozygous deletion of C-terminal activation domain of Fli1. First, mRNA levels of Fli1-responsive genes were determined in skin tissue samples by quantitative RT-PCR. The mRNA level of PLOD2 was elevated, while decorin, fibromodulin, and lumican mRNA levels were decreased in Fli1^{rec/rec} mice compared with wild type mice. There was no difference in the mRNA levels of LOXL2. Since sLRPs play a central role in collagen fibrillogenesis, we next examined the ultrastructure of collagen in the back skin by transmission electron microscopy. In Fli1^{rec/rec} mice, in addition to fibrils with normal diameter there were much thicker, irregularly shaped fibrils. Mean fibril diameter was 118.6 +/- 39.2 in Fli1^{rec/rec} mice and 92.7 +/- 34.5 in wild type ($P < .01$). Furthermore, Fli1^{rec/rec} mice skin showed a wide range of fibril diameter in cross section (22-302 nm in wild type and 15-511 nm in mutants), indicating the increase of immature thin collagen fibrils as well as thick fibrils. These *in vitro* and *in vivo* findings demonstrated that Fli1 is involved in regulation of collagen processing and fibrillogenesis, in addition to its role as a repressor of fibrillar collagen genes. Moreover, this study supports the view that the absence of Fli1 plays a pathological role in scleroderma skin fibrosis.

29. Prevalence of female sexual dysfunction among women with systemic sclerosis

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Purpose: To determine the prevalence of sexual dysfunction in female systemic sclerosis (SSc) patients enrolled in the Connective Tissue Diseases Database (CTD) at the Medical University of South Carolina

Methods: Thirty women with SSc enrolled in the CTD were asked to complete the Female Sexual Function Index, a validated 19-item questionnaire. Associations between domain scores and clinical characteristics were examined and tested using Spearman rank correlations and Wilcoxon rank sum tests. Respondents' scores were also compared to published values of healthy controls.

Results: A total of 30 questionnaires were distributed to SSc patients, and 17 questionnaires were completed (57%). There were no significant differences found between responders and non-responders with respect to age or disease classification; however, responders did have significantly higher total Rodnan skin scores. Compared to published results for healthy controls, SSc patients had significantly ($P < .001$) lower levels of desire, arousal, lubrication and satisfaction. Pain scores were also worse among the SSc patients. None of the domains of sexual dysfunction were significantly associated with responders' age, disease classification, or total skin score.

Conclusion: The prevalence of sexual dysfunction among female SSc patients is high. In our study, patients with SSc had more difficulty with sexual desire, arousal, lubrication, orgasm, satisfaction, and pain than healthy controls. Specific areas of sexual dys-

function were not significantly associated with responders' clinical characteristics. Previous work in this area by Bhadauria et al (*Am J Obstet Gynecol.* 1995;172:580-7) also noted that decreased orgasmic function appears to be a common finding in SSc. Our study is unique in that we utilized a validated, standardized questionnaire to assess female sexual function in these patients. Additionally, we noted that SSc patients had significantly lower levels of sexual desire, arousal, lubrication, and overall satisfaction with their sexual life. Our study, although relatively small, suggests that sexual dysfunction is a significant problem in female patients with SSc. We believe that further studies are indicated to determine the etiology of these symptoms, whether it be physical, emotional, or both. In addition, determining the cause of sexual dysfunction and finding appropriate treatment modalities is necessary in order to provide these individuals with functional sexual health throughout the course of their chronic disease.

30. Endothelin axis polymorphisms in scleroderma patients

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Background: In the context of Systemic sclerosis (SSc) endothelins and their receptors have been implicated in both vascular and fibrotic pathologies. Elevated circulating levels of Endothelin 1 (ET-1), differential expression of ET subtypes receptors in tissues and an altered response to ET-1 have been described in SSc patients. Underlying cause of the differential expression is unknown but may be related to genetic polymorphisms.

Objective: To evaluate the distribution of polymorphisms in Endothelin 1 (EDN1), Endothelin receptor A (EDNRA) and Endothelin receptor B (EDNRB) gene in systemic sclerosis and disease subsets.

Methods: Two hundred and five systemic sclerosis patients and 255 healthy controls were screened for polymorphism in EDN1, EDNRA and EDNRB by SSP-PCR. The polymorphisms studied were at positions -1370 (T-1370G), exon 1 +138 (+138 A/-), exon 3 +85 (E106E) and exon 5 +23 (K198N) for EDN1; exon 1 -231 (G-231A), exon 6+ 6(H323H) and +105 (E335E) of EDNRA and exon 2 +2841 (EDNRB-3), exon 3 -2547 (EDNRB-2) and -2446 (EDNRB-1) exon 3 of EDNRB. The genotype, allele carriage and allele frequency were determined by direct counting. Logistic regression was used to compare the distribution of polymorphism between SSc and Control groups. Comparisons between the mutually exclusive autoantibody subgroups were performed by using polytomous logistic regression. A *P* value less than .05 was considered to be significant.

Results: All the studied polymorphisms were in Hardy-Weinberg equilibrium. No significant difference was found for any of the investigated polymorphisms in EDN1, EDNRA and EDNRB

between the SSc group as a whole and control subjects. However, compared to patients with limited skin disease, patients with diffuse involvement had increased frequency of allele carriage of EDNRB-1A allele (76.8% vs. 54.4%, *P* = .002), EDNRB-2A (79.7% vs. 60.2%, *P* = .006) and EDNRB-3G (79.7% vs. 56.6%, *P* = .001). A significantly increased allele carriage frequencies for EDNRA alleles H323H/C and E335E/A was found in SSc patients with anti-RNA polymerase antibodies (ARA), both compared to ARA negative SSc patients (*P* < .05) and to control subjects (*P* < .005).

Conclusion: The finding of significant genetic differences in the endothelin axis members within SSc population and the association between endothelin receptor A and B with distinct clinical and immunological SSc subsets supports the role of endothelin and its receptors in the pathogenesis of SSc. However, further studies are needed before the role of these SNPs in the pathogenesis of SSc can be fully appreciated.

31. A repressor in the first intron of the human alpha2(I) collagen gene may be defective in scleroderma?

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Fibrotic diseases are characterized by an abnormally high amount of collagen deposition, resulting in an accumulation of scar tissue, which can cause organ failure and death. There is no effective therapy for fibrotic disorders, in part because the aetiology of these diseases is unknown. The chronic disease scleroderma (systemic sclerosis; SSc) is characterized by scarring of the skin, lungs and internal organs. The human gene that code for the alpha 2 chain of collagen I (*COL1A2*) is driven by a combination of a proximal promoter (-378 bp) and an upstream enhancer (-20 kb). The complex and highly combinatorial nature of the regulatory network governing *COL1A2* We have investigated the transcriptional contribution of the unique open chromatin site in the first intron of *COL1A2* using a transgenic mouse model. DNase I footprinting identified a cluster of three distinct areas of nuclease protection that span from nucleotides +647 to +759, relative to the transcription start site, and which contain consensus sequences for GATA and IRF transcription factors. Gel mobility shift and chromatin immunoprecipitation assays corroborated this last finding by documenting binding of GATA-4 and IRFs 1 and 2 to the first intron sequence. Moreover, a short sequence encompassing the three footprints was found to inhibit expression of transgenic constructs containing the *COL1A2* proximal promoter and far-upstream enhancer in a position-independent manner. Mutations inserted into each of the footprints restored transgenic expression to different extents. These results therefore indicate that the unique open chromatin site of *COL1A2* corresponds to a repressor, the activity of which seems to be mediated by the concerted

action of GATA and IRF proteins. These studies speculate that the problem with fibrotic diseases and SSc in particular is the inability to switch off collagen transcription because of defects in the transcription factors regulating the repressor or the combination of key factors in fibroblast overexpression of the mammalian alpha 2(I) collagen gene.

32. A novel TGF β , response element located in the human collagen type I-2 far upstream enhancer is SMAD independent

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Background: A SMAD-dependent TGF-, response element (TbRE) in the proximal promoter of the human collagen type I-2 (COL1-2) gene is well characterized. However previous studies in the TGF β , response of COL1-2 do not include the far upstream enhancer (FUE) which is situated more than 20kb upstream of the proximal promoter. The COL1-2 FUE is crucial for the temporal and tissue-specific transcriptional regulation of collagen type I in embryonic development and controls COL1-2 expression in tissue remodeling, repair and fibrosis. As activation of the TbRE in the proximal promoter occurs rapidly after the onset of TGF β , treatment and the role of the enhancer is to sustain collagen expression in the longer term, we hypothesized the existence of an alternative TbRE within the enhancer. Our aim is to study the TGF β , response of the COL1-2 FUE and characterize the mode of action of putative TbREs.

Methods: DNA constructs of the FUE fused to reporter genes and constructs containing mutations of specific sequences were used in transient transfection assays in explanted skin fibroblasts from normal and scleroderma (SSc) patients or mouse dermal fibroblasts from wild type and SMAD3 null mice, in the presence/absence of TGF β , and a cmvSMAD7 expression vector. AP1 inhibitors and expression vectors of AP1 family members were also used. EMSA were performed with labeled oligonucleotides and ChIP assays using AP1 antibodies and PCR primers to the FUE and proximal promoter.

Results: Reporter gene constructs in transient transfection assays of fibroblasts (normal and SSc) with SMAD7 in the presence/absence of TGF β , resulted in suppressed TGF β , induction of the construct containing the proximal promoter alone but only partially inhibited the activity of the construct containing both enhancer and promoter. A construct which replaced the COL1-2 proximal promoter with the thymidine kinase (TK) promoter (not TGF β , inducible) but contained the FUE was activated with TGF β . Transfections with these constructs in SMAD3 null cells confirmed the presence of a SMAD independent TbRE in the FUE. The novel TbRE was localized within the 2.1kb COL1-2

FUE to a 235bp sequence. Its mode of action studied by cotransfections with expression vectors, EMSA and ChIP showed that activation requires specific AP1 family members. Mutagenesis showed that two AP1 sites in the FUE were involved in the TGF β , response. Activation occurs approximately 30 hours after the onset of TGF β , treatment *in vitro* in contrast to the much earlier activation of the SMAD-dependent TbRE response. Data show that in SSc fibroblasts, the FUE TbRE is constitutively active without exogenous TGF β .

Conclusions: A novel TbRE has been identified in the FUE region of the COL1-2 gene. The FUE TbRE is SMAD-independent and requires specific AP1 members for activation. The late activation after TGF-, treatment in normal fibroblasts suggests that it may play a role in the sustained expression of COL1-2. In SSc fibroblasts, the FUE TbRE appears to be constitutively active.

33. Mechanisms regulating CTGF transcription in systemic sclerosis

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Background: Enhanced expression of CTGF is a fibrogenic characteristic of fibroblasts from patients with systemic sclerosis (SSc). We and others have shown that the activity of transcription factors Sp1 and CBF is enhanced in SSc, and that high basal CTGF expression is dependent on Sp1. This effect was mapped to a GC-rich site in the proximal promoter, adjacent to a putative CBF binding site (CCAAT-box). CBF is known to synergize with Sp1, possibly involving MAPK pathways, however little is known regarding the molecular mechanisms underlying the over-expression of CTGF in SSc. The purpose of this study was to further characterise protein interactions at the promoter and to elucidate signalling pathways leading to enhanced CTGF activation in SSc.

Methods: Human primary adult fibroblasts were isolated from skin and lung tissue and used at passages 4-7. Transcriptional activity was assessed by transient transfection of CTGF promoter constructs into fibroblasts, and measurement of luciferase reporter activity by chemiluminescence. MAPK signalling pathways were assessed using specific pharmacological inhibitors, and the expression and activity of kinases determined by western blot analysis. DNA-protein interactions were demonstrated by electrophoresis mobility shift assay (EMSA) using nuclear extracts and radio-labelled gene-specific double-stranded oligonucleotides.

Results: We have previously shown that a proximal Sp1 site is necessary for the high CTGF expression of CTGF in SSc. Here we investigate the underlying signalling pathways involved. SSc fibroblasts were found to exhibit constitutively active JNK1 and ERK1/2, but not p38. Using specific inhibitors of JNK and ERK activation, we found that repression of ERK1/2, but not JNK,

potently inhibited CTGF expression in SSc fibroblasts. In parallel, protein interaction at the proximal Sp1 site was reduced. In addition to the proximal Sp1 site, we have identified a high affinity binding site for Sp3 at position -755 upstream of the transcription start-site. Site-directed mutagenesis of this site enhanced transcription about 3 fold, suggesting a potent repressor. The over-expression of Sp3, reducing the activity of the wild-type CTGF promoter by about 50%, but having no effect on a mutated -755 Sp3 site, suggests a novel repressor site for the CTGF gene. This was further confirmed *in vivo* by chromatin immuno-precipitation. Protein interaction to a previously uncharacterised inverted CCAAT-box in the proximal promoter of the CTGF gene was investigated using EMSA. Distinct complexes were formed, and competition and antibody supershift assays demonstrated that CBF is the major factor binding at this site. Since enhanced CBF activity has been observed in SSc fibroblasts, this site is likely to play an important role in the over-expression of CTGF in SSc. The cooperative role of these sites in SSc is under investigation.

Conclusions: Our results provide evidence to suggest that Sp1/Sp3 and CBF are important regulators of CTGF and that signalling pathways, inappropriately activating these factors in SSc, may be useful targets for novel therapeutic strategies.

34. Over-expression of CTGF in Tsk2 mouse skin in the absence of a mononuclear cell infiltrate

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Background: Scleroderma is an autoimmune connective tissue disease characterized by fibrosis of the skin and internal organs. While the etiology of scleroderma remains unknown, a variety of animal models have been used to study the mechanism of fibrosis. Connective tissue growth factor (CTGF) has been identified as a major fibrotic mediator and elevated levels of CTGF mRNA and protein are observed in the skin of scleroderma patients. The tight skin 2 (Tsk2) mutation is autosomal dominant, localized on chromosome 1 and mutant mice are characterized by a tightness of the skin in the interscapular region. The cutaneous phenotype is associated with an excessive accumulation of collagen and the presence of mononuclear cells in the dermis and underlying adipose layer has been reported.

Aims: The goal of this study was to determine if the cutaneous fibrosis observed in the Tsk2 mice is associated with an elevation in CTGF expression and the possible relationship of the mononuclear infiltrate to the fibrotic condition.

Materials and Methods: The Tsk2/+ mutant mice used in this study were obtained from the MRC Radiobiology Unit Laboratories, Chilton, UK in a F₁ hybrid background (C3H/He X 101/H). From these initial breeders, two separate sub lines were established; a hybrid line maintained by crossing Tsk2/+ mice to normal (C3H/He X C57BL/6) mice and an inbred line developed by backcrossing to C57BL/6 (>N10). CTGF protein expression was evaluated by immunohistochemistry of frozen skin sections isolated from 2-3 month old Tsk2/+; C57BL/6 and +/-; C57BL/6.

To evaluate the presence of inflammatory cells in Tsk2/+ mice, skin samples were taken from Tsk2/+ mice both in the hybrid background (C3H/He X C57BL/6) and the line backcrossed to C57BL/6. Paraffin sections were examined from three age groups - 10 days old, 2-3 months and 7-8 months of age. As a control for the detection of mononuclear cells, Tsk2/+ and normal control mice were injected daily with 100 ml of bleomycin or PBS. Subcutaneous injections of bleomycin induce a dermal fibrosis that is preceded by an infiltration of mononuclear cells. Skin samples were collected from Tsk2/+ and normal controls after 8 days of bleomycin injections. Frozen sections were examined for the presence of inflammatory cells as well as stained with anti Mac-3 antibody that specifically detects macrophages.

Results: Examination of the skin sections stained with anti-CTGF antibody revealed an increased number of cells in the hypodermis of Tsk2/+ mice where the majority of cells are expressing CTGF in contrast to control samples. While examining the sections, we did not observe a mononuclear cell infiltrate in Tsk2/+ skin preparations collected from mice of any age group or genetic background. To test the sensitivity of our method for the detection of inflammatory cells, skin sections were examined from Tsk2/+ and normal control mice after 8 days of daily subcutaneous injections with bleomycin. Inflammatory cells were observed following bleomycin injections and, further, these cells stained positive with anti Mac-3 antibody indicating that these cells were macrophages. While bleomycin treatment resulted in abundant inflammatory cells observed in both Tsk2/+ and control skin, they are not detected in samples collected from mice treated with PBS.

Conclusions: CTGF expression is elevated in the hypodermis of Tsk2 mice, however, this up-regulation is not associated with an infiltration of inflammatory cells.

35. Parabiosis and transplantation models do not show the presence of circulating dermal fibroblast progenitors in bleomycin induced skin fibrosis

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Background: Systemic sclerosis (SSc) is a connective tissue disease that is characterized by fibrosis of the skin and various internal organs. Although the molecular and cellular mechanisms associated with the dysregulation of dermal fibroblast function are not clearly understood, one hypothesized mechanism is the abnormal activation of a subset of dermal fibroblasts to produce excess matrix. Since scleroderma is largely adult in presentation, this activation could occur at the level of a fibroblast progenitor cell leading to the production of a sub-population of hyperactive matrix producing cells. The first step toward addressing this question involves understanding basic information on dermal fibroblast progenitor cells. Are dermal fibroblast progenitor cells strictly dermal in origin or do they have an extra-dermal origin or perhaps a combination of these two?

Aim: The aim of this study was to test the hypothesis that dermal fibroblasts that participate in the development of skin fibrosis are

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BRIEF SUMMARY

The following is a brief summary of the Full Prescribing Information for Ventavis (iloprost) Inhalation Solution. Please review the Full Prescribing Information prior to prescribing Ventavis.

INDICATIONS AND USAGE

Ventavis is indicated for the treatment of pulmonary arterial hypertension (WHO Group I) in patients with NYHA Class III or IV symptoms. In controlled trials, it improved a composite endpoint consisting of exercise tolerance, symptoms (NYHA Class), and lack of deterioration (see **CLINICAL PHARMACOLOGY, Clinical Trials** section of the Full Prescribing Information).

CONTRAINDICATIONS

There are no known contraindications.

WARNINGS

Ventavis is intended for inhalation administration only via either of two pulmonary drug delivery devices: the I-neb[™] AAD[®] System or the Prodose[®] AAD[®] System (See **DOSAGE AND ADMINISTRATION** section of the Full Prescribing Information). It has not been studied with any other nebulizers.

Vital signs should be monitored while initiating Ventavis. In patients with low systemic blood pressure, care should be taken to avoid further hypotension. Ventavis should not be initiated in patients with systolic blood pressure less than 85 mmHg. Physicians should be alert to the presence of concomitant conditions or drugs that might increase the risk of syncope. Syncope can also occur in association with pulmonary arterial hypertension, particularly in association with physical exertion. The occurrence of exertional syncope may reflect a therapeutic gap or insufficient efficacy, and the need to adjust dose or change therapy should be considered.

Should signs of pulmonary edema occur when inhaled iloprost is administered in patients with pulmonary hypertension, the treatment should be stopped immediately. This may be a sign of pulmonary venous hypertension.

PRECAUTIONS

General: Ventavis solution should not be allowed to come into contact with the skin or eyes; oral ingestion of Ventavis solution should be avoided.

Direct mixing of Ventavis with other medications in the I-neb[™] AAD[®] System or the Prodose[®] AAD[®] System has not been evaluated.

Ventavis has not been evaluated in patients with chronic obstructive pulmonary disease (COPD), severe asthma, or with acute pulmonary infections.

Information for Patients: Patients receiving Ventavis should be advised to use the drug only as prescribed with either of two pulmonary drug delivery devices, the I-neb[™] AAD[®] System or the Prodose[®] AAD[®] System, following the manufacturer's instructions (see **DOSAGE AND ADMINISTRATION** section of the Full Prescribing Information). Patients should be trained in proper administration techniques including dosing frequency, ampule dispensing, I-neb[™] AAD[®] System or Prodose[®] AAD[®] System operation, and equipment cleaning. Patients should be advised that they may have a fall in blood pressure with Ventavis, so they may become dizzy or even faint. They should stand up slowly when they get out of a chair or bed. If fainting gets worse, patients should consult their physicians about dose adjustment.

Patients should be advised that Ventavis should be inhaled at intervals of not less than 2 hours and that the acute benefits of Ventavis may not last 2 hours.

Drug Interactions: In studies in normal volunteers, there was no pharmacodynamic interaction between intravenous iloprost and either nifedipine, diltiazem, or captopril. However, iloprost has the potential to increase the hypotensive effect of vasodilators and antihypertensive agents. Since iloprost inhibits platelet function, there is a potential for increased risk of bleeding, particularly in patients maintained on anticoagulants. During clinical trials, iloprost was used concurrently with anticoagulants, diuretics, cardiac glycosides, calcium channel blockers, analgesics, antipyretics, nonsteroidal anti-inflammatories, corticosteroids, and other medications. Intravenous infusion of iloprost had no effect on the pharmacokinetics of digoxin. Acetylsalicylic acid did not alter the clearance (pharmacokinetics) of iloprost.

Although clinical studies have not been conducted, *in vitro* studies of iloprost indicate that no relevant inhibition of cytochrome P450 drug metabolism would be expected.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Iloprost was not mutagenic in bacterial and mammalian cells in the presence or absence of extrinsic metabolic activation. Iloprost did not cause chromosomal aberrations *in vitro* in human lymphocytes and was not clastogenic *in vivo* in NMRI/SPF mice. There was no evidence of a tumorigenic effect of iloprost clathrate (13% iloprost by weight) in Sprague-Dawley rats dosed orally for up to 8 months at doses of up to 125 mg/kg/day (Cmax of 45 ng/mL serum), followed by 16 months at 100 mg/kg/day, or in CrI:CD-1@((ICR)BR albino mice dosed orally for up to 24 months at doses of up to 125 mg/kg/day (Cmax of 156 ng/mL serum). The recommended clinical dosage regimen for iloprost (5 mcg) affords a serum Cmax of 0.16 ng/mL. Fertility of males or females was not impaired in Han-Wistar rats at intravenous doses up to 1 mg/kg/day.

Pregnancy: Pregnancy Category C. In developmental toxicity studies in pregnant Han-Wistar rats, continuous intravenous administration of iloprost at a dosage of 0.01 mg/kg daily (serum levels not available) led to shortened digits of the thoracic extremity in fetuses and pups. In comparable studies in pregnant Sprague-Dawley rats which received iloprost clathrate (13% iloprost by weight) orally at dosages of up to 50 mg/kg/day (Cmax of 90 ng/mL), in pregnant rabbits at intravenous dosages of up to 0.5 mg/kg/day (Cmax of 86 ng/mL), and in pregnant monkeys at dosages of up to 0.04 mg/kg/day (serum levels of 1 ng/mL), no such digital anomalies or other gross-structural abnormalities were observed in the fetuses/pups. However, in gravid Sprague-Dawley rats, iloprost clathrate (13% iloprost) significantly increased the number of non-viable fetuses at a maternally toxic oral dosage of 250 mg/kg/day and in Han-Wistar rats was found to be embryolethal in 15 of 44 litters at an intravenous dosage of 1 mg/kg/day. There are no adequate and well-controlled studies in pregnant women. Ventavis should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether Ventavis is excreted in human milk. In studies with Han-Wistar rats, higher mortality was observed in pups of lactating dams receiving iloprost intravenously at 1 mg/kg daily. In Sprague-Dawley rats, higher mortality was also observed in nursing pups at a maternally toxic oral dose of 250 mg/kg/day of iloprost clathrate (13% iloprost by weight). It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Ventavis, a decision to discontinue nursing should be made, taking into account the importance of the drug to the mother.

Pediatric Use: Safety and efficacy in pediatric patients have not been established.

Geriatric Use: Clinical studies of Ventavis did not include sufficient numbers of subjects age 65 and older to determine whether they respond differently than younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, dose selection for an elderly patient should be cautious, usually starting at the low end of the dosing range, reflecting the greater frequency of decreased hepatic, renal, or cardiac function and of concomitant disease or other drug therapy.

Hepatic or Renal Impairment: Ventavis has not been studied in patients with pulmonary hypertension and hepatic or renal impairment, both of which increase mean AUC in otherwise normal subjects (see **CLINICAL PHARMACOLOGY, Special Populations** section of the Full Prescribing Information).

ADVERSE REACTIONS

Safety data on Ventavis were obtained from 215 patients with pulmonary arterial hypertension receiving iloprost in two 12-week clinical trials and two long-term extensions. Patients received inhaled Ventavis for periods of from 1 day to more than 3 years. The median number of weeks of exposure was 15 weeks. Forty patients completed 12 months of open-label treatment with iloprost.

The following table shows adverse events reported by at least 4 iloprost patients and reported at least 3% more frequently for iloprost patients than placebo patients in the 12-week placebo-controlled study.

Adverse Events in Phase 3 Clinical Trial

Adverse Events	Iloprost	Placebo	Placebo
	(n=101)	(n=102)	subtracted %
Vasodilation (flushing)	27	9	18
Cough increased	39	26	13
Headache	30	20	10
Trismus	12	3	9
Insomnia	8	2	6
Nausea	13	8	5
Hypotension	11	6	5
Vomiting	7	2	5
Alk phos increased	6	1	5
Flu syndrome	14	10	4
Back pain	7	3	4
Abnormal lab test	7	3	4
Tongue pain	4	0	4
Palpitations	7	4	3
Syncope	8	5	3
GGT increased	6	3	3
Muscle cramps	6	3	3
Hemoptysis	5	2	3
Pneumonia	4	1	3

In a small clinical trial (the STEP trial, see **CLINICAL TRIALS** section of the Full Prescribing Information), safety trends in patients receiving concomitant bosentan and iloprost were consistent with those observed in the larger experience of the Phase 3 study in patients receiving only iloprost.

Serious adverse events reported with the use of inhaled iloprost and not shown in the table above include congestive heart failure, chest pain, supraventricular tachycardia, dyspnea, peripheral edema, and kidney failure.

Adverse events with higher doses: In a study in healthy volunteers (n=160), inhaled doses of iloprost solution were given every 2 hours, beginning with 5 mcg and increasing up to 20 mcg for a total of 6 dose inhalations (total cumulative dose of 70 mcg) or up to the highest dose tolerated in a subgroup of 40 volunteers. There were 13 subjects (32%) who failed to reach the highest scheduled dose (20 mcg). Five were unable to increase the dose because of (mild to moderate) transient chest pain/discomfort/tightness, usually accompanied by headache, nausea, and dizziness. The remaining 8 subjects discontinued for other reasons.

OVERDOSAGE

In clinical trials of Ventavis, no case of overdose was reported. Signs and symptoms to be anticipated are extensions of the dose-limiting pharmacological effects, including hypotension, headache, flushing, nausea, vomiting, and diarrhea. A specific antidote is not known. Interruption of the inhalation session, monitoring, and symptomatic measures are recommended.

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extradermal in origin and enter the dermis from the circulation.

Materials and Methods: Parabiosis is the surgical connection of two mice to generate a pair of individuals with a common circulation. Experimental parabiotic pairs consisted of one transgenic mouse expressing a green fluorescent protein (GFP) under the control of the *Coll1a1* 3.6 promoter, while the parabiotic partner was C57BL/6. This combination would permit the tracking of the movement of dermal progenitors from the circulation to the skin, through the expression of GFP driven by a *Coll1a1* promoter expressed in dermal fibroblasts. After a common circulation was established, skin fibrosis was induced in both mice by repeated subcutaneous injections of bleomycin. Control pairs were injected with saline. Four weeks after injection with bleomycin or saline, skin and lung samples were evaluated for GFP expression and stained with Masson trichrome to determine the degree of fibrosis. To assess the hypothesis that bone marrow could be an extra-dermal source of cells participating in dermal fibrosis, bone marrow chimeras were created in which non-transgenic recipient mice received injections of bone marrow cell preparations isolated from transgenic pOBCol3.6GFP mice. After bone marrow chimerism had been successfully established, fibrotic lesions in the skin were again induced by local bleomycin injections.

Results: In parabiotic pairs treated with bleomycin, skin samples from both parabionts showed a dense deposition of collagen and inflammatory cell infiltrates in the thickened dermis in comparison with parabiotic pairs treated with saline. Although, in all cases, continuous injection of bleomycin for 4 weeks induced skin fibrosis, only a few GFP positive cells were detected in skin samples from some of the treated non-transgenic mice. Unexpectedly similar results were observed in saline-treated controls. Interestingly, the infrequently detected GFP expressing cells were localized around hair follicles and had a dendritic shape. In the bone marrow transplantation experiments donor GFP expressing cells were not observed in the bleomycin induced fibrotic lesions in recipient mice. However, a significant number of GFP expressing cells were observed in the lung preparations from all recipient mice, both treated and controls. These round GFP expressing cells were also detected in parabiotic pairs, however in that setting the number of GFP positive cells was very low. Mac-3 antibody immunostaining confirmed a macrophage phenotype for these GFP expressing cells suggesting expression of the *Col3.6GFP* transgene in a non-collagen-producing cell.

Conclusion: Based on these observations, there is no evidence of circulating dermal fibroblast progenitors that participate in the development of bleomycin induced skin fibrosis.

36. Inducible fibroblast-specific disruption of TGF β signaling in vivo replicates features of late-stage systemic sclerosis

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Background: Altered expression of TGF β receptors (T β R) has been observed in skin fibroblasts of systemic sclerosis (SSc). To

replicate this in an animal model we have deleted T β RII in fibroblasts of mice post-natally, using a Cre-Lox method that avoids the embryonic lethality of a conventional knock-out for this gene.

Methods: Transgenic mice with fibroblast-specific expression of tamoxifen-dependent Cre-recombinase (driven by a lineage-specific *Colla2* expression cassette) were crossed with animals homozygous for a floxed allele of T β RII. Short-term tamoxifen administration was used to delete T β RII in compound mutant progeny. Macroscopic phenotype, skin biopsy histology, biochemical properties of explanted fibroblasts and healing of excisional skin wounds in mice lacking T β RII in fibroblasts (T β RII-null-fib) were compared with matched controls.

Results: Genomic deletion of the floxed allele was confirmed by PCR analysis of tail biopsy DNA. From 2 weeks after activation of Cre-recombinase, mice lacking T β RII developed progressive digital contractures at the interphalangeal joints of all limbs. They also failed to thrive, attaining significantly lower body mass (mean \pm sem) at 6 weeks of age. Thus, young adult male T β RII-null-fib mice weighed 13.4 ± 2.5 g, compared with 21.1 ± 2.6 g for control littermates ($P = .05$, $n=12$). Immunohistochemical staining of skin sections, and western blot analysis of cell lysates, confirmed absence of T β RII in fibroblasts, but not other cell types. Fibroblasts cultured from T β RII-fib-null mice showed altered expression of TGF β regulated gene products. The predominant biochemical phenotype was one of diminished TGF β signaling and responsiveness. Thus, there was significantly lower constitutive expression (mean \pm sem) of type I procollagen (12 ± 6 RDU) by T β RII-null fibroblasts compared with littermate controls (39 ± 5 , $P = .003$). PAI-1 protein expression was also lower in mutant (55 ± 9 RDU) than wildtype fibroblasts (170 ± 4 , $P = .001$). Recombinant TGF β 1 (2 ng/mL) increased type I procollagen secretion 4-fold in wildtype fibroblasts but less than 1.5-fold in T β RII-null-fib cells. Moreover, the prototypic TGF β regulated protein CTGF was induced in control cells (156 ± 20 , $P = .005$) but not in T β RII-null fibroblasts (28 ± 9 , $P = .6$), confirming refractoriness of the mutant cells to TGF β 1. Healing of 4 mm excisional skin wounds was significantly delayed at 7 days ($P = .0001$) in T β RII-null-fib mice (wound diameter 2.70 ± 0.2 mm, $n=8$) compared with control mice (0.80 ± 0.1 , $n=8$). There was impaired wound contraction and myofibroblast differentiation was deficient. Ki67 proliferation marker demonstrated greater expression in the epidermis of null mice confined to the wound edges compared to controls which showed expression in the granulation tissue. An in vitro scratch wound assay confirmed that fibroblast migration was impaired in T β RII-null-fib mice.

Conclusions: The T β RII-null-fib strain replicates 2 key aspects of late-stage SSc; severe digital contractures and slow healing of full-thickness skin wounds (skin ulcers). Our findings are consistent with a model of TGF β overactivity in early SSc but defective responsiveness of fibroblasts to TGF β ligand in established disease.

37. Renal outcomes and mortality from scleroderma renal crisis—a 15 year retrospective

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Background: Scleroderma renal crisis (SRC) has a substantial morbidity including the frequent need for renal replacement therapy, although some patients recover sufficient renal function to discontinue dialysis. We have reviewed outcome in SRC cases treated over the past 15 years during which time use of angiotensin converting enzyme inhibitors (ACEI) has been routine.

Methods: Cases of SRC from 1990-2005 were ascertained from databases in the departments of rheumatology, histopathology and renal medicine. Case notes were audited, and patients with new onset hypertension (>150/85) and renal impairment (GFR decrease by >30%) included. Patients with renal biopsies showing other diagnoses were excluded. Renal outcome was assessed by patients need for dialysis, and by calculated GFR (MDRD method).

Results: One-hundred ten patients were identified, a frequency of 3.8% of patients under follow up. Seventy-eight percent had diffuse disease (16.9% of all dcSSc cases) and 22% limited disease (1.9%). Mean age 50.7, range 24-82. There was an excess of patients with fine speckled ANA pattern and negative ENA (consistent with RNA polymerase antibodies), with a relative risk for developing SRC of 7.7 (95% CI 5.5-11.1). Twenty patients were taking ACEI or ATII blockers prior to SRC. Thirty-eight (35%) did not require any renal support acutely (no dialysis ND). Twenty-four (23%) required dialysis and subsequently recovered sufficient renal function to discontinue dialysis (dialysis-recovery DR). Forty-four (42%) required dialysis and did not recover (dialysis no recovery DNR). Only three of the patients who did not require dialysis or recovered from dialysis subsequently required renal replacement. Recovery from dialysis occurs over 1-34 months (median 11, IQR 5-15 – in all but one case by 24 months). The GFR in patients not on dialysis increases after the SRC for at least 3 years (year 1 12.8 mL/min/year (95% CI 8.3-17.4) year 2 4.1 mL/min/year (0.80-7.4), year 3 1.89 mL/min/year (0.21-3.6)). Systolic and diastolic blood pressure correlate with renal outcome, with high presenting blood pressure associated with better outcomes (mean ND 205/122, DR 196/114, DNR 177/103 ANOVA $P < .002$). Advanced age at SRC is associated with poor outcome of those patients requiring dialysis (mean age DR 44 DNR 55 $P < .05$), but is not different between those requiring dialysis and those not requiring dialysis (mean age ND 49). Use of ACEI/ATII blockers prior to SRC was associated with a trend towards a higher rate of poor outcome (relative risk of DNR cases 1.71 (95% CI 0.92-3.19)). There was no association with steroid use, presence of MAHA, or antibody profile. Survival at 1 year is 81%, 3 years 71%, 5 years 58%, 10 year 47%. Death rates are highest in the DNR group (31% 5 year survival), lowest in the ND group (68%), and lowest in the DR group (94%). Increasing age is associated with higher mortality (alive 48.6 years, dead 53.9, P

= .027). There is no correlation between disease subset and mortality. **Conclusions:** SRC occurs in both SSc subsets. Overall, 35% cases do not require dialysis, 23% require temporary and 42% permanent dialysis. Renal function improves for at least 3 years after renal crisis. Severe hypertension at presentation is associated with good renal outcome from SRC. Age and poor renal function pre-SRC are poor prognostic markers in patients requiring dialysis. ACEI/ATII receptor antagonist treatment prior to SRC may be associated with poorer renal outcomes. Overall mortality remains high for at least 10 years.

38. Vasculature in systemic sclerosis

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Systemic sclerosis (Ssc) is a connective tissue autoimmune disorder that has been hypothesized as a progressive inflammatory vascular disease. The pathogenesis of Ssc involves a vasculopathy characterized by intimal hyperplasia and capillary rarefaction. The current widespread belief among researchers of Ssc is that the vascular changes are triggered by endothelial cell injury, death and replacement of the endothelial cell layer with an associated defect in angiogenesis. To investigate the vasculature of Ssc we compared skin biopsies from 8 Ssc patients and 7 normal controls. We assessed the state of the vasculature and the surrounding tissue with vessel associated markers using various methods including RNA *in situ* and immunohistochemistry. The results of these studies show a remarkable phenotypic change in endothelial cells in Ssc, an increase and change of smooth muscle gene expression and distribution, significant loss of blood vessels, and change in functional markers but no sign of endothelial apoptosis or proliferation. We conclude that endothelial injury, death and replacement, if present, occur earlier than our biopsies were taken. The loss of blood vessels in Ssc implies that rarefaction occurs at some earlier point since there was no evidence of blood vessel turnover. The changes we observe in various vascular markers imply changes in vascular permeability, stiffness, flow regulation, immune activation, and endothelial function consistent with the vascular hypothesis.

39. Association of the IL-1 beta C-511T polymorphism with the presence of restrictive lung physiology in Italian SSc patients

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Background: Interleukin-1 (IL-1) cluster gene single nucleotide polymorphisms (SNPs) have been implicated in the pathogenesis of some interstitial lung diseases, such as sarcoidosis or pneumoconioses, most likely favouring local inflammation or promoting the deposition of intercellular matrix and tissue remodelling.¹

Recently, we have also observed that the T/C polymorphism at position -889 of the IL-1 α promoter gene influences the response to therapy in patients with systemic-sclerosis (SSc)-associated fibrosing alveolitis (FAS).² Whilst the T-889C polymorphism seems to play a role in the progression of restrictive lung disease in patients with FAS despite treatment with oral cyclophosphamide and medium-dose steroids, other genetic factors might be relevant in the onset of restrictive lung disease (ILD) in SSc patients, comprising those without overt lung inflammation. The present retrospective study was conducted to evaluate whether IL-1 cluster gene polymorphisms are associated with the presence of restrictive lung disease in SSc.

Material and Methods: IL-1 α T-889C, IL-1 β +3962T, IL-1 β C-511T, IL-1R Cpst1970T, and IL-1Ra Cmspal11100T polymorphisms were determined in 182 consecutive SSc patients. The presence/absence of a restrictive lung physiology (RLP), defined as a forced vital capacity (FVC) < 60% of the predicted values, was retrospectively assessed: all the patients routinely undergo a standard evaluation with the execution of pulmonary function testing every 6 months and thus the onset of RLP can be easily reconstructed. Cox regression analysis was used to assess the risk for RLP; disease subset, gender, IL-1 cluster gene polymorphisms and the previous use of CYC were chosen as covariates. The time to the onset of the event was defined as the lapse of time between the onset of the disease (ie, the time of the appraisal of the first non-Raynaud symptom) and the detection of a FVC < 60% of the predicted.

Results: Patients were mostly females (n=137, 75.3%), with the limited cutaneous form (lcSSc) of the disease (n=162, 89%), aged 48 \pm 13.7 years at the onset of SSc. IL-1 cluster gene polymorphisms genotype frequencies were the following:

IL-1 α T-889C – CC=107 (58.8%), CT=59 (32.4%), TT=16 (8.8%);
IL-1 β +3962T – CC=86 (47.3%), CT=78 (42.9%), TT=18 (9.9%);
IL-1 β C-511T – CC=108 (59.3%), CT=61 (33.5%), TT=13 (7.1%);
IL-1R Cpst1970T – CC=83 (45.6%), CT=21 (42.9%), TT=21 (11.5%);
IL-1Ra Cmspal11100T – CC=8 (4.4%), CT=72 (39.6%), TT=102 (56%).

Thirty-six out of 182 patients (19.8%) developed RLP after a mean of 11 \pm 7.0 years from the onset of the disease. Among the considered variables, only disease subset and the IL-1 β C-511T polymorphisms were relevant to the development of RLP. Patients with the dcSSc showed an increased risk for presenting a reduction in FVC % of predicted values as compared to lcSSc patients and the TT genotype increased the risk as compared to CT or CC genotypes: dcSSc vs lcSSc – $P < .001$, OR=4.83 (CI₉₅=2.27-10.26); IL-1 β C-511T polymorphism, TT vs CC – $P = .011$, OR=5.66 (CI₉₅=1.48-21.36); IL-1 β C-511T polymorphism, TT vs CT – $P = .016$, OR=4.52 (CI₉₅=1.32-15.44). IL-1 cluster gene polymorphisms were not correlated with disease subset or total skin score.

Conclusions: In Italian SSc patients, the IL-1 β C-511T polymorphism is associated with the onset of restrictive lung disease, independently of disease subset. Presently, it is not possible to determine whether this polymorphism is simply a marker for a linked disease associated locus or it is a direct disease causing allele.

1. Yucesoy B, Vallyathan V, Landsittel DP, et al. Cytokine polymorphisms in sili-

cosis and other pneumoconioses. *Mol Cell Biochem.* 2002;234:219-24.

2. Beretta L, Cappiello F, Barili M, et al. T-889C IL-1 α promoter polymorphism influences the response to oral cyclophosphamide in scleroderma patients with alveolitis. *Clin Rheumatol.* 2006 Apr 25 [epub ahead of print].

40. Akt positively regulates collagen in dermal fibroblasts via a Smad3 independent pathway

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Increased collagen deposition, along with increased deposition of other ECM components, is a characteristic feature of SSc fibroblasts. Excessive collagen accumulation in these cells is not only due to increased synthesis, but also to a decreased degradation rate. Despite intense efforts, the signaling pathways that regulate collagen synthesis in SSc are not entirely understood. Published data has shown that Akt is constitutively activated in the fibroblasts of SSc patients and that TGF β increases the phosphorylation status of this protein in cultured cells (Jun et al, 2005). Furthermore, Akt1 knockdown mice have a substantially reduced density of collagen fibrils and a reduced amount of total collagen in their skin (Chen et al, 2005). The aim of this study was to examine the role of Akt in the regulation of collagen gene expression in cultured dermal fibroblasts. Dermal fibroblasts were treated with a specific Akt inhibitor in the presence or absence of TGF β and collagen gene expression was evaluated by Western Blot or Quantitative Real Time PCR. Three days after treatment, basal and TGF β stimulated collagen protein levels were almost completely abolished, basal COL1A1 and COL1A2 mRNA levels were reduced by 70% ($P < .001$) and 75% ($P < .001$) respectively and TGF β stimulated COL1A1 and COL1A2 mRNA levels were reduced by 70% ($P < .01$) and 52% ($P = .07$) respectively. Transient overexpression of a dominant negative form of Akt reduced basal and TGF β induced COL1A2 promoter activity by 75% ($P < .001$) and 78% ($P < .05$) respectively, while overexpression of a constitutively active form of Akt increased it by 72.5% ($P = .053$) and 58% ($P < .05$) respectively, suggesting that Akt is required and sufficient to stimulate collagen gene transcription. Taken together these data suggests that Akt is a positive regulator of basal and TGF β stimulated collagen synthesis. To determine if Akt is involved in collagen upregulation by affecting the canonical TGF β /Smad3 pathway, we evaluated by Western Blot the effects of different doses of an Akt inhibitor on Smad3 phosphorylation in the presence and absence of TGF β . There were no changes in Smad3 phosphorylation with Akt inhibition after 30 min of TGF β treatment, suggesting a Smad3 independent effect of Akt on collagen gene regulation. Likewise, TGF β stimulated phosphorylation of Smad1 was not affected. To further explore the mechanism by which Akt regulates collagen accumulation, we assessed the effects of Akt inhibition on the expression of MMP-1 using Western Blot and Quantitative Real Time PCR analysis. According to our data, sustained Akt inhibition upregulates basal MMP1 protein levels, with a 10x increase in mRNA levels. Interestingly, the inhibitory effect of TGF β on MMP1 was not only abrogated after Akt inhibition, but this combination resulted

in a much potent stimulation of MMP1 protein and mRNA levels (20x) than the inhibitor alone. Therefore, in the absence of Akt signaling, TGFβ becomes a positive regulator of MMP1 synthesis.

Conclusion: Our study shows that Akt contributes to increased deposition of collagen by human dermal fibroblasts via two mechanisms: direct upregulation of collagen synthesis and inhibition of collagen degradation via MMP1. Constitutive activation of Akt pathway may directly contribute to elevated collagen in SSc fibroblasts.

41. COMP as a marker of active fibrosis in SSc: immunohistochemical analysis of skin, lung, and kidney tissue.

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Background: We have previously found Cartilage Oligomeric Matrix Protein (COMP) accumulating in lesional (L) and non-lesional (NL) Scleroderma (SSc) skin. The COMP gene is upregulated by TGFβ, thus its putative role in fibrosis could be modulated by TGFβ autocrine stimulation. Murine fibroblasts (fb) engineered to express human COMP demonstrated that COMP leads to excess deposition of collagen and fibronectin in the matrix, even in the absence of TGFβ. To further characterize the role of COMP in SSc fibrosis, we investigated COMP protein distribution in histological sections from several patient tissues as well as from differing disease subsets of SSc.

Methods: 40 forearm skin samples were obtained by 6mm punch biopsy, including 16 L/NL pairs and 5 L from SSc patients, one from a patient with morphea, and 2 from healthy controls. All patients met the ACR criteria for the diagnosis of SSc. Four serial biopsies were obtained after 6 months from skin adjacent to the previous biopsy. Additionally, 2 SSc lung biopsies from the basal fibrotic lobe and apical noninvolved lobe, and 2 kidney biopsies were obtained from post mortem tissue from one SSc patient. COMP protein was evaluated using rat anti human COMP antibody, and TGFβ using mouse anti-human antibody. Collagen distribution in the skin was revealed using Masson's Trichrome staining.

Results: COMP protein appears to be distributed amongst collagen bundles in the papillary and deep dermis in 89% of SSc skin sections and seems to be tightly overlapping collagen distribution shown by Masson's staining. COMP protein was strikingly expressed in fibrotic areas of SSc lung and in SSc kidney interstitia, but barely detected in nonfibrotic areas of SSc lung. Morphea, control skin biopsies and non SSc kidney didn't express COMP protein. COMP was also expressed in fb cytoplasm in a group of skin biopsies. TGFβ protein was detected in a large number of fb especially in sections where fb were strongly positive for COMP staining. TGFβ, COMP, and collagen analyses on serial skin biopsies showed a drastic reduction in TGFβ and COMP staining in 2 patient samples after 6 months, but no significant change in 2 other biopsies.

Conclusion: The analysis of COMP in skin biopsies revealed its presence in both limited and diffuse SSc, in L and N/L areas, and in involved organs such as lung and kidney. Its distribution follows that of collagen in vivo, supporting our previous hypothesis that COMP contributes to the development of fibrosis by assembling collagen fibrils and increasing matrix deposition. COMP study on serial biopsies suggests that its presence in SSc skin is not stable but tends to disappear perhaps due to quiescence of the disease. These findings suggest that COMP expression in vivo may represent an effect of TGFβ stimulation thus it may be a marker of ongoing fibrosis and underlying SSc disease activity.

42. Immunologic disturbances or vascular dysfunction in systemic sclerosis - what comes first? Searching for answer with prostaglandin E1

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Background: Complex correlations of microvascular dysfunction, increased immune activation and tissue fibrosis in systemic sclerosis (SSc) encourage searching for therapeutic options comprising various elements of disease pathogenesis. The aim of the study was to assess the effect of prostaglandin E1 (PGE1) on immunologic activity of systemic sclerosis, endothelial damage resulting from apoptosis and cytotoxic reactions, as well as its correlations between disturbed microcirculation and immunologic activity.

Methods: 50 patients with SSc and 18 matched healthy controls were included in the study. Serum levels of IL-6, sIL-2R, TGF-beta, fractalkine, granzyme A, endothelin 1 and sFas ligand was assessed with ELISA, and lymphocyte expression of CD11a and CD49d was evaluated by flow cytometry. In 25 patients treated with PGE1 these parameters were assessed before and after PGE1 infusions (20 ug - 40 ug - 60 ug on 3 consecutive days). Isolated lymphocytes were cultured with PGE1, supernatant levels of IL-6, sIL-2R, TGF-beta, granzyme A and expression of CD11a and CD49d on lymphocyte were assessed. Lymphocyte count in peripheral blood was evaluated.

Results: A significant reduction ($P < .05$) of serum increased IL-6, sIL-2R, TGF-beta, fractalkine, granzyme A, endothelin 1 and sFas ligand after PGE1 treatment was observed in SSc patients. The treatment resulted in marked reduction ($P < .05$) of CD11a and CD49d lymphocyte expression. In vitro, PGE1 significantly ($P < .05$) reduced supernatant levels of sIL-2R, TGF-beta, granzyme A and lymphocyte expression of CD11a and CD49d, while it increased supernatant level of IL-6. PGE1 significantly ($P < .05$) increased peripheral lymphocyte count.

Conclusions: PGE1 decreases lymphocytes' transmigration and reduces immunologic activation and endothelial damage. The



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effects of PGE1 result from its direct influence on lymphocytes as well as on lymphocyte-endothelium interaction. Direct and indirect immunomodulatory effect PGE1 might influence immunologically induced fibrosis.

43. The German network for systemic scleroderma: establishment of a register encompassing 1350 patients leading to new data on organ involvement and immunosuppressive therapy

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To improve clinical care and provide well characterized biologic material for basic research on systemic sclerosis in Germany a nationwide network was established. In order to guarantee recruitment of all major disease variants the network gathers the princi-

pal subspecialties involved, ie, dermatologists, rheumatologists, pulmonologists and nephrologists. To date the network consists of 29 centers which have entered more than 1350 patients in a detailed register concerning diagnosis, organ involvement and therapy. Of these, 46% suffer from the limited form of the disease, 33% from the diffuse form and 10% from an overlap syndrome. Median Rodnan skin score of patients with limited SSc was 5.1 and with diffuse SSc was 11. Pulmonary hypertension was present in both groups (17 % diffuse, 11 % limited), whereas pulmonary fibrosis was more frequent in diffuse SSc patients (57% vs. 23 %). Renal dysfunction was reported in 15% of the diffuse and 13% of the limited patients, whereas renal crisis was rare with a frequency of 1%. However, using the new sensitive technique of urine-microelectrophoresis pathological results indicating early renal damage could be detected in up to 50% of patients.

To date no proven effective immunosuppressive therapy for systemic sclerosis (SSc) is known. Also, the use of corticosteroids has been shown to be potentially damaging to kidney function. However, it is not known to which extent in clinical practice patients are treated with these agents. Therefore data were collected within the register of the Network referring to the type and dosage of corticosteroids and immunosuppressants used. 1009 patients within the network were available for analysis showing that 40.9 % received corticosteroid therapy. Among those individuals receiving steroids, more than one third (36.7 %) received doses higher than 7.5 mg/d prednisone equivalents. Corticosteroid use was reported in 48 % of patients with diffuse and in 31.6 % of

patients with limited disease ($P < .0001$). Immunosuppressive therapy was administered to 30.6 % of patients. Immunosuppressants used included methotrexate (9.6%), azathioprine (7.3 %), cyclophosphamide (7 %), chloroquine (2.8 %), cyclosporine A (1.7 %) and others. Use of immunosuppressants was reported in 44 % of patients with diffuse and 19.1 % of patients with limited disease ($P < .0001$).

Conclusion: These data clearly demonstrate that despite lacking evidence for a disease-modifying potential in systemic sclerosis, immunosuppressants and corticosteroids are widely used. The established network and register constitutes an ideal framework to intensify related basic research and follow-up the outcome of the development of diagnosis and treatment recommendations and their implementation.

44. Defining a role for fibroblasts in the persistence of chronic inflammation

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One of the most important but as yet unanswered questions in inflammation research is not why chronic inflammation occurs but why it does not resolve. Current models of inflammation stress the role of antigen-specific lymphocyte responses and attempt to address the causative agent. However recent studies have begun to challenge the primacy of the lymphocyte and have begun to focus on an extended immune system in which stromal cells, such as macrophages and fibroblasts play a role in the persistence of the inflammatory lesion. In this lecture I will illustrate how fibroblasts play an important role in regulating the switch from acute resolving to chronic persistent inflammation associated with the pathology of diseases such as rheumatoid arthritis. In chronic inflammation the normal physiological process of the death and emigration of unwanted inflammatory cells becomes disordered leading to accumulation of leucocytes within lymphoid aggregates that resemble those seen in lymphoid tissue. I will describe how fibroblasts from the rheumatoid joint provide survival and retention signals for leucocytes leading to their inappropriate and persistent accumulation within inflamed tissue. Our work suggests that targeting the stromal microenvironment is likely to be an important strategy for future anti-inflammatory therapies.

45. Oncostatin M as a trigger and therapeutic target for fibrotic disease

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Rationale: Oncostatin M (OSM), a cytokine in the IL-6 family, has been reported to be upregulated in patients with fibrotic diseases such as scleroderma, a debilitating, systemic disease charac-

terized by excessive dermal fibrosis with progression to internal organs. Major aspects of this multi-organ disease include not only fibrosis, but also immune dysregulation as evidenced by inflammatory cell infiltrates. To investigate the role of OSM in fibrotic disease, we have evaluated the expression of OSM in an established, chemically induced model of lung fibrosis, and further analyzed the ability of OSM to promote fibrosis and inflammation *in vivo*.

Methods and Results: As reported in the literature, subcutaneous administration of bleomycin results in an inflammatory and fibrotic response in the lungs of mice. Upregulation of OSM and a downstream marker of OSM, Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) were evident in the BAL fluid from bleomycin-treated mice. In order to determine whether OSM is sufficient to induce an inflammatory and fibrotic response in the skin and lungs independent of additional triggering agents, recombinant murine OSM was administered 1) intradermally (1 ug, 3x/week for up to 3 weeks) or 2) intranasally (2 ug, daily for up to 2 weeks). When administered intradermally, OSM results in a significant increase in dermal fibrosis, inflammatory infiltrates of mononuclear cells and eosinophils, and epithelial cell hyperplasia. There is also a marked increase of collagen, evident by histology and the sircol assay, a quantitative measure of collagen. Intranasal dosing of OSM, in comparison to control mice, results in a significant inflammatory infiltrate into the lung and collagen deposition.

Conclusions: We have demonstrated that OSM is upregulated in a bleomycin-induced model of lung fibrosis and is a potent inducer of inflammation and fibrosis *in vivo*. Current treatments for fibrotic diseases target the inflammatory cascade, but do not suppress the fibrotic process and show little efficacy in modulating the disease course. The expression of OSM in human disease, coupled with its ability to promote both inflammation and fibrosis, may provide a rationale for therapeutically targeting OSM signaling in fibrotic diseases.

46. Rate of skin thickness progression in early systemic sclerosis with diffuse cutaneous involvement: associations with organ involvement and 5-year survival

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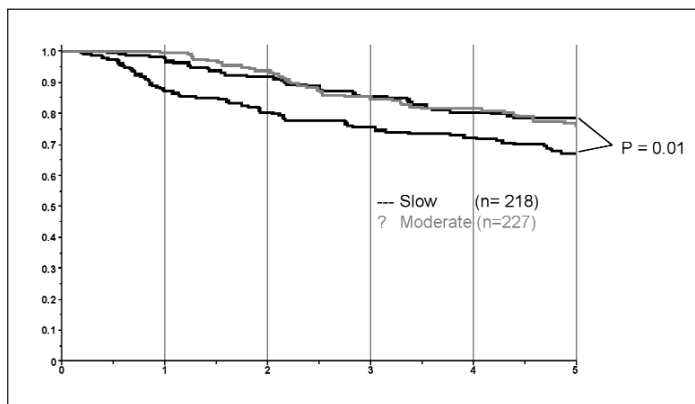
Patients with Systemic Sclerosis (SSc) and diffuse cutaneous involvement (skin thickening proximal to elbows or knees) have an increased risk of developing internal organ involvement early in their disease. Physicians need guidelines for assessment of these risks at the initial visit.

Purpose: To evaluate the rate of skin thickness progression judged by history and physical exam, and its association with SSc internal organ involvement (frequency, severity and time to development) and 5 year cumulative survival rate (CSR).

Methods: We evaluated SSc patients from the University of

Pittsburgh cohort who had the following characteristics: 1) first visit between 1980 and 2004, 2) diffuse cutaneous (dc) SSc at first visit, 3) duration of skin thickening less than two years, and 4) only one identified autoantibody (anti-RNA polymerase III, anti-topoisomerase I, or anti-U3 RNP antibody) or none of these, categorized as other. Skin thickness progression rate (STPR) was defined as total skin thickness score by the modified Rodnan method at first visit divided by the time from onset of skin thickness (by history) until the first visit. For example, a total skin score (TSS) of 24 acquired over 8 months was considered the equivalent of 36 TSS 'units' per year. All STPRs were divided by tertiles into three categories: 1) rapid (>45/year), 2) intermediate (25 – 45/year), and 3) slow (<25/year). The frequency and time to internal organ involvement (kidney, lung, heart and GI tract), frequency of death secondary to individual organ involvement and 5-year CSR from onset of skin thickening were assessed.

Results: 668 patients meeting criteria were identified. The 5 year CSRs by STPR tertile group for all patients are depicted in **Figure 1**.



The mean STPR and 5 year CSR by autoantibody are shown in the **Table 1**. The CSRs parallel the STPR, but in the opposite direction general clinical impression would predict.

Table 1:

Autoantibody Group	Mean STPR (units/year)	Mean 5 Year CSR
Anti-RNA Pol III (n=282)	47.1	81%
Anti-Topo (n=172)	42.0	66%
Anti-U3 RNP (n=20)	30.0	47%
Other (n=194)	42.5	74%

The slow and intermediate groups had similar 5 year CSR across autoantibody, and thus were grouped and compared to the rapid STPR group. For anti-RNA polymerase III patients, rapid STPR revealed significant associations for the proportion of patients dying from scleroderma renal crisis (SRC) ($P = .0142$) and a reduced 5 year CSR ($P = .0001$). There were trends toward increased frequency of SRC, and development of SRC within the

first year after onset of skin thickening ($P = .07$). For anti-topoisomerase I patients, rapid STPR was not correlated with the frequency of interstitial lung disease (ILD), but was associated with the development of ILD within 1 year after onset of skin thickening ($P = .001$). Associations with rapid STPR and frequency of internal organ involvement included cardiac involvement ($P = .009$) and a trend for SRC ($P = .09$). The number of anti-U3 RNP patients was too small for meaningful comparisons. However, there were trends for association of rapid STPR in this group with increased frequency of cardiac involvement ($P = .09$) and reduced 5 year CSR ($P = .09$). The other dcSSc patients showed no associations with STPR and organ involvement or survival.

Conclusions: Rapid STPR at the time of initial evaluation in dcSSc patients with disease duration less than 2 years has significant associations with the frequency and rate of internal organ system involvement and 5 year cumulative survival. Notably, the major autoantibody subsets have different profiles of association with STPR, internal organ involvement and survival. Assessment of risk in individual dcSSc patients and planning of clinical trials enrolling these patients should take the simply obtained STPR measurement into consideration.

47. PDGF_β receptor activation is essential for fibroblast and pericyte recruitment during cutaneous wound healing

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Introduction: Connective tissue remodeling provides mammals with a rapid mechanism to repair wounds following injury. Inappropriate activation of these reparative mechanisms are central to the development of pathological scarring and tissue fibrosis seen in scleroderma (SSc). Over-expression of platelet derived growth factor-beta receptors (PDGFR_β) is a prominent characteristic in both wound repair and SSc tissue. As conventional deletion of PDGFR_β results in embryonic lethality, it has not been possible to fully assess the contribution of PDGFR_β signaling to adult connective tissue repair and the development of fibrotic lesions in SSc. Here we studied the effects of PDGFR_β blockade *in vivo* using the PDGFR_β inhibitor, imatinib mesylate on tissue repair.

Methods: Full thickness excisional wounds were made in mice harboring the -17kb promoter sequence of the murine collagen 1-2 gene linked to a β -galactosidase reporter gene. Animals were sacrificed at defined time points post-wounding. A series of *in vitro* and *in vivo* analyses were carried out to assess the effects of PDGFR_β blockade on wound closure, cell proliferation and migration and the synthesis of extracellular matrix components.

Results: Healing of wounds was delayed with significantly impaired granulation tissue contraction ($P < .05$) and concomitant reduction in myofibroblast frequency, expression of fibronectin ED-A and collagen type I. Inhibiting PDGFR, activation restricted the distribution of collagen synthesizing cells to wound margins and dramatically reduced cell proliferation *in vivo* ($P < .01$). Blocking PDGFR, signaling did not prevent the differentiation of myofibroblasts *in vitro*, but potently inhibited fibroblast proliferation and migration ($P < .01$). Additionally, PDGFR, inhibition *in vivo* was accompanied by abnormal microvascular morphogenesis reminiscent of that observed in PDGF,-/- mice with significantly reduced immunostaining of the pericyte marker, NG2. Imatinib treatment also inhibited pericyte proliferation and migration *in vitro*.

Conclusions: This study highlights the significance of PDGFR, signaling for the recruitment, proliferation and functional activities of fibroblasts and pericytes during wound healing and provides further insights on how PDGFR, signaling contributes to the development of fibrosis.

48. Defining the Role of Integrin $\alpha 11\beta 1$ in Wound Healing and Fibrosis

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Fibrosis is the replacement of healthy tissue with excess collagenous matrix. The collagen-binding integrins are key regulators of collagen production and have been implicated in the development of fibrosis. We have found that the recently described integrin alpha 11 (Itga11) was induced in response to the profibrotic cytokine TGF β , and is over-expressed in fibrotic tissues. To better understand the role of Itga11 we have developed an Itga11 null mouse and have examined the behavior of Itga11 null cells *in vitro*. Itga11 was strongly expressed in primary adult mouse dermal fibroblasts and was localized to focal adhesions. When dermal fibroblasts from Itga11 null mice were cultured in collagen lattices, an *in vitro* model of force transduction and wound healing, they were deficient in contracting the gel compared to wild type cells, and also responded less to stimulation by TGF β . These data suggest that Itga11 has an important role in the adhesion of fibroblasts to the extracellular matrix and is involved in the force production required for wound contraction. To investigate downstream signaling from Itga11, we expressed full-length protein in K562 cells, a line that does not express collagen-binding integrins. Stable transfection of Itga11 resulted in cell surface expression of $\alpha 11\beta 1$ protein and allowed the cells to bind to collagen. Interestingly, expression of Itga11 in K562 cells caused a decrease in proliferation rate and an increase in the differentiation state of the cells, effects not seen on expression of Itga1 or Itga2. To ensure that these effects of Itga11 were due to the cytoplasmic domain we made a chimeric protein consisting of the extracellular

and transmembrane domains of Itga1 fused to the intracellular portion of Itga11. Expression of this construct gave identical results to expression of Itga11. These data indicate that Itga11 initiates intracellular signaling events that are unique among the collagen binding integrins and provides a simple system in which to identify downstream signaling pathways from Itga11. Taken together our data suggest that Itga11 may have an important role to play in wound healing and the development of fibrosis, and could be an attractive target for antifibrotic therapies.

49. Evidence for subsets in the gene expression patterns of scleroderma skin biopsies

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Systemic Sclerosis (SSc; Scleroderma) is a complex, highly variable and in many cases, fatal disease. The disease is characterized by vascular dysfunction (Raynaud's phenomenon, vascular dropout and intimal proliferation), tissue fibrosis, internal organ dysfunction, and immune dysfunction resulting in autoantibody production; cause and effect among these different pathological processes is not known. The complexity of the disease and the lack of accurate model systems mean clinical samples are our best window into the biological processes occurring. We have analyzed the genome-wide patterns of gene expression with whole-genome DNA microarrays in skin biopsies from 18 patients with diffuse scleroderma, 7 patients with limited scleroderma, 3 patients with morphea and 6 normal controls. Sixty-one skin biopsies have been analyzed in total; the addition of 14 technical replicates results in a total of 75 microarrays analyzed to date. Using unsupervised hierarchical clustering methods and Principle Component Analysis (PCA), we again find nearly identical patterns of gene expression in the clinically affected forearm and unaffected back skin biopsies of scleroderma patients. 11/15 forearm-back pairs from patients with diffuse skin involvement and 6/8 forearm-back pairs from patients with limited skin involvement cluster side by side, emphasizing the systemic nature of the disease. Using this property of the gene expression, we have selected the 995 most 'intrinsic' genes with an estimated False Discovery Rate (FDR) of 4.0%. These genes are those most consistently expressed within the forearm-back pairs and technical replicates for the same patient, but show the most variability across all patients and normal controls. Using this list of genes, we have analyzed the inherent data driven groupings and show evidence for multiple subgroups that can be distinguished by their unique gene expression patterns. Distinct patterns of gene expression distinguish patients with limited skin involvement from those with diffuse skin involvement and both are easily distinguished from normal controls. Most importantly, our data provide evidence for multiple distinct subgroups among the patients with diffuse skin involvement and these groups are independent of disease

activity, age of onset, or disease duration suggesting the differences are rooted in fundamental biology.

This work has been supported by grants from the Scleroderma Research Foundation to MLW and MKC. MLW is supported by Howard Hughes Medical Institute Biomedical Research Support Award #76200-560801 to Dartmouth College.

50. Genome-wide characterization of the TGF β -activated gene expression signature in normal and scleroderma dermal fibroblasts

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The hallmark of scleroderma is fibrosis of the skin, which is characterized by thickening, and tightening of the dermis as a result of excessive synthesis and deposition of extracellular matrix (ECM) components such as collagen. Dermal fibroblasts are the primary cell type responsible for ECM deposition in the skin and as such are the primary candidates for investigating fibrotic mechanisms in scleroderma. An extensive body of evidence has implicated the potent pro-fibrotic cytokine TGF β in scleroderma pathogenesis and much work has been done to show that this signaling pathway is deregulated in scleroderma fibroblasts *in vitro*. We have captured the response of both normal and scleroderma cultured dermal fibroblasts to TGF β over the course of 24 hours using DNA microarrays. In doing so, we have defined a genome-wide expression signature for the 'TGF β -activated' response in both normal and scleroderma dermal fibroblasts. We report here that normal and scleroderma fibroblasts show similar responses to TGF β , although the baseline level of expression of TGF β inducible genes is higher in scleroderma fibroblasts than in normal fibroblasts. Preliminary data interrogating the overlap between the TGF β -activated signature and those genes differentially expressed between diffuse scleroderma and normal skin biopsies, suggest a subset of the TGF β signature is differentially expressed in subgroups of the scleroderma skin biopsies *in vivo*.

This work has been supported by grants from the Scleroderma Research Foundation to MLW and MKC. MLW is supported by Howard Hughes Medical Institute Biomedical Research Support Award #76200-560801 to Dartmouth College.

51. Regulation of fibrotic responses in mouse fibroblasts lacking CTGF/CCN2

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Background: Transforming growth factor- β (TGF- β) induces

profibrotic responses in fibroblasts, and is implicated in scleroderma. The cysteine-rich TGF- β -inducible protein CTGF/CCN2 is a matricellular protein of unknown physiological function that is markedly up-regulated in scleroderma lesions. CCN2 has been functionally implicated as a down-stream mediator of the profibrotic effects of TGF- β . In order to gain insight into its physiologic role in fibrosis, we studied CCN2 expression *in vivo*, and the regulation of TGF- β induced fibrogenic response in CCN2-null fibroblasts *in vitro*.

Methods: CCN2 expression in a mouse model of scleroderma was examined by immunohistochemistry. TGF- β responses in fibroblasts (MEFs) from heterozygous and homozygous CCN2-null mouse embryos and wildtype controls were analyzed by immunohistochemistry, Northern and RT-PCR analysis, Western blot and transient transfection assays. Myofibroblasts were identified with antibody to α -smooth muscle actin (α -SMA).

Results: Bleomycin-induced murine scleroderma was associated with elevated CCN2 levels in lesional skin. *In vitro*, CCN2 expression was rapidly induced by TGF- β in wildtype MEFs. CCN2-null MEFs appeared normal, but showed a 50% reduction in proliferation. TGF- β -dependent early (90 min) activation of Smad2/3 (phosphorylation and nuclear accumulation) and Smad4 were unaffected by loss of cellular CCN2. Transient transfection assays with the Smad-responsive pSBE₄-luc reporter construct confirmed intact Smad-mediated transcriptional activity in both wildtype and CCN2-null MEFs. Northern and RT-PCR analysis revealed significant TGF- β stimulation of COL1A2 and fibronectin mRNA expression in CCN2-null MEFs, and Western analysis indicated similar induction of Type I collagen, fibronectin and PAI-1. Furthermore, TGF- β -induced a comparable increase of α -SMA expression and stress fiber organization in wildtype and CCN2-null MEF.

Conclusion: CCN2 expression was up-regulated in fibrotic lesions *in vivo*, and its expression in fibroblasts *in vitro* was stimulated by TGF- β . While CCN2-null MEFs showed reduced proliferation, TGF- β -Smad-mediated intracellular signal transduction remained intact. Furthermore, endogenous CCN2 did not appear to be required for TGF- β stimulation of collagen, fibronectin and PAI-1 expression. These results suggest that endogenously induced CCN2 is not indispensable for mediating profibrotic responses elicited by TGF- β .

52. Possible future therapy for digital ischaemia in systemic sclerosis. Whole finger iontophoresis?

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Introduction: Systemic sclerosis (SSc)-related digital ischaemia can result in ulceration, scarring and gangrene and is a major source of morbidity. Intravenous prostanoids are the treatment of choice; however, they require admission to hospital, can be associated with vasodilatory side effects and are not always effective. Therefore, a safe and effective treatment, which does not cause systemic adverse effects, is badly needed.

Iontophoresis is a noninvasive process whereby ions of a drug or chemical are ‘driven’ into the skin by the application of a low current. Previous pathophysiological studies of iontophoresis with acetylcholine chloride (ACh) or sodium nitroprusside (NaNP), to assess vasodilatory (dys)function, have shown significant localized increases in cutaneous perfusion in response to both ACh and NaNP, even in patients with thickened skin (1-3). Their results raised the question as to whether iontophoresis might be used therapeutically in patients with SSc.

Methods: *Patients:* Eight patients (6 female, 2 male; median age 56 [range 39-73] yr) and 8 healthy controls (6 female, 2 male; 62 [42-76] yr) were recruited into the study. Four patients were on oral vasodilator therapy.



Figure 1. Whole finger iontophoresis chamber.

Protocol: Following acclimatization (20 min at 23°C) a baseline perfusion scan of the digit to be iontophored was taken using laser Doppler imaging (LDI). The digit was then iontophored for 2 min at 200 µA, with a specially designed chamber (Figure 1).

Following iontophoresis repeat LDI scans were performed for 10 min or until blood flow

returned to baseline values. The 2 min treatment was carried out bilaterally. Iontophoresis was then repeated for 5 min on one digit only (the order of the digit treatment was randomly assigned to left or right) and repeat scans performed for up to 25 min. Treatment was with either 0.5% NaNP or 1% ACh, and the whole procedure then repeated with the other vasodilator. The order of the chemical delivered was randomly assigned. The maximum perfusion increase from baseline (MAX) and the area under the time perfusion curve (AUC) normalized for baseline, were calculated. Data were compared with a 3-way ANOVA test.

Results: Values for MAX and AUC are shown in Table 1. MAX and AUC values were significantly higher for the control than the

marked vasodilation, there was a definite blood flow increase with both ACh and NaNP in the patient group, indicating that this local approach to vasodilation was effective in increasing digital blood flow. Increasing the time of treatment causes a more sustained vasodilation. Further studies are now indicated to investigate a possible therapeutic effect in patients with severe digital ischaemia and/or ulceration.

53. Gene expression analysis of scleroderma peripheral blood cells reveals putative molecular markers and a possible decrease in circulating monocytes

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Systemic Sclerosis is a complex disease with clear systemic manifestations. We have analyzed the genome-wide patterns of gene expression from peripheral blood cells taken from patients with scleroderma and normal controls. Total peripheral blood cells were collected using the PAXgene Blood RNA isolation system which minimizes sample handling; RNA was analyzed on Agilent Technologies oligonucleotide microarrays. Analysis of the patterns of gene expression reveals clear and distinct patterns of expression that distinguish individuals with scleroderma from normal individuals. Using CBC and differential counts we have identified patterns of gene expression that are correlated with monocyte counts and these data suggest a decrease in circulating monocytes in individuals with scleroderma. These data provide evidence that genes indicative of the disease process are differentially expressed in scleroderma peripheral blood cells, which may provide insight into the pathogenesis of this complex disease.

Table 1. Maximum increase from baseline (MAX) and area under the perfusion curve (AUC), normalised for baseline

	AmNaNP 0.5% conc, 200				AmACh 1.0% conc, 200			
	2 min		5 min		2 min		5 min	
	MAX	AUC	MAX	AUC	MAX	AUC	MAX	AUC
Control N=8	2.2 (1.6-2.7)	15.3 (13.1-18.6)	2.6 (2.4-3.0)	28.7 (26.9-36.1)	2.0 (1.7-2.3)	12.5 (11.2-16.5)	2.5 (1.9-2.8)	24.2 (22.1-30.6)
Patient N=8	1.3 (1.1-1.6)	11.2 (10.2-12.6)	1.4 (1.2-2.3)	21.6 (20.5-24.0)	1.5 (1.2-1.8)	12.4 (10.6-12.9)	1.1 (1.0-1.7)	20.2 (20.0-21.4)

Median (interquartile range). Each subject's left and right 2 min data was averaged.

patient group ($p_{MAX}=0.001$, $p_{AUC}=0.008$ respectively). Values were significantly higher for the 5 min treatment compared to the 2 min treatment ($p_{MAX}=0.011$, $p_{AUC}=0.001$). No significant differences were found between the use of NaNP and ACh.

Discussion: Although the control group demonstrated more

54. Increased expression of Siglec-1 (Sialoadhesin, CD169) in systemic sclerosis (SSc) monocytes

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Purpose: Systemic sclerosis (SSc) shares many features with another autoimmune disease, systemic lupus erythematosus (SLE). Recently, increased expression of interferon (IFN)-regulated genes were found by multiple investigators to be increased in the peripheral blood mononuclear cells (PBMCs) of patients with SLE. We postulated a similar finding would be present in SSc.

Methods: PBMCs were obtained from SSc patients with early diffuse disease (onset of 1st non-Raynaud's symptom within 6 months) and healthy controls. RNA from PBMCs were purified and hybridized to an Affymetrix U133 Plus 2.0 microarrays. RNA

from PBMCs isolated from these and additional SSc patients with limited and diffuse disease were used to validate gene expression by Real-Time PCR utilizing an ABI 7700 and Siglec-1 probes from Applied Biosystems. Flow cytometry of PBMC and immunohistochemistry (IHC) of skin biopsy tissue was performed using mouse anti-human Siglec-1 mAb.

Results: As in SLE, a subset of SSc patients showed a pattern of increased IFN-regulated gene expression. In particular, the activated macrophage-specific gene Siglec-1 was markedly increased in SSc patients. This finding was verified by Real-Time PCR. Flow cytometry of PBMC in SSc patients and healthy controls demonstrated that increased expression of Siglec-1 was specific for CD14+ monocytes. Siglec-1 expression was induced on healthy donor monocytes by IFN or TLR stimulation. IHC revealed a population of Siglec-1+ macrophages in the dermis of SSc patients in both lesional and non-lesional skin that was absent in healthy controls.

Conclusions: Siglec-1 expression is increased in SSc and suggests type-I IFN-mediated activation occurs in monocytes in SSc. Our *in vitro* experiments indicate a potential role for TLR activation of IFN secretion, possibly via nucleic acid containing immune complexes.

55. Dihydro sphingosine 1 phosphate (dhS1P) has antifibrotic activity in dermal fibroblast through interference with TGFβ signaling pathway

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Fibrosis is a complex disease characterized by the excessive deposition of collagen and other extracellular matrix (ECM) components. The net accumulation of collagen in tissue fibrosis is a result of an imbalance between the factors regulating collagen deposition and collagen degradation. Transforming growth factor β (TGFβ) and Tumor necrosis factor alpha (TNFα) have contrasting effects on ECM deposition. Profibrotic effects of TGFβ involve upregulation of collagen synthesis and inhibition of MMP1 production, while TNFα inhibits collagen and stimulates MMP1. We have recently shown that dhS1P is a principal mediator of the TNFα induction of MMP1 (Bu et al, *Faseb J*, 2006). In this study, we investigated whether dhS1P also plays a role in regulation of collagen production in dermal fibroblasts. TNFα stimulation of fibroblasts leads to a rapid activation of Sphingosine kinase (SphK), which catalyzes generation of dhS1P and sphingosine 1-phosphate (S1P), two bioactive lipids that mediate distinct biologic response of TNFα. The present study shows that TGFβ-induced collagen synthesis and TGFβ-induced Smad2/3 phosphorylation are inhibited by TNFα. Using SphK1 specific siRNA we demonstrate that this inhibition requires SphK1. Furthermore, treatment of fibroblasts with low doses of dhS1P (500 nM) is sufficient to recapitulate these inhibitory effects. While dhS1P inhibits TGFβ-induced Smad2/3 phosphorylation, it does not affect TGFβ receptor phosphorylation status, suggesting that a post-receptor mechanism is involved in these effects. In contrast, S1P increases collagen levels and stimulates Smad2/3 phosphorylation. Preliminary data indicate that SSc fibroblasts are much more responsive than control fibroblasts to the SphK1-mediated collagen inhibition at the basal level, suggesting possible alterations of the sphingolipid signaling in SSc. In conclusion, this study demonstrates that the relative ratio between dhS1P and S1P may have a direct role in regulating the balance between matrix degradation and matrix deposition and that the imbalance of these mediators may play a role in SSc fibrosis.

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56. Modulation of collagen and MMP-1 gene expression in fibroblasts by the immunosuppressive drug rapamycin: a direct role as an antifibrotic agent?

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We have examined whether rapamycin, an immunosuppressive drug, may exert parts of its antifibrotic activity by directly targeting fibroblast extracellular matrix deposition. Incubation of human lung fibroblast (WI-26) cultures with rapamycin led to dose- and time-dependent reduction in the expression of type I and III collagens, both at the protein and mRNA levels. Rapamycin had no effect on collagen promoter activity but accelerated mRNA decay, indicating post-transcriptional control of collagen gene expression. In contrast, rapamycin significantly enhanced the expression of interstitial collagenase (MMP-1), both at the mRNA level and transcriptionally. We determined that rapamycin efficiently activates AP-1-driven transcription, by rapidly inducing *c-jun*/AP-1 phosphorylation by activation of the Jun-N-terminal kinase (JNK) cascade, resulting in enhanced binding of AP-1/DNA complex formation and AP-1 dependent gene transactivation. Conversely, the JNK inhibitor SP600125 inhibited rapamycin-induced *MMP-1* gene transactivation and AP-1/DNA interactions. A *c-jun* antisense expression vector efficiently prevented rapamycin-induced *MMP-1* gene transcription. Pharmacologic inhibition of either ERK or p38/MAPK pathways was without effect on rapamycin-induced *MMP-1* gene expression. It thus appears that rapamycin may exert direct antifibrotic activities independent from its immunosuppressive action.

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

Have you seen this patient?

- Rapidly progressing systemic sclerosis (SSc)
- Extensive skin involvement (modified Rodnan skin score ≥ 16)
- Early internal-organ involvement

If you want to learn more about SCOT or have a potential patient to refer, please call:

1.866.909.SCOT (7268)
www.sclerodermatrial.org

SCOT is a pivotal, prospective, randomized, clinical research study reviewed by the FDA and supported by the NIH. A total of 226 subjects between the ages of 18 and 65 years (inclusive) will be enrolled across North America over a 3-year period and randomly assigned in a 1:1 ratio to one of the following:

- High-dose immunosuppressive therapy (HDIT) with autologous stem cell transplantation, or
- Monthly IV pulse cyclophosphamide for 12 months

Event-free survival at 44 months after randomization is the primary endpoint.

The SCOT study is a research effort that has been funded in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, under Contracts No. N01-AI-25481 and N01-AI-05419.





The best way to help scleroderma patients is to fund research that will lead to better therapies and, eventually, a cure.



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