

## *Six Highest Ranking Abstracts*

## ABSTRACTS

### **Th17 and Th1 Subsets are increased in Patients with Systemic Sclerosis**

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### **The nuclear autoantigen centromere protein B (CENP-B) displays cytokine-like activities towards vascular smooth muscle cells**

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### **Caveolin-1 scaffolding domain peptide inhibits the monocyte to fibrocyte differentiation of normal and scleroderma peripheral blood mononuclear cells.**

*Elena Tourkina, Mathieu Richard, James Oates, Richard M. Silver, and Stanley Hoffman Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina, USA*

### **Therapeutic angiogenesis by local autologous progenitor cell implantation for ischemic digits in patients with systemic sclerosis.**

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### **Cloning of agonistic autoantibodies specific for the PDGF receptor from the B cell repertoire of SSc patients**

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### **Intercellular adhesion molecule-1 expression on fibroblasts contributes to the development of skin fibrosis in Tight-Skin Mice**

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### **1. Investigation of the role of Rac1 in a bleomycin-induced scleroderma model using fibroblast-specific Rac1 knockout mice**

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**Background** Activated adhesive signaling is a hallmark of fibroblasts isolated from scars of scleroderma (systemic sclerosis; SSc) lesions. Rac1 plays a key role in adhesive signaling. The aim of the present study was to examine the role of Rac1 in bleomycin-induced scleroderma using mice bearing a fibroblast-specific deletion of Rac1.

**Materials and Methods** Cutaneous sclerosis was induced by subcutaneous injection of bleomycin. Control groups were treated with phosphate buffered saline (PBS). Mice bearing a fibroblast-specific deletion of Rac1 and control mice were investigated. Dermal thickness, inflammation, collagen production and the number of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)-positive cells were determined. The quantity of the collagen-specific amino acid hydroxyproline was also measured. The effects of Rac inhibition were assessed on primary scleroderma fibroblasts.

**Results** Bleomycin treatment induced marked cutaneous thickening, inflammation and fibrosis in control mice. Deletion of Rac1 resulted in resistance to bleomycin-induced fibrosis and inflammation. Rac inhibition alleviated the persistent fibrotic phenotype of scleroderma fibroblasts

**Conclusion** Rac1 expression by fibroblasts is required for fibrogenesis. Inhibition of Rac1 may be a viable method to alleviate the development of cutaneous sclerosis.

### **2. Th17 and Th1 Subsets are increased in Patients with Systemic Sclerosis**

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**Background:** Systemic sclerosis (SSc) is characterized by vasculopathy, chronic inflammation and fibrosis. T cells participate in the pathogenesis of this disease. The Th1/Th2 paradigm was not very helpful to explain several aspects of this disease and the description of T helper 17 (Th17) cells and their biology opened new possibilities in the study of the pathogenesis of SSc. The cytokine profile of Th17 cells is IL-17 A/F, TNF- $\alpha$ , IL-1, IL-6 and GM-CSF. This profile is proinflammatory, and the overall effects of these cytokines could lead to the inflammation and fibrosis that are hallmark of SSc. Several authors have demonstrated elevated production of IL-17, TNF- $\alpha$ , IL-1 and IL-6 by peripheral blood mononuclear cells of patients with SSc. Here we present the first

report of the analysis of CD4<sup>+</sup> T cell subpopulations in patients with SSc and its correlation with SSc subtypes and organ involvement.

**Patients and Methods:** Blood samples from 97 patients (n=8 men) with SSc were obtained. Clinical evaluation and organ involvement were determined using the Medsger's severity scale. Controls were collected from age- and sex-matched healthy volunteers (n=16, 3 men; mean age=36.7±12.2 years). Mononuclear cells were analyzed by flow cytometry to determine Th1 (CD4<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>), Th2 (CD4<sup>+</sup>/IL-4<sup>+</sup>), Th17 (CD4<sup>+</sup>/IL-17<sup>+</sup>) and Treg (CD4<sup>+</sup>/CD25<sup>+</sup>/FOXP3<sup>+</sup>) subsets. Patients were classified in two groups: (a) diffuse (n=39; age=43.6±13.2 years; disease evolution=8.6±6.1 years) and (b) limited (n=58; age=48.5±14.3 years; disease evolution=10.7±9.5 years) SSc. Statistical analysis was performed using Mann-Whitney Rank Sum Test, student's t test and Chi square test.

**Results:** Th17 subset was 4-5-fold increased in SSc groups vs. control group (Diffuse=2.9±0.3; Limited=3.0±0.3; vs. Control=0.6±0.03; p=0.001). Meanwhile Th1 was 1.5 to 1.8-fold increased in SSc vs. control group (p<0.05). Treg subpopulation was scarce but not significantly decreased in SSc patients vs. controls. There were no statistical differences in age, time of evolution and treatment between groups.

**Conclusion:** Th17 and Th1 subsets are increased while Treg subset is slightly decreased in patients with diffuse and limited SSc. These alterations can be regarded as an imbalance of the immune response that leads to chronic inflammation, fibrosis and favour autoimmunity, all of which are prominent features of SSc.

### 3. Conditional deletion of GSK3 $\beta$ results in enhanced tissue repair and fibrogenesis in vivo via an endothelin-1-dependent mechanism

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**Background:** It has been previously hypothesized that Wnt/ $\beta$ -catenin signaling pathway may play a key role in driving the fibrogenic responses in disease such as scleroderma. However, till date, no exact role of this pathway in driving fibrogenesis has been elucidated. To test this hypothesis, we targeted glycogen synthase kinase-3 (GSK-3), a key component of Wnt/ $\beta$ -catenin signaling pathway. GSK-3 $\beta$  normally phosphorylates  $\beta$ -catenin causing  $\beta$ -catenin to be targeted for degradation. In the absence of GSK-3,  $\beta$ -catenin is translocated into the nucleus to activate transcription. To investigate the contribution of GSK-3 $\beta$  in fibrogenesis, we generated mice containing a fibroblast-specific deletion of GSK-3 $\beta$  and then subjected these mice to a model of wound repair.

**Materials and Methods:** To generate mice containing a fibroblast-specific deletion of GSK-3 $\beta$  mice that carry a tamoxifen-inducible Cre-recombinase under the control of a fibroblast-specific regulatory sequence from the pro $\alpha$ 2(I) collagen gene were crossed

with mice that carry homozygous conditional LoxP-GSK-3 $\beta$  allele to generate Cre/GSK-3 $\beta$  heterozygote mice. The second cross obtained Cre/GSK-3 $\beta$  mice. To delete GSK-3 $\beta$ , mice (age, 3 weeks) were given ip injections of the tamoxifen and deletion of GSK-3 $\beta$  was tested by PCR. These mice were then subjected to the full thickness incisional model (skin punch biopsy) of dermal wound healing. Wound healing parameters were studied over a period of 28 days post-wounding. Dermal fibroblasts from these mice were isolated and cultured for *in vitro* analysis.

**Results:** In this study, for the first time we show that GSK-3 $\beta$  conditional knockout mice show protracted enhanced wound closure, excessive collagen production, elevated levels of pro-fibrotic  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and myofibroblast formation during the process of wound healing. GSK-3 $\beta$ -deficient mice were unable to terminate tissue repair, resulting in excessive scarring. In cultured GSK-3 $\beta$ -deficient fibroblasts, adhesion, spreading, migration and contraction were significantly enhanced. GSK-3 $\beta$ -deficient mice and fibroblasts showed elevated endothelin-1 (ET-1) production and expression. Antagonizing ET-1 by bosentan (an endothelin receptor antagonist) reversed the fibrotic phenotype of GSK-3 $\beta$ -deficient fibroblast *in vitro* and GSK-3 $\beta$ -deficient animals *in vivo*. Thus GSK-3 $\beta$  controls the progression of wound healing and fibrosis, via modulating ET-1 levels.

**Conclusions:** Our results show that targeting the GSK3- $\beta$  pathway or ET-1 may be of benefit in controlling tissue repair and fibrogenic responses in conditions such as scleroderma.

### 4. The nuclear autoantigen centromere protein B (CENP-B) displays cytokine-like activities towards vascular smooth muscle cells

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**Background:** *In vitro* studies have demonstrated that some autoantigens have an additional biological function when they are released in the extracellular environment during injurious insults resulting in cell death. Indeed, it was previously suggested that extracellular autoantigens participate in normal wound repair processes by acting like cytokines and/or chemokines and subsequently display pathogenic activities that contribute to the development of autoimmune diseases. Our present findings suggest that centromere protein B (CENP-B), a nuclear autoantigen specifically targeted in the limited cutaneous subset of systemic sclerosis (SSc), can be added to this set of bifunctional molecules.

**Materials and methods:** In SSc, autoantibodies to CENP-B are associated with the occurrence of prominent vascular manifestations such as pulmonary arterial hypertension (PAH), that in turn appears to be caused by intimal migration and proliferation of vascular smooth muscle cells (SMC). However the factors driving this vascular remodelling are unknown. Thus we examined the biological effects of extracellular CENP-B on human pulmonary artery smooth muscle cells (HPASMC).

**Results:** Purified CENP-B and CENP-B released from apoptotic endothelial cells bound specifically to the surface of HPASMC with a greater affinity for the contractile than for the synthetic type. CENP-B binding subsequently stimulated the migration of HPASMC

*in vitro*, and stimulated the release of the pro-inflammatory cytokines and chemokines IL-6 and IL-8, respectively. The mechanism by which CENP-B mediated these effects involves the FAK, Src, ERK1/2, and p38 MAPK pathways. The migration induced by CENP-B was sensitive to pertussis toxin treatment, thus implicating one or several G protein-linked receptors in this process.

**Conclusions:** CENP-B has all the hallmarks of a bifunctional molecule that may participate in normal and pathogenic mechanisms where SMC are particularly involved. Our data support the concept that the primary role of autoantigens may be to alert the immune system to danger signals from invaded and damaged tissues to facilitate repair, and an autoimmune response can result from a failure to turn off the reparative immune response that occurs only in subjects with impaired immunoregulatory functions. The discovery of CENP-B as a potential cytokine initiates a new field of investigation and opens up a new perspective for studying the pathogenic role of anti-CENP-B autoantibodies present in limited cutaneous SSc patients. *Supported by the Canadian Institutes of Health Research and Sclérodémie Québec.*

#### **5. Intercellular adhesion molecule-1 expression on fibroblasts contributes to the development of skin fibrosis in Tight-Skin Mice**

M. Hasegawa<sup>1</sup>, Y. Matsushita<sup>1</sup>, T. Matsushita<sup>1</sup>, M. Fujimoto<sup>1</sup>, D.A. Steeber<sup>2</sup>, T.F. Tedder<sup>3</sup>, K. Takehara<sup>1</sup> & S Sato<sup>4</sup>,

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**Background:** The tight-skin (TSK/+) mouse, a genetic model for systemic sclerosis (SSc), develops cutaneous fibrosis. Although a fibrillin 1 gene mutation and immunological abnormalities have been demonstrated, the roles of adhesion molecules have not been investigated.

**Materials and methods:** To directly assess roles of adhesion molecules in skin fibrosis, TSK/+ mice lacking L-selectin and/or intercellular adhesion molecule (ICAM)-1 were generated. Using these mice, the roles of L-selectin and ICAM-1 for the skin sclerosis were examined both *in vivo* and *in vitro*.

**Results:** The deficiency of ICAM-1 but not L-selectin significantly suppressed (~48%) the development of skin sclerosis in TSK/+ mice. Similarly, ICAM-1 antisense oligonucleotides inhibited skin fibrosis in TSK/+ mice. Although T cell infiltration was modest into the skin of TSK/+ mice, ICAM-1 deficiency down-regulated this migration which is consistent with the established roles of endothelial ICAM-1 in leukocyte infiltration. In addition, altered phenotype or function of skin fibroblasts was remarkable and dependent on ICAM-1 expression in TSK/+ mice. ICAM-1 expression was augmented on TSK/+ dermal fibroblasts stimulated with interleukin (IL) -4. Although growth or collagen synthesis of TSK/+ fibroblasts cultured with IL-4 was up-regulated, it was suppressed by the loss or blocking of ICAM-1. Collagen expression was dependent on the strain of fibroblasts but not on the strain of co-cultured T cells.

**Conclusions:** Immune cell infiltration via ICAM-1 expression on endothelial cells and subsequent production of cytokines such as IL-4 may be a trigger or stimulate fibroblast proliferation and collagen synthesis in TSK/+ mice. However, our findings more strongly indicate that altered function of TSK/+ skin fibroblasts is the primary cause of skin sclerosis and ICAM-1 expression on fibroblasts is critical for fibroblast function.

#### **6. The prospective juvenile systemic sclerosis inceptions cohort**

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**Introduction:** Juvenile systemic sclerosis (jSSc) is a rare disease. Currently just only retrospective data exist regarding organ involvement, and evolution of the disease, without standardized assessment of the patients. We developed a prospective assessment protocol for disease involvement manifestations and progression of jSSc, which may become accepted as a it presents the standard of a good clinical care.

**Objectives:** To learn about the evolution of organ involvement, the reliability of proposed assessment tools to measure change in organ involvement, and the outcome of patients in an early jSSc cohort

**Methods:** Early jSSc patients, enrolled within 18 months after the first non-Raynaud's symptom of the disease, will be followed over 36 months using a standardized assessment protocol. No specific therapy will be suggested. An Internet platform was created to make the project accessible- [www.juvenile-scleroderma.com](http://www.juvenile-scleroderma.com). Interested colleagues can request the protocol, assessment tools, and a model consent form to apply for local IRB approval. After they receive local IRB approval, they will receive an access code to the internal side of the homepage, where the detailed protocol of the project and the assessment sheets for the visits in PDF format are available. Data entry of the patients is de-identified. The completed assessment sheets can be sent to the coordinating centre via E-mail, fax or mail. The study nurse of the coordinating centre is in charge of data entry and data control. The data will be summarized every 6 to 12 months and presented at rheumatology meetings. The principal investigator of each center will be listed as co-author according to the number of enrolled patients. Every 12 months the assessment tools will be evaluated, with the help of a biostatistician, according the OMERACT criteria.

**Conclusion:** This project will represent the first prospectively followed cohort of jSSc patients, and will enable us to learn about evolution disease and about the reliability of the proposed assessment tools. The results of this study will not only advance our

understanding of jSSc, but will also help to guide other physicians in their care of children with systemic sclerosis and will enable us to plan therapeutic studies.

### **7. Characteristics of juvenile onset systemic sclerosis patients in an adult single centre cohort Does this patient population present a survival bias?**

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**Introduction:** Juvenile systemic sclerosis (jSSc) is a rare autoimmune disease in childhood, with a presumed incidence of 0.05 per 100 000. 2-10% of patients of all SSc cases develop the disease in childhood. Currently data regarding long-term outcome of jSSc is scarce. We evaluated a large single centre cohort to learn more about the characteristics of jSSc patients in an adult cohort. Patients with disease onset before the age of 17 years were selected from the systemic sclerosis patient cohort of the centre. Demographic characteristics and pattern of organ involvement were evaluated to assess outcome.

From more than 1800 cases of SSc, 46 adults with jSSc were identified. The median age of onset was 13.06 years (range 5 to 16). 35 (76%) of the 46 patients were female. Median age at last visit was 32.67 years (range 16 to 71). The median disease duration was 21.15 years (range 3 to 58). 39% of the patients had a diffuse and 61% a limited subtype of SSc. 20 (43.5%) of the 46 patients showed overlap features of other connective tissue diseases, the most common overlap was with polymyositis in 10 of the patients. Three (6.5%) of the 46 patients had anticentromere antibodies. 12 (26%) of the 46 patients were anti-Scl 70 positive. The most common organ involvements were oesophageal in 33 patients (72%), pulmonary fibrosis in 22 patients (47%), bowel involvement in 9 patients (20%) and pulmonary hypertension in 7 patients (15%). Interestingly 7 patients (15%) did not have any major organ involvement beside skin and vascular involvement. The survival of the 46 patients after 15, 20 and 25 years was 97%, 93% and 83%. Seven of the 46 patients died during the observation period in the cohort. The mean disease duration of these patients was 28.86 years (range 17 to 47).

This patient population has similar organ involvement and disease subtype characteristics as expected from an adult SSc cohort. It is interesting to see the high proportion of patients with overlap features. It is likely the study cohort of patients reflect a survival bias, representing the classical paediatric pattern of SSc but with under-representation of the diffuse subtype due to higher early mortality of this subset. The antinuclear antibody pattern, with only 6.5% of all patients anticentromere positive, contrasts markedly with adult SSc where this is a very common hallmark reactivity.

### **8. Mandibular function is severely impaired in Systemic Sclerosis Patients**

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**Background:** The temporomandibular (TMJ) has never been evaluated objectively and systematically in Systemic Sclerosis. Therefore, the objective of this study is to evaluate the TMJ function in SSc patients.

**Methods:** 35 SSc(ACR criteria) women and 30 age/sex-matched healthy-controls were selected. Helkimo's index was performed and includes: anamnesis index(Ai) clinical dysfunction index(Di) and mandibular mobility index(MI). Skin involvement was scored by the Modified Rodnan Skin Score(MRSS).

**Results:** Ai dysfunction was more frequent in SSc patients (80%) compared to controls (50%,  $p<0.001$ ). Di dysfunction was also more frequent in SSc patients (91.4%) than in controls (50%,  $p<0.001$ ). The degree of Di was distinct in patients (8.6% normal, 48.6% mild, 22.8% moderate and 20% severe) and controls (50% normal, 33.3% mild 16.7% and moderate,  $p<0.001$ ). Diffuse SSc patients ( $n=9$ ) with moderate/severe Di had a trend of higher face MRSS score than those with mild ( $n=12$ ) Di dysfunction ( $p=0.06$ ). More than 80% of the SSc patients with severe Di dysfunction (86%) were on cyclophosphamide treatment (cutaneous fibrosis), contrasting with the remaining patients ( $p<0.001$ ). Abnormal Mi index was universal in SSc patients and more frequent than controls (100% vs. 66.7%,  $p<0.001$ ). The Mi dysfunction was severe in 77.1% and mild in 22.9% of the cases contrasting with controls (13.3% severe, 53.3% mild and 33.4% normal),  $p<0.001$ . Approximately half of SSc patients with severe Mi index (47%) were on cyclophosphamide treatment (cutaneous fibrosis), contrasting with the mild group ( $p=0.02$ ).

**Conclusion:** Temporomandibular dysfunctions are very frequent in SSc and are possibly related to skin fibrosis. The concept that this clinical problem involves more than the TMJ and masticatory muscles is certainly relevant for future therapeutic strategies.

**9. Caveolin-1 scaffolding domain peptide inhibits the monocyte to fibrocyte differentiation of normal and scleroderma peripheral blood mononuclear cells.**

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**Background:** Peripheral blood mononuclear cells (PBMC) play an important role in inflammation and fibrosis. They also are involved in fibrosis by serving as progenitors for fibrocytes, a circulating population of collagen-expressing cells that enter tissues and differentiate into fibroblasts. We previously showed that caveolin-1 is a key signaling molecule in the regulation of collagen expression by scleroderma lung fibroblasts. In the current experiments we have determined that caveolin-1 is also a key signaling molecule in the differentiation and function of monocytes and fibrocytes in scleroderma lung disease patients.

**Materials and Methods:** PBMC were isolated from the blood of healthy volunteers and scleroderma patients, and treated with the CSD (caveolin-1 scaffolding domain) peptide or scrambled, control peptide. Caveolin-1 and signaling molecules activities/levels were determined by Western Blotting. MMP-9 secretion levels were determined by gelatin zymography. Monocyte to fibrocyte differentiation was evaluated by morphology and flow cytometry.

**Results:** 1) Less caveolin-1 is present in PBMC isolated from scleroderma patients than in these cells from healthy volunteers (caveolin-1 levels were only  $41 \pm 5$  % as high in scleroderma PBMC as in normal cells), and the expression/activity of MAP kinases family members regulated by caveolin-1 is also altered. 2) Activation of normal monocytes with  $TNF\alpha$  and  $TGF\beta$  decreases the expression of caveolin-1 and increases the activation of ERK, JNK, and p38 kinases. 3) CSD peptide treatment inhibits  $TGF\beta$ -induced MMP-9 secretion in normal monocytes and in normal and scleroderma fibrocytes. 4) Fibrocytes derived from the PBMC of scleroderma patients exhibit low caveolin-1 and high ASMA levels compared with fibrocytes isolated from the PBMC of healthy individuals. 5) The percentage of scleroderma monocytes that differentiate into fibrocytes in vitro is enhanced two-fold compared to normal monocytes. 6) CSD peptide treatment inhibits the transformation of normal and scleroderma monocytes to fibrocytes.

**Conclusions:** These observations suggest that the low level of caveolin-1 present in several cell types in scleroderma patients plays an important role in the progression of inflammation and fibrosis, and that the CSD peptide can provide remarkable protection against inflammation and fibrosis.

**10. Cardiac Conduction and Morphological Changes by Electrocardiogram (ECG) in patients with Systemic Sclerosis (SSc) related Interstitial Lung Disease (ILD).**

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**Methods and Patients:** ECG findings of 163 patients with SSc and ILD recruited for a multi-center trial were reviewed. The mean subject age was  $52.3 \text{ years} \pm 11.6$ , male: female ratio was 1:3.1, mean disease duration was  $6.4 \text{ years} \pm 6.5$ . Of the 163 subjects, 95 had diffuse SSc (58%) and 68 (42%) were classified as limited SSc. Subjects had ILD by HRCT and were enriched for “active” ILD by using worsening FVC, DLco and dyspnea criteria. Standard 12 lead ECG was recorded at rest with the patient in supine position for 5 minutes at randomization. Heart rate (HR), PR, QT, QT corrected for HR (QTc) and QRS intervals were recorded. ECG abnormalities related to rate, rhythm, morphology and repolarization were recorded. Screening echocardiography was utilized to exclude overt pulmonary arterial hypertension.

N = 159	%
Normal	60.40
Sinus bradycardia	1.25
First degree atrio-ventricular block	2.50
Intraventricular conduction delay	1.25
Right bundle-branch block	2.50
Left bundle-branch block	2.50
Left anterior hemi-block	2.50
Left ventricular hypertrophy	3.75
Left atrial hypertrophy	2.50
Right ventricular hypertrophy	2.50
Low voltage	1.25
Nonspecific ST/T wave abnormality	5.75
T wave inversion	5.00
Myocardial infarction sequelae	2.50
Atrial ectopy	1.25
Ventricular ectopy	4.50

**Background:** Cardiac involvement in SSc is complex and includes pericardial disease, myocardial disease and disorders of rhythm and conduction. Electrocardiographic

evaluation is a non-invasive and accessible measure. Its utility, sensitivity and specificity have not been established in well characterized SSc populations.

**Results:** 159 ECGs were available for analysis: mean HR was 76 bpm ± 12, mean PR interval was 157 msec ± 27, mean QT interval was 375 msec ± 31, mean QTc interval was 406 msec ± 37 and mean QRS was 89 msec ± 19.

ECG were normal by all features in 96 (60.4%) of subjects

**Conclusion:** ECG abnormalities of all types were prevalent in this chosen population of patients with mild to moderate “active” SSc-ILD. 75% of the abnormalities were related to conduction defects. Measures reflective of cardiac morphology (e.g. LVH, LAH, RVH) were noted in 22% of the abnormal ECGs. It is important to note that our protocol excluded pulmonary hypertension of all types and severe ILD (FVC<40%). In this population felt to be representative of early, active SSc-ILD, ECG conduction abnormalities are common and may reflect concomitant myocardial involvement.

### 11. The utility of the SHAQ-DI and VAS-Breathing as a subjective rating of Respiratory Function in Systemic Sclerosis (SSc) Patients with Interstitial Lung Disease (ILD)

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**Background:** Several physiological and subjective measures exist for the measurement of pulmonary involvement in SSc. These outcome measures have not been fully validated. This study examined the utility of 6MWT, SHAQ-DI, and VAS Breathing.

**Methods and Patients:** 163 patients with SSc-ILD participated in a multi-center, randomized double-blind clinical trial. Baseline data is presented for the subgroup of 86 patients whom were randomized to the placebo group. Data gathered included: 6MWD, FVC, DLco, Borg dyspnea, and SHAQ-DI. 39 (45.3%) had limited and 47 (54.7%) diffuse SSc. 64 (74.4%) were female. Mean age was 54.5 y ± 11 and disease duration 5.2 ± 6.6. Mean distance walked was 404.85m (SD 86.3m).

**Results:** Highest correlation with 6MWD was found with SHAQ-DI (-.48, p<.001). A regression analyses was conducted including the following variables: SHAQ-DI, 6 MWD, BORG, height, weight, and age. When plotting the residuals 2 distinct subgroups were found: those with SHAQ-DI score below/above 1.50. Although only 16 patients fell into the category >1.5, t-test analyses showed that these groups were significantly different on the following variables: 6MWD (M= 418.2m, SE=10.15 vs M=350.4m, SE=19.16), Borg (M=2.46, SE=.214 vs M=3.94, SE=.62), SHAQ-Activity (M=.79, SE=.08 vs M=2.56, SE=.15), VAS Breathing (M=51.33, SE=4.31 vs M= 94.87, SE=10.26), TLC (M=4.2, SE=.13 vs M=3.61, SE=.19), FEV1(M=2.17, SE=.08 vs M= 1.7, SE=.09), and FVC (M=2.65, SE=.10 vs M=2.14, SE=.14).

Correlations between 6MWD, SHAQ-DI, VAS Breathing, and some objective measures of lung function for the total group were:

Selected Correlations between Physiologic and Subjective Measures of Lung Disease									
	6MWD	FVC	DLco	Borg	SHAQ-DI	SHAQ Activity	SHAQ Walking	SHAQ Severity	SHAQ Breathing
6MWD	1.00	.26*	.20	-.29**	-.48**	-.45**	-.33**	-.35**	-.30**
SHAQ-DI	-.48**	-.22*	-.36**	.39**	1.00	.87**	.63**	.67**	.54**
VAS Breathing	-.30**	-.28*	-.26*	.44**	.54**	.56**	.57**	.83**	1.00

\*P≤ .01 (2-tailed), \*\* P≤.05 level (2-tailed)

**Conclusion:** 6MWD had significant correlations with all other measures in this study except DLco (selected results shown). The highest correlation for 6MWD was with SHAQ-DI. This functional outcome measure correlated as high or higher with measures of lung functioning than the physiologic lung measures. The 1-item VAS-Breathing variable had similar or higher correlations than 6MWD with FVC, DLco, and Borg.

These analyses also showed that even in a homogeneous sample of SSc- patients with ILD meeting strict inclusion criteria, substantial differences between groups exist when dividing the group along SHAQ-DI scores. Given their ease of administration, SHAQ-DI and VAS-Breathing may serve as inclusion criteria for clinical trials focused on cohort enrichment.

### 12. Therapeutic angiogenesis by local autologous progenitor cell implantation for ischemic digits in patients with systemic sclerosis.

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**Background:** Microvascular abnormality is the most common finding in systemic sclerosis (SSc) that causes reduced blood flow and tissue ischemia, leading to digital ulcers. Insufficient vascular repair has been recently proposed to contribute to this process, thereby autologous progenitor cells implanted to promote angiogenesis could be a promising therapeutic strategy for SSc ischemic complications. **Objectives:** This study was aimed to evaluate the short-term and long-term (3-6 months) effects of local bone marrow CD34-positive and mononuclear cell (MNC) implantation on clinical, functional and morphological characteristics of peripheral vascular disease in SSc patients.

**Methods:** Five dSSc patients with multiple intractable digital ulcers in both lower and upper extremities were treated with bilateral local injections of CD34-positive cells from peripheral blood (PB) after mobilization by G-CSF (case 1) and bone marrow (BM) (case 2,3,4,5) for ischemic skin ulcers in hands, while MNCs were implanted in lower extremities of the same patients. Ischemic status was evaluated by measuring ulcer healing, Raynaud's Condition Score (RCS), visual analog pain, Raynaud's phenomenon (RP) and ulcer scales. To evaluate vasculoprotective action of the implanted cells, we

studied weekly during the first month and monthly later the changes in endothelial function, using measurement of flow-mediated brachial artery reactivity by high-resolution ultrasonography and vascular endothelial injury markers, circulating endothelial precursors (CD34+VEGFR2+, CD133+VEGFR2+ CEP) by FACS analysis, cutaneous blood flow (laser Doppler flowmetry), skin surface temperature (thermograph), peripheral arterial diameter and blood flow characteristics by Duplex ultrasonography, morphological signs of microangiopathy by nailfold videocapillaroscopy.

**Results:** Both CD34-positive cells ( $11.2 \pm 0.3 \times 10^6$ , purity of selection using the M.A.C.S. technique – 91.3-96%) and MNCs showed rapid and evident beneficial effect on vascular symptoms in SSc patients: remarkable decrease in daily frequency and duration of RP attacks, RCS, VAS for RP, ulcers and pain. 15 out of 18 ulcers were completely healed and the mean surface area of the others significantly decreased. Physical function and disability measured with HAQ and SHAQ improved in line with improved hand function (decreased finger-palm and increased interdigital indices). Therapeutic efficacy of stem cell therapy was associated with restoration of altered endothelial function and significant (150 fold) increase in blood levels of early immature CD133+VEGFR2+ CEPs, known as cell effectors of angiogenesis. Laser Doppler flowmetry parameters that reflect the functional microvascular damage, such as increased biological “zero” and T1/2 of blood flow recovery after occlusion, fell to normal values, indicating the improved vessel reactivity and cutaneous blood flow. No differences in blood flow were found at the level of large and medium peripheral arteries in terms of both amplitude and kinetics, as well as in thermograph before and after cell implantation. We did not notice any side effects or safety problems with BM aspiration, administration of G-CSF and the injection of autologous cells.

**Conclusion:** We demonstrated the safety, feasibility and efficacy of local therapeutic angiogenesis in SSc. Improved endothelial function, stimulatory effects on CEP kinetics and augmentation of microcirculatory blood flow may contribute into therapeutic potential of the implanted cells.

### 13. Arthropathy in systemic sclerosis

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**Background:** Articular involvement is one of the most common manifestations and the major determinant of disability in patients with systemic sclerosis (SSc), but the clinical features of articular disease in limited (lSSc) and diffuse (dSSc) subtypes, underlying morphological findings, as well as diagnostic value and clinical outcome remain unknown.

**Objectives:** The aim of the study was to characterize the articular involvement in SSc patients.

**Methods:** 217 consecutive SSc patients fulfilled ACR criteria were clinically assessed and prospectively followed up (mean 7 yrs) for clinical features of articular disease, the spectrum of underlying pathological lesions of articular and extra-articular structures using by magnetic resonance imaging (MRI) and ultrasonography (US) studies,

conventional radiography and histological study of 80 knee synovial biopsy specimens in comparison with those from 93 rheumatoid arthritis pts. HLA-DRB1 genotyping in 120 SSc pts, 166 RA and 135 healthy controls was performed by oligonucleotide hybridisation of enzymatically amplified DNA allowing low-resolution HLA-DRB1 genotyping comprising specificities DRB1\*01 to DRB1\*17. **Results:** Articular symptoms were observed in 70% of SSc pts at an early stage of disease. Athralgia and joint contractures prevailed, the clinical evidence of synovitis was found in 12% of pts. The frequency (68% vs 75%) and pattern of affected joints did not differ between dSSc and lSSc. 52% of SSc pts showed oligoarticular and 48% -polyarticular involvement. In lSSc pts articular symptoms more often preceded skin edema (26% vs 9%,  $p < 0.05$ ). In dSSc group articular manifestations appeared simultaneously with the onset of skin changes in 52% of cases or accompanied the progression of skin involvement (39%), both indicating a high disease activity. Imaging techniques and morphological study revealed a high prevalence of sub-clinical synovitis (30-48%) with 12 histological features specific for SSc, local bone destruction (21-38%) and periarticular tissue involvement (tenosynovitis 36%). Having analyzed the clinical evolution and radiographic progression, we found that SSc pts experienced two variants of arthritis: 1) predominance of fibrotic induration of articular and periarticular soft tissues, leading to joint contractures, accompanied by persistent low-grade pain and mild morning stiffness; at follow-up, radiologically, joint space narrowing (76%) and ankylosis (20%); most often variant; onset at any time during SSc course; 2) symmetric US-confirmed inflammatory joint lesions that mimic RA clinically, but with less severe inflammation and in the most cases without destructive joint changes characteristic for RA; most pronounced in early and active SSc. Among the last group, 19 SSc pts fulfilled ACR criteria for both RA and SSc, 81% showed hand deformities, ulnar hand deviation was found in 44% of pts and muscle atrophy - in 94%; 82% of them had X-ray signs of joint destruction with multiple bone erosions in 32% of cases. These patients as a group, when compared with the rest, showed the predominance of limited skin involvement, more protracted disease course, milder visceral pathology, rare peripheral ischemic complications, positivity for anti-CCP and RF, as well as a strong genetic association with HLA-DRB1\*01. **Conclusion:** Articular involvement is frequent and early manifestation of SSc characterized by a high prevalence of sub-clinical synovitis with typical histological features. Two variants of SSc arthritis exist with different modes of presentation, clinical features, pace of progression and outcome. SSc patients who develop deforming erosive arthritis represent a distinct subset with clinical and immunogenetic peculiarities that facilitate its early recognition.

### 14. An objective method of measuring skin elasticity in Systemic Sclerosis: Results from a pilot study

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**Background:** Systemic sclerosis (SSc) is an autoimmune disorder with characteristic fibrosis of various organs, including the skin. The modified Rodnan score, used to assess skin involvement, is a subjective and user dependent method. As prognosis and response to therapy can be evaluated by assessing skin involvement, it is important to have an objective and reproducible technique available to measure this. The aim of this study was to test a newly designed skin torsion device in measuring skin elasticity in patients with systemic sclerosis as compared to healthy controls.

**Material and Methods:** 16 patients with systemic sclerosis and 71 healthy controls were recruited for the study. Skin elasticity was measured on the back of their hands and forearms with the newly designed hand held portable device. The device gently rotates the skin for 15 seconds to a maximum of 40 degrees. Total and localised (back of hands and forearms) modified Rodnan scores were also assessed.

**Results:** A statistically significant difference in the skin elasticity of the hands (1.9 degrees/second versus 2.31 degrees/second, Mann Whitney  $p < 0.0001$ ) and forearms (1.9 degrees/second versus 2.5 degrees/second, Mann Whitney  $p < 0.0001$ ) was observed between patients and controls. On doing further linear regression analysis, the only significant predictor of the skin elasticity scores was having the disease.

**Conclusion:** In the present pilot study we found the portable skin torsion device to be a reliable non-invasive method that can be readily used in patients with systemic sclerosis to assess skin involvement. Further work is planned in this area.

### 15. Adenosine A<sub>2A</sub> Receptor Occupancy Promotes Dermal Fibrosis by Modulating IL-13 and Fli1 Expression

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We have previously reported that adenosine enhances dermal matrix production. Adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R)-deficient mice are resistant to dermal fibrosing stimuli such as bleomycin. To further clarify the mechanisms by which A<sub>2A</sub> receptor stimulation induces dermal matrix accumulation, we explored the effects of A<sub>2A</sub> receptor occupancy on key fibrogenic mediators in the dermis.

IL-13 is a potent fibrogenic cytokine. IL-13 levels were significantly increased in mice with elevated dermal levels of adenosine (ADA-deficient mice) (1.47±0.06 vs. 2.54±0.21 pg/mg protein, ADA WT vs. ADA KO, ELISA,  $p < 0.001$ ). This increase was reversed by treatment with the A<sub>2A</sub>R antagonist, ZM241385 (10 μM,  $p < 0.01$ ), suggesting that induction of IL-13 production by adenosine is mediated by A<sub>2A</sub>R. IL-13Rα1 mRNA expression was also upregulated by A<sub>2A</sub>R agonist CGS21680 (10 μM) in human dermal fibroblasts (DF) (real-time PCR), and IL-13Rα1 protein expression was increased by CGS21680 in membrane cell preparations from DF (Western blot, 24 hrs). *In vivo*, treatment of ADA KO mice with ZM241385 significantly decreased IL-13Rα1 mRNA ( $p < 0.01$ ,  $n = 4$ ).

Fli1 is a known transcriptional repressor of fibrillar collagen genes and connective tissue growth factor (CTGF/CCN2) in DF. A<sub>2A</sub>R stimulation with CGS21680 (10 μM) suppressed Fli1 mRNA expression by 47.0±18.2% (vs. control) in DF nuclear extracts (4hrs, real-time PCR,  $n = 4$ ,  $p < 0.05$ ). Fli1 protein was also significantly reduced by CGS21680 (24hrs, 31.9±12.8% reduction, Western,  $n = 5$ ,  $p < 0.05$ ). Furthermore, IL-13

also stimulates a 38±7% ( $p < 0.05$ ,  $n = 4$ ) reduction in Fli1 protein in the nucleus. CGS21680 also increased CCN2 secretion by DF (2.6-fold).

**Conclusion:** A<sub>2A</sub>R occupancy promotes dermal matrix production by (1) inducing the expression of the profibrogenic cytokine IL-13, (2) suppressing expression of the transcriptional repressor Fli1 in DF, and (3) augmenting CCN2 secretion by DF. These findings suggest that modulation of A<sub>2A</sub>R function may be a novel therapeutic approach to limit dermal fibrosis as seen in conditions such as scleroderma.

### 16. Dermal reconstitution following injury requires Integrin-Linked Kinase, Manon C. Zweers<sup>1</sup>, Markus Schmitz<sup>1</sup>, Andreas Peters<sup>1</sup>, Christopher P. Denton<sup>2</sup>, Reinhard Fässler<sup>3</sup>, Donald Gullberg<sup>4</sup>, Thomas Krieg<sup>1</sup> and Beate Eckes<sup>1</sup>,

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The extracellular matrix is a key regulator of cell functions. Binding of matrix macromolecules to integrins initiates the assembly of an intracellular multiprotein complex, the focal adhesion, of which integrin-linked kinase (ILK) is a central component. ILK binds to the intracellular tail of β1 integrins and recruits adaptor proteins, thus connecting the outside environment to the actin cytoskeleton. Focal adhesions are force-transducing structures important for cell adhesion/migration and to counteract stress from the environment, such as during the contraction of wound granulation tissue.

We showed previously that fibroblasts plated on collagen require integrin α2β1 for proper focal adhesion architecture and collagen lattice contraction *in vitro*. However, in mice, formation and contraction of granulation tissue proceed largely unaltered in the absence of collagen binding integrins α2β1 or α11β1.

By contrast, absence of ILK in fibroblasts profoundly not only disturbs focal adhesion and stress fiber formation, collagen lattice contraction and migration *in vitro*, but also results in strongly impaired granulation tissue formation in mice that we generated with inducible, fibroblast-restricted ablation of ILK. Wounds contain fewer myofibroblasts and express lower α-smooth muscle actin levels, due to reduced proliferation and/or impaired TGF-β production and signalling. In line with abnormal TGF-β response, greatly disturbed granulation tissue formation was observed.

We believe that fibroblasts deficient in ILK, but not in collagen-binding integrins, are unable to transmit external forces, which in wild type fibroblasts induce an activated, collagen-producing myofibroblastic phenotype. Thus, our results strongly indicate that ILK plays an important role in fibroblasts as mechanical link in dermal cell-matrix interactions and in conveying environmental force cues.

### 17. Gravitational Stress in Linear Scleroderma of limbs

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**Background and Rationale:** Linear Scleroderma (LS) is the most common morphea subtype in children and young people. LS is a cutaneous disease of unknown etiology. The pathogenesis is not clear but the process of disease involves vascular damage, immunological mechanisms and fibroblast activation. It is a disorder that characteristically causes skin induration and pigmentary changes in a linear distribution, which runs down an arm or a leg. LS may involve the deep dermis, subcutaneous tissue, muscle and the underlying bone causing in some cases severe limb deformities, contractures and functional disabilities. In previous studies we have demonstrated that the endothelium critically situated at the blood-tissue interface, is an important target of gravitational stress (GS), which constitutes a mechanical stimulus over the vessel wall that enhances Prostacyclin (PGI<sub>2</sub>) and nitric oxide (NO) synthesis. No therapy is universally accepted for the fibrotic stage of scleroderma. The evidence that EDRFs can modulate fibroblast properties led us to the present study.

**Material and Methods:** Seven cases of linear scleroderma involving limbs were reported (5F, 2M). The mean age at the entrance to GS protocol was 15+ 7 (range, 6 to 28 years). Disease duration at the entering ranged from 1 to 7 years. Clinical examination: The upper limb was involved in 3 patients, the lower limb in 4 pts and both upper and lower limbs ipsilateral in one patient. Linear scleroderma involving fat, fascia, muscle, and bone, was presented in two patients. Linear scleroderma and morphea coexisted in one patient. Joint contractures, arthralgias, flexion contractures, limb atrophy, leg length discrepancy, limb pain and impaired functional capacity were presented in four patients. Six patients had not received any treatment prior to entering the study. A pediatric patient, a 12 years old girl, with severe LS involving her left lower leg, confirmed by skin biopsy, underwent low-dose prednisone daily during 15 months prior the entrance to gravitational stress therapy. The main outcome measure was clinical evaluation before and after treatment using a skin severity score (SSS) to grade regional skin thickness and mobility (0 to 3 point scale). Laboratory and radiological studies were also done. The Ethics Committee of the Center approved the study and all patients or parents gave their informed consent.

**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, two times per week, over three months.

**Results** From the onset of GS treatment, the disease stopped progressing in all patients. Improvement in skin score in these patients with linear scleroderma was **associated with significant softening of sclerosis and functional** recovery of limbs. A significant improvement regarding mobility and muscle strength, hand function, joint contractures and overall functional ability of limbs was also observed. This clinical improvement after gravitational therapy was maintained during a follow-up period of 5.0+2.7 years (range 2 to 10 years).

**Conclusions** Gravitational Stress as a therapy provides improvement in the management of linear scleroderma, reducing sclerosis and improving functional disabilities of lower or upper limbs related to pain, joint involvement, muscle force, growing and inducing the functional recovery of limbs. These data suggest that GS as a therapy is beneficial and safe in the treatment of patients with LS.

## 18. Cardiac involvement in Systemic Sclerosis

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**Background:** Systemic Sclerosis (SSc) is a multisystemic disorder characterized by the fibrosis of the skin and internal organs. Patients with SSc may have cardiac involvement, but no cardiac symptoms. Objective: to evaluate the frequency and type of cardiac involvement and other organs in a population of patients with SSc.

**Materials & Methods:** Two hundred patients (176 F, 24 M, mean age 49 + 15 years) who fulfilled the criteria for systemic sclerosis according the American Rheumatism Association criteria were included in the study. According to skin involvement 59 patients had diffuse cutaneous sclerosis dSSc (involvement of the trunk and face in addition to the extremities) and 141 pts had limited cutaneous sclerosis (involvement of the extremities distal to the elbows and knees + face) according the criteria of Le Roy et al. Screening of cardiac involvement included 12 lead ECG, 24-h ECG Holter monitoring and echocardiography with echo-Doppler. Lown classification for ventricular premature beats (VPB) was used.

**Results:** The female/male ratio was 7:1, the mean age at onset of symptoms was 44 (range 1-82), and the mean age at recruitment was 49 years (range 2-85). The duration of the disease was 8 + 7 years (range 9 months - 35yrs). In this population Raynaud's phenomenon was present in 182 pts (91%), digestive tract disorders evidenced by endoscopic studies in 134 (67%), lung affection evidenced by CT chest and pulmonary function tests in 44 (22%), joint affection in 106 (53%), high blood pressure in 23 (11,5%), diabetes mellitus in 3 (1,5%). Only 6 pts (3%) had cardiac symptoms. Abnormal ECG was seen in 47 patients (23%) and abnormal echocardiograms in 24 patients (12%). ECG abnormalities included conduction system disturbances 21 (10,5%), low voltage in 3 (1,5%), signs of infarction 2 (1%), non-specific ST and T-wave changes in 11 (5,5%), supraventricular arrhythmia in 20 pts (10%), atrial premature beats (APB) were found in 19 pts (9,5%) and paroxysmal supraventricular tachycardia in 1 pt (0,5%), ventricular arrhythmia in 33 pts (16,5%), simple ventricular premature beats (VPB-Lown I) in 24 pts (12%), bigeminy of VPB Lown II 2 pts (1%), multiformes of VPB Lown III 4 pts (2%), complexed arrhythmias (> III°): 1 patient couplets of VPB-Lown IVa, and 2 pts ventricular tachycardia Lown IVb . In one patient of 27 years old with diffuse SS, presyncopal episodes, left posterior and right heart bundle block and a permanent pacemaker was implanted. Echocardiographic abnormalities included: LVH in 8 pts (4%), RVH in 5 pts (2,5%) pulmonary hypertension in 8 pts (4%), systolic and / or diastolic dysfunction in 10 pts (5%) and pericardial effusion in 11 pts (5,5%).

**Conclusions:** Patients with SSc may have cardiac involvement, but no cardiac symptoms. ECG, 24 h ECG Holter monitoring and ECHO examination were valuable methods for detection of clinically silent cardiac involvement in patients with systemic sclerosis.

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## 19. Increased Prevalence of Obstructive Sleep Apnea in Patients with Systemic Sclerosis and Pulmonary Hypertension

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**Background:** Obstructive sleep apnea (OSA) can lead to pulmonary hypertension (PH) or increase its severity when PH is already present. While many PH centers screen for OSA when faced with a new PH diagnosis, suspicion for OSA in Systemic Sclerosis (SSc) and PH is usually low. Our center's experience suggests that the prevalence of OSA in patients with SSc may be higher than expected compared to the general population.

**Methods:** A large academic PH center database (1/00-7/07) was reviewed. Patients fulfilling criteria for PH by right heart catheterization (RHC) or echocardiogram were included. OSA was defined as apnea hypopnea index (AHI) >5/hr. Data are presented as mean  $\pm$  SD.

**Results:** We identified 43 patients (38 female, average age 62, range 40-79) with PH and SSc. PH was confirmed by RHC in 33 patients and by echocardiogram in 10. As part of their initial PH workup, a sleep study was performed in 16 patients based on suspicion for OSA (snoring, witnessed apneas, obesity and/or daytime sleepiness). OSA was present in 13 of them with 30% prevalence for the entire cohort. Mean AHI was 11.6, SD 6.11 (range 5-22). Mean body mass index for the OSA group was 34.3, SD 6.0 (range 22-45) compared to 33.8, SD 9.4 (range 24-43) for those in whom sleep study excluded OSA.

**Conclusion:** Compared with a 2-4% OSA prevalence in the general population, the prevalence of OSA in this select group of SSc with PH appears to be higher than expected. Given that OSA carries an increased risk for cardiopulmonary complications - hypoxemia, pulmonary vasoconstriction, systemic hypertension, diastolic dysfunction - clinicians treating these patients should have a lower threshold to diagnose and treat this comorbid condition. Additional studies to define the incidence/prevalence of OSA in patients with SSc and PH, as well as factors which predict the presence of this disorder are warranted.

## **20. Associations with In-Hospital Mortality of Scleroderma Patients from 1990-2006**

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**Background:** Studies examining clinical associations of in-hospital mortality in scleroderma patients have yielded varied results, although have examined periods of only one to two years. Literature from single-center scleroderma cohorts have suggested that causes of death in have changed over recent decades. We hypothesized that earlier conflicting results for in-hospital mortality may reflect secular trends. The objective of our study was to evaluate for changes in the associations of in-hospital mortality in scleroderma patients from 1990 to 2006.

**Methods:** We used data from the United States National Hospital Discharge Survey from 1990 to 2006. This survey is conducted annually by the US National Center for Health Statistics and includes approximately 270,000 inpatient records from 500 hospitals. Data collected include demographic characteristics, diagnoses and

procedures performed during hospitalization. We evaluated the outcomes and comorbid diagnoses of hospitalizations in patients with a discharge diagnosis of scleroderma (ICD9=710.1; n=2857) from three time periods: 1990-1995, 1996-2001 and 2002-2006. Differences between time periods were examined with chi-square analysis. Associations of in-hospital mortality were examined using logistic regression analysis.

**Results:** The median age at hospitalization was 61 years (IQR 49, 72) and 85% were female; 75% of patients were Caucasian, 20% black and 5% of other race. The median length of stay was 5 days (IQR 3,8). A total of 5.6% of hospitalizations ended in death. The proportion of in-hospital deaths has decreased over time (5.9%, 5.8%, 5.1%,  $p < 0.0001$ ). Associations of comorbid medical conditions with in-hospital mortality are depicted in Table 1.

**Table 1. Associations of Comorbid Medical Conditions with In-Hospital Mortality of Scleroderma Patients OverTime**

	1990-1995 OR (95% CI) <sup>†</sup>	1996-2001 OR (95% CI)	2002-2006 OR (95% CI)	p-value for trend
<b>Age (years)</b>				0.49
> 65	1.15 (1.07-1.23)	1.46 (1.38-1.55)	0.67 (0.6200.72)	
50-65	1.09 (1.01-1.17)	1.37 (1.29-1.46)	1.59 (1.49-1.70)	
16-49*	1.0	1.0	1.0	
<b>Male</b>	2.17 (2.03-2.32)	2.41 (2.28-2.55)	1.81 (1.69-1.95)	0.36
<b>Black</b>	1.38 (1.29 – 1.49)	2.02 (1.90-2.14)	2.26 (2.10-2.43)	0.24
<b>Pulmonary Fibrosis</b>	1.72 (1.58-1.88)	2.80 (2.64-2.96)	2.55 (2.41-2.71)	0.05
<b>Pulmonary Hypertension</b>	3.35 (3.03-3.70)	4.74 (4.20-5.29)	0.87 (0.78-0.97)	0.53
<b>Heart Failure</b>	4.82 (4.53-5.12)	2.00 (1.89-21.12)	2.57 (2.43-2.71)	0.28
<b>Renal Disease</b>	12.96 (11.96-14.03)	5.06 (4.76-5.37)	8.66 (8.14-9.21)	0.24
<b>Malabsorption</b>	5.12 (3.81-6.86)	NS	NS	0.27
<b>Respiratory Infections</b>	9.78 (9.13-1.49)	NS	2.28 (2.14-2.42)	0.66
<b>Respiratory Failure</b>	6.38 (5.70-7.14)	19.77 (18-68-20.92)	37.10 (34-90-39.44)	0.12

† confidence interval \*reference group

**Conclusions:** The association of pulmonary fibrosis with in-hospital mortality in scleroderma has increased over time. Although most other comorbidities revealed nonsignificant temporal changes, the trends observed are consistent with current perceptions of the relative changes in cause of death over time.

## 21. Silica-induced resistance of cultured fibroblasts to Fas-induced apoptosis: implications for scleroderma

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**Background:** Systemic sclerosis (SSc) is a rare autoimmune disease that has been associated with exposure to specific chemicals, particularly silica and certain solvents. The mechanisms by which certain chemicals may cause SSc are unknown. Dermal

fibroblasts cultured from SSc patients show an altered phenotype that includes the over-expression of collagen and a reduced susceptibility to Fas-induced apoptosis, both of which may exacerbate fibrosis. Fas-resistant populations of fibroblasts can be selected using repeated cytokine treatment. Treatment of cells with silica has previously been reported to induce expression of transforming growth factor (TGF)β1 and other cytokines which may provide a pathway from exposure to resistance. Hence the hypothesis of this study was that repeated treatments with silica may be able to induce/select for the resistant phenotype.

**Materials and Methods:** MRC-5 human lung fibroblasts were treated repeatedly with medium alone (negative control), quartz silica, sonicated quartz silica (disrupted with ultrasound), or CH11 (an antibody stimulating Fas-induced apoptosis, positive control). Cells were treated at a concentration that was previously determined to cause 50% cell death. An MTT cytotoxicity assay was carried out following each treatment to determine the level of Fas-induced cell death in response to CH11.

**Results:** Following repeat treatments, average cell death induced by CH11 varied between cells treated with medium only and medium plus chemical. The cell death induced in medium treated cells was 34.5% ±4.0%, unsonicated silica 35.5% ±6.0%, sonicated silica 22.8% ±2.8% and CH11 23.6% ±1.8%. The results for sonicated silica and CH11 are statistically significant compared to the medium-treated control, with p<0.01. This effect was apparent following a minimum of two treatment cycles.

## Conclusions:

1. An increased level of resistance to apoptosis was induced or selected for following repeated treatments with sonicated silica compared to unsonicated silica or medium only.
2. This suggests a possible mechanism by which cells in genetically susceptible individuals may become more resistant following a prolonged low-level exposure to silica.
3. Sonication exposes fresh surfaces of the silica, resulting in a greater production of reactive oxygen species, which is a possible cause of this effect.

## 22. First analysis of complex metabolic composition of synovial fluid in scleroderma by proton magnetic resonance spectroscopy

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**Background:** Magnetic resonance spectroscopy (MRS) is suitable for the simultaneous detection and measurement of the metabolic components of a biologic sample. Because of the difficulty of synovial fluid prelevation in scleroderma, previous studies have given little information and we tried to depict possible correlation between disease's pathogenesis and complex composition of synovial fluid.

**Materials and methods:** We realized the first analysis of the complex metabolic environment of the joints affected by diffuse scleroderma in 5 patients with knee and elbow arthritis in comparison with rheumatoid arthritis (RA) analysis. Our previous studies used MRS method for the simultaneous detection and measurement of the metabolic composition of synovial fluid in different pathologies. NMR spectra have been recorded on a Bruker 400 MHz spectrometer

**Results:** This method led to the possibility to attribute the signals for glutamine, threonine, lactate, hydroxybutyrate, glycine, dimethylamine and lipoprotein-associated fatty acids, ceramide and citrulline in synovial fluid. We have shown extremely weak signal intensity for citrulline at 3.15 ppm (highly specific for RA synovial fluid - sensitivity 80%, specificity 100% in our previous study, n=30patients) and decreased concentration of glucose. Despite the difficulty of performing statistical analysis the overall aspect of synovial spectrum is completely different from RA.

**Conclusions:** MRS investigation of synovial fluid provides valuable information consisting in simultaneous detection of different metabolites in sclerodermic synovial fluid.

We can correlate the presence of lipids components with the endothelial dysfunction of synovial vessels, hypersecretion of endothelin and possible early progression of atherosclerosis in scleroderma patients. Oxidation of increased lipid components inside the joint by chemically reactive oxygen species has been postulated to be a key step in pathogenesis of atherosclerosis.

The amount of valuable information surpasses the actual possibilities to correlate the presence of so many metabolites to their pathological relevance.

### 23. Two dimensional tear protein electrophoresis for differential diagnosis of ocular manifestation in scleroderma

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**Background:** The eye is frequently involved in patients with scleroderma. Most often, this involvement consists in cutaneous abnormalities of the eyelids, resulting in tightness of the lids and blepharophimosis. Also, keratoconjunctivitis sicca (KCS) with a Sjogren's-like picture has been described. Our study analyzed and compared electrophoretic tears patterns of normal subjects and patients with scleroderma with and without clinical manifestations of KCS. Thus, we try to determine the disease| biomarkers that can be used for noninvasive diagnosis of KCS in patients with scleroderma.

**Materials and methods:** We analyzed three groups of subjects: patients with scleroderma without KCS (n=6), patients with scleroderma with KCS (n=6) and a control group comprising healthy volunteers (n=10). Tears were sampled using the Schirmer method. Tears were eluted in 40 microL of elution solution containing sodium dodecyl sulphate, urea, EDTA, beta-mercaptoethanol and bromphenol blue.

Tear proteins were separated by two-dimensional electrophoresis (in the combination of isoelectric focusing with sodium dodecyl sulphate-poliacrylamide gel electrophoresis), and protein bands were stained with silver.

**Results:** We have depicted more than 20 bands, main components (tear-specific pre-albumin, lactoferrin, lysozyme, secretory immunoglobulin A and immunoglobulin G) being identified using a marker of molecular weight. Isoelectric points of all proteins separated were determined by comparison with isoelectric point standards. The densitometric analysis of electrophoretic lanes was performed with ordinary flat scanner.

The tear protein patterns of patients with scleroderma and KCS are different in number and intensity of spots from those of healthy subjects. Patients with KCS had decreased levels of lysozyme and lactoferrin in tears ( $p<0.03$ ;  $p<0.05$  at densitometric quantification).

**Conclusion:** Two-dimensional electrophoretic analysis of tear protein patterns of patients with scleroderma is a fast, reproducible and simple method that provided information for a rapid diagnosis of KCS in these patients.

### 24. Predictive Value of Microvascular Imaging Techniques for SSc-Spectrum Disorders

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**Background:** Systemic sclerosis (SSc) affects both microvascular structure and function. Laser Doppler and thermal imaging can be used to measure cutaneous blood vessel function. Nailfold capillaroscopy (NC) measures capillary morphology. The aim of this study was to investigate the relationship between capillary morphology and blood flow, and to determine which combination of techniques allows best discrimination between patients with SSc, primary Raynaud's phenomenon (PRP) and healthy controls (HC).

**Methods:** Following acclimatisation, NC was performed in 16 patients with SSc, 14 with PRP and 16 healthy controls. In addition, participants underwent cold stimulus with cold water (15 °C, one minute). Hands were imaged for 15 minutes to monitor re-warming and reperfusion. Nailfold morphological features (width, density<sup>1</sup> and tortuosity) were measured and baseline images and re-warming curves analysed (area under the curve, maximum temperature/blood flow after re-warming, initial gradient).

**Results:** Significant differences were found between groups (ANOVA) for capillary morphological features and re-warming curve characteristics. Correlation ( $p<0.001$ ) was found between laser Doppler and thermal imaging for baseline (0.667) and maximum (0.729) blood flow and skin temperature and for the areas under the re-warming curves (0.684).

ROC curves generated using logistic regression indicated that nailfold microscopy, thermal imaging and laser Doppler imaging allowed 89%, 74% and 72% respectively of SSc patient data to be correctly classified versus PRP patients and controls. A combination of all three techniques gave positive and negative predictor values of 93% and 94%.

**Conclusions:** Nailfold microscopy, thermal and laser Doppler imaging each independently provide good discrimination between patients with SSc and those with PRP and healthy controls. Laser Doppler and thermal imaging give equivalent information on dynamic changes in the cutaneous microcirculation, however these only weakly correspond to capillary morphology. Nailfold microscopy is the most suitable technique for classifying patient groups; however classification is further improved if all three techniques are combined.

#### 25. A 3 month prospective study of nailfold capillary architecture changes with time

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**Background:** Structural microvascular disease is a hallmark of systemic sclerosis [SSc]-spectrum disorders and is well demonstrated using the technique of nailfold microscopy. We have developed software to allow automated measurements of nailfold microvessel architecture (width, density<sup>-1</sup>, tortuosity and derangement). It is not known over what time period SSc related microvascular derangement evolves nor whether changes also occur in healthy controls. The aim of this sub-study was to assess changes in nailfold capillary architecture over a three month period, as part of a larger 2 year prospective study.

**Methods:** 19 patients with primary Raynaud's phenomenon (3 male, median [range] age 41 [21 to 74] years), 12 patients with undifferentiated connective tissue disease ([UTCD], all female, 51 [33 to 68] years), 38 patients with SSc (32 with limited cutaneous and 6 with diffuse cutaneous subtypes, 8 male, 55 [31 to 74] years) and 31 healthy controls (11 male, 42 [26 to 70] years) were recruited into the study. All patients were asked to refrain from smoking and caffeine for 4 hours prior to examination. Following acclimatisation for 20 minutes at room temperature (23°C), the ring finger nailfold of the non-dominant hand was imaged with video microscopy at 300x magnification. Features were extracted from images by automated software.

**Results:** As previously shown, capillary density and dimensions differed between disease and healthy control groups. There were no significant changes within any of the 4 subject groups over the 3 month period.

**Conclusions:** 1) No within group changes were observed over 3 months, however, some individuals did demonstrate change over 3 months; 2) The computerised nailfold system was able to track changes in capillaries over time, lending further support to its potential as an outcome measure of microvascular change; 3) Longer term studies, in progress, will clarify the nature of microvascular disease progression in patients with SSc-spectrum disorders.

#### 26. Mechanism of action of agonistic PDGFR auto-antibodies isolated from scleroderma patients

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**Background:** Systemic sclerosis (SSc) is characterized by fibrosis of the skin and visceral organs. We have identified stimulatory IgG auto-antibodies to the PDGF receptor that are capable of converting normal fibroblasts into SSc-like cells inducing excessive oxygen species (ROS) production by activating membrane NADPH oxidase complex. In order to elucidate the earliest mechanism involved in the molecular pathogenesis of systemic sclerosis we characterize the interactions between PDGFR and other components of the active NADPH system in the presence of PDGF or SSc IgGs and the functional role of the lipid raft in the formation of the complex.

**Material and Method:** The receptor activation by PDGF or SSc IgGs was monitored immunoprecipitating total cellular extract of stimulated fibroblasts with anti PDGFR $\alpha$  and PDGFR $\beta$  antibodies and analysed by Western blotting with phospho-tyrosine antibody. The interaction between PDGFR and NADPH oxidase complex subunits was evaluated by immunoprecipitation with anti PDGFR, anti gp91phox and anti Ha-Ras antibodies. For the membrane localization of the complex we pretreated the cells with  $\beta$ -cyclodextrin for the cholesterol depletion of membrane and assayed the sample for tyrosine phosphorylation

**Results:** We have found that SSc IgGs from several different patients selectively immunoprecipitate gp91 phox in addition to PDGFR. PDGFR autoantibodies stabilized the interaction between PDGFR and the NADPH oxidase subunit, which is otherwise assembled transiently in response to PDGF, and lead to permanent activation of the signalling cascade. The receptor gets stabilized by these antibodies and form a stable membrane complex protected from degradation. This interaction is localized in the lipid compartment of the plasma membrane (lipid raft) and contains also Ha-Ras protein. Prolonged ROS production can be explained by the persistence of the receptor in the membrane bound to the NADPH subunit, gp91 phox.

**Conclusions:** These data illustrate how stimulatory auto-antibodies from SSc patients are able to induce prolonged ROS production. SSc IgGs prevent down regulation of PDGF receptor and lock the receptor in the active configuration. This results in persistent ROS production and appearance of the disease pathological signatures.

#### 27. Reproducible and stable subsets in serial skin biopsies taken from patients treated in an open-label trial of rituximab

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**Background:** The purpose of this study was to assess the response of patients with diffuse systemic sclerosis (dSSc) to rituximab therapy, using genome wide gene expression profiling of skin biopsies. The other purpose of this study was to further

characterize the global gene expression of dSSc skin to extend and validate previous results.

**Methods:** Using whole-genome DNA microarrays, gene expression was measured in skin biopsies from 13 patients with early diffuse cutaneous SSc (dcSSc), and 4 healthy controls. Both lesional forearm and non-lesional back biopsies were analyzed for each patient; for 6 patients, samples were taken at pre-treatment and at 6-months post-rituximab therapy. A single patient underwent biopsies at base, 6 and 12 months.

**Results:** We were unable to detect significant changes in the gene expression profiles before and after rituximab treatment. This is consistent with the observation that the mean change in mRSS for treated patients was not significantly different between baseline (20.6) and 6-months (20.2). Using an intrinsic gene identifier algorithm to select the intrinsic genes in SSc, we recapitulated three 'intrinsic' subsets (proliferation, inflammatory and normal-like) identified in a previous study of 24 patients with scleroderma. Analysis of skin biopsies from 6 early dSSc patients over the course of 12 months, and one patient over 18 months showed consistent and stable patterns of gene expression.

**Conclusions:** Rituximab treatment had little effect on the gene expression of skin biopsies from dSSc patients. Lesional and non-lesional skin from patients with SSc showed nearly identical patterns of gene expression, recapitulating the findings of two prior gene expression studies. The 'intrinsic' subgroups recapitulated here provide validation that these subgroups are an inherent feature of the disease. Serial biopsies taken from SSc patients at six-month intervals show nearly identical patterns of gene expression demonstrating that, over the time scale analyzed, the intrinsic subsets are a stable component of SSc gene expression.

## 28. Developing a Canadian Scleroderma Damage Index

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**Background:** Disease severity or activity scales for systemic sclerosis have been developed in Europe, Sweden, and the United States. A global damage index is under development in Canada.

**Materials and methods:** Data consist of over 1,000 variables collected on nearly 700 patients in the National Patient Registry of the Canadian Scleroderma Research Group. Statistical methods include multiple linear regression and multivariate procedures including but not limited to factor analysis and canonical correlation analysis.

**Results:** Challenges include data completeness and differences in diagnostic variables measured by Canadian rheumatologists compared to rheumatologists in other nations.

**Conclusions:** The development of the Canadian Scleroderma Damage Index is in process. Variables in the National Registry are being assessed and variables not yet in this Registry are being considered.

## 29. Fibroblast-directed CTGF over-expression in vivo promotes systemic connective tissue fibrosis reminiscent of fibroproliferative diseases

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**Background:** Connective tissue growth factor (CTGF/CCN2) is a cysteine-rich secreted protein involved in wound healing and tissue repair. Its expression has been associated with many different forms of fibrosis.

### Materials and Methods:

We have developed transgenic mice with a high level of fibroblast-specific over-expression of CTGF/CCN2 using the *Col1a2* enhancer/promoter sequence.

**Results:** These mice exhibit pronounced fibrosis of the skin, lung, kidney and small arteries and recapitulate clinical, histological and biochemical symptoms of many fibrotic diseases. Elevated collagen deposition in the dermis of these mice was mirrored by elevated collagen production and secretion by transgenic dermal fibroblasts in vitro. Examination of skin biopsies revealed enhanced fibroblast proliferation and a significant expansion of the myofibroblast cell population. Transgenic dermal fibroblasts also showed increased proliferation, enhanced migration in scratch wounds and increased remodeling of 3-dimensional collagen gels compared to wild-type controls. Gene expression analysis by Northern and Western blotting revealed elevated expression of endogenous *CTGF*, *Col1a1*, *TIMP 1* and *3*, *osteopontin*, *Fibronectin* and  $\alpha$ -*SMA*, and a series of other biochemical markers and gene clusters consistent with a fibrogenic response. Both phospho-p38 and phospho-Erk1/2 were increased in transgenic mouse fibroblasts whereas no change occurred in phospho Smad3 compared to wild-type fibroblasts. Transfection experiments using SRE, CTGF and CAGA promoters showed significantly increased basal activity compared to wild-type control fibroblasts, and enhanced induction in the presence of TGF- $\beta$  for the CTGF and CAGA promoters.

**Conclusions:** These data suggest that high levels of fibroblast-specific CTGF/CCN2 expression in vivo promote connective tissue fibrosis. Enhanced CTGF/CCN2 levels appear to synergize with non-canonical TGF- $\beta$  signaling, modulating fibroblast responses to resemble a fibrogenic phenotype characteristic of many fibrotic diseases.

## 30. Systemic sclerosis- Particularities related to capillaroscopy findings, Raynaud's phenomenon characteristics and disease-specific autoantibodies

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**Background:** Among all connective tissue diseases, systemic sclerosis (SSc) has the most widespread microvasculature abnormalities and also the greatest prevalence of Raynaud's phenomenon (RP).

**Objectives:** The aim of this study was to assess the relationship between SSc microangiopathy using nailfold capillaroscopy (NCS) and 1) the characteristics of RP; 2) the presence of digital ulcers (DU) and 3) the presence of SSc-specific autoantibodies.

**Methods:** 25 consecutive patients [M/F 3/22], median age 50.6 years (range 30-72) with mean disease duration 7.2±6.2 years, fulfilling the ACR classification criteria for SSc, were enrolled in the study. Patients were assessed for DU. NCS was performed in all patients for each finger excluding the thumb, using an Olympus SZX7 stereomicroscope. We classified NCS findings in "early stage" (ES)- capillary enlargement and minimum reduction of their density, "late stage" (LS)- multiple avascular areas with significant reduction of capillaries and few or none enlarged capillaries, "florid active stage" (FAS)-polymorphic capillaries with tortuosities, megacapillaries, bushy/ramified capillaries, hemorrhages and moderate avascular areas, and "soft active stage" (SAS) - presence of 3 items from FAS. Patients fulfilled a RP questionnaire regarding 3 issues: changes in hands color - white (W), blue (B) and red (R), duration of crisis (minutes) and intensity of crisis - VAS scale [0-10]. Antitopoisomerase I antibodies (Scl-70) and anticentromere antibodies (ACA) were determined in all patients.

**Results:** A. *Capillaroscopy findings.* NCS stage frequency and SSc autoantibodies were as follows: ES -13 patients, all women: 5 ACA+, 4 Scl70+; SAS - 6 patients, all women: 3 ACA+, 3 Scl70+; FAS - 5 patients: 2 men, both Scl70+, and 3 women - two Scl70+ and one ACA+; late stage: 1 patient, male, Scl70+. Interestingly, the left hand presented more NCS abnormality than the right: 12 vs. 2 patients, equal NCS changes in 11 patients. Finger IV was most affected (13 patients), followed by fingers II (3 patients), III (5 patients) and V (4 patients).

B. *Digital ulcers (DU).* Eight patients had at least one hand DU (excluding those from the articular surface): 5 were ACA+ and 3 were Scl70+. Five out of 18 fingers with DU had FAS or SAS particularities. None of them had LS particularities. Four patients had toe ulcers, all being Scl70+, all with FAS finger capillaroscopy changes. Most frequent pathologic NCS finding were megacapillaries, followed by moderate avascular areas, bushy capillaries, hemorrhages, and multiple avascular areas.

C. *RP findings:* Score > 4 on VAS scale: 10 patients - 6 Scl70+, 3 ACA+. Three of them had severe NCS stages. On 19 subjects finger first involved in - and finger most exposed to RP had less severe or equal NCS findings than at least one of the others 7 fingers examined. Fifteen patients had RP duration of < 8 years; 8 of them had minimum SAS. Ten patients had duration of RP > 8 years; 4 of them had minimum SAS. Of 9 patients who described color in crisis as B or B+R, 8 had minimum SAS. In another 9 patients who described color in crisis as W+R or only R, only 2 had minimum SAS and 7 ES. Of 7 patients describing W+B+R color changes, 3 had minimum SAS. Eight patients had puffy fingers; 7 of these had minimum SAS.

#### **Conclusions:**

- Left hand and finger IV were the most frequently affected. Early stage NCS changes were met in half of the patients. More than 80% of patients with FAS and LS were Scl70+.
- Fingers with DU did not display severe NCS findings. Toe ulcers correlated with severe NCS findings.
- Higher intensity RP crisis scores were met more frequently in Scl70+ patients. Higher scores did not correlate with severe stages. Finger first involved in- and finger most exposed to RP did not correlate with the most severe NCS findings. None of the stages were strongly related to the duration of RP.

Patients with blue color alone or blue and red color of the hands in RP crisis most frequently had SAS. On the contrary, white plus red color associated with ES, while a complete RP had all kind of stages. Puffy fingers matched severe NCS findings.

Our results suggest there are 2 different microvascular involvement patterns in SSc patients: one associating digital ulcers and ACA, the other associating cyanosis, puffy fingers and severe NCS findings.

#### **31. Cross-Sectional Study in Systemic Sclerosis**

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**Objectives:** This study aims to overview the current clinical assessment of hospitalized scleroderma (ScS) patients in three rheumatology units from Bucharest between 01-2007-01-2008.

**Methods:** Fifty-three ScS patients were included (8 men and 46 women); Mean age was 49, 97±11,33years; Fifty patients fulfilled ACR criteria for ScS. The others fulfilled Le-Roy-Medsgar (2001) criteria for early limited ScS. Our tools were: EUSTAR- Minimal Essential Data Set( Version August 2004) and supplementary 7 parameters from the extensive disease-specific clinical data-base.

**Results:** A. Quantitative: Forty-six parameters were evaluated to each subject; Another five were partial evaluated: DLCO-to 34 (64,1%)patients, pulmonary hypertension ECHO(PHT)-to 38 (71,6%)patients, ANA-to 48 (90,5%) subjects, ACA to 33(62%)patients, SCL-70 to 44(83%)patients. Mean disease evolution since first non Raynaud's symptom was 5,48±5,72 years. Mean age to onset of Raynaud's was 39, 49±13,69 years. Twenty seven patients (50, 9%)had diffuse-ScS (dc-ScS), 16 patients (30,2%) had limited-ScS(lc-ScS)and 10 patients (18,9%)had other subsets. B. Qualitative- Most frequent parameters were: Raynaud's syndrome (100%), ANA(97,9%), sclerodactily (94,3%), esophageal symptoms(88,7%), DLCO <80%(88,2%), hiper and hipo-pigmentation (79,2%), telangiectasy (75,5%), pulmonary fibrosis plain x-Ray (PFx):77,4%. Less frequent parameters were: friction rubs(11,3%), proteinuria (11,3%), reduced ventricular ejection fraction(9,4%), conduction blocks(9,4%), intestinal symptoms (9,4%), C.K.elevation(6,3%), distal amputations(5,7%), renal crisis (0%). ScS was active in 37,7% of cases; Overlap syndrom had 9,4%(one with myositis, 3 rheumatoid arthritis, one- Systemic lupus erythematosus). ACA were present in 33%; Distribution of ACA : 82%lc-ScS, 9%dc-ScS, 9%-other subsets. From all ACA patients -11% had PHT. SCL-70 were present in 61,4%; Distribution of SCL-70: 74% dc-ScS, 15% other, 11% lc-ScS. From all SCL-70 patients -47,6% had PHT. PHT was met in 34,2% cases; Fifty percents of men had PHT and 30,33% of women had PHT. From patients with TLCO<80%, 30,76% had PHT and 93,3% had PFx. Patients who had PHT, PFx, respective Rodnan score >14 have the probability (OR>1)of 5.10

,1.407 respective 1.33 to be exposed to chemical agents, while those who had PFX have equal chances to be smokers (OR=1) as those who did not have PFX in our scleroderma group. Next parameters: friction rubs, elevated phase reactants and men were met frequent in patients with both PHT and PFX. From those with active disease 60% were SCL-70 positive.

**Conclusion:** The overall disease activity was rather low in our group. Patients with active disease were mostly SCL-70 positive. Patients SCL-70 positive were predominant in dc-SSc. PHT was more frequent in patients group with SCL-70 positive. Men were more affected of PHT. As a test DLCO <80% had good sensitivity for pulmonary fibrosis on X-ray in the scleroderma group. The risk of exposure to chemical agents was higher in PHT, skin fibrosis and PFX patients from our scleroderma group. Our results confirm the higher prevalence of pulmonary involvement in SCL-70 positive patients.

### 32 Oxidative DNA damage in systemic sclerosis

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**Background:** Oxidative stress is implicated in the causation and perpetuation of tissue damage in scleroderma (SSc). Several studies have shown increased lipid peroxidation in SSc<sup>1</sup>. There is some evidence to suggest that oxidative DNA damage is increased in systemic lupus erythematosus<sup>2</sup>, but there is no published data in SSc.

**Objective:** To investigate whether oxidative DNA damage is increased in SSc.

**Subjects and Methods:** Ten patients fulfilling the ACR criteria for SSc and attending the Connective Tissue Diseases Clinic, Chris Hani Baragwanath Hospital, Soweto, and 15 age- and ethnically-matched healthy controls were studied. *In vitro* baseline oxidative DNA damage and repair following hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced DNA damage were measured in peripheral mononuclear cells using the single cell gel electrophoresis or 'Comet' assay as described by Singh *et al*<sup>3</sup>. Oxidative DNA damage was also assessed by measuring urinary 8-hydroxydeoxyguanosine (8-OHdG) adducts using an ELISA assay (Assay Designs).

**Results:** No significant differences in baseline percentage (%) tail DNA in mononuclear cells was observed between the SSc patients (11.1%) and controls (10.8%). Mononuclear cells from both groups were able to repair H<sub>2</sub>O<sub>2</sub> induced DNA damage after removal of the toxicant and culturing in fresh media at 37°C for 3 hours. However, DNA damage repair expressed as a decrease in % tail DNA to baseline levels was significantly faster in the control group compared to the SSc group (p=0.007). Urinary excretion of 8-OHdG was significantly higher in SSc group than in the control group (0.85±0.54µg/mg creatinine vs. 0.2±0.136µg/mg creatinine, respectively, p=0.0003).

**Conclusion:** Our preliminary results suggest comparable baseline DNA damage levels in SSc patients and healthy controls, but patients exhibited delayed repair of free radical induced strand breaks *in vitro* suggesting a defect in the DNA damage repair mechanism in patients with SSc. The increased rate of excretion of 8-OHdG in the SSc patients is further supportive evidence of oxidative stress in SSc.

### References:

#### 33. Defects in matrix increase oxidative stress and endothelial mesenchymal transition

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**Background:** Systemic scleroderma (SSc) increases oxidative stress, fibrosis and microvascular rarefaction. Tight skin (Tsk<sup>+/+</sup>) mice have a defect in fibrillin-1, causing the mice to duplicate many of the cardiovascular features in humans with SSc. Previously we showed that D-4F, an apoA-I mimetic, reduces fibrosis and oxidative stress in hearts of Tsk<sup>+/+</sup> mice. Here we reasoned that defects in matrix might increase oxidative stress and inhibit EC function.

**Materials and Methods:** Microfibrils were isolated from ~6 month old C57BL/6 mice, Tsk<sup>-/-</sup> mice and Tsk<sup>+/+</sup> mice treated with D-4F (1 mg/kg 6-8 weeks) and were examined for 4-hydroxynonenal (4-HNE) content. Human umbilical vein endothelial cells (EC) were cultured on microfibril preparations and examined for effects on proliferation and expression of fibroblast specific protein-1 (FSP-1) and expression of twist and slug, transcription factors involved in mesenchymal transition. Finally, microvascular EC and apoptotic cells in hearts of mice were immunostained and quantified.

**Results:** Tsk<sup>-/-</sup>-microfibrils contained markedly higher levels of 4-HNE than C57BL/6- or D-4F-Tsk<sup>+/+</sup> microfibrils. Tsk<sup>+/+</sup>-microfibrils impaired EC proliferation while C57BL/6- and D-4F-Tsk<sup>+/+</sup> microfibrils did not (p<0.05). Tsk<sup>+/+</sup>-microfibrils increased EC expression of FSP-1 as well as twist and slug that were either markedly reduced or absent in EC on C57BL/6- and D-4F-Tsk<sup>+/+</sup> microfibrils. Finally, Tsk<sup>+/+</sup> hearts had fewer microvascular EC and more apoptotic cells than hearts from C57BL/6 or D-4F-treated Tsk<sup>+/+</sup> mice (p<0.05).

**Conclusions:** Defects in matrix increase oxidative modification of microfibrils *in vivo* that in turn impairs EC proliferation and increases endothelial mesenchymal transition. Thus, oxidative stress represents a second hit in SSc that promotes the transition of vascular EC into mesenchymal cells. Such changes in vascular EC function begin to explain why SSc increases microvascular rarefaction.

#### 34. Cloning of agonistic autoantibodies specific for the PDGF receptor from the B cell repertoire of SSc patients

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**Background:** Systemic sclerosis (SSc) is a disorder characterized by fibrosis of skin and visceral organs. We have provided evidence that serum of SSc patients contains

stimulatory autoantibodies directed to the PDGF receptor (PDGFR) that elicit Ha-Ras-ERK 1/2 signaling and collagen production in normal fibroblasts.

**Materials and methods:** IgG-positive B lymphocytes obtained from peripheral blood of SSc patients were immortalized by EBV infection. Supernatants of single lymphocyte clones were screened for their ability to react selectively with F alpha cells (murine fibroblasts expressing the human PDGFR alpha) but not with F-/- cells (mock-transfected fibroblasts) by immunofluorescence and flow cytometry. Positive clones were further screened for the production of antibodies stimulating reactive oxygen species (ROS) and collagen production in normal human fibroblasts. Positive clones were expanded in serum-free medium, IgGs were purified from supernatants by A/G protein and tested to confirm both binding and biological activity on fibroblasts. mRNA was obtained from such positive single lymphocyte clones for sequencing and cloning of antibody variable regions.

**Results:** We isolated clones producing IgGs that i) reacted with F alpha cells, but not with F-/- cells, ii) stimulated ROS production, iii) induced Ha-Ras-ERK 1/2 cascade and type I collagen gene, iv) converted normal human primary fibroblasts into myofibroblasts. Several variable heavy and light chain IgG sequences were obtained by cloning selected cDNA fragments from total mRNA.

**Conclusions:** Stimulatory PDGFR monoclonal autoantibodies were isolated from the immunoglobulin repertoire of scleroderma patients. These antibodies bind to PDGFR, induce ROS and collagen I production in normal fibroblasts and display the biological features identified in the total immunoglobulin pools purified from serum of SSc patients.

### 35. Association of Interferon Regulatory Factor 5 (IRF5) polymorphisms with Systemic Sclerosis (SSc)

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**Background:** Type-I interferon (IFN) signature has been shown to be the hallmark peripheral blood gene expression pattern in systemic lupus erythematosus (SLE). More recent studies have also noted a similar type-I IFN signature in systemic sclerosis (SSc). The transcription factor interferon regulatory factor 5 (*IRF5*) is a component of this IFN-gene expression signature and regulates the expression of other genes involved in cell-cycle regulation, cell adhesion, apoptosis and immune responses. *IRF5* gene polymorphisms have been reported to be associated with SLE, rheumatoid arthritis, Sjögren's syndrome, psoriasis, multiple sclerosis and inflammatory bowel diseases. The purpose of this work was to investigate the possible association between *IRF5* polymorphisms with SSc.

**Methods:** We performed SNP genotyping for 3 SNPs on *IRF5* gene using the Taqman Assay in 1,391 Caucasian, African-American, and Hispanic SSc patients along with 1,027 race-matched controls. All SSc patients fulfilled ACR criteria or had at least 3 of the 5 CREST features. Chi-square, Fishers exact and logistic regression analyses were used for statistical comparisons. Illumina Human-REF8 arrays were used for peripheral blood gene expression analysis.

**Results:** After HWE verification and correcting for multiple testing, two SNPs (rs2004640 & rs752637) showed significant association with White SSc patients. The TT genotype for the SNP rs2004640 had a frequency of 34.5% in White SSc patients as compared to 27.1% in White controls which was statistically significant. The bestfit model for the rs2004640 SNP was an additive model and was used for all comparisons. Logistic regression analysis controlling for gender and race showed that the TT genotype was an independent risk factor for SSc, including anti-topoisomerase-I antibody positive SSc and SSc with fibrosing alveolitis (Table 1). *IRF5* was the topmost differentially expressed gene based on the rs2004640 SNP genotypes in peripheral blood arrays of SSc patients ( $p$ -value  $1.39 \times 10^{-5}$ ) (Fig. 1).

**Conclusion:** These data suggest an important role of this *IRF5* polymorphism in its susceptibility to SSc. The TT genotype causes a splice variant containing exon IB whereas the GG genotype contains exon IA and exon IC. This *IRF5* polymorphism leading to this isoform, upon stimulation, may facilitate expression of genes encoding type-I IFNs and other proinflammatory cytokines, thus increasing the risk for development of SSc.

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**Introduction:** Tissue fibrosis caused by the excessive deposition of extracellular matrix is a common feature of many connective tissue diseases, notably scleroderma (systemic sclerosis; SSc). Evidence suggests that complex intercellular interactions involving immune cells, endothelial cells and fibroblasts are important pathogenic events. However, the role of cellular events involving epithelial cells in initiating and maintaining fibrosis has not been extensively explored. The epidermal keratinocyte is a major source of pro-inflammatory and pro-fibrotic mediators. Antibodies directed against these cells have been reported in a number of other autoimmune diseases, and appear to lead to keratinocyte activation and secretion of potent soluble inflammatory/fibrotic mediators. We have previously shown that in early SSc the epidermis exhibits a phenotype resembling that observed in wound healing, and expresses a number of markers characteristic of epidermal differentiation.

**Objective:** We studied the presence of anti-keratinocyte antibodies in SSc and examined the influence of the keratinocyte autoantibodies on the secretion of interleukin-1 $\alpha$  (IL-1  $\alpha$ ).

**Method:** Sera were obtained from 30 diffuse cutaneous SSc (dcSSc), 30 limited cutaneous SSc (lcSSc) patients and 30 healthy controls and evaluated for antibody binding to keratinocyte cells by cell-based ELISA. Immunoglobulin-G (IgG) was purified from 3 SSc patient and 3 controls selected from cell-based enzyme-linked immunosorbent assay (ELISA). Pre-incubation of IgG from dcSSc and from control with keratinocytes was assessed for intracellular and extracellular expression of IL-1  $\alpha$ . Interaction of IgG with keratinocyte binding and internalization was assessed using immunofluorescence. Biopsy sections were stained for human IgG. IL-1 $\alpha$  expression in scleroderma epidermis was assessed by ELISA.

**Results:** We found that IgG purified from SSc patients bound to nucleolar antigens in keratinocytes compared to that of control IgG. In addition, pretreatment of human keratinocyte cells with purified IgG from scleroderma sera led to the induction and the secretion of IL-1  $\alpha$  from normal human keratinocytes. Staining of human IgG in biopsy section from scleroderma patients showed cell membrane staining in the epidermis compared to control biopsies. Furthermore, IL-1  $\alpha$  is overexpressed in dcSSc epidermis compared to control epidermis.

**Conclusion:** We have demonstrated the over-expression of IL-1 $\alpha$  in dcSSc epidermis compared to that of control epidermis. Pre-treatment of keratinocytes with IgG purified from the sera of patient with SSc resulted in time dependent increase in the secretion of IL-1 $\alpha$ . This data suggest over-expression of IL-1 $\alpha$  by epidermal cells due to antibody-mediated activation plays a key role in the abnormal function of both dermal and epidermal cells in dcSSc. The possibility exists that the epidermis in SSc is being activated by an autoimmune-disease driven mechanism.

### 37. Antibody profile of patients enrolled in the scleroderma lung study

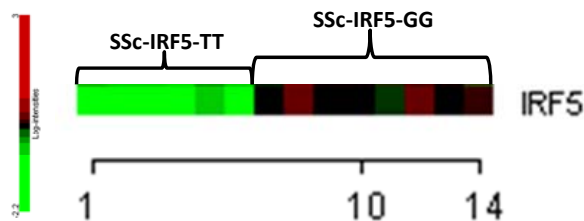
Victoria K. Shanmugam<sup>1</sup>, Aida Manu<sup>1</sup>, Ning Li<sup>2</sup>, Donald P. Tashkin<sup>2</sup>, Richard M. Silver<sup>3</sup>, Philip J. Clements<sup>2</sup>, Virginia D. Steen, <sup>1</sup>Georgetown University Hospital,

Table 1. Estimated risk of TBX21 SNP rs17699436 in SSc patients versus controls, by logistic regression analysis\*

	Wald Chi-Square	p-value	Genotypes	OR (95% CI)
SSc versus controls	12.6	0.0004	TT vs GG TG vs GG	1.56 (1.3-2.0) 1.24 (0.99-1.6)
ACA-positive SSc versus controls	4.4	0.037	TT vs GG TG vs GG	1.53 (1.1-2.3) 1.37 (0.96-1.9)
ATA-positive SSc versus controls	11.0	0.0009	TT vs GG TG vs GG	1.96 (1.4-2.9) 1.36 (0.93-2.0)
RNA POL III-positive SSc versus controls	3.8	0.051	TT vs GG TG vs GG	1.51 (1.1-2.3) 1.34 (0.92-1.9)
LIMITED SSc versus controls	8.1	0.005	TT vs GG TG vs GG	1.54 (1.2-2.1) 1.29 (0.98-1.7)
DIFFUSE SSc versus controls	6.6	0.010	TT vs GG TG vs GG	1.48 (1.1-2.0) 1.19 (0.91-1.6)
Fibrosing alveolitis	11.1	0.0008	TT vs GG TG vs GG	1.58 (1.2-2.04) 1.30 (1.05-1.6)

\* The analysis was controlled for the confounding effects of sex and race. SSc = systemic sclerosis; OR = odds ratio; 95% CI = 95% confidence interval; ACA = anticentromere antibody; ATA = anti-topoisomerase I antibody; RNA POL III = anti-RNA polymerase III antibody  
Control subjects are used as reference for all comparisons.

Fig. 1 Heatmap of IRF5 gene and SSc samples



### 36. IgG autoantibodies from scleroderma patients induces Interleukin-1 alpha secretion

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**Background:** The scleroderma lung study (SLS) was a double-blind randomized placebo-controlled trial that demonstrated modest but significant beneficial effects on pulmonary function, dyspnea, skin thickening, and health-related quality of life in scleroderma patients treated with one year of oral cyclophosphamide. The current study involved antibody analysis of patients enrolled in SLS to evaluate whether autoantibody status was associated with differences in baseline characteristics.

**Materials and Methods:** Of the 158 patients enrolled in SLS, 107 had serum available for autoantibody testing. Assays for anti-Scl-70, anti-centromere, and anti-RNA polymerase III (Pol3) were performed using enzyme immunoassay. Statistical analysis was performed using chi-square test to compare the mean and standard deviation of sex, race, and presence of positive bronchoalveolar lavage. Disease duration, skin score, high-resolution CT scan scores for fibrosis, ground glass and honeycombing, Mahler dyspnea index, and disability index of the scleroderma health assessment questionnaire score (HAQ-DI) were compared using wilcoxon rank-sum test. Finally, age and components of the pulmonary function tests were compared using the two sample t-test.

**Results:** Of the 107 patients with antibody results available, one patient had both Scl-70 antibody and Pol3 and was excluded from further analysis. Although 41.5% of patients were classified as having limited cutaneous scleroderma, only 3 patients in this study had positive anti-centromere antibodies. Anti-Scl-70 antibody was positive in 29 patients, 66.67% of whom had diffuse cutaneous scleroderma. These patients were significantly younger than anti-Scl-70 negative patients ( $p=0.0051$ ). Disease duration was similar in patients with and without anti-Scl-70 antibody. Anti-Scl-70 antibody was associated with higher baseline ground glass score ( $p=0.0133$ ) and a trend toward a higher frequency of alveolitis by bronchoalveolar lavage ( $p=0.0960$ ). In contrast, 93.75% of anti-Pol3 antibody positive patients had diffuse cutaneous scleroderma. Anti-Pol3 positivity was associated with higher baseline skin score ( $p=0.0019$ ) and higher HAQ-DI ( $p=0.0495$ ) but lower baseline fibrosis score ( $p=0.0576$ ).

**Conclusions:** Scl-70 and Pol3 antibody status may account for some of the differences in baseline characteristics of patients enrolled in the SLS. Outcome analysis is ongoing, but it is hoped that antibody status might help identify patients more likely to respond to therapy in scleroderma-associated lung disease.

### 38. The German Network for Systemic Scleroderma: new data on disease subsets and organ involvement from more than 2000 patients

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**Background:** Systemic scleroderma (SSc) is a rare, heterogeneous disease, which affects different organs and therefore requires interdisciplinary diagnostic and therapeutic management. The German Network for Systemic Scleroderma (DNSS) was

founded three years ago for basic and clinical research. Presently, it comprises dermatologists, rheumatologists, pulmonologists and nephrologists from 40 medical centers. To improve detection and follow up of patients presenting with early stages of the disease or overlap-syndromes, patients were classified in 5 subsets, i.e. limited, diffuse systemic sclerosis, overlap-syndrome, undifferentiated Scleroderma and scleroderma sine scleroderma. Recent analyses revealed that 48% of patients suffer from limited SSc (ISSc), 31% from diffuse SSc (dSSc) and 11% of patients were diagnosed with an overlap-syndrome. 8% had an undifferentiated form while Scleroderma sine scleroderma was present in 1% of patients. Pulmonary fibrosis was significantly more frequent in dSSc than in ISSc (61% vs. 24%). Accordingly, pulmonary arterial hypertension was more common in dSSc (20%) compared to ISSc (14%). Muscular involvement was typical for overlap-syndromes (69%). The onset of initial skin changes, following first attacks of RP, occurred earlier in dSSc than in ISSc. A family history of rheumatic diseases was associated with early disease onset. Follow up data revealed that the prevalence of joint contractures, hypertension and diastolic dysfunction increases significantly within one year.

In this register a classification of patients with disease manifestations characteristic of systemic sclerosis in 5 groups allows to include a broader spectrum of patients with features of systemic sclerosis and to gather new insights into disease evolution.

### 39. TGF $\beta$ induced $\alpha$ -smooth muscle actin expression and extracellular matrix contraction in fibroblasts requires TAK1

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**Background:** The pro-fibrotic protein transforming growth factor-beta (TGF $\beta$ ) is found in elevated amounts in scleroderma (SSc) patients. TGF- $\beta$ -activated kinase 1 (TAK1) is thought to be a key signaling pathway of TGF $\beta$ . The effect of loss of TAK1 on transcriptional responses in fibroblasts is unclear. In this report, we use wild-type fibroblasts and those deficient in TAK1 to probe the contribution of TAK1 to mediate transcriptional responses in fibroblasts in responses to TGF $\beta$ .

**Materials and methods:** Dermal fibroblasts ( $n=6$ ) were obtained from control and SSc tissue. Fibroblasts derived from wild-type (WT), TAK1-/- (KO) embryos were used. The effect of TGF $\beta$  on the phenotype of TAK1 WT and KO was assessed by MOE430 Affymetrix gene arrays analyzed by Genespring software, real-time polymerase chain reaction (RT-PCR) and Western blot analysis. In addition, the ability of TGF $\beta$ -1 to influence matrix remodelling in collagen contraction models was also examined.

**Results:** TGF $\beta$  induced 265 transcripts greater than 2-fold in WT fibroblasts. Of these 194 were not induced greater than 2-fold in tak1-/- fibroblasts. Cluster analysis revealed that expression pro-fibrotic transcripts were reduced in response to TGF $\beta$  in TAK1-deficient cells, including thrombospondin 1 (tsp1), TIMP3, vinculin, and several collagen genes. Results were verified using real-time PCR analysis of RNA isolated from WT and KO-/- treated with and without TGF $\beta$  for 6 hours ( $p<0.05$ ). TGF $\beta$  cannot induce matrix

contraction or a cohort of fibrotic genes, including  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in TAK1-deficient cells. TGF $\beta$  significantly enhanced the ability of WT to contract collagen lattices ( $p < 0.05$ ), whilst only marginally modulated TAK1 $^{-/-}$  contraction. Western blot analysis shows that the ability of TGF $\beta$  to phosphorylate TAK1 is reduced by the FAK/src inhibitor PP2. The ability of TGF $\beta$  to induce JNK phosphorylation is impaired in the absence of TAK1. Activated, phosphorylated TAK-1 is present in the majority of SSC fibroblasts but not in normal fibroblasts.

**Conclusions:** Our results uncover new insights into the contribution of TAK1 to tissue repair and remodeling responses in fibroblasts. These data show that TAK1 operates downstream of FAK/src in mediating fibrogenic responses and that targeting TAK1 may be a viable anti-fibrotic strategy in SSC.

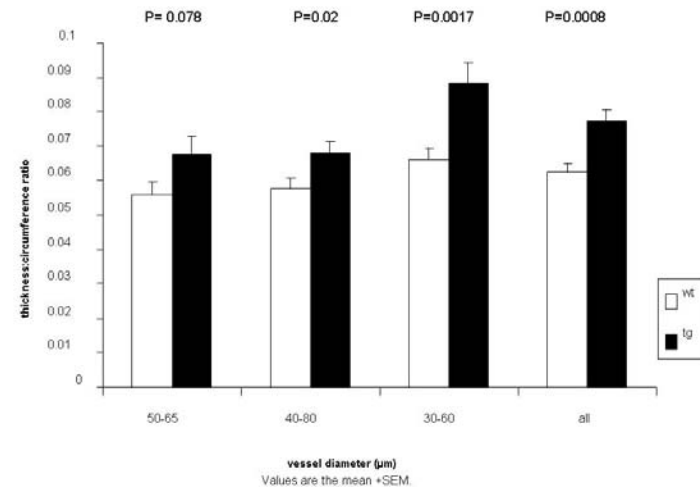
#### 40. Pulmonary and Systemic Vasculopathy in a TGF- $\beta$ Dependent Mouse Model of Systemic Sclerosis

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**Purpose:** Vascular complications of systemic sclerosis (SSc) are a major cause of mortality and morbidity. A robust mouse model of SSc-related vasculopathy is yet to be described. We have systematically examined vascular structure in a genetically determined model of SSc characterised by ligand-dependent activation of TGF- $\beta$  signalling in fibroblasts.

**Methods:** The transgenic mouse strain T $\beta$ RII $\Delta$ k-fib expresses a kinase-deficient type II TGF- $\beta$  receptor linked to a fibroblast-specific promoter leading to balanced ligand-dependent upregulation of TGF- $\beta$  signaling. Comparisons between transgenic and wildtype heart, lung and kidney were performed. We used immunohistochemistry to confirm excess TGF- $\beta$  and T $\beta$ RII and evaluated vascular and perivascular architecture by H&E and special stains. Activation of TGF- $\beta$ -dependent signaling pathways in cultured fibroblasts was confirmed by qPCR measurement of CTGF, PAI-1 and Col1a1. Confirmatory aortic smooth muscle cell proliferation and phenotype assays, including signaling responses to exogenous TGF- $\beta$  and endothelin-1, were performed.

**Results:** TGF- $\beta$ 1 expression was increased in transgenic lung vessels and endothelin-1 and its specific receptors ET-RA and ET-RB were also upregulated. Figure 1 shows that transgenic intimal diameter, measured in 1000 vessels from 10 littermate pairs, was significantly increased, and particularly noted in the smaller 30-60 $\mu$ m pulmonary arterial vessels with an increase in intimal smooth muscle being the major contributing factor.



$\alpha$ -SMA was also visible in very small ( $< 20 \mu$ m) pulmonary arterioles in transgenic but rarely in wildtype samples (mean 3.0 per HPF transgenic, 1.8 wildtype,  $P = 0.02$ ). Elastin deposition was disordered in the pulmonary vascular bed and downstream TGF- $\beta$ -mediated signaling pathways were increased. Cardiac collagen content, measured by Sircol assay, was increased in transgenic males.

**Conclusions:** Alterations in vascular and perivascular architecture were seen in the transgenic vessels. Some of the changes are indistinguishable from those seen in human pulmonary arterial hypertension. Our results support a role for TGF- $\beta$  overactivity in the vasculopathy of systemic sclerosis.

#### 41. Thrombospondin 1 is a key mediator of TGF $\beta$ -mediated cell contractility in systemic sclerosis via a MEK/ERK-dependent mechanism

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**Background:** Scleroderma (SSc) is a connective tissue disease of unknown aetiology. Abnormal connective tissue metabolism is believed to play a central role in the pathogenesis of scleroderma resulting in excessive deposition and contraction of extracellular matrix (ECM) by resident fibroblasts. The mechanism underlying the ability of fibroblasts to contract a collagen gel matrix is largely unknown. Fibroblasts from scarred (lesional) areas of patients with the fibrotic disease scleroderma show

enhanced ability to contract collagen relative to healthy fibroblasts. Thrombospondin-1 (TSP-1), an activator of latent TGF $\beta$ , is over-expressed by scleroderma fibroblasts. In this study we investigated whether activation of latent TGF $\beta$  by TSP-1 played a key role in matrix contraction by normal and scleroderma fibroblasts.

**Materials and Methods:** The matrix contraction of normal and SSc fibroblasts to response altered TSP-1 activities were assayed by the fibroblast populated collagen lattices (FPCL) model via the multi station tensioning Culture Force Monitor.

**Results:** We have demonstrated that interfering with TSP1/TGF $\beta$  binding and knockdown of TSP-1 expression suppressed the contractile ability of normal and scleroderma fibroblasts basally and in response to TGF $\beta$ . During mechanical stimulation in the FPCL system using the mst-CFM we observed that TSP-1 expression and p-ERK activation in fibroblasts was enhanced. Inhibiting TSP1 activity reduced the elevated activation of MEK/ERK and expression of key fibrogenic proteins. TSP-1 may potentially mediate fibroblasts responses to PDGF in the pathogenesis of SSc via MEK/ERK pathway.

**Conclusion:** TSP-1 is a key mediator of matrix contraction of normal and SSc fibroblasts via a MEK/ERK dependant mechanism.

#### 42. Serial Anti-RNA-Polymerase Antibody Levels in Patients with Systemic Sclerosis

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**Background:** Anti-RNA-polymerase antibodies (ARA) occur in up to 20% of patients with systemic sclerosis (SSc), are strongly associated with the diffuse cutaneous (dc) subset of the condition and particularly with scleroderma renal crisis (SRC). We analysed ARA levels in SSc patients over time looking for relationship with clinical presentation and disease outcome.

**Materials and methods:** Subjects had definite SSc. A commercially available ELISA method was used to measure ARA levels.

**Results:** We included 33 patients who had ARA levels measured between 3 and 19 times over a follow-up period of between 21 and 142 months. Of them 88% had dcSSc; 48% had SRC, 30% had pulmonary fibrosis (PF), 9% developed pulmonary arterial hypertension (PAH) and 6% had clinically significant cardiac involvement. Over the follow-up period there were 4 deaths and survival at 3 and 5 years was 94% and 91% respectively.

We observed considerable inter- and intra-patient variability in ARA levels (11-188U/ml, mean $\pm$ SD - 85 $\pm$ 42U/ml). We divided subjects into subgroups according to degree of change in ARA levels over the first 36 months of disease and according to cumulative antibody levels. We could not demonstrate any difference in survival or number of internal organ complications among the patients in the different subgroups. There was no significant correlation between absolute or peak ARA levels and onset of SRC.

Twenty-six patients had received immunosuppressive treatment during the assessment period. Although there was no significant difference in ARA levels when on and off

treatment, subgroup analysis demonstrated a trend towards lower ARA levels with Mycophenolate mofetil (MMF) compared to no treatment or therapy with other immunosuppressants (p=0.058).

**Conclusions:** Despite the strong association of ARA with SRC, there is no clinically significant correlation between ARA levels and development of internal organ complications in patients with SSc. Change in ARA levels over the first 3 years of the disease occur but do not appear to have major clinical significance. Antibody levels may be affected by treatment with MMF.

#### 43. Scleroderma finger clawing. Putative new quantitative measures. Estimates in normal individuals and scleroderma patients.

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*Westmead Hospital, Sydney; Australia, \*Flinders Medical Centre, Adelaide, Australia*

**Background:** Scleroderma finger clawing (Sfc) is associated with variable – sometimes profound - morbidity.

We have devised new measures to semiquantitatively and quantitatively measure Sfc, describe these values in individuals with normal hands and in those with scleroderma.

**Materials and Methods:** 'Sydney Harbour Bridge [SHB]' = arc/chord ie dorsal finger length/volar linear finger length; 'Perth'=[hand height x 10/open span]

Finger clawing grade: Hands opposed: Grade 0=mcp's and pip's contact; Grade 1= mcp's contact; Grade 2= no mcp contact angle between plane of distal phalanx and palm $\leq$ 90°; Grade 3 =no mcp contact and angle between plane of distal phalanx and palm>90°.

Individuals with normal hands (structurally/functionally); 100 1- per gender per decade 20-69yrs. Scleroderma patients:n=13.

##### Results:

##### 1. Normal values

Perth median = 1.44 [5,95 percentile 1.07-1.92].

SHB median = 1.00 [5-95th percentiles 0.96-1.07]

##### 2. Correlation

R vs L SHB (Pearson =0.77; p<0.0001);R/L SHB vs Perth ( Pearson 0.05-0.005;p=0.63-0.97)

##### 3. SHB/Perth– effect of age and gender:SHB age-dependent in males [p<0.0001].

**Conclusions:** SHB less variable in normal individuals than 'Perth'.Symmetry of hand involvement reflected by good correlation between L and R SHB. Poor correlation between SHB and 'Perth' implies a weak relationship between finger abduction/ hand height (Perth) and finger flexion/extension (SHB). SHB and Perth independent of gender. SHB possibly age dependent (role of Heberden's nodes?). 'Perth' greater sensitivity than SHB in scleroderma hand. Both quantitative measures simple, quick, cheap. SHB widely adaptable.

#### 44. Antitopoisomerase antibody positivity predates nailfold capillaroscopic changes in scleroderma

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**Background:** At the time of clinical presentation, antinuclear antibody is usually present in high titre and nailfold capillaroscopy is classically abnormal in patients with scleroderma. Indeed, the presence of autoantibody positivity in conjunction with abnormal nailfold capillaroscopy is often used to delineate primary from secondary Raynaud's in patients with evolving connective tissue disease.

The temporal evolution of scleroderma before disease diagnosis – including the temporal relationship between autoantibody positivity and abnormal nailfold capillaroscopy – remains incompletely determined. The preclinical staging of the disease is also undefined.

We wish to present a patient's history in which the temporal relationship between antitopoisomerase antibody positivity and nailfold capillaroscopy changes were fortuitously observed. We wish to tentatively propose a temporal staging of the prediagnostic phase of scleroderma.

**Materials and Methods: Case Report:** A 25 year old female first presented in May 2005 with a history of increased fatigue, weight loss, and stiff, swollen painful upper and lower extremities. Antitopoisomerase antibody was noted. Nailfold capillaroscopy was normal. The patient then developed biphasic Raynaud's, symptomatic synovitis and finger clawing. Scleroderma was diagnosed in December 2005 by which time she had sclerodactyly, slowly pitting skin oedema over the fingers, periungual erythema, and mild neck flexor weakness. The remainder of the clinical examination was normal. Repeat nailfold capillaroscopy on this occasion showed dilatation of capillaries with an obvious degree of capillary irregularity with increased intravascular red cell aggregation.

**Conclusions:** In this patient with early scleroderma, the serological abnormality of antitopoisomerase antibody positivity predated vessel wall changes as evidenced by nailfold capillaroscopy changes. Whether a similar temporal pattern of autoantibody positivity preceding microvascular pathology noted on nailfold capillaroscopy is borne out by further observations remains to be determined.

We tentatively propose 5 prediagnostic stages for this disease of multifactorial aetiology.

Stage 1: Conception. Genetic predisposition. Raynaud's/ HLA/other.

Stage 2: Phase between genetic predisposition and environmental exposure.

Stage 3: Phase after initial environmental exposure – with no perturbation of body status.

Stage 4: Phase characterized by asymptomatic clinical changes.

During this phase autoantibody positivity may predate microvascular changes noted on nailfold capillaroscopy.

Stage 5: Symptomatic prediagnostic phase.

#### 45. CTGF Contributes to Collagen Type I Expression in the Bleomycin Model of Lung Fibrosis and in Human Pulmonary Fibroblasts by MAPK Dependent Transcriptional Activation

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**Background:** Connective tissue growth factor (CTGF) has emerged as a key candidate fibrogenic factor in autoimmune rheumatic diseases such as systemic sclerosis (SSc). CTGF is rapidly and strongly induced by transforming growth factor- $\beta$  (TGF- $\beta$ ) and is a downstream mediator of TGF $\beta$ -dependent profibrotic effects *in vitro*. Here we aim to investigate the role of CCN2 in enhanced collagen type I expression in the bleomycin model of lung fibrosis and possible underlying mechanisms.

**Materials and methods:** Transgenic mice carrying collagen type I  $\alpha 2$  (Col1a2) or CTGF promoter/reporter constructs were utilised in combination with intra-tracheal instillation of bleomycin, and CTGF blocking agents *in vivo* and *in vitro*. Inflammatory and fibrotic markers were measured. Western blot analysis, transient transfections and electrophoretic mobility shift assays (EMSA) were used to identify underlying molecular mechanisms in mouse and human pulmonary fibroblasts *in vitro*.

**Results:** Maximal levels of CTGF expression and promoter activity were observed in lung tissue one week after bleomycin instillation, whereas maximal collagen type I expression and Col1a2 promoter activity followed two weeks post-challenge. Anti-CTGF antibody applied three times over a nine day period after bleomycin treatment had a modest, but statistically significant, effect on the induction of Col1a2 promoter activity (26% reduction,  $p < 0.01$ ), as measured in lungs two weeks after the challenge. Fibroblasts isolated from lungs two weeks after bleomycin, retained their fibrotic phenotype *in vitro*, and displayed greatly elevated CTGF protein expression and promoter activity. Inhibition of CCN2 action by siRNA, reduced collagen expression, and anti-CTGF antibody treatment *in vitro* inhibited collagen protein expression and Col1a2 promoter activity (41% reduction,  $p < 0.001$ ). The enhanced Col1a2 promoter activity in fibroblasts from bleomycin treated lungs was partly Smad dependent. However, the contribution from CTGF was independent of the Smad response element and occurred via sequences around the proximal inverted CCAAT-box in an ERK1/2 and JNK dependent manner, with a possible involvement of CREB and c-jun. Finally, promoter induction by recombinant CTGF via these mechanisms was confirmed using a human COL1A2 promoter construct in human lung fibroblasts.

**Conclusions:** The data presented suggest a significant contribution by CTGF in the development of lung fibrosis, and reveal putative underlying mechanisms of CTGF action.

#### 46. Anti-Endothelial Cell Antibody Levels are Low in Scleroderma Internal Organ Vasculopathies.

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**Introduction:** Systemic sclerosis is associated with fibrosis and vasculopathy. Scleroderma renal crisis (SRC) and scleroderma-associated pulmonary arterial hypertension (PAH) are exemplars of vasculopathy in scleroderma. Anti-endothelial cell antibodies (AECA) have been found at high titres in SSc and a variety of other connective tissue diseases. They have been associated with disease activity and subsets and subsets of disease.

**Methods:** Serum samples diluted 1:100 from 18 age-matched controls, 26 cases of SSc without SRC or PAH, 17 PAH, 27 SRC, 12 prior to onset of SRC, and 48 samples >2 months after SRC were assayed for AECA on 3<sup>rd</sup> passage HUVEC. Results were expressed as an index where pooled serum is 1, and negative control is 0. The upper limit of normal was taken as mean of controls +2SD.

**Results:** AECA index was elevated in SSc compared with controls (index 2.35 vs 1.23, 77% vs 0% above ULN, p<0.001). Levels were lower in PAH than SSc controls (mean 1.64, 35% above ULN, p=0.07). Levels were lower in SRC than SSc controls (mean 1.56, 26% above ULN, p=0.001). Levels before SRC are not different to SSc controls, and rise after SRC (mean 2.09, 71% above ULN). There is a negative correlation with skin score, but no difference in those with or without significant pulmonary fibrosis.

**Conclusions:** AECA are high in SSc. The levels drop in cases with internal organ vasculopathy and rise when vasculopathy recovers. This phenomenon may be due to increased binding of the antibodies to activated endothelium.

#### 47. Proteasome inhibitor bortezomib overrides TGFbeta effect in human fibroblasts

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**Background:** Extracellular matrix (ECM) provides a controlled environment for cellular differentiation and tissue development. Its integrity is maintained through a balance between ECM components deposition and degradation. Excessive ECM deposition occurs in fibrotic diseases. One of the predominant ECM components found in fibrotic lesions is type I collagen (COL1A2 and COL1A1). ECM accumulation is controlled by relative expression levels of collagen, ECM degrading enzymes such as matrix metalloproteinases (MMPs) and their inhibitors, named Tissue Inhibitor of MMPs (TIMPs). We previously published that bortezomib, a proteasome inhibitor, exerts an *in vitro* anti-fibrotic activity, dominant over the pro-fibrotic phenotype induced by TGFbeta. We report here an extensive study of the transcriptional regulation of ECM genes in human dermal fibroblasts.

**Materials and methods:** Variation in ECM mRNA and protein levels was determined by RT-PCR, enzyme-linked immunosorbent assay (ELISA) and Western blotting. Promoter

activity of COL1A1 and MMP-1 genes was measured by reporter gene assay. Increase in binding of various transcription factors to specific promoter region of ECM genes was performed *in vivo* via chromatin immunoprecipitation (ChIP) and *in vitro* via electrophoretic mobility shift assay (EMSA).

**Results:** Bortezomib activated transcription of MMP-1 via increased binding to AP-1 site. Analogous response to bortezomib treatment was observed for MMP-13, whereas MMP-2 and MMP-9 were not affected. TGFbeta activated transcription of COL1A1 or COL1A2 via increased binding to AP-2 or SP1 sites, respectively. While bortezomib did not affect TGFbeta-induced binding of AP-2 to COL1A1 promoter, it completely abolished TGFbeta-induced binding of SP1 to COL1A2 promoter.

**Conclusions:** We identified elements of MMP-1 and COL1A1 promoters in fibroblasts, essential for bortezomib- or TGFbeta-mediated activation. Bortezomib treatment triggers converging signals: activation of MMP-1 and MMP-13 transcription, due to increased occupancy of AP-1 site and repression of TGFbeta-mediated induction of COL1A2 transcription on the SP1 site. These signals result in an *in vitro* anti-fibrotic phenotype in human fibroblasts.

#### **48. Detection of possible risk factors for digital ulcers in Systemic Sclerosis**

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**Background:** Digital ulcers (DU) are a major complication in the course of systemic sclerosis (SSc). In recent years, efficacious, but expensive vasoactive therapies (e.g. iloprost, sildenafil or bosentan) have shown to improve healing or to reduce recurrence of DU. In order to deliberate timely or even prophylactic treatment it would be useful to identify potential risk factors for DU in patients with SSc. Such statistical analyses have been rare, because they require sufficiently high numbers of patients.

**Materials and methods:** The German Network for Systemic Sclerosis (DNSS) encompasses a nation-wide patient registry of patients with SSc. We evaluated data of 1881 patients included by August 2007. We assessed potential risk factors for DU by comparing patients with (n= 408) and without active DU at time of their entry into the network.

**Results:** Multivariate analysis revealed that male gender, presence of pulmonary arterial hypertension (PAH), involvement of oesophagus, diffuse skin sclerosis (only when PAH was present), anti-Scl70 antibodies, young age at onset of Raynaud's phenomenon (RP), and elevated sedimentation rate (ESR) present independent factors associated with DU.

Certain combinations increase the patients' probability to present with DU, with the highest probability (88%) for male patients with early onset of RP, ESR above 30, anti-Scl70 antibodies and PAH. Patients with DU developed RP, skin sclerosis and organ involvement approximately 2 to 3 years earlier than patients without DU.

**Conclusions:** The results reveal possible risk factors and risk factor combinations for occurrence of DU in SSc. Since these DU are prone for local complications, it may be justified to consider prophylactic vasoactive treatment for these patients.