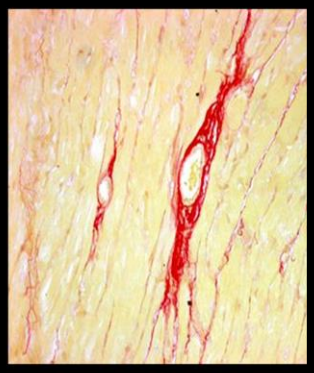
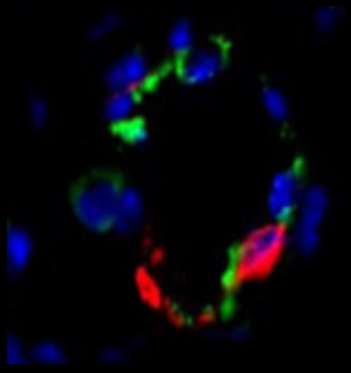
# SCLERODERMA RESEARCH

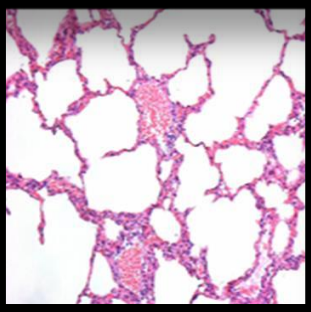
14th International Workshop on

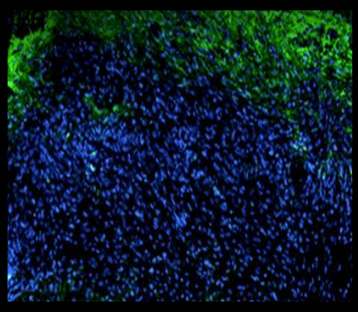
Understanding the pathogenesis of Scleroderma,



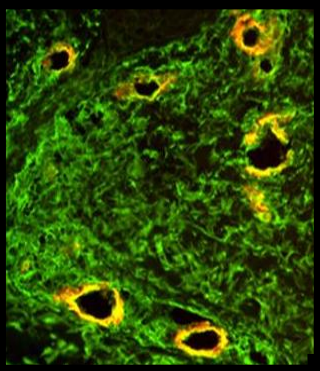


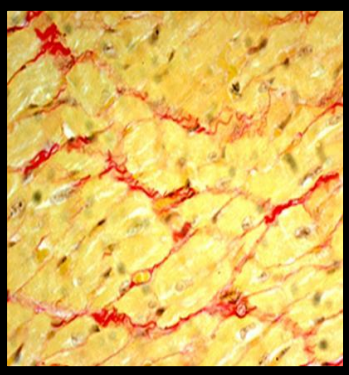
Utilising current therapies and





Identifying new targets and treatment strategies,





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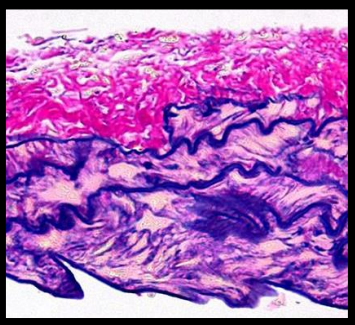
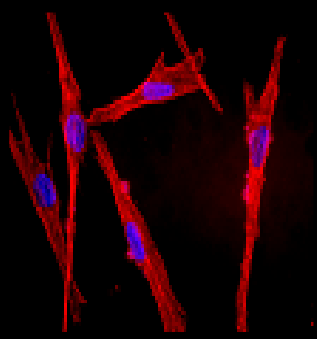




better management for patients with Scleroderma.

1-5 AUGUST 2015 ◆ ST. JOHN'S COLLEGE ◆ CAMBRIDGE ◆ UK

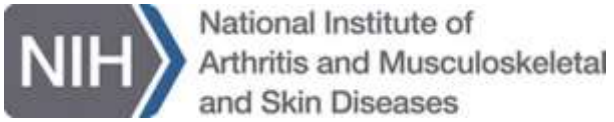
Advancing Scleroderma Research and Translational Medicine

*The Scientific Steering Committee would like to thank the following for their generous support of the Workshop:*







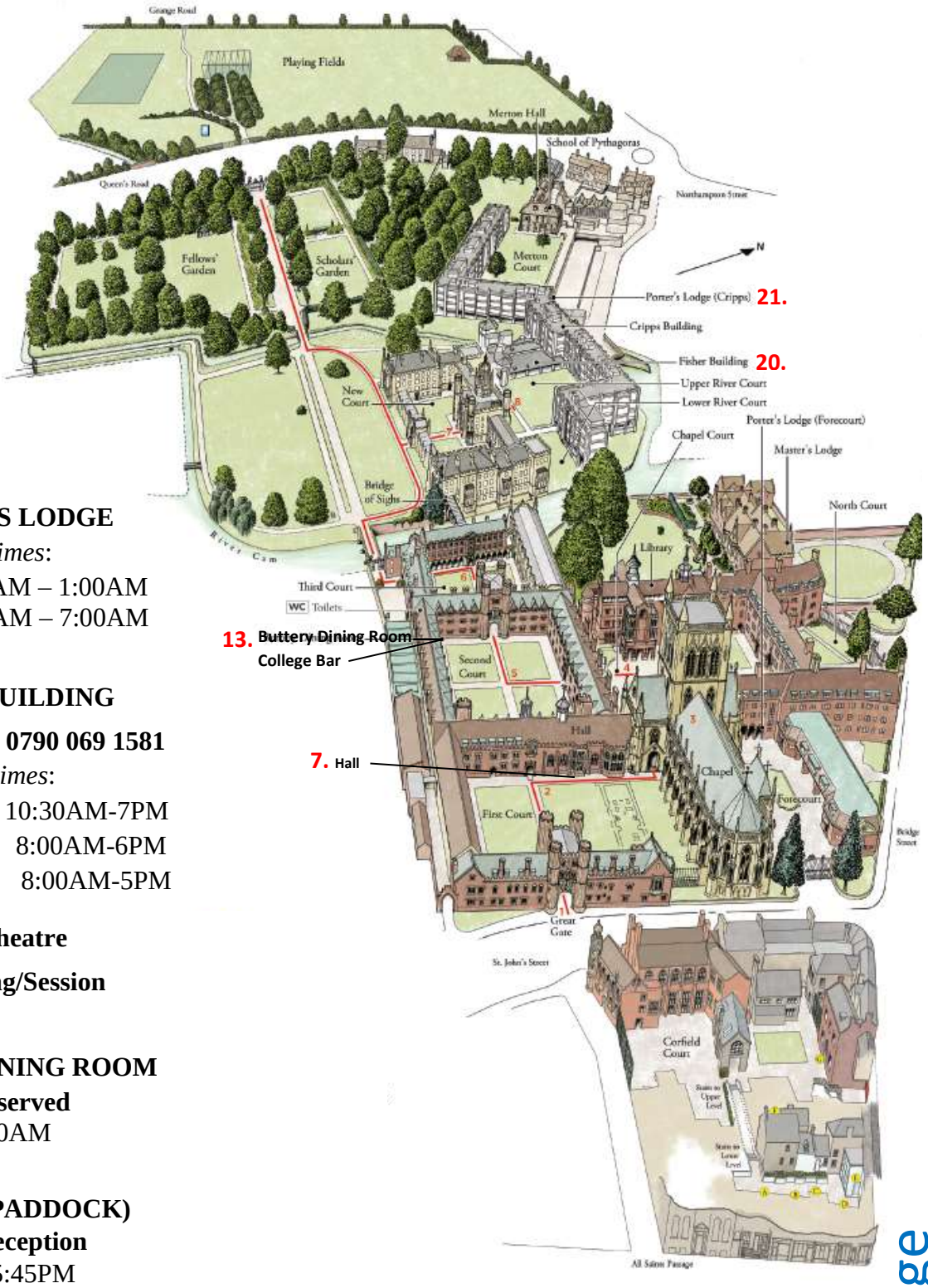








# Acknowledgements




**PORTER'S LODGE**

*Opening times*:

Forecourt: 7:00AM – 1:00AM

21. Cripps: 1:00AM – 7:00AM

**20. FISHER BUILDING**

**Conference Office: 0790 069 1581**

*Opening times*:

| | |
|---|---|
| Saturday | 10:30AM-7PM |
| Sunday-Tuesday | 8:00AM-6PM |
| Wednesday | 8:00AM-5PM |

**Lecture Theatre**

**Poster Viewing/Session**

**13. BUTTERY DINING ROOM**

**Breakfast served**

From 7.30AM

**THE BACKS (PADDOCK)**

**Welcome Reception**

Sunday 5:45PM

**7. HALL**

**Lunch served**

(See Programme for times)

**Banquet**

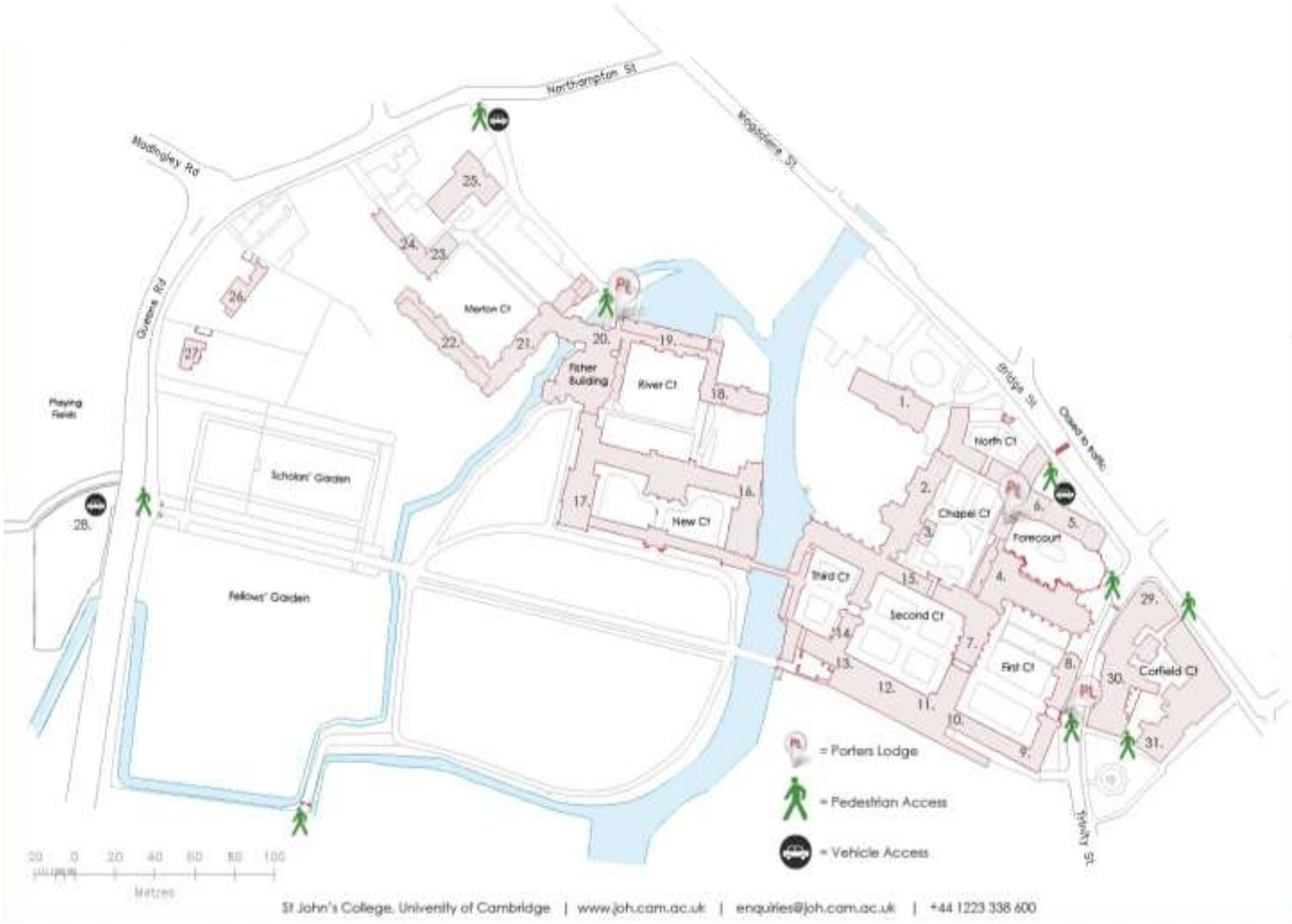
Tuesday 7.00PM

**13. COLLEGE BAR**

Coffee and bar snacks also available

Open until 11.00PM

St. John's College




St John's College, University of Cambridge | www.joh.cam.ac.uk | enquiries@joh.cam.ac.uk | +44 1223 338 600

Main Site:

1. Master's Lodge
2. College Office
3. Library
4. Chapel
5. Lady Superintendent's Office
6. Computer Support Office
7. Hall
8. Old Music Room
9. SBR
10. Parsons Room, Wordsworth Room
11. Catering and Conference Office
12. Wilberforce Room
13. Buttery
14. JCR
15. Development Office
16. Senior Guest Rooms
17. Bursary
18. 'A' staircase Cripps
19. 'B' & 'C' staircase Cripps
20. 'D' staircase Cripps
21. 'E' & 'F' staircase Cripps
22. 'G' & 'H' staircase Cripps
23. School of Pythagoras
24. Merton Hall
25. Maintenance Dept.
26. Merton House
27. Merton Cottage
28. Playing Fields car park
    Occasional use - for details contact the Domestic Bursar's office
29. Junior Guest Rooms
30. Old Divinity School
31. 1 All Saints Passage
    Teaching & Meeting Rooms

Advancing Scleroderma Research and Translational Medicine

## *SATURDAY 1 AUGUST* — *Fisher Building*

| **10.30am-7:00pm** | **REGISTRATION** | **Conference Office** |
|---|---|---|
| 6:00pm | Welcome and Introduction | Carol Black and Robert Lafyatis |
| | **KEYNOTE LECTURE**<br>***Horizons in Proteomics*** | *Chair: Thomas Krieg*<br>**Matthias Mann**<br>The Max Planck Institute of Biochemistry |
| 7:00pm | Opening Reception | |

## *SUNDAY 2 AUGUST*

| **8am-6pm** | **REGISTRATION** | **Conference Office** |
|---|---|---|
| 8:30 | Welcome and Introduction | Carol Black and Robert Lafyatis |
| | **SESSION 1**<br>**Exploring advances in pathobiology and clinical trials**<br>Chairs – Thomas Medsger and Frank van den Hoogen | |
| 8:40 | Horizons in Scleroderma Pathobiology | Antony Rosen |
| 9:10 | Early stage scleroderma trial design in the 21st Century | James Seibold |
| 9:40 | BREAK | |
| | **SESSION 2**<br>**Integrative and systems biology**<br>Chairs – Michael Whitfield and Maureen Mayes | |
| 9:55 | Introduction | |
| 10:00 | Unravelling genes in Scleroderma | Daniel Kastner |
| 10:35 | The 100,000 Genome Project | Clare Turnbull |
| 11:05 | Genomics in Scleroderma | Javier Martin |
| 11:30 | MORNING COFFEE | FOYER |
| | **SESSION 3**<br>**Ageing, autophagy and inflammation**<br>Chairs – Carol Feghali-Bostwick and Carol Artlett | |
| 11:55 | Introduction | |
| 12:00 | Autophagy in fibrosis: lessons from Crohn's disease | Alison Simmons |
| 12:30 | Immune senescence and ageing | Arne Akbar |
| 1:15 | Discussion | |
| 1:30 | LUNCH | HALL |
| | **SESSION 4**<br>**Host microbiome interactions**<br>Chairs – Armando Gabrielli and Richard Silver | |
| 2:25 | Introduction | |
| 2:30 | Translating microbiome science in to clinical medicine | Vanya Gant |
| 3:00 | The microbiome in inflammation | Michael Curtis |
| 3:30 | Discussion | |
| 3:45 | AFTERNOON TEA | FOYER |

Programme

| | | |
|---|---|---|
| **SESSION 5** | | |
| **Epigenomics in fibrosis** | | |
| Chairs – Peter Merkel and Bashar Kahaleh | | |
| 4:10 | Introduction | |
| 4:15 | Epigenetics and fibrosis | Michael Zeisberg |
| 4:45 | Epigenetic modifications as novel targets in tissue fibrosis | Derek Mann |
| 5:15 | Discussion | |
| 5:45 | END OF SESSION | |
| **5:45- 7PM** | **WELCOME RECEPTION** | |
| | **The Backs (Paddock)** | |
| | **POSTER VIEWING** | |

## *MONDAY 3 AUGUST*

| | | |
|---|---|---|
| **SESSION 6** | | |
| **Young Investigators – Selected abstracts** | | |
| Chairs – Carol Black and Robert Lafyatis | | |
| 8:55 | Introduction | |
| 9:00 | Jessica Gordon | Reliability and Validity of the Total Joint Count and Swollen Joint Count in Early Diffuse Systemic Sclerosis |
| 9:15 | Sule Yavuz | Subclinical biventricular systolic function is impaired in patients with systemic sclerosis with real time 3-D Echpcardiography:1 year follow-up study |
| 9:30 | Antonia Valenzuela | Calcinosis is associated with digital ulcers and osteoporosis in patients with Systemic Sclerosis: A Scleroderma Clinical Trials Consortium Study |
| 9:45 | Stephen O'Reilly | IL-13 mediated fibrosis is mediated through microRNA-135b in systemic sclerosis |
| 10:00 | Yun Zhang | Poly(ADP-ribose) polymerase-1 (PARP-1) suppresses the profibrotic effects of Transforming Growth Factor β in Systemic Sclerosis |
| 10:15 | Pier Paoli | Liver Damage Induces Changes to Skin Wound Healing in a Transgenerational Model |
| 10:30 | MORNING COFFEE | FOYER |
| **SESSION 7** | | |
| **Autoimmunity and cancer** | | |
| Chairs – Yannick Allanore and Ami Shah | | |
| 10:55 | Introduction | |
| 11:00 | An Update on Cancer and Scleroderma | Ami Shah |
| 11:30 | Lymphocyte signaling in autoimmunity and cancer | Klaus Okkenhaug |
| 12:00 | Discussion | |
| 12:30 | LUNCH | HALL |
| **SESSION 8** | | |
| **Mechanobiology and sensing** | | |
| Chairs – Thomas Krieg and Francesco del Galdo | | |
| 1:55 | Introduction | |
| 2:00 | Matrix – Cytoskeletal dynamics in tissue repair | Boris Hinz |
| 2:30 | Actin dynamics mechanosensing in fibroblasts | Barbara Smith |
| 3:00 | Discussion | |
| 3:30 | AFTERNOON TEA | FOYER |

| | | |
|---|---|---|
| **SESSION 9** | | |
| **Vascular disease in Scleroderma** | | |
| Chairs – Gerry Coghlan and Ariane Herrick | | |
| 3:55 | Introduction | |
| 4:00 | **KEYNOTE LECTURE** | |
| | ***The FGF/TGF Beta Axis in Vascular Health and Disease*** | **Michael Simons**<br>Yale School of Medicine |
| 4:40 | Vascular disease in systemic sclerosis | Marco Matucci-Cerinic |
| 5:10 | Current mechanisms and treatments for PAH | Nicholas Morrell |
| 5:40 | Discussion | |
| 6:10 | END OF SESSION | |
| | **POSTER VIEWING** | |

# *TUESDAY 4 AUGUST*

| | | |
|---|---|---|
| **SESSION 10** | | |
| **Exploring lessons from IPF** | | |
| Chairs – Peter Merkel and Jörg Distler | | |
| 8:55 | Introduction | |
| 9:00 | Are IPF and Scleroderma the same disease? | Erica Herzog |
| 9:30 | Translational medicine and outcome measures | Athol Wells |
| 10:00 | Pathobiology of IPF | Rachel Chambers |
| 10:30 | Discussion | |
| 11:00 | MORNING COFFEE | FOYER |
| **SESSION 11** | | |
| **Translational Partnerships, Industry Interactions and Biomarkers** | | |
| Chairs – Daniel Furst and Alan Tyndall | | |
| | **This session provides a forum for our industrial partners to discuss and present aspects of academic-industry interactions** | |
| **Discovery partnerships with Industry** | | |
| 11:30 | Safety and Efficacy of Subcutaneous Tocilizumab in Adults With Systemic Sclerosis: Week 48 Data From the FaSScinate Trial | Thierry Sornasse |
| 11:45 | Targeting IL13 in Lung Fibrosis | Lynne Murray |
| 12:00 | Hyper-immune serum in established scleroderma | Chris Denton |
| 12:15 | Fresolimumab in systemic sclerosis | Robert Lafyatis |
| 12:30 | LUNCH | HALL |
| **Biomarkers and Patient Stratification** | | |
| 1:30 | Assessment of Extra cellular Matrix (ECM)remodeling in fibrotic diseases: The role and damage of the basement and interstitial membranes | Morten Karsdal |
| 1:45 | Uncovering complex biology and pharmacology with SOMAscan, a highly multiplexed proteomic assay | David MacAllan |
| 2:00 | The ELF Test in scleroderma | Francesco Del Galdo |
| 2:15 | Clinical utility of serial KL-6 measurement in interstitial lung disease associated with systemic sclerosis | Yuichiro Shirai |
| 2:30 | Skin collagen synthesis rates distinguish between early and late diffuse scleroderma patients | Claire Emson/Scott Turner |

| | | |
|---|---|---|
| **SESSION 12** | | |
| **Fibrosis and TGFβ revisited** | | |
| Chairs – Robert Lafyatis and Kristofer Rubin | | |
| 2:45 | Introduction | |
| 2:50 | Emerging mechanisms of fibrosis | Andrew Tager |
| 3:20 | TGF beta revisited | Gisli Jenkins |
| 3:50 | Discussion | |
| 4:15 | END OF SESSION | |
| **4:15 onwards** | **POSTER SESSION** | |
| **7:00** | **BANQUET**<br>**The Hall** | |

## *WEDNESDAY 5 AUGUST*

| | | |
|---|---|---|
| **SESSION 13** | | |
| **Mesenchymal cell differentiation** | | |
| Chairs – Luc Mouthon and Maria Trojanowska | | |
| 8:55 | Introduction | |
| 9:00 | Mesenchymal stem cells in autoimmune disease | Alan Tyndall |
| 9:30 | Mesenchymal cells and regeneration | Bruno Péault |
| 10:00 | Discussion | |
| 10:30 | MORNING COFFEE | FOYER |
| **SESSION 14** | | |
| **Monocytes/macrophages and mediators in disease** | | |
| Chairs – Masataka Kuwana and Jacques Behmoaras | | |
| 10:55 | Introduction | |
| 11:00 | Resolution of Inflammation | Derek Gilroy |
| 11:30 | IL-6 signaling drives fibrosis in unresolved inflammation | Simon Jones |
| 12:00 | Discussion | |
| 12:30 | LUNCH | HALL |
| **SESSION 15** | | |
| **Scleroderma Clinical Trials Consortium : Issues in Modern Trial Design** | | |
| Moderators – Virginia Steen and Murray Baron | | |
| **1. Cohort Enrichment in Skin Trials** | | |
| 1:30 | EUSTAR experience | Britta Maurer |
| 1:45 | US experience | Robyn Domsic |
| 2:00 | Royal Free experience | Christopher Denton |
| 2:15 | Discussion | |
| **2. Group 3 PH - How to Advance the Field** | | |
| 2:30 | Lessons from PHAROS | Virginia Steen |
| 2:45 | Proposal for Trial Design | Gerry Coghlan |
| 3:00 | Discussion | |
| 3:30 | END OF SESSION | |
| | **END OF WORKSHOP** | |


# Abstracts

## Oral and Poster Presentations

**Reliability and Validity of the Total Joint Count and Swollen Joint Count in Early Diffuse Systemic Sclerosis**

Gordon JK[1], Girish G[2], Berrocal V[2], Zhang M[1], Hatzis C[1], Assassi S[3], Bernstein E[4], Domsic RT[5], Hant FN[6], Hinchcliff M[7], Schiopu E[2], Steen V[8], Frech T[9], Khanna D[2]

[1]Hospital for Special Surgery, New York, NY; University of Michigan, Ann Arbor, MI; [2]University of Texas Health Science Center at Houston, Houston, TX;[3]Columbia University, New York, NY;[4]University of Pittsburgh, Pittsburgh, PA;[5]Medical University of South Carolina, Charleston, SC;[6]Northwestern University, Chicago, IL;[7]Georgetown University, Washington, DC;[8]University of Utah, Salt Lake City, UT.

**Background:** Clinical trials in diffuse cutaneous Systemic Sclerosis (dcSSc) sometimes include the tender joint count (TJC) and swollen joint count (SJC) as secondary outcome measures; however, these outcomes have not yet been validated in dcSSc. Our objectives were to assess inter and intrarater reliability of the TJC and SJC and to compare physician joint examinations with musculoskeletal ultrasound (MSK-US) as a gold standard.

**Methods:** On a single day 7 patients in the Prospective Registry of Early Systemic Sclerosis (PRESS) cohort underwent 2 separate TJC/SJC by 10 rheumatologists and had MSK-US of the bilateral hands and wrists (22 joints). We computed inter and intrarater reliability for TJC/SJC and compared the TJC/SJC to the MSK-US to determine sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

**Results:** The mean TJC was 4.2 (2.0). The interobserver reliability for the TJC was 0.97, and the intraobserver reliability for the TJC was 0.99, showing almost perfect agreement. The mean SJC was 1.3 (0.8). The interobserver reliability for the SJC was 0.24, showing fair agreement, and the intraobserver reliability for the SJC was 0.71, showing substantial agreement.

9.7% (15/154) of joints showed synovitis or synovial thickening on MSK-US. Sensitivity, specificity, PPV and NPV are shown below.

| | Sensitivity – mean(SD) | Specificity – mean(SD) | PPV – mean(SD) | NPV – mean(SD) |
|---|---|---|---|---|
| SJC1 | 0.020 (0.045) | 0.956 (0.028) | 0.039 (0.076) | 0.894 (0.009) |
| SJC2 | 0.014 (0.045) | 0.947 (0.048) | 0.017 (0.047) | 0.869 (0.008) |
| TJC1 | 0.093 (0.034) | 0.778 (0.033) | 0.046 (0.014) | 0.881 (0.013) |
| TJC2 | 0.035 (0.037) | 0.900 (0.063) | 0.046 (0.048) | 0.866 (0.005) |

**Conclusion**: We noted excellent inter and intrarater reliability for the TJC and acceptable intrarater reliability for the SJC in patients with early dcSSc. TJC/ SJC showed low sensitivity, but high specificity when compared with MSK-US. In this group with low prevalence of MSK-US abnormalities, the PPV of the TJC/SJC was low, and the NPV was high. There was poor agreement between the TJC/SJC and MSK-US in early dcSSc.

**Subclinical biventricular systolic function is impaired in patients with systemic sclerosis with real time 3-D Echpcardiography: 1 year follow-up study**

Taylan Sahin[1], Selen Yurdakul[1], Neslihan Yilmaz[2], Yonca Cagatay[2], Saide Aytekin[1], Sule Yavuz[2]

[1] Bilim University Faculty of Medicine, Cardiology Department, [2] Bilim University Faculty of Medicine, Rheumatology Department

**Background**: Silent myocardial involvement is associated with poor prognosis in patients with systemic sclerosis (SSc). In the present study we aimed to evaluate subclinical left ventricular (LV) and right ventricular (RV) systolic dysfunction in SSc patients without any cardiovascular disease, by using both strain imaging method, "speckle tracking echocardiography" (STE) and real time 3-D Echocardiography .

**Methods**: Fifthy-five SSc patients were screened, 7 patients were excluded because of ischemic heart disease. We studied 48 patients with SSc and 25 age and sex-matched healthy controls(HC), without any cardiac disease and with preserved LV-EF. Conventional echocardiography, STE-based strain imaging and real time 3-D echocardiography (Bothell,WA,USA) were performed to assess biventricular deformation analyse. Association with anti-Scl 70 was sought in patients with SSc.

**Results:** In SSc patients (Female/Male: 44/4) the mean age was 47.7 years. Anti Scl-70 seropositivity was 22 (45.8%). Left ventricular conventional echocardiographic measurements (LV end diastolic diameter, LV end systolic diameter and LV EF) were similar between SSc and HC (table1). Both LV and RV longitudinal peak systolic strain/ strain rate were significantly impaired in SSc, demonstrating subclinical LV and RV systolic dysfunction ($p \leq 0.001$). LVESV was significantly increased in SSc (44.3±7.3 vs 37.8±2.4; SSc vs HC, $p < 0.001$).

Systolic PAB was negatively correlated with both LV and RV longitudinal peak systolic strain/strain rate (LV: r=-0.552 and r= -0.637, respectively, $p<0.001$ and RV: r=-0.547 and r=-0.638, respectively, p=0.001). Anti Scl -70 positive patients had impaired LV longitudinal peak systolic strain and strain rate values, compared to the others, however the difference did not reach statistical significance (13.01±1.26 % to 13.04±1.90 %, p=0.96 for strain; 0.30±0.06 1/s to 0.31±0.15 1/s, p=0.79 for strain rate). There was a trend for decreasing left ventricular strain and increasing LEVSV in 1 year analysis of SSc patients but it did not reach statistical significance.

**Conclusions**: SSc is associated with myocardial systolic dysfunction. Both deformation analysis by STE-based strain imaging and end systolic left ventricular volume analysis by real time 3-D echocardiography are promising modalities that allow us for non-invasive, comprehensive analysis of early deterioration in biventricular systolic function in patients with SSc.

**Table 1** Speckle tracking echocardiography (STE) and real time 3-D echocardiography results of SSc patients and healthy controls.

| | **SSc n=48** | **HC n=25** | **p value** |
|---|---|---|---|
| LV longitudinal peak systolic strain (%) | 13.3± 0.82 | 20.35±3.05 | 0.0001 |
| LV strain rate (1/s) | 0.91±0.21 | 1.70±0.47 | 0.0001 |
| RV longitudinal peak systolic strain (%) | 11.68±1.61 | 14.63±2.35 | 0.001 |
| RV strain rate (1/s) | 0.31±0.01 | 2.73±0.4 | 0.0001 |
| LVEDV (ml) | 104.6±16.2 | 106±17.5 | 0.63 |
| LVESV (ml) | 44.3±7.3 | 37.8±2.4 | 0.0001 |

*Values were presented as mean ±SD. LV; left ventricul, RV; right ventricul, LVEDV;left ventricular end diastolic volume,LVESV; left ventricular end systolic volume*

**Calcinosis is associated with digital ulcers and osteoporosis in patients with Systemic Sclerosis: A Scleroderma Clinical Trials Consortium Study**

Antonia Valenzuela[1], Murray Baron[2], the Canadian Scleroderma Research Group, Ariane Herrick[3], Susanna Proudman[4], Wendy Stevens[5], the ASIG rubric, Tatiana S. Rodriguez-Reyna[6], Alessandra Vacca[7], Thomas A. Medsger Jr.[8], Monique Hinchcliff[9], Vivien Hsu[10], David Fiorentino[11], Lorinda Chung[1]

[1]Department of Immunology and Rheumatology, Stanford University School of Medicine, [2]Department of Rheumatology, Jewish General Hospital McGill University, [3]Department of Rheumatology, University of Manchester, [4]Rheumatology Unit, Royal Adelaide Hospital North Terrace, [5]Department of Rheumatology, St. Vincent's Hospital Melbourne, [6]Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, [7]Unit and Chair of Rheumatology, University Hospital of Cagliari, [8]Department of Medicine/Rheumatology, University of Pittsburg School of Medicine, [9]Department of Rheumatology, Northwestern University, [10]Department of Medicine, Rutgers-RWJ Medical School, [11]Department of Dermatology, Stanford University School of Medicine

**Background:** Calcinosis is a debilitating cutaneous complication of systemic sclerosis (SSc) with no effective treatments. We sought to determine the clinical factors associated with calcinosis in an international multi-center collaborative effort with the Scleroderma Clinical Trials Consortium (SCTC).

**Methods:** This is a retrospective cohort study of 5180 patients with SSc from 9 centers within the US, Australia, Canada, United Kingdom, Italy, and Mexico. Calcinosis was defined as the presence of calcium deposition in the skin and/or subcutaneous tissues as determined by physical examination and/or radiography. Logistic regression was used to obtain odds ratios (OR) relating calcinosis to various clinical features in multivariate analyses.

**Results:** A total of 1290 patients (24.4%) had calcinosis. Patients with calcinosis were older than patients without calcinosis (59.4±12.8 vs. 56.8±13.4), more likely to be female (89.1% vs. 83.5%), and had longer disease duration from first non-Raynaud symptom (13±10.4 vs. 8.3±9.2) ($p$<0.0001). Patients with calcinosis were more likely to have digital ulcers (65.5% vs. 34.3%), telangiectasias (88.3% vs. 64.6%), and acro-osteolysis (26.7% vs. 9.4%), cardiac disease (18.1% vs. 12.5%, $p$<0.0001), pulmonary hypertension (16.4% vs. 13.8%, $p$=0.02), gastrointestinal involvement (74.5% vs. 63.2%, $p$<0.0001), and arthritis (29.9% vs. 26.7%, $p$=0.04), but less likely to have myositis (8.8% vs. 12.3%, p=0.0009). Osteoporosis was much more common in patients who had calcinosis (22.8% vs. 2.8%, $p$<0.0001). Scl-70, RNA-polymerase-III, and RNP autoantibodies were significantly less common in patients with calcinosis, while anticentromere (ACA), PM-1, and anticardiolipin were more frequent. In multivariate analysis, the strongest associations with calcinosis were digital ulcers (OR 3.7, 95%CI 2.6-5.3, $p$<0.0001), and osteoporosis (OR 3.9, 95%CI 2.1-7.4, $p$<0.0001)(Table). After controlling for steroid use and BMI in the model, the association with osteoporosis persisted in stratified analyses in non-obese patients (OR 6.5, 95%CI 1.8-23.8, $p$=0.004).

**Conclusion:** Almost one quarter of patients with SSc have calcinosis. Our data support a strong association of calcinosis with digital ulcers and osteoporosis in non-obese patients. This may shed light on the pathogenesis of calcinosis and guide the development of future therapies.

**Table. Non-stratified univariate and multivariate analyses**

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95%CI | p-value | OR | 95%CI | p-value |
| Age at last visit | 1.02 | 1.01 – 1.02 | <0.0001 | | | |
| Disease duration | 1.05 | 1.04 – 1.05 | <0.0001 | - | - | - |
| Female | 1.6 | 1.3 – 1.9 | <0.0001 | 2.8 | 1.4 – 5.7 | 0.0038 |
| Obese | 0.8 | 0.6 – 0.9 | 0.0133 | | | |
| Steroids use ever | 0.7 | 0.6 – 0.8 | <0.0001 | | | |
| mRSS >11 | 1.1 | 0.98 – 1.3 | 0.0759 | | | |
| Raynaud's phenomenon | 2.9 | 1.7- 4.9 | <0.0001 | | | |
| Digital ulcers | 3.6 | 3.2 – 4.2 | <0.0001 | 3.7 | 2.6- 5.3 | <0.0001 |
| Digital pitting scars | 3.0 | 2.7 - 3.5 | <0.0001 | | | |
| Loss of digital pulp | 2.9 | 2.5 - 3.4 | <0.0001 | | | |
| Nailfold capillary changes | 2.7 | 2.3- 3.2 | <0.0001 | | | |
| Puffy fingers | 0.8 | 0.7 - 0.9 | 0.0232 | 0.6 | 0.4 - 0.9 | 0.0179 |
| Sclerodactyly | 1.6 | 1.3 - 2.0 | <0.0001 | | | |
| Acroosteolysis | 3.5 | 2.5 – 4.9 | <0.0001 | | | |
| Telangiectasias | 4.1 | 3.4 – 4.9 | <0.0001 | 3.5 | 2.1 – 5.7 | <0.0001 |
| Osteoporosis | 10.2 | 6.9 – 15.0 | <0.0001 | 3.9 | 2.1 – 7.4 | <0.0001 |
| Cardiac disease | 1.6 | 1.3 – 1.9 | <0.0001 | 1.9 | 1.1 – 3.0 | 0.0136 |
| PAH | 1.2 | 1.0 – 1.5 | 0.0231 | | | |
| GI disease | 1.7 | 1.4 – 1.96 | <0.0001 | 1.9 | 1.1 – 2.5 | 0.0265 |
| Myositis | 0.7 | 0.6 – 0.9 | 0.001 | - | - | - |
| Arthritis | 1.2 | 1.0 – 1.4 | 0.0416 | 1.5 | 1.0 - 2.2 | 0.0323 |
| Positive Scl-70 | 0.6 | 0.5 - 0.8 | <0.0001 | | | |
| Positive anticentromere | 1.7 | 1.4 – 2.0 | <0.0001 | 2.4 | 1.6 – 3.5 | <0.0001 |
| Positive PM-1 | 1.8 | 1.2 – 2.5 | 0.0028 | | | |
| Positive RNA polymerase III | 0.8 | 0.6 - 0.9 | 0.0159 | | | |
| Positive RNP | 0.5 | 0.4 - 0.8 | 0.0002 | | | |
| Positive Anticardiolipin | 1.7 | 1.2 – 2.2 | 0.0005 | | | |

Abbreviations: mRSS=modified Rodnan skin score, PAH=pulmonary artery hypertension, GI=gastrointestinal, ACA=anticentromere, RNP= Ribonucleoprotein

Multivariate model included: disease duration from first non-Raynaud symptoms, gender, modified Rodnan skin score (mRSS), digital ulcers, puffy fingers, telangiectasias, osteoporosis, cardiac disease, GI disease, myositis, arthritis, and anticentromere antibody

**IL-13 mediated fibrosis is mediated through microRNA-135b in systemic sclerosis**

Steven O'Reilly

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**Background:** Systemic sclerosis is an autoimmune connective tissue disease characterised by a T helper type 2 response and the increase of th2 type cytokines. These Th2 type cytokines include IL-4 and IL-13 and these are elevated in the serum and also induce fibrosis. Although IL-13 is known to mediate fibrosis its exact mechanism is still unknown. The aim of this study was to examine the effects of IL-13 on dermal fibroblasts and to examine its molecular mechanism.

**Materials and Methods:** Healthy dermal fibroblasts were cultured and dermal fibroblasts from clinically diagnosed patients were also cultured from punch biopsies. Cells were cultured at low passage in DMEM and stimulated with recombinant IL-13 and qRT-PCR was performed for extracellular matrix genes. STAT6 was inhibited with both small interfering RNA (siRNA) and a small molecule inhibitor to examine its role in IL-13 fibrosis. The microRNA-135b was measured by qRT-PCR and miR-135b was also transfected into dermal fibroblasts. miR-135b was also measured in healthy and patients serum. MiR-135b was also measured in skin samples from mice that were exposed to bleomycin to induce skin fibrosis.

**Results:** IL-13 dose dependently induces collagen1A1 expression in dermal fibroblasts. This IL-13 mediated induction of collagen was not dependant on TGF-β1 signalling. IL-13 induced collagen was dependant on STAT6 as siRNA and chemical inhibition abrogated the IL-13 mediated induction. miR-135 transfection also modulated the induction of collagen1A1 and reduced STAT6 levels. Bioinformatics analysis suggested that miR135b targets STAT6 in its Untranslated Region (UTR). Analysis of fibroblasts from scleroderma patients showed lower levels of miR-135b in dermal fibroblasts and its target STAT6 was also elevated, suggesting a direct link. Serum levels of miR-135b in patients were also lower too. Using the bleomycin model of fibrosis in mice measurement of miR-135b also demonstrated lower levels compared to control treated mice.

**Conclusions:** IL-13 is a critical molecule in mediating fibrosis and is independent of TGF-β signalling. IL-13 is dependent on STAT6 signalling to mediate its effects in dermal fibroblasts. Epigenetic modifications also play a role as miR135b targets STAT6 and is reduced in scleroderma patient's cells and serum. The measurement of miR-135b is sera could be a potential biomarker.

**Poly(ADP-ribose) polymerase-1 (PARP-1) suppresses the profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis**

Yun Zhang(1), Katrin Palumbo-Zerr(1), Christian Beyer(1), Pawel Zerr(1), Ruifang Liang(1), Clara Dees(1), Oliver Distler(2), Georg Schett(1), Jörg H.W. Distler(1).

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**Background:** The enzyme poly(ADP-ribose) polymerase-1 (PARP-1) transfers negatively charged ADP-ribose units from the donor β-NAD onto various substrate proteins either as mono- or oligomeric moieties or as linear or branched poly (ADP-ribose) (PAR) chains. Those modifications can have pronounced regulatory effects on the half-life or the enzymatic activity of target proteins. Recent studies demonstrated that PARP-1 can poly(ADP-ribosyl)ates (PARylates) members of the Smad family of transcription factors. However, the role of PARP1 in the pathogenesis of SSc has not been investigated.

**Materials and Methods:** The expression of PARP1 in human skin and in experimental fibrosis was determined by qPCR and immunohistochemistry. TGFβ signalling was analysed by Smad reporter assays and target gene analysis after 1mM selective PARP1 inhibitor 3-Aminobenzamide (3AB). Bleomycin-induced skin fibrosis and Tsk-1 mice were used to evaluate the effect of PARP deficiency and PARP inhibition (10mg/kg/d 3AB) in vivo.

**Results:** Decreased expression of PARP1 was detected by immunohistochemistry in skin sections of SSc patients, particularly in fibroblasts. Inhibition of PARylation by 3AB augmented the stimulatory effects of TGFβ on fibroblasts in vitro. PARP1 inhibition increased Smad dependent transcription in reporter assays and promoted the transcription of TGFβ/Smad target genes. Treatment with 3AB also stimulated the collagen release and fostered the expression of contractile proteins with increased expression of α-smooth muscle actin (α-SMA) and enhanced formation of stress fiber formation compared to fibroblasts stimulated with TGFβ alone. Inhibition of PARylation also exacerbated experimental fibrosis in vivo. Treatment with 3AB induced a more severe fibrotic response to bleomycin with increased dermal thickening (by up to 103%, $p<0.0001$), hydroxyproline contents and myofibroblast counts compared to control mice ($p<0.0001$ and $p=0.0059$). Inhibition of PARylation also strongly exacerbated fibrosis in Tsk-1 mice. Meanwhile, after bleomycin injection, dermal thickening, hydroxyproline contents and myofibroblast counts of PARP1 knockout mice are increased by 85% ($p=0.0046$), 67% ($p=0.0098$) and 56% ($p=0.0043$) compared to wild-type mice.

**Conclusion:** We demonstrate that PARP1 negative regulates canonical TGFβ signalling. The down-regulation of PARP1 in SSc fibroblasts may thus directly contribute to hyperactive TGFβ signalling and to persistent fibroblast activation in SSc.

**Liver Damage Induces Changes to Skin Wound Healing in a Transgenerational Model**

Pier P. Paoli[1], Mujdat Zeybel[1], Jelena Mann, Eleana Pappa and Derek A Mann[1]

[1]Institute of Cellular Medicine, Faculty of Medical Sciences, 4th Floor, William Leech Building, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.

Wound healing is a complex multistep and multicellular process that operates to restore tissue architecture and function after trauma or damage caused by environmental insults. Previously, we have found that a history of liver damage led to suppressive adaptation in further generations (Zeybel et al., 2012). Further to this we investigated the effects of adaptation in the skin as a model for wound healing and repair.

A standard hole-punch assay was used to assess the rate of wound healing between control and animals that had been epigenetically adapted from protection to liver fibrosis through multiple generations . Skin wounds were measured internally, and externally, at eight days post-injury. Cell migration was measured with a scratch assay using isolated fibroblasts and observed for a total of 48hrs. A panel of genes involved in wound healing and repair were assayed using qPCR in both whole skin and fibroblasts at three days and eight days post-injury. A miRNA profile was also generated for the same samples to complement the transcript data. Identification and analysis of the miRNA transcripts was achieved using the Seq-Imp pipeline (http://www.ebi.ac.uk/research/enright/software/kraken). Finally, a DNA methylation profile for the skin fibroblasts was produced and analysed using a combination of bismark and R scripts to plot differentially methylated regions (DMRs).

Skin from adapted animals took longer to close than control females after eight days post-injury (13 and 20mm$^2$ respectively). In the same animals, CTGF and IL-1β were found to be significantly down regulated in whole skin samples compared to the control group. In the fibroblasts, αSMA, TGFβ-1 and TIMP-1 were also downregualted in the adapted group. The miRNA profiling revealed a total of 12 differentially regulated miRNA in the adapted group in whole skin samples, and 15 in fibroblasts compared to the control animals. Significant changes in the DNA methylation landscape were also found between the two groups in the fibroblasts.

In conclusion, animals adapted to liver damage showed slower recovery to sustained injury than non-adapted animals, on both cellular and transcript levels. In addition, damage to the liver has implications in the skin in a similar manner, and may be a potential method to assess liver fibrosis non-invasively.

References:

Müjdat Zeybel, Timothy Hardy, Yi K Wong, John C Mathers, Christopher R Fox, Agata Gackowska, Fiona Oakley, Alastair D Burt, Caroline L Wilson, Quentin M Anstee, Matt J Barter, Steven Masson, Ahmed M Elsharkawy, Derek A Mann & Jelena Mann (2012). Multigenerational epigenetic adaptation of the hepatic wound-healing response. *Nature Medicine* 18, 1369–1377.

## 1. Sexual function and quality of life in women with Systemic Sclerosis

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**Keywords:** Systemic sclerosis; quality of life; sexual function

**Introduction:** Sexuality is an often neglected area in patients with rheumatic disease. The aim of this study is to assess sexual functioning and quality of life in a group of married women with Systemic Sclerosis (SSc).

**Methods:** This is a horizontal study for descriptive and analytical purposes. Married women with SSc were interviewed about their sexual functioning and their quality of life.

**Results:** A total of ten patients who met the criteria have accepted to participate to the study. Their mean age was 52, 4± 8,2 years. Eight women thought that the disease had affected their sexual activity. All patients reported a decrease in the frequency of intercourse since the onset of their disease. Eight of the sample reported a diminished desire for a sexual relationship. The reasons were fatigue, altered body image and pain. The assessment of sexual functioning using the Female sexual function index (FSFI) showed a mean FSFI score at 14,2±7,8 with nine women scoring in the range associated with sexual dysfunction (SD) (<26). All the subscales were affected. Our patients reported a mean total score on WHOQOL-brief (World Health Quality of Life-Brief Version) of 60 out of 120 indicating a moderate altered quality of life. Depression has been identified as determinants of impaired sexual function.

**Conclusion:** The prevalence of SD in women with SSc is high when a specific questionnaire is used to assess it. These results indicate that in daily practice, inquiring about sexuality and screening for depressive symptoms is indicated for every patient with SSc.

## 2. MeCP2 exerts global control over the myofibroblast transcriptome and reveals new *regulators of fibrosis*

*E. Moran-Salvador[1], P.P. Paoli[1], G. Smith[1], A. Page[1], R. Howarth[1], F. Oakley[1], J. Mann[1] and D.A. Mann[1]**

*[1]Institute of Cellular Medicine, Faculty of Medical Sciences, 4th Floor, William Leech Building, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK*

**Background:** Hepatic stellate cells (HSCs) are considered the central extracellular matrix (ECM)-producing cells in the wound-healing response process driven by a persistent liver injury. Previous studies in our laboratory identified MeCP2 (methyl-CpG binding protein 2) as the epigenetics master regulator of HSCs activation in liver fibrogenesis. Nowadays, the epigenetic machinery embraces an extensive network of non-coding RNAs (ncRNAs), such as long non coding RNAs (lncRNAs) and microRNAs (miRNAs). The transcription of ncRNAs establishes new regulatory systems affecting chromatin structure and gene expression. The aim of this study was to identify potential targets and a novel ncRNA regulatory network controlled by MeCP2 in HSCs.

**Materials and methods:** RNA from Wt (n=3) and MeCP2-null (n=3) mice HSCs was subjected to a microarray based profiling analyses (Arraystar Mouse LncRNKeyA Microarray V2.0 platform) and to a small RNA next-generation sequencing (NGS) (Illumina platform).

**Results:** Using the microarray platform we found 161 upregulated and 83 downregulated lncRNAs in MeCP2-null HSCs. We focused our attention to Gm3893 and AK080187; their deregulated expression was confirmed by qPCR and correlated with the expression of their associated gene transcripts (cytokines Ccl21b/c and IL11ra2 receptor and the profibrogenic marker Acta2, respectively). Pathway analysis based on KEGGs database denoted that most of 284 downregulated mRNAs in MeCP2-null HSCs were significantly enriched at DNA replication and cell cycle pathways, while most of 124 upregulated mRNAs were associated with pathways involved in the immune system and metabolism. Most importantly, MeCP2-null HSCs presented a robust downregulation in five members of the MCM protein complex and a remarkable decrease in other components of the replisome multisubunit complex. The resulting altered pattern of mRNAs also emphasized the downregulation of the hyaluronan synthase 2 (Has2) upon loss of MeCP2 in HSCs. In liver, Has2 was predominantly expressed in RNA of HSCs and its protein expression increased over the HSCs transdifferentiation process. In primary HSCs, small interfering RNA for Has2 decreased the gene expression of ECM proteins. Of note, miRNA-328-3p pinpointed from the 14 significantly altered miRNA identified by NGS analyses as has been proved to target and reduce the hyaluronic acid receptor CD44 expression.

**Conclusions:** Our findings successfully evidence a vast ncRNA network controlled by MeCP2 and highlight the complex role that MeCP2 elicits in the HSC transdifferentiating process.

**3. Sequencing the miR-nome: Identifying the Role of miRNA in Hepatic Stellate Cell (HSC) Activation**

*Luigi Locatelli[1], Derek A Mann[1], Tim Hardy[1], Rachel Howarth[1], Fiona Oakley[1], Pier P Paoli[1]*

*[1]Institute of Cellular Medicine, Faculty of Medical Sciences, 4th Floor, William Leech Building, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.*

**Materials and Methods** Cellular transdifferentiation is a critical step in many physiological processes (e.g. wound healing), and is implicated in pathologies such as cancer and tissue fibrosis. To-date the role of miRNAs in transdifferentiation has not been fully addressed. Here, we use a cell culture model for transdifferentiation of human and rat hepatic stellate cells (HSCs) into myofibroblasts to determine functional changes in miRNAs. The aims of this study were: a) perform Next Generation Sequencing (NGS) to generate a miRNA profile in human and rat activated HSCs to identify potential miRNA-mRNA regulatory networks in common with both; b) biological validation of specific miRNA target genes and miRNA function.

Data generated by sequencing were analysed via the Seq-Imp pipeline

(http://www.ebi.ac.uk/research/enright/software/kraken) to identify individual miRNAs, and to perform and an initial statistical analysis. Targets for each miRNA were identified from TargetScan, microCOSM, and miRTar, and assigned by picking genes that were in at least two out of three databases.

A profile of 11 significantly upregulated and 11 downregulated miRNAs were identified that were identical in both activated human and rat HSC. Within the differentially regulated miRNAs, we focused our attention on mir-150-5p, which regulates a series of target proteins such as the Zinc finger E-box binding homeobox (ZEB1) involved in the cell cycle, cellular development, and apoptosis. Particularly mir-150-5p has been found to be downregulated in both human and rat activated HSCs. A significant inverse correlation between ZEB1 and mir-150-5p was found at the RNA and protein level. *In vitro* we showed that the overexpression of mir-150-5p in human and rat HSCs by a mir-150-5p mimic was able to downregulate the expression of ZEB1 with an observed reduction of extracellular matrix proteins collagen type I (COL1A1) and α-smooth muscle actin (α-SMA). Moreover, the level of mir-150-5p in the serum of 10 Alcoholic Liver Disease (ALD) patients was found to be significantly downregulated compared to healthy controls.

**Conclusion,** HSC transdifferentiation is associated with discrete alterations in the expression of miRNAs, and in particular we have identified mir-150-5p as a regulator of the myofibroblast phenotype and potential biomarker of fibrosis in ALD patients.

**4. Liver Damage Induces Changes to Skin Wound Healing in a Transgenerational Model**

*Pier P. Paoli[1], Mujdat Zeybel[1], Jelena Mann, Eleana Pappa and Derek A Mann[1]*

*[1]Institute of Cellular Medicine, Faculty of Medical Sciences, 4th Floor, William Leech Building, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.*

**Background:** Wound healing is a complex multistep and multicellular process that operates to restore tissue architecture and function after trauma or damage caused by environmental insults. Previously, we have found that a history of liver damage led to suppressive adaptation in further generations (Zeybel et al., 2012). Further to this we investigated the effects of adaptation in the skin as a model for wound healing and repair.

**Materials and Methods:** A standard hole-punch assay was used to assess the rate of wound healing between control and animals that had been epigenetically adapted from protection to liver fibrosis through multiple generations. Skin wounds were measured internally, and externally, at eight days post-injury. Cell migration was measured with a scratch assay using isolated fibroblasts and observed for a total of 48hrs. A panel of genes involved in wound healing and repair were assayed using qPCR in both whole skin and fibroblasts at three days and eight days post-injury. A miRNA profile was also generated for the same samples to complement the transcript data. Identification and analysis of the miRNA transcripts was achieved using the Seq-Imp pipeline

(http://www.ebi.ac.uk/research/enright/software/kraken). Finally, a DNA methylation profile for the skin fibroblasts was produced and analysed using a combination of bismark and R scripts to plot differentially methylated regions (DMRs).

**Results:** Skin from adapted animals took longer to close than control females after eight days post-injury (13 and 20mm$^2$ respectively). In the same animals, CTGF and IL-1β were found to be significantly down regulated in whole skin samples compared to the control group. In the fibroblasts, αSMA, TGFβ-1 and TIMP-1 were also downregualted in the adapted group. The miRNA profiling revealed a total of 12 differentially regulated miRNA in the adapted group in whole skin samples, and 15 in fibroblasts compared to the control animals. Significant changes in the DNA methylation landscape were also found between the two groups in the fibroblasts.

**Conclusion:** Animals adapted to liver damage showed slower recovery to sustained injury than non-adapted animals, on both cellular and transcript levels. In addition, damage to the liver has implications in the skin in a similar manner, and may be a potential method to assess liver fibrosis non-invasively.

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**5. Calcinosis is associated with digital ulcers and osteoporosis in patients with Systemic Sclerosis: A Scleroderma Clinical Trials Consortium Study**

*Antonia Valenzuela[1], Murray Baron[2], the Canadian Scleroderma Research Group, Ariane Herrick[3], Susanna Proudman[4], Wendy Stevens[5], the ASIG rubric, Tatiana S. Rodriguez-Reyna[6], Alessandra Vacca[7], Thomas A. Medsger Jr.[8], Monique Hinchcliff[9], Vivien Hsu[10], David Fiorentino[11], Lorinda Chung[1]*

*[1]Department of Immunology and Rheumatology, Stanford University School of Medicine, [2]Department of Rheumatology, Jewish General Hospital McGill University, [3]Department of Rheumatology, University of Manchester, [4]Rheumatology Unit, Royal Adelaide Hospital North Terrace, [5]Department of Rheumatology, St. Vincent's Hospital Melbourne, [6]Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, [7]Unit and Chair of Rheumatology, University Hospital of Cagliari, [8]Department of Medicine/Rheumatology, University of Pittsburg School of Medicine,[9]Department of Rheumatology, Northwestern University, [10]Department of Medicine, Rutgers-RWJ Medical School, [11]Department of Dermatology, Stanford University School of Medicine*

**Background:** Calcinosis is a debilitating cutaneous complication of systemic sclerosis (SSc) with no effective treatments. We sought to determine the clinical factors associated with calcinosis in an international multi-center collaborative effort with the Scleroderma Clinical Trials Consortium (SCTC).

**Methods:** This is a retrospective cohort study of 5180 patients with SSc from 9 centers within the US, Australia, Canada, United Kingdom, Italy, and Mexico. Calcinosis was defined as the presence of calcium deposition in the skin and/or subcutaneous tissues as determined by physical examination and/or radiography. Logistic regression was used to obtain odds ratios (OR) relating calcinosis to various clinical features in multivariate analyses.

**Results:** A total of 1290 patients (24.4%) had calcinosis. Patients with calcinosis were older than patients without calcinosis (59.4±12.8 vs. 56.8±13.4), more likely to be female (89.1% vs. 83.5%), and had longer disease duration from first non-Raynaud symptom (13±10.4 vs. 8.3±9.2) ($p$<0.0001). Patients with calcinosis were more likely to have digital ulcers (65.5% vs. 34.3%), telangiectasias (88.3% vs. 64.6%), and acro-osteolysis (26.7% vs. 9.4%), cardiac disease (18.1% vs. 12.5%, $p$<0.0001), pulmonary hypertension (16.4% vs. 13.8%, $p$=0.02), gastrointestinal involvement (74.5% vs. 63.2%, $p$<0.0001), and arthritis (29.9% vs. 26.7%, $p$=0.04), but less likely to have myositis (8.8% vs. 12.3%, p=0.0009). Osteoporosis was much more common in patients who had calcinosis (22.8% vs. 2.8%, $p$<0.0001). Scl-70, RNA-polymerase-III, and RNP autoantibodies were significantly less common in patients with calcinosis, while anticentromere (ACA), PM-1, and anticardiolipin were more frequent. In multivariate analysis, the strongest associations with calcinosis were digital ulcers (OR 3.7, 95%CI 2.6-5.3, $p$<0.0001), and osteoporosis (OR 3.9, 95%CI 2.1-7.4, $p$<0.0001) (Table).

After controlling for steroid use and BMI in the model, the association with osteoporosis persisted in stratified analyses in non-obese patients (OR 6.5, 95%CI 1.8-23.8, $p$=0.004).

**Conclusion:** Almost one quarter of patients with SSc have calcinosis. Our data support a strong association of calcinosis with digital ulcers and osteoporosis in non-obese patients. This may shed light on the pathogenesis of calcinosis and guide the development of future therapies.

Table. Non-stratified univariate and multivariate analyses

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95%CI | p-value | OR | 95%CI | p-value |
| Age at last visit | 1.02 | 1.01 – 1.02 | <0.0001 | | | |
| Disease duration | 1.05 | 1.04 – 1.05 | <0.0001 | - | - | - |
| Female | 1.6 | 1.3 – 1.9 | <0.0001 | 2.8 | 1.4 – 5.7 | 0.0038 |
| Obese | 0.8 | 0.6 – 0.9 | 0.0133 | | | |
| Steroids use ever | 0.7 | 0.6 – 0.8 | <0.0001 | | | |
| mRSS >11 | 1.1 | 0.98 – 1.3 | 0.0759 | | | |
| Raynaud's phenomenon | 2.9 | 1.7- 4.9 | <0.0001 | | | |
| Digital ulcers | 3.6 | 3.2 – 4.2 | <0.0001 | 3.7 | 2.6- 5.3 | <0.0001 |
| Digital pitting scars | 3.0 | 2.7 - 3.5 | <0.0001 | | | |
| Loss of digital pulp | 2.9 | 2.5 - 3.4 | <0.0001 | | | |
| Nailfold capillary changes | 2.7 | 2.3- 3.2 | <0.0001 | | | |
| Puffy fingers | 0.8 | 0.7 - 0.9 | 0.0232 | 0.6 | 0.4 - 0.9 | 0.0179 |
| Sclerodactyly | 1.6 | 1.3 - 2.0 | <0.0001 | | | |
| Acroosteolysis | 3.5 | 2.5 – 4.9 | <0.0001 | | | |
| Telangiectasias | 4.1 | 3.4 – 4.9 | <0.0001 | 3.5 | 2.1 – 5.7 | <0.0001 |
| Osteoporosis | 10.2 | 6.9 – 15.0 | <0.0001 | 3.9 | 2.1 – 7.4 | <0.0001 |
| Cardiac disease | 1.6 | 1.3 – 1.9 | <0.0001 | 1.9 | 1.1 – 3.0 | 0.0136 |
| PAH | 1.2 | 1.0 – 1.5 | 0.0231 | | | |
| GI disease | 1.7 | 1.4 – 1.96 | <0.0001 | 1.9 | 1.1 – 2.5 | 0.0265 |
| Myositis | 0.7 | 0.6 – 0.9 | 0.001 | - | - | - |
| Arthritis | 1.2 | 1.0 – 1.4 | 0.0416 | 1.5 | 1.0 - 2.2 | 0.0323 |
| Positive Scl-70 | 0.6 | 0.5 - 0.8 | <0.0001 | | | |
| Positive anticentromere | 1.7 | 1.4 – 2.0 | <0.0001 | 2.4 | 1.6 – 3.5 | <0.0001 |
| Positive PM-1 | 1.8 | 1.2 – 2.5 | 0.0028 | | | |
| Positive RNA polymerase III | 0.8 | 0.6 - 0.9 | 0.0159 | | | |
| Positive RNP | 0.5 | 0.4 - 0.8 | 0.0002 | | | |
| Positive Anticardiolipin | 1.7 | 1.2 – 2.2 | 0.0005 | | | |

Abbreviations: mRSS=modified Rodnan skin score, PAH=pulmonary artery hypertension, GI=gastrointestinal, ACA=anticentromere, RNP= Ribonucleoprotein
Multivariate model included: disease duration from first non-Raynaud symptoms, gender, modified Rodnan skin score (mRSS), digital ulcers, puffy fingers, telangiectasias, osteoporosis, cardiac disease, GI disease, myositis, arthritis, and anticentromere antibody.

**6. A Longitudinal Biomarker for the Extent of Skin Disease in Patients with Diffuse Cutaneous Systemic Sclerosis**

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**Background:** The goal of this study was to define a pharmacodynamic biomarker based on gene expression in skin that would provide a biological measure of disease extent in patients with diffuse cutaneous systemic sclerosis (dcSSc) and that could be used to monitor skin disease longitudinally.

**Methods:** Skin biopsies taken from a cohort of dcSSc patients that included longitudinal samples were analyzed by microarray. Expression of genes correlating with the modified Rodnan skin score (MRSS) were examined by nanostring for change over time, and a generalized estimating equation used to define and validate longitudinal, pharmacodynamic biomarkers composed of multiple genes.

**Results:** Microarray analysis of genes parsed to include only genes correlating with the MRSS revealed prominent clusters of profibrotic/TGFβ-regulated, IFN-regulated/proteasome, macrophage and vascular marker genes. Using genes changing longitudinally with the MRSS, two multigene, pharmacodynamic biomarkers were defined. The first was defined mathematically, applying a generalized estimating equation to longitudinal samples. This modeling method selected cross-sectional THBS1 and longitudinal THBS1 and MS4A4A genes. The second model was based on a weighted selection of genes, including additional genes with statistically significant change over time: CTGF, CD163, CCL2 and WIF1. Biomarker levels calculated using both models correlated highly with the MRSS in an independent validation dataset.

**Conclusion:** Skin gene expression can be used effectively to monitor SSc skin disease change over time. We have implemented these relatively simple models on a nanostring platform permitting highly reproducible assays that can be applied directly to samples from patients or collected as part of clinical trials.

**7. IL-13 mediated fibrosis is mediated through microRNA-135b in systemic sclerosis**

*Steven O'Reilly*

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**Background:** Systemic sclerosis is an autoimmune connective tissue disease characterised by a T helper type 2 response and the increase of th2 type cytokines. These Th2 type cytokines include IL-4 and IL-13 and these are elevated in the serum and also induce fibrosis. Although IL-13 is known to mediate fibrosis its exact mechanism is still unknown. The aim of this study was to examine the effects of IL-13 on dermal fibroblasts and to examine its molecular mechanism.

**Materials and Methods:** Healthy dermal fibroblasts were cultured and dermal fibroblasts from clinically diagnosed patients were also cultured from punch biopsies. Cells were cultured at low passage in DMEM and stimulated with recombinant IL-13 and qRT-PCR was performed for extracellular matrix genes. STAT6 was inhibited with both small interfering RNA (siRNA) and a small molecule inhibitor to examine its role in IL-13 fibrosis. The microRNA-135b was measured by qRT-PCR and miR-135b was also transfected into dermal fibroblasts. miR-135b was also measured in healthy and patients serum. MiR-135b was also measured in skin samples from mice that were exposed to bleomycin to induce skin fibrosis.

**Results:** IL-13 dose dependently induces collagen1A1 expression in dermal fibroblasts. This IL-13 mediated induction of collagen was not dependant on TGF-β1 signalling. IL-13 induced collagen was dependant on STAT6 as siRNA and chemical inhibition abrogated the IL-13 mediated induction. miR-135 transfection also modulated the induction of collagen1A1 and reduced STAT6 levels. Bioinformatics analysis suggested that miR135b targets STAT6 in its Untranslated Region (UTR). Analysis of fibroblasts from scleroderma patients showed lower levels of miR-135b in dermal fibroblasts and its target STAT6 was also elevated, suggesting a direct link. Serum levels of miR-135b in patients were also lower too. Using the bleomycin model of fibrosis in mice measurement of miR-135b also demonstrated lower levels compared to control treated mice.

**Conclusions:** IL-13 is a critical molecule in mediating fibrosis and is independent of TGF-β signalling. IL-13 is dependent on STAT6 signalling to mediate its effects in dermal fibroblasts. Epigenetic modifications also play a role as miR135b targets STAT6 and is reduced in scleroderma patient's cells and serum. The measurement of miR-135b is sera could be a potential biomarker.

**8. Pilot study of multispectral imaging to measure skin oxygenation in healthy controls and in patients with systemic sclerosis**

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**Introduction:** Systemic sclerosis (SSc) causes blood flow dysfunction in skin and internal organs. There is a drive to identify non-invasive measures of pathophysiology to facilitate clinical trials of novel drugs. Multispectral imaging (MSI) provides images taken at different wavelength bands allowing measurement of skin properties such as oxygenation. Our aim was to ascertain whether MSI could be used to measure changes in oxygenation, in response to digital occlusion, in healthy controls (HC) and patients with SSc.

**Methods:** 10 healthy controls (HC, median age 40, interquartile range (IQR) [31-47] years), 10 patients with LcSSc (age 58 [53-63] years; duration of Raynaud's phenomenon [RP] 22 [14-27] years; duration of non- RP symptoms 18 [12-22] years) and 7 patients with DcSSc (age 54 [49-60]; duration of RP 4 [2-6] years; duration of non-RP symptoms 3 [2-4] years) were recruited for the study. Participants underwent multispectral imaging of the hand at baseline, during digital arterial occlusion (2 mins, 200 mmHg), at occlusion release and 1 min after release. Multispectral data (500–710 nm) were collected. All patients gave informed consent. Data were analysed using custom software. The change on release and 1 minute later was compared between groups using linear regression. Differences in overall response to the protocol were assessed with a test of interaction in a generalised least squares model.

**Results:** All groups showed decreased oxygenation during occlusion from baseline (Table 1). Upon occlusion release hyperaemic oxygenation increase was observed in all groups. The greatest decrease in oxygenation under occlusion and largest reactive hyperaemia was in the HC group. One minute after occlusion release, oxygenation levels were decreasing back to baseline in the HC group but not the SSc groups. MSI was able to measure differences in the amount of change in oxygenation levels between groups ($p<0.0001$).

**Conclusion:** HC undergo significantly increased oxygenation during hyperaemic response and SSc groups experience a prolonged return to baseline; both may indicate structural vessel changes in patients with SSc. MSI is able to detect differences in oxygenation changes between groups implying sensitivity to change and is a suitable technique for skin oxygenation measurement.

*Funding: Arthritis Research UK and EPSRC.*

Table1. Shows mean skin tissue oxygenation measurements over a region of interest in arbitrary units across all groups. Change on release ($p < 0.001$) and change 1 minute post-release ($p = 0.02$) were found to systematically differ between groups.

| Group | Oxygenation change on occlusion (occlusion - baseline) | Oxygenation change on release (release-occlusion) | Oxygenation change 1 min post (1 minute-release) | Oxygenation change 5 mins post release (5 mins-1min post release) |
|---|---|---|---|---|
| Healthy<br>Median<br>IQR | <br>-1.63<br>-1.5 to -1.8 | <br>3.5<br>3.3 to 3.5 | <br>-0.9<br>-1.1 to -0.6 | <br>-0.3<br>0.1 to -0.5 |
| lcSSc<br>Median<br>IQR | <br>-1.4<br>-1.0 to -1.8 | <br>2.0<br>0.5 to 2.2 | <br>0.2<br>-0.2 to 0.4 | <br>-0.3<br>0.1 to -0.4 |
| dcSSc<br>Median<br>IQR | <br>-0.9<br>-0.5 to -1.3 | <br>2.2<br>1.1 to 2.3 | <br>0.1<br>-0.1 to 0.7 | <br>-0.6<br>-0.34 to -0.72 |

## 9. Investigation of the relationship between fibrosis and microvascular structural and functional changes in systemic sclerosis

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**Aims:** Systemic sclerosis (SSc) is a multisystem connective tissue disease causing fibrosis, microvascular dysfunction and changes to capillary structure. Changes occur both internally and in skin; the skin allows us to monitor these changes non-invasively. SSc is sub-grouped according to skin involvement into Limited or diffuse cutaneous SSc (lcSSc or dcSSc). The data from this cross-sectional study represents first year data from a longitudinal study to assess relationships between structural and functional microvascular abnormalities and fibrosis.

**Methods:** Hand images from 58 healthy controls, 84 patients with lcSSc and 40 patients with dcSSc were assessed. Measurements were taken, at the finger, of dermal thickness (high frequency ultrasound), relative blood flow (laser Doppler imaging post local heating), and capillary density (nailfold capillaroscopy).

**Results:** Dermal thickness (mm) was lower in the control group than SSc groups: Controls, median 1.31 (IQR 1.02-1.46); lcSSc, 1.40 (1.19-1.58) and dcSSc, 1.39 (1.23-1.61). Blood flow (arbitrary perfusion units) was higher in the control group than SSc groups: Controls, 433.60 (314.62-620.05); lcSSc, 407.20 (283.15-542.38); dcSSc 318.60 (200.90-529.75). Capillary density at the nailbed was higher in the control group than SSc groups: Controls 13.34, (10.71-16.58); lcSSc, 8.13 (5.34-10.77); dcSSc, 7.07 (4.64-9.82). Weak negative correlations were identified between dermal thickness and both blood flow (-0.144 [p=0.033]) and nailfold capillary density (-0.249 [p=0.000]).

**Conclusions:** Patients with SSc have increased dermal measurements due to fibrosis and decreased perfusion and capillary density due to microvascular dysfunction and structural changes. As fibrosis increases both perfusion and capillary density decreases, implying a relationship between both aspects of the SSc disease process.

*Funding: Arthritis Research UK.*

## 10. Soluble small molecule inhibitors of the IL-1 receptor abrogate fibrosis

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**Background:** Scleroderma (SSc) is a fatal fibrotic disease of the vasculature, skin, and internal organs. It has been classified an orphan disease by the FDA. It is the most lethal rheumatic disease and is associated with significant loss of health related quality of life. Patients die of scarring and dysfunction of organs due to uncontrolled expression of collagen and other extracellular matrix proteins. Recently, we found that fibrosis in SSc is dependent on the inflammasome regulation of IL-1 and proposed that by blocking the IL-1 receptor we would abrogate fibrosis.

**Materials and methods:** Using a hybrid structure based in silico screening method, we identified soluble small molecules that bind into the IL-1 receptor at the same sites as the human IL-1 receptor antagonist. The lead candidate molecules were tested against IL-1 and SSc fibroblasts cell lines. Analogs of the lead molecules were also selected based on structure, solubility, and drug like properties; and assessed for $IC_{50}$ in bleomycin induced hydroxyproline assays.

**Results:** In validation studies, we demonstrate in normal fibroblasts that we can block IL-1α and IL-1β induction of collagen. Furthermore, we found that we could down regulate IL-6 as this is dependent on IL-1 signaling. In SSc fibroblasts, we show amelioration of collagen synthesis measured by hydroxyproline and western blotting. Secondary screening for analogs of the lead molecules resulted in several molecules with low nanomolar $IC_{50}$ values that can be further developed for in vivo proof of concept efficacy studies.

**Conclusions:** The recent development of biological drugs (Kineret™, Rilonacept™, Ilarist™) inhibiting IL-1 signaling has been successful in treating a number of autoimmune and orphan diseases, however these drugs are not without adverse events such as injection site reactions and immunogenicity. We have identified soluble small molecules and these have advantages over pre-existing biologicals in that they do not require injection, they will not elicit immunogenicity, and can be used in the nanomolar range. We demonstrate efficacy using the lead candidate molecules on SSc fibroblasts that results is a significant reduction in collagen synthesis, suggesting that the IL-1 receptor could be a successful therapeutic target for the treatment of SSc.

## 11. Statins and Microvascular Endothelial Function in Early Diffuse Systemic Sclerosis

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**Background:** Systemic sclerosis (SSc) pathogenesis involves a complex interplay of vascular injury, immune system activation and excessive fibrosis. Current theories suggest endothelial cell injury and dysfunction as an inciting event. Statins have beneficial pleiotropic effects on the vasculature and may modify all three aspects of SSc pathogenesis. We hypothesized that statins may modify endothelial dysfunction in patients with early diffuse SSc.

**Materials and methods:** We identified patients presenting to a Scleroderma Clinic with early diffuse SSc, defined as < 2 years since the first symptom attributable to SSc and skin thickening proximal to the elbows or knees. We assessed microvacular endothelial dysfunction using three methods: 1) Endo-PAT™, which measures the pulse wave amplitude before and after reactive hyperemia with a pneumatic probe placed on the index finger, 2) laser speckle contrast imaging (LSCI) of the hand using the PeriCam PSI system™ before and after hyperemia, and 3) LSCI before and after nitroglycerin (NTG). LSCI assesses continuous measurement of blood perfusion at the capillary level. EndoPAT calculates a reactive hyperemia index (RHI). A RHI of < 1.67 has been previously validated as reflecting endothelial dysfunction.

**Results:** Thirteen SSc patients participated. The mean age was 50, 62% were female and the mean disease duration was 1.3 (± 0.5) years. 12/13 had Raynaud phenomenon, 4 had a history of digital ulcers, but no active ulcers. In those patients on statins (for hyperlipidemia) the EndoPAT mean RHI was significantly higher at 1.66±0.47 compared to SSc patients not on a statin (0.99±0.53 (p=0.04)). Using the RHI cut-off of < 1.67 for endothelial dysfunction, only 40% of SSc patients on statins had microvascular endothelial dysfunction compared to 100% not on a statin (p=0.03). Of 5 SSc patients on statins and 8 patients not on statins, those not taking statins had a larger response to exogeneous NTG (Figure 1).

**Conclusions:** Patients with early diffuse SSc taking statins for hyperlipidemia had better microvascular endothelial function than those not taking a statin. The data showing less microcirculatory flow response to exogeneous NO in statins users suggests that statins may modify microvascular endothelial dysfunction through greater endogeneous NO availability.

## 12. Expression and function of the P2X7 receptor in dermal fibroblasts from patients with Systemic Sclerosis

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**Background:** Systemic Sclerosis (SSc) is a connective tissue disease characterized by vasculopathy in multiple vascular beds, immunological alterations and fibrosis in the skin and visceral organs.

P2X7 receptor (P2X7R) is a nucleotide-gated ion channel, chiefly involved in the inflammatory response triggered by passive release of ATP from damaged cells. It is largely expressed in monocytes and plays a key role in promoting the release of IL-1β and IL-6. As recent evidence suggests that P2X7R is also expressed in fibroblasts thus possibly having a role in promoting tissue fibrosis in different body districts, we hypothesized that the P2X7R may be involved in the pathogenesis of SSc.

Aim of the study was to evaluate the expression and function of the P2X7R in cultured human dermal fibroblasts (HDFs) deriving from SSc patients in comparison with normal HDFs.

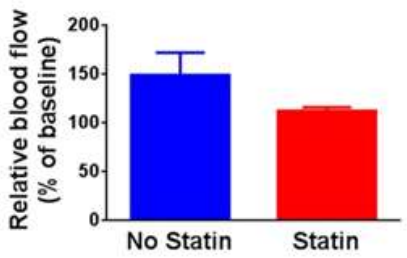
**Materials and methods:**

HDFs were obtained by punch biopsy from the forearm of 7 patients affected by limited SSc and from 6 healthy volunteers. P2X7R expression was evaluated by flow cytometry together with the effects of P2X7R agonist and/or antagonist on Ca2+ flux (single-cell fluorescent microscopy), on collagen production (ELISA) and cytokines (IL-1β, IL-6) release (ELISA).

**Results:** When compared to normal human dermal fibroblasts, SSc fibroblasts displayed (i) a higher P2X7R surface expression, (ii) an enhanced P2X7R function in terms of both Ca2+ flux and collagen production. No significant changes in supernatant levels of IL-1β, but an increase of IL-6 was observed.

**Conclusions:** Our results seem to provide the evidence that in SSc fibroblasts both the expression and function of the P2X7R are increased. In particular, by enhancing collagen production from SSc fibroblasts, P2X7R may promote the fibrotic process associated with the disease. These findings increase our knowledge on the pathophysiology of SSc, also suggesting a role for the P2X7R as a new attractive target for pharmacological modulation.

Figure 1: Statins reduce microcirculatory flow response to sublingual NTG in SSc patient



**13. Low levels of S-nitrosothiols in plasma of patients with Raynaud's phenomenon and Systemic Sclerosis**

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**Background:** Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterised by vasculopathy, prolonged inflammation and extensive tissue remodelling leading to excessive scaring and fibrosis.

The involvement of free radical nitric oxide (NO) has been suggested in the pathogenesis of SSc (1). NO, generated constitutively is short lived (1< sec) and is involved in cell signalling and redox regulation of vascular function. S-nitrosothiols (RSNOs) are bioactive forms of NO and at physiological pH stabilize bioactivity of NO. RSNOs may be involved in protein posttranslational modifications and regulate the function of broad spectrum of proteins in the cells by S-nitrosylation (2), the addition of an NO group to a Cys (cysteine) thiol of protein. A breakdown product of RSNO is $NO_2^-$.

**Objective:** In this study we have measured plasma concentrations of circulating S-nitrosothiols (s-nitrosoalbumin) in patients with systemic sclerosis (SSc) and Primary Raynaud's phenomenon.

**Patients and methods**: Venous blood was collected from 16 patients with RP, 45 with SSc; 34 limited SSc (lcSSc) and 11 diffuse cutaneous disease (dcSSc)). Twenty-six healthy subjects were used as controls. Plasma S-nitrosothiol concentrations were measured by chemiluminescence (3). The measurements were related to the extent of biological age, capillary/skin scores and disease duration.

**Results**: In the patient groups, plasma RSNO levels with RP (2.12 $\pm$ 0.6nM) and those in SSc (1.17 $\pm$ 0.5nM) compared to the concentrations in control subjects (6.0$\pm$ 0.8nM). In RP and SSc, plasma S-nitrosothiols was often below the level of detection (1nM).

**Conclusions:** Low level of S-nitrosothiols observed in the blood of RP and SSc patients may play a key role in its progress with the profound disturbances in NO metabolism. The evaluation and study of the concentration of RSNOs in plasma of patients in RP and SSc and correlation with some clinical status may help in safe treatment of NO donor drugs. Acknowledgement: We acknowledge the Arthritis Research UK for support and Prof K. Moore for the helpful suggestions.

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**14. Increased CD44v6-NOX4 signaling is involved in pathogenesis of interstitial lung disease**

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**Background:** Increase in TGFβ1 results in activation of the CD44v6-hyaluroan pathway and leads to activation of myofibroblasts that accumulate at sites of tissue remodeling in lung fibrosis [1]. CD44 is an important reactive oxygen species (ROS)-sensitive gene expressed during inflammation in vascular cells [2]. NADPH-oxidase-4 (NOX4)-derived ROS mediates myofibroblast activation during lung injury [3]. Studies have shown that NOX subunits are regulated by AP-1 in vascular cells. However, the effect of AP-1 on the regulation of NOX4/p22PHOX and CD44v6, and whether CD44v6 regulates NOX4-mediated fibrogenic responses in interstitial lung disease (ILD) is unknown.

**Materials and Methods:** Using isolated lung fibroblast culture from the murine model of lung fibrosis, and from the ILD patients, we investigated the potential role of CD44v6/NOX4 pathway in the regulation of myofibroblast activation in lung injury.

**Results:** Lung fibroblasts treated with TGFβ1 shows a significant increase in mRNA and protein expression of NOX4 and this response requires upregulation of CD44v6 and hyaluronan. This study is the first to identify: 1) increased CD44v6 expression induces NOX4 expression through AP-1 activity in a model of lung fibrosis; 2) In lung fibroblasts exposed to TGFβ1, inhibition of AP-1 related pathways reduces CD44v6 expression and NOX4 activity; 3) NOX4- increases the CD44v6 promoter activity, both of which contribute to a positive feedback loop that amplifies NOX4 expression and enhances myofibroblast apoptotic resistance; 4) blockade of CD44v6-hyaluronan interaction inhibits TFβRI kinase activity and this inhibition can be recovered by exogenous addition of TGFβ1 leading to Smad3 phosphorylation necessary for stimulation of NOX4 in lung fibroblasts; and 5) Genetic or peptide based targeting of CD44v6 abrogates NOX4 expression and fibrogenic responses in cell fibrosis stimulated by NOX activity in fibrogenic lung tissue of ILD Patients.

**Conclusions**: The physical interaction of AP-1 with CD44v6 gene promoter facilitates NOX regulation. These studies support a function for CD44v6 in lung tissue fibrogenesis and CD44v6 may represent a potential therapeutic target for the treatment of ILD fibrosis.

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**15. Increased Hyaluronan-CD44 signaling regulate cellular fibrosis stimulated by NADPH Oxidase in sclerotic skin fibroblasts in SSc patients**

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**Background:** Systemic sclerosis (scleroderma, SSc) is a connective tissue disorder with excessive deposition of extracellular matrix (ECM) molecules, resulting in fibrosis of skin and other organs. Among ECM proteins, the transforming growth factor β1 (TGFβ1) is regarded as a master regulator of the SSc disease process, since it potently accelerates skin fibrosis by inducing differentiation of fibroblasts to myofibroblasts, which increases type 1 collagen (COL1A1) production leading to fibrosis (1, 2). The pathways for TGFβ1-induced myofibroblast differentiation are complex and not completely understood. Thus, there is a great need to clarify aspects of myofibroblast differentiation underlying the pathogenesis of SSc.

**Materials and Methods:** Using isolated skin fibroblast culture from the murine model of skin fibrosis, and from the Scleroderma patients, we investigated the potential role of hyaluronan/reactive oxygen species (ROS) pathway in the regulation of myofibroblast activation in skin injury.

**Results:** Our recent published studies *in vitro* and *in vivo* show that hyaluronan-CD44v6 signaling is upregulated in SSc lung fibroblasts and that this signaling is critical for induction of COLA1 and α-smooth muscle actin (α-SMA) (2). Our recent studies provide insights into the hyaluronan-CD44 signaling in the context of activation of SSc skin fibroblasts (SScSFbs) in scleroderma. Our data indicate that : (i) The pathway by which TGFβ1 stimulates NADPH OXIDASE (NOX) derived ROS requires upregulation of synthesis of hyaluronan-synthase-2 (HAS-2); (ii) TGFβ1-induced HAS-2 expression is mediated by transcription factor SP-1 in a NOX dependent manner; (iii) GKT137831 a specific inhibitor of NOX1- and NOX4-containing NADPH oxidase activity, or hyaluronan-CD44 antagonist CD44v6shRNA attenuated ROS generation, and decreased HAS2 gene expression; and (iv) a hyaluronan-CD44v6 pathway upregulates RhoA in order to induce the NOX/ROS mediated fibrogenic response in SSc skin tissue and fibroblasts.

**Conclusion:** These results represent, to our knowledge, the first report to provide one of the mechanisms whereby increased hyaluronan-CD44v6 signaling regulate cell fibrosis stimulated by NOX activity in sclerotic skin fibroblasts of Scleroderma Patients.

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**16. Reduction of T-bet in the nucleus leads to up-regulation of GATA-3 and interleukin-13 in CD8+ T cells from systemic sclerosis patients**

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**Background:** The type-2 cytokine interleukin (IL)-13 is a main pro-fibrotic factor in systemic sclerosis (SSc), a connective tissue disease characterized by vasculopathy, inflammation and fibrosis. In previous studies we demonstrated that IL-13 over-production by SSc CD8+ T cells is caused by up-regulation of the Th2-specific transcription factor GATA-3. Here we investigated the molecular mechanism underlying the GATA-3 up-regulation in these cells. We focused on the role played by the Th1-specific transcription factor, T-bet, which induces IFN-$\gamma$ production and inhibits Th2 cytokines, including IL-13, by antagonizing GATA-3 expression and/or function.

**Materials and Methods:** Quantitative PCR, flow cytometry, western blot, and immunohistochemistry were used to determine expression of nuclear factors by CD8+ T cells from the blood and skin of SSc patients. Co-immunoprecipitation and fluorescent microscopy were employed to assess physical interactions between proteins. Specific siRNAs were used for gene silencing, and chromatin immunoprecipitation analysis was employed to determine binding of GATA-3 to the IL-13 promoter.

**Results:** CD8+ T cells from patient blood express high levels of IL-13 and GATA-3 but similar levels of IFN-$\gamma$ and T-bet compared to controls. However, the levels of the active phosphorylated form of T-bet as well as the binding between T-bet and GATA-3 are reduced in the nucleus of SSc CD8+ T cells, allowing more GATA-3 to bind to the IL-13 promoter and to induce IL-13 expression.

**Conclusions:** Increased IL-13 expression by SSc CD8+ T cells results from reduced down-regulation of GATA-3 by T-bet. Our new insights will establish novel biomarkers of immune dysfunction in SSc patients that can be used as therapeutic targets.

**17. CD8+CD28- T cells are cytotoxic and pro-fibrotic in patients with systemic sclerosis**

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**Objective**: Loss of CD28 expression by CD8+ T cells occurs with age and during chronic inflammatory conditions. CD8+CD28- T cells are a heterogeneous cell subpopulation whose function ranges from immunosuppressive to effector. We showed previously that Interleukin (IL)-13-producing CD8+ T cells are implicated in the pathogenesis of systemic sclerosis (SSc). Here we analyzed the role of CD8+CD28- T cells in SSc pathogenesis in an age- and disease-controlled cohort of patients.

**Methods:** Flow cytometry was employed to determine cell surface phenotype, cytokine production and cytotoxicity function of peripheral blood CD8+CD28- T cells from SSc patients and healthy controls. Proliferation and apoptosis properties of CD8+ T-cell subsets were measured by CFSE and Annexin V staining. Immunofluorescence and immunohistochemistry were used to assess expression of cytokines and markers of cytotoxicity by CD8+ T cells in the affected skin of patients.

**Results**: We found that the proportion of CD8+CD28- T cells is increased in patients with SSc independent of patient age and correlates with the extent of skin fibrosis. SSc CD8+CD28- T cells are found in the effector and effector/memory subsets, secrete IL-13, and are cytotoxic. Moreover, they exhibit normal levels of proliferation and susceptibility to apoptosis. Finally, IL-13-producing CD8+ T cells that express markers of cytotoxicity are found in the sclerotic skin of patients.

**Conclusions**: We provide new insights into the pathogenesis of SSc by identifying a pathogenic CD8+ T-cell subset characterized by profibrotic IL-13 overproduction and direct cellular cytotoxicity. These novel molecular targets may open avenues for future therapeutic intervention.

**18. Poly(ADP-ribose) polymerase-1 (PARP-1) suppresses the profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis**

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**Background:** The enzyme poly(ADP-ribose) polymerase-1 (PARP-1) transfers negatively charged ADP-ribose units from the donor β-NAD onto various substrate proteins either as mono- or oligomeric moieties or as linear or branched poly (ADP-ribose) (PAR) chains. Those modifications can have pronounced regulatory effects on the half-life or the enzymatic activity of target proteins. Recent studies demonstrated that PARP-1 can poly(ADP-ribosyl)ates (PARylates) members of the Smad family of transcription factors. However, the role of PARP1 in the pathogenesis of SSc has not been investigated.

**Materials and Methods:** The expression of PARP1 in human skin and in experimental fibrosis was determined by qPCR and immunohistochemistry. TGFβ signalling was analysed by Smad reporter assays and target gene analysis after 1mM selective PARP1 inhibitor 3-Aminobenzamide (3AB). Bleomycin-induced skin fibrosis and Tsk-1 mice were used to evaluate the effect of PARP deficiency and PARP inhibition (10mg/kg/d 3AB) in vivo.

**Results:** Decreased expression of PARP1 was detected by immunohistochemistry in skin sections of SSc patients, particularly in fibroblasts. Inhibition of PARylation by 3AB augmented the stimulatory effects of TGFβ on fibroblasts in vitro. PARP1 inhibition increased Smad dependent transcription in reporter assays and promoted the transcription of TGFβ/Smad target genes. Treatment with 3AB also stimulated the collagen release and fostered the expression of contractile proteins with increased expression of α-smooth muscle actin (α-SMA) and enhanced formation of stress fiber formation compared to fibroblasts stimulated with TGFβ alone. Inhibition of PARylation also exacerbated experimental fibrosis in vivo. Treatment with 3AB induced a more severe fibrotic response to bleomycin with increased dermal thickening (by up to 103%, $p<0.0001$), hydroxyproline contents and myofibroblast counts compared to control mice ($p<0.0001$ and $p=0.0059$). Inhibition of PARylation also strongly exacerbated fibrosis in Tsk-1 mice. Meanwhile, after bleomycin injection, dermal thickening, hydroxyproline contents and myofibroblast counts of PARP1 knockout mice are increased by 85% ($p=0.0046$), 67% ($p=0.0098$) and 56% ($p=0.0043$) compared to wild-type mice.

**Conclusion:** We demonstrate that PARP1 negative regulates canonical TGFβ signalling. The down-regulation of PARP1 in SSc fibroblasts may thus directly contribute to hyperactive TGFβ signalling and to persistent fibroblast activation in SSc.

**20. Subclinical biventricular systolic function is impaired in patients with systemic sclerosis with real time 3-D Echpcardiography: 1 year follow-up study**

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**Background**: Silent myocardial involvement is associated with poor prognosis in patients with systemic sclerosis (SSc). In the present study we aimed to evaluate subclinical left ventricular (LV) and right ventricular (RV) systolic dysfunction in SSc patients without any cardiovascular disease, by using both strain imaging method, "speckle tracking echocardiography" (STE) and real time 3-D Echocardiography.

**Methods**: Fifthy-five SSc patients were screened, 7 patients were excluded because of ischemic heart disease. We studied 48 patients with SSc and 25 age and sex-matched healthy controls(HC), without any cardiac disease and with preserved LV-EF. Conventional echocardiography, STE-based strain imaging and real time 3-D echocardiography (Bothell,WA,USA) were performed to assess biventricular deformation analyse. Association with anti-Scl 70 was sought in patients with SSc.

**Results:** In SSc patients (Female/Male: 44/4) the mean age was 47.7 years. Anti Scl-70 seropositivity was 22 (45.8%). Left ventricular conventional echocardiographic measurements (LV end diastolic diameter, LV end systolic diameter and LV EF) were similar between SSc and HC (table1). Both LV and RV longitudinal peak systolic strain/ strain rate were significantly impaired in SSc, demonstrating subclinical LV and RV systolic dysfunction ($p \leq 0.001$). LVESV was significantly increased in SSc (44.3±7.3 vs 37.8±2.4; SSc vs HC, $p < 0.001$ )

Systolic PAB was negatively correlated with both LV and RV longitudinal peak systolic strain/strain rate (LV: r=-0.552 and r= -0.637, respectively, $p<0.001$ and RV: r=-0.547 and r=-0.638, respectively, p=0.001). Anti Scl -70 positive patients had impaired LV longitudinal peak systolic strain and strain rate values, compared to the others, however the difference did not reach statistical significance (13.01±1.26 % to 13.04±1.90 %, p=0.96 for strain; 0.30±0.06 1/s to 0.31±0.15 1/s, p=0.79 for strain rate). There was a trend for decreasing left ventricular strain and increasing LEVSV in 1 year analysis of SSc patients but it did not reach statistical significance.

**Conclusions**: SSc is associated with myocardial systolic dysfunction. Both deformation analysis by STE-based strain imaging and end systolic left ventricular volume analysis by real time 3-D echocardiography are promising modalities that allow us for non-invasive, comprehensive analysis of early deterioration in biventricular systolic function in patients with SSc.

***Table 1.*** Speckle tracking echocardiography (STE) and real time 3-D echocardiography results of SSc patients and healthy controls.

| | **SSc n=48** | **HC n=25** | **p value** |
|---|---|---|---|
| LV longitudinal peak systolic strain (%) | 13.3± 0.82 | 20.35±3.05 | 0.0001 |
| LV strain rate (1/s) | 0.91±0.21 | 1.70±0.47 | 0.0001 |
| RV longitudinal peak systolic strain (%) | 11.68±1.61 | 14.63±2.35 | 0.001 |
| RV strain rate (1/s) | 0.31±0.01 | 2.73±0.4 | 0.0001 |
| LVEDV (ml) | 104.6±16.2 | 106±17.5 | 0.63 |
| LVESV (ml) | 44.3±7.3 | 37.8±2.4 | 0.0001 |

*Values were presented as mean ±SD. LV; left ventricul, RV; right ventricul, LVEDV;left ventricular end diastolic volume,LVESV; left ventricular end systolic volume*

## 21. The Homeoprotein Engrailed-1 regulates canonical TGF- β signaling in experimental systemic sclerosis

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**Background:** Homeobox containing transcription factor, Engrailed-1 plays a key role in embryonic development and has also been linked to disease, including cancer. EN-1 is induced by proinflammary cytokines and oxidative stress which play an important role in Systemic Sclerosis

**Methods:** The expression of EN-1 in skin tissue and in human dermal fibroblasts was determined by real-time PCR, Western blot and immunohistochemistry. Knock-down and overexpression strategies were used to evaluate the effect of EN-1 on fibroblast activation. The outcome of mice with fibroblast-specific knockout of EN-1 was evaluated in bleomycin-induced skin fibrosis, fibrosis induced by overexpression of a constitutively active TGF-β receptor I (TBRIact) and in the Tsk model which resembled the later stages of SSc.

**Results:** An increased expression of EN-1 was detected in the upper layer of the dermis of SSc patients on fibroblasts double stained for EN-1 and anti-prolyl-4-hydroxylase. EN-1 expression was induced by TGF-β in cultured fibroblasts and treatment with the TBR inhibitor SD-208 prevented the induction of EN-1 in experimental fibrosis. EN-1 fibroblast specific knockdown fibroblasts were less sensitive to the pro-fibrotic effects of TGF-β with impaired induction of collagen mRNA and protein. In the model of bleomycin-induced fibrosis dermal thickening, hydroxyproline content and myofibroblast counts were significantly decreased in EN-1 knockdown mice compared to wildtype littermates. EN-1 knockdown mice were also protected from TBRIact-induced fibrosis and genetic fibrosis in Tsk model. Function studies demonstrated that EN-1 is induced by Smad3 and regulates the pro-fibrotic effects of TGF-β.

**Conclusion:** We demonstrate for the first time a role of EN-1 in fibroblast activation and tissue fibrosis in SSc. Inactivation of the EN-1 reduced the stimulatory effect of TGF-β on fibroblasts by interfering with canonical Smad and protected from experimental fibrosis in different mouse models. Considering the potent anti-fibrotic effects observed in this study, EN-1 might be a candidate for molecular targeted therapies of SSc.

## 22. M10, a Caspase Cleavage Product of the HGF Receptor MET, Interacts with Smad2 and Demonstrates *In Vitro* and *In Vivo* Anti-Fibrotic Properties

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**Objective:** Hepatocyte growth factor receptor, also known as cellular mesenchymal-epithelial transition factor (c-MET, MET), is an important antifibrotic molecule that protects various tissues, including lung, from injury and **fibrosis. The intracellular cytoplasmic tail of MET contains a caspase-3 recognition motif DEVD-T that** upon cleavage by caspase-3 **generates a 10 amino acid peptide, TRPASFWETS,** designated as "M10". **This study was undertaken to investigate antifibrotic mechanisms of M10 in lung and skin fibroblasts.**

**Materials and Methods:** Lung fibroblasts were derived from lung tissues obtained at autopsy. Skin fibroblasts were isolated from the biopsy samples obtained from the involved forearm skin of SSc patients and from age-, sex-, and race-matched healthy adult donors. Antifibrotic *in vivo* effects of M10 (10 mg/kg, intraperitoneal, every 48h) were studied in the bleomycin-induced murine model of pulmonary fibrosis. Potential peptide-protein interactions were modulated *in-silico* and investigated by co-immunoprecipitation and protein interaction assays. Protein localization, expression, and phosphorylation were determined by immunoblotting, immunohistochemistry, and immunofluorescent studies.

**Results:** M10 contains at its N-terminus the uncharged amino acid proline (P) directly after a cationic amino acid arginine (R) which favors the transport of the peptide through membranes. M10, when added to cell culture medium, remains in the cytoplasm and nuclei of cells for up to 24 hours. M10 effectively decreases collagen, connective tissue growth factor (CTGF, CCN-2), and smooth muscle α-actin (SMA) in scleroderma and TGFβ-stimulated normal lung and skin fibroblasts. M10 interacts with the MH2 domain of Smad2 and inhibits TGFβ-induced Smad2 phosphorylation, suggesting that the antifibrotic effects of M10 are mediated in part by counteracting Smad-dependent fibrogenic pathways.

In the bleomycin-induced murine model of pulmonary fibrosis, M10 noticeably reduced fibrotic lung parenchyma. A semi-quantitative evaluation of histopathology by Ashcroft scale demonstrated a substantial decrease in bleomycin-induced fibrosis of M10-treated mice as compared to mice treated with scrambled peptide (5.63±1.72 and 1.67±1.01, respectively), reflecting a pronounced antifibrotic effect of M10.

**Conclusions:** M10 peptide interacts with Smad2 and demonstrates strong antifibrotic effects *in vitro* and *in vivo* in an animal model of lung fibrosis and should be considered as a potential therapeutic agent for systemic sclerosis and other fibrosing diseases.

## 24. Systems-genetics approaches in macrophages during fibrosis

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**Introduction:** Disruption of the normal wound healing cascade contributes to the development of fibrotic diseases. Inflammation is common to all major types of fibrosis and there is now mounting evidence that fibrosis does not occur in the absence of a primary inflammatory insult. Macrophages are immune cells playing an effector role in the inflammatory pathway leading to fibrosis. Their activation has been associated with physiological wound healing and many fibrotic diseases including scleroderma.

**Aims:** This study aims to correlate gene expression and genotype in bone marrow derived macrophages (BMDM) from 230 genetically outbred mice showing large phenotypic variability in wound healing after ear punch. This will identify macrophage-specific expression quantitative trait loci (eQTLs) and co-expression networks regulating fibrosis. As a translational approach, the same workflow is used to study gene expression and co-expression networks in monocyte-derived macrophages derived from scleroderma patients. These approaches focus on the macrophage function during wound healing (mice) and pathological fibrosis (human scleroderma).

**Methods:** A comprehensive systems-genetics approach is underway where BMDM gene expression (RNA-sequencing), genome variation (low coverage whole-genome sequencing), and quantitative wound healing phenotypes from 115 ‘high-healing’ and 115 ‘low-healing’ outbred mice are integrated. In parallel, RNA-sequencing in macrophages from different groups of scleroderma patients (limited and diffuse) and healthy controls is first used to identify differentially expressed genes and co-expression gene networks. eQTL mapping will then be performed using Bayesian variable selection approaches.

**Conclusion:** These approaches will reveal new loci associated with inflammation in fibrosis.

**25. EPHRIN-B2: A novel mediator of fibrogenesis**

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**Rationale:** Fibrotic diseases including systemic sclerosis (SSc) are characterized by fibroblast differentation into myofibroblasts and excessive extracellular matrix deposition. Mechanisms driving fibroblast activation are not fully known. Microarray studies show that ephrin-B2 ligand (member of largest subfamily of receptor protein-tyrosine kinases) is overexpressed in SSc and idiopathic pulmonary fibrosis (IPF) fibroblasts. However it's role in fibrogenesis is unknown.

**Methods:** Mice were injected subcutaneously with recombinant ephrin-B2/Fc (100µg/Kg/mouse) daily for two weeks and development of skin fibrosis was assessed. Fibroblast-specific ephrin-B2 KO mice were generated and subjected to bleomycin-induced skin and lung fibrosis models. Ephrin-B2 levels were determined by western-blot and ELISA in BAL fluids from these mice and IPF patients. 96-Multiwell Insert System was used to measure chemotaxis of mouse skin fibroblasts transfected with 20nM siRNA targeting EphB2, EphB3, EphB4 receptors.

**Results:** Ephrin-B2 is overexpressed in SSc fibroblasts compared to controls. Fibroblast-specific ephrin-B2 KO mice were significantly protected from bleomycin-induced lung and skin fibrosis, associated with reduced collagen synthesis and profibrotic gene expression. Further, we identified a soluble form of ephrin-B2 (sEphrin-B2) containing the ectodomain of the full length protein, which showed elevation in BAL fluid from bleomycin-challenged mice. Soluble ephrin-B2 induced fibroblast chemotaxis through EphB3/EphB4 but not EphB2 receptor. Further, mice treated (SQ) with recombinant mouse ephrin-B2/Fc, comprising of just the ectodomain of this protein, exhibited significant skin fibrosis associated with enhanced collagen deposition. Our preclinical studies also indicate that sEphrin-B2 levels are elevated in both BAL and plasma from IPF patients, suggesting its potential as both biomarker and therapeutic target.

**Conclusion:** Our study has identified ephrin-B2 as a novel pro-fibrotic mediator and targeting ephrin-B2 ligand or its receptors EphB3/EphB4 may serve as a new therapeutic strategy in counteracting fibrosis in SSc and IPF diseases.

**27. Title: Proving the Fibrotic Effect of the C33S Mutation in *Col3a1* and the Potential Role of the TWEAK/FN14 Pathway.**

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The tight skin 2 (Tsk2/+) mouse is a genetic model of systemic sclerosis (SSc). This model has several similarities to human disease including tight skin, increased collagen, and alterations in the extracellular matrix. In this mouse, increased collagen is detectable in the skin of 10 week mice, but an earlier 'tight' phenotype can be detected at 2 weeks. Until recently, the mutation responsible for the Tsk2/+ phenotype had yet to be elucidated. In collaboration with the Whitfield and Ehrlich labs we found a SNP within the second exon of collagen alpha-1(III) chain (*Col3a1*) that causes a cysteine to serine (C33S) amino acid change and is responsible for the Tsk2/+ phenotype.

***Confirming lethality****:* To confirm that the C33S mutation is responsible for the lethality of our *Tsk2/Tsk2* homozygotes, we crossed our mouse to a $Col3a1_{HET}$, hypothesizing that without a normal Col3a1 allele $Tsk2/Col3a1_{KO}$ offspring won't be viable. Indeed, this mating produced no $Tsk2/Col3a1_{KO}$ offspring.

***Confirming fibrosis:*** To confirm that $Col3a1_{Tsk2}$ could induce fibrosis we transfected $Col3a1_{KO}$ fibroblasts with $Col3a1_{WT}$ or $Col3a1_{Tsk2}$ plasmid vectors. $Col3a1_{Tsk2}$ transfectants had increased mRNA and protein expression of COL1A1 (a known fibrosis meditator), mRNA expression of pro-fibrotic signatures, and phosphorylated p38. Furthermore inhibition of p38 decreased protein levels of COL1A1 in $Col3a1_{Tsk2}$ transfectants, indicating several potential pathways of interest including TGF-β and TWEAK/FN14. These pathways are altered i*n vivo* in Tsk2/+ mice and are up-regulated in human scleroderma. Both of these pathways can phosphorylate p38 and we are exploring if inhibition of these pathways can prevent the pro-fibrotic signature in $Col3a1_{Tsk2}$ transfectants. Additionally, we have crossed Tsk2/+ mice to $Fn14_{KO}$ mice to determine if the TWEAK/FN14 fibrosis pathway is necessary for development of Tsk2/+ fibrosis.

***Conclusions***: We have confirmed that the C33S SNP in *Col3a1* is responsible for lethality by a cross to a $Col3a1_{HET}$ mouse. We have also confirmed that transfection of $Col3a1_{Tsk2}$ causes an increase in fibrotic signatures, proving this mutation can induce a pro-fibrotic phenotype. Early work shows that the TWEAK/FN14 and TGF-β pathways are up-regulated in *Tsk2*/+ mice and that their interruption may be key to inhibiting fibrosis in this mouse.

**28. Mitochondria contribution to oxidative stress in fibroblasts isolated from patients affected by Systemic Sclerosis**

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**Background:** Oxidative stress plays an important role in the development of fibrosis under various pathological conditions. Fibroblasts isolated from patients with Systemic Sclerosis (SSc) overproduce reactive oxygen species (ROS), overexpress type I collagen and α-smooth muscle actin (α-SMA) and show DNA damage and activation of checkpoint kinase ATM (1, 2). NADPH oxidase (NOX) is an important source of ROS, and its overactivity or overexpression are often associated with chronic diseases, characterized by tissue damage and fibrosis. Recently, we have demonstrated that NOX2 and NOX4 are critical components of NADPH oxidase complex in SSc fibroblasts and ROS generated by NOX play a primary role in the pathological activation of dermal fibroblasts (3). Since a recent study reported that NOX4 localizes to membranes and mitochondria and contributes to the generation of mitochondrial ROS (4), we investigated whether mitochondria also play a role in ROS production in SSc fibroblasts.

**Methods:** ROS production was analysed by confocal microscopy using dihydroethidium (DHE, to assess all intracellular superoxide), or MitoSOX™ Red (to detect mitochondria superoxide), or using a microplate reader following Amplex® Red kit protocol (to measure $H_2O_2$). Mitochondria were purified using a mitochondria isolation kit. For protein expression, mitochondrial and cytosolic fractions were subjected to western blot with specific antibodies.

**Results**:SSc fibroblasts incubated with the mitochondria-targeted antioxidant MitoQ showed a reduced MitoSOX™ Red staining, and a partially decreased DHE fluorescence, suggesting that mitochondria contribute to the redox state in SSc fibroblasts. Mitochondria purified from SSc fibroblasts generated significantly higher levels of ROS compared to controls. Incubation of normal cells with PDGF, a profibrotic cytokine able to activate a SSc-like phenotype, led to a significant increase of total and mitochondrial ROS levels compared to unstimulated cells. Mitochondrial and cytosolic fractions from SSc and activated normal fibroblasts were also analyzed to evaluate different protein expression patterns.

**Conclusions:** In this study we demonstrated that mitochondria contribute to the abnormal redox state of SSc fibroblasts and activated normal cells. Further studies may clarify whether mitochondrial ROS are generated by a mitochondrial NOX isoform or are the result of the interplay between mitochondria and NOX enzymes located outside the organelles.

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## 29. Effect of *MUC1* genotype on KL-6 levels in SSc-ILD

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**Background:** More than 50% of patients with systemic sclerosis develop interstitial lung disease (SSc-ILD). SSc-ILD disease behaviour varies widely from only limited pulmonary involvement with ongoing stability to severe rapidly progressing pulmonary fibrosis. Development of a tool to predict disease progression, and thus inform treatment regimens, in individual patients is therefore imperative. Elevated serum levels of Krebs von den Lungen-6 (KL-6), a glycoprotein expressed by type II alveolar cells, are associated with the presence and severity of pulmonary fibrosis in SSc-ILD, making it a candidate bio-marker for use in a prognostic index. The rs4072037 single nucleotide polymorphism (SNP) in the KL-6 gene *MUC1* has been associated with altered KL-6 serum levels.

**Materials and Methods:** SSc-ILD patients attending clinics at the Royal Brompton and Royal Free Hospitals, London were genotyped for rs4072037 (n=241). Serum KL-6 levels were measured by ELISA (n=160). Genotype frequencies were compared to those published by the International HapMap project, and compared between patient sub-groups by chi-squared tests. Effect of genotype on KL-6 levels was assessed by a Mann-Whitney test and multivariate regression analysis.

**Results:** Genotype and allele frequencies of rs4072037 in the UK SSc-ILD cohort were similar to those in the HapMap population of European descent. No genotype or allele differences were observed in the patient cohort according to SSc cutaneous type, or autoantibody. KL-6 levels were significantly higher in patients carrying the C allele (median ± interquartile rage) (782 IU/ml ± 438-1491) than those not carrying the C allele (438 IU/ml ± 280-719), p=0.002. On multivariate regression analysis, genotype remained independently correlated with serum KL-6 levels, even after adjustment for age, gender, smoking status, and either CPI or extent of lung fibrosis on HRCT (p≤0.001 and p≤0.005, respectively).

**Conclusions:** The results of this preliminary study suggest that the *MUC1* variant does not itself predispose to development of SSc-ILD or to any of the disease sub-types/phenotypes tested. We report that the rs4072037 genotype is significantly associated with serum KL-6 levels independently of a number of patient variables, including ILD severity. Further studies are needed to assess whether this genetic variant needs to be incorporated into diagnostic/prognostic KL-6 thresholds.

## 30. Clinical utility of serial KL-6 measurement in interstitial lung disease associated with systemic sclerosis

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***Objective:*** Interstitial lung disease (ILD) is the leading cause of mortality in patients with systemic sclerosis (SSc). KL-6, a mucin-like glycoprotein highly expressed on type II pneumocytes, is known as a circulating biomarker for lung injury and various forms of ILD. Previous cross-sectional studies have shown that circulating KL-6 is elevated in patients with SSc-ILD and is inversely correlated with percent predicted forced vital capacity (%FVC). We have recently found that elevated KL-6 at baseline is an independent predictor of %FVC decline and mortality. In this study, we investigated clinical utility of serial KL-6 measurement in patients with SSc-ILD.

***Methods:*** We enrolled 46 consecutive patients who were diagnosed as having SSc between 2006 and 2012. These patients were selected from our SSc database, based on disease duration ≤8 years, follow-up period > 3 years, availability of pulmonary function test (PFT) and chest high-resolution CT (HRCT) at diagnosis, and availability of serial PFT results in an interval of <2 years. Serum KL-6 was measured using a monoclonal antibody-based kit (Eidia, Japan) at every visit. All clinical information had been prospectively recorded on the database.

***Results:*** Fifteen (33%) were classified as diffuse cutaneous SSc, and 27 (59%) had ILD evaluated by HRCT. Baseline serum KL-6 was significantly elevated in SSc patients with ILD than in those without ($P < 0.003$). Among patients with ILD, KL-6 was higher in extensive than in limited disease ($P < 0.009$). KL-6 levels were pretty stable during the disease course in patients without ILD, but changed with variation in patients with ILD (37% increased, 30% unchanged, and 33% decreased). There was a trend toward a correlation between rise in KL-6 and reduction of %FVC during follow-up ($P = 0.051$). In 11 patients with ILD who received treatment with oral or intravenous cyclophosphamide, tocilizumab, etanercept, or corticosteroids (>20 mg daily of prednisolone), KL-6 were significantly reduced after treatment ($P = 0.01$), although there was no correlation between changes of KL-6 and %FVC.

***Conclusion:*** Serial measurement of KL-6 may be useful in predicting progression of pulmonary function in patients with SSc-ILD.

**31. The Relationship Between Vascular Biomarkers and Disease Characteristics in Systemic Sclerosis: Elevated MCP-1 is Associated with Predominantly Fibrotic Manifestations**

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**Background:** To determine the relationship between vascular biomarkers reflecting the vascular injury and organ involvement in systemic sclerosis (SSc).

**Materials and Methods:** Seventy-two SSc patients (66 females) fulfilling 2013 ACR/EULAR Criteria were evaluated. Serum samples of patients were collected for flow-cytometric analysis of CD40L, tPA, MCP-1, sE-selectin, IL-8, IL-6, VEGF, sP-selectin, TGF-β1 and VCAM levels (Bender MedSystems) in SSc patients and 20 healthy controls. Results were compared with Pearson chi-square / Fischer's and Mann Whitney-U tests.

**Results:** The mean age of the patients was 44.9 and disease duration from the appearance of first non-Raynaud symptom was 3,2±2.4 years. Of the patients 23 (32%) had diffuse and 49 (68%) limited cutaneous involvement, 15 (21%) were anti-centromere (+) and 34 (47%) were anti-Scl70 (+). In SSc patients, levels of tPA (p=0.02), MCP-1 (p=0.001), sE-selectin (p=0.008), TGF-β1 (p=0.001) were significantly higher, sP-selectin (p=0.011) and IL-8 (p=0.001) were lower than healthy controls (table-1). In the subgroup analyses, levels of MCP-1 was elevated in patients with dcSSc, patients with flexion contractures, FVC<80%, DLCO<80%, pulmonary fibrosis and high acute phase response (p=0.002, p=0.005, p=0.045, p=0.005, p=0.036, p=0.006, respectively), TGF-β1 in patients under immunosupressives (p=0.001), sE-selectin in patients with high acute phase response (p=0.028), sCD40L in smokers (p=0.032) and lcSSc (p=0.011). MCP-1 and sE-selectin levels were weakly correlated with disease activity (r=0.243, p=0.040 and r=0.303, p=0.010).

**Conclusions:** MCP-1, t-PA, TGF-β1, sE-Selectin, sP-Selectin and IL-8 were differently regulated in SSc patients. MCP-1 was the prominent biomarker correlated with manifesatations related to fibrosis and may be a surrogate marker for fibrotic disease activity. Treatment and smoking may have an effect on cytokine profile. Vascular biomarkers can be used to predict the characteristics and severity of SSc warranting prospective studies.

*Notes:*

*The abstract was a poster presentation in ACR 2014*
*This study was supported by Actelion-Turkey*

**Table-1: Vascular Biomarkers in Healthy Controls and Systemic Sclerosis**

| ***Biomarker Levels (mean±SD)*** | **Healthy Controls (n=20)** | **Systemic Sclerosis (n=72)** |
|---|---|---|
| **sCD40L** (pg/ml) | 24620±13051 | 27847±33315 |
| **tPA** (pg/ml) | 2415±1279 | 4036±6961* |
| **MCP-1** (pg/ml) | 907±300 | 1302±550** |
| **sE-selectin** (ng/ml) | 205±78 | 269±106** |
| **IL-8** (pg/ml) | 49±73** | 22±80 |
| **IL-6** (pg/ml) | 0 | 0.6±2.8 |
| **VEGF** (pg/ml) | 704±363 | 776±591 |
| **sP-selectin** (ng/ml**)** | 364±137* | 287±86 |
| **TGF-β1** (pg/ml) | 2421±4785 | 8277±8592** |
| **VCAM** (pg/ml) | 3231±1435 | 3945±1754 |

**p<0.05, **p<0.01 When healthy controls and sytemic sclerosis patients were compared with Mann-Whitney test*

**32. Integrative, multi-organ network analysis of systemic sclerosis reveals a macrophage signature associated with disease severity**

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**Background:** Systemic sclerosis (SSc) is an autoimmune disease characterized by multi-organ involvement and clinical heterogeneity. Recent efforts have produced an unprecedented amount of SSc gene expression data from different tissues and sampling distinct SSc-associated pathophenotypes. Simultaneously, “big data” analysis has yielded powerful tools to infer tissue-specific pathobiology. Here, we present an integrative meta-analysis of SSc microarray data that identifies common disease drivers and leverages the different SSc disease stages that have been sampled in expression studies to infer the sequence of pathological events. In particular, we present a model of SSc-associated pulmonary fibrosis (SSc-PF) progression—a major cause of SSc mortality with no disease-modifying treatment.

**Materials and methods:** We employed a novel data mining procedure that identified conserved coexpression patterns between ten datasets from four different tissues (skin, lung, esophagus, blood) with multiple clinical manifestations (pulmonary arterial hypertension [PAH], PF, limited and diffuse subtypes) represented. We identified expression patterns that were conserved across all solid tissues and were upregulated in pulmonary manifestations of SSc. We used these modules to query tissue-specific gene-gene interaction networks and analyzed the resulting lung- and skin-specific networks to infer common fibro-inflammatory pathways as well as distinct tissue-specific signatures.

**Results:** We identified similar gene expression patterns underlying PAH and PF in lung and the inflammatory molecular subsets in skin and esophagus indicative of macrophages capable of participating in extracellular matrix (ECM) remodeling. In-depth study of the tissue-specific networks revealed a coupling of inflammatory and ECM processes.

Projection of genes that were upregulated in early and late SSc-PF onto the lung network emphasized an alveolar macrophage lipid trafficking signature that was up in early, but not late disease. We also found evidence for a shift in the balance of apoptotic processes in late disease.

**Conclusions:** We find evidence in our analysis and present a model of SSc-PF for an initial insult that results in an interferon response, followed by lipid uptake by alveolar macrophages that appear alternatively activated and may secrete TGF-β. Genes that bridge multiple molecular processes (e.g. ECM remodeling, apoptosis) are important in animal models of PF and are attractive targets for therapeutic intervention.

**33. Endothelial Fli1 deficiency impairs angiogenesis through activation of the FOXO3a pathway**

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**Background:** Endothelial cell injury and deficiency of angiogenesis contribute to Systemic Sclerosis vasculopathy. Although the mechanisms of the injury remain poorly understood, there is strong evidence that the transcription factor Fli1 plays a pivotal role in this process. Our previous study showed that mice with a conditional knockout of Fli1 in endothelial cells display abnormal skin vasculature, and increased permeability. The aim of this study was to further investigate the biological consequences of Fli1 downregulation in endothelial cells and its effect on angiogenesis.

**Materials and methods:** Human dermal microvascular endothelial cells (HDMECs) were isolated from foreskin tissues and stimulated with TGFβ or various TLR ligands. Fli1 knockdown in HDMECs was performed using Fli1siRNA. Expression levels of Fli1 and FOXO3a were determined by qPCR and Western blot. Direct binding of Fli1 to the FOXO3a promoter was determined by CHiP assay. Proliferation was examined with the Essen BioScience IncuCyteTM Live-Cell Imaging system. Angiogenic potential was assessed in *ex vivo* organ culture of dermal tissue obtained from foreskins.

**Results:** Fli1 protein level in HDMECs was decreased in response to TGFβ, TLR3 (Poly I:C) and TLR7 (Imiquimod) ligands, and increased in response to TLR9 (ODN2395) ligand. Treatment with TGFβ, Poly(I:C), and Imiquimod as well as Fli1siRNA decreased the basal proliferation index of HDMECs. Moreover, we observed further reduction of proliferation in Fli1 deficient cells treated with TGFβ, Poly(I:C), or Imiquimod. In contrast, ODN2395 treatment increased the basal proliferation index in HDMECs. Downregulation of Fli1 in HDMECs resulted in increased mRNA and protein levels of FOXO3a. Moreover, using a CHiP assay we showed that Fli1 directly binds to the FOXO3a promoter. In the *ex vivo* dermal culture we observed decreased total number of branching tubules in skin tissue treated with Fli1siRNA, TGFβ, Poly(I:C) and Imiquimod, while ODN2395 treatment increased number of sprouts.

**Conclusion:** This work demonstrates that Fli1 deficiency impairs proliferation of HDMECs by activating expression of FOXO3a. Furthermore, treatment with TGFβ, TLR3 and -7 ligands decreased proliferation of HDMECs at least in part by downregulating Fli1 protein level. Together these findings strongly suggest that activation of TGFβ and innate immune pathways underlie scleroderma vasculopathy.

**34. Peripheral B lymphocytes secrete both interleukin 6 and transforming growth factor-beta and potentiate fibroblast activation in systemic sclerosis**

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**Background** Systemic sclerosis (SSc) is a rare connective tissue disease characterized by fibroblasts activation, increased extra-cellular matrix synthesis and autoantibodies. Little is known regarding the potential role of B lymphocytes in the pathophysiology of SSc.

**Patients and Methods**: Peripheral blood B cell subpopulation phenotyping, activation and inhibitory receptor expressions, IL-6 and TGF-β productions were characterized by flow cytometry and multiplex assay. Proliferation of fibroblasts and collagen production by fibroblasts after culture in the presence of B cell supernatants isolated from patients or controls were assessed using XTT, BrdU, Ki67 immunofluorescence staining and collagen assay, respectively.

**Results**: Eighty patients with SSc fulfilling the ACR/EULAR criteria, including 18 males (22 with diffuse SSc (dSSc) and 58 with limited cutaneous SSc (lSSc), and 21 healthy controls (HC) were studied. The proportion of $IgD^-$ $CD27^+$ B cells was significantly lower in SSc patients (Mean±SD 19.33%±11.85) than in HC (25.91%±11.25) (p=0.041). Increased proportions of B cells expressing CD69 (8.29%±7.77 vs 2.36%±2.29 p=0.0017) and CD95 (47.02%±19.82 vs 28.93%±11.21 (p=0.0004)) were found in both lSSc and dSSc. Compared to HC and lSSc, B lymphocytes from dSSc patients also expressed higher proportions of CD5 (24.12%±7.93 vs 14.09%±6.58 for lSSc (p=0.0296) and 14.21%±5.34 for HC), CD86 (39.89%±22.11 for dSSc vs 17.72%±13.98 for lSSc (p=0.0007) and 11.68%±11.09 for HC) , IL-6R (33.64%±23.12 for dSSc vs 17.91%±13.62 for lSSc (p<0.0001) and 12.08±8.68 for HC) and IL-21R (32.55%±20.19 for dSSc vs 5.76%±4.40 for lSSc (p<0.0001) and 5.93%±3.29 for HC). Intracellular flow cytometry identified a significantly increased proportion of IL-6 (24.53%±6.69 vs 13.23%±4.71 (p<0.0001)) and TGF-β (18.38%±10.22 vs 6.31%±3.62 (p<0.0001)) positive B lymphocytes in patients with SSc as compared to HC. By multiplex assay, we detected higher production of IL-6 (314.3 pg/ml±317.8 vs 6.10pg/ml±2.58 (p=0.0007)) and TGF-β (1020pg/ml±569 vs 163.8pg/ml±98.69 (p=0.0011)) and lower IL-10 production (8.67pg/ml±6.22 vs 333.1pg/ml±123.7 (p=0.0008)) by stimulated isolated B lymphocytes from SSc patients compared to HC. Fibroblast proliferation and collagen production in the presence of B cell supernatant from SSc patients were significantly increased as compared to HC.

**Conclusions**: Peripheral B lymphocytes secrete both interleukin 6 and transforming growth factor-beta, and activate fibroblasts in patients with SSc.

**35. Targeting Biomechanical Survival Circuits with the BH3 mimetic ABT-263 Reverses Established Fibrosis**

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**Background:** Resistance to apoptosis is a hallmark of collagen-producing myofibroblasts in scleroderma fibrosis. We have recently shown that myofibroblast mechanotransduction of signals from increased dermal matrix stiffness promotes their persistence. We previously demonstrated that inhibition of focal adhesion kinase (FAK) mechanosignalling induces myofibroblast apoptosis and reverses established fibrosis in mouse models of scleroderma. The mechanism by which FAK inhibition induces myofibroblast apoptosis has yet to be elucidated.

**Materials and methods:** We used atomic force microscopy to measure matrix stiffness in skin samples harvested after 28 daily subcutaneous injections of either saline or bleomycin. We tested the effect of matrix stiffness on fibroblast apoptosis using collagen-coated polyacrylamide hydrogels with the stiffnesses observed in normal and fibrotic skin. Fibroblast apoptosis was determined by caspase 3/7 activity and tunnel staining. To evaluate the therapeutic potential of targeting FAK, or BCL-2 or BCL-$X_L$, we administered PF-562,271 (25 mg/kg daily), or the BH3 mimetics ABT-199 or ABT-263 (100 mg/kg daily) to mice in the bleomycin model of scleroderma.

**Results:**Dermal matrix stiffness increased in bleomycin-induced skin fibrosis *in vivo*. Plating fibroblasts *in vitro* on biomimetic substrates mimicking the stiffness of fibrotic dermis promoted their survival. Pharmacological inhibition of FAK induced myofibroblast apoptosis and reversed established fibrosis *in vivo*. Apoptosis is regulated by the dynamic interaction of pro-apoptotic and anti-apoptotic BCL-2 family proteins in the mitochondrial outer membrane. We found that FAK inhibition specifically prevents matrix stiffness-induced expression of the pro-survival protein BCL-$X_L$ but not BCL-2, which promotes the apoptosis of these cells. BCL-2 and BCL-$X_L$ proteins are endogenously inhibited by BH3 domain-containing pro-apoptotic proteins, and small molecule BH3 mimetics can similarly inhibit the pro-survival activities of BCL-2 and BCL-$X_L$. We found that BH3 mimetic ABT-199, which specifically inhibits the pro-survival effects of BCL-2, failed to reverse established skin fibrosis in mice. Treatment with the BH3 mimetic ABT-263 (navitoclax), a dual BCL-2/BCL-$X_L$ inhibitor, in contrast dramatically induced dermal myofibroblast apoptosis *in vivo* and mitigated established dermal fibrosis.

**Conclusions:** Our results demonstrate that selective pharmacological inhibition of BCL-$X_L$ with the BH3 mimetic ABT-263 has potential to be a novel therapeutic strategy for scleroderma fibrosis.

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## 36. Exposure to quartz dust and the risk of development of connective tissue diseases

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***Background:*** Exposure to quartz (crystalline silica) has been associated with the occurrence of connective tissue diseases.

***Methods:*** In a systematic review based on PRISMA criteria and involving searches in 4 databases 1162 articles were identified. Our eligibility criteria led to an inclusion of 22 studies covering 24 analyses on the relation between quartz exposure and the risk of systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis and small vessel vasculitis.

***Results:*** A disease-specific meta-analyses showed that quartz exposure was associated with systemic sclerosis [meta-odds ratio (OR) 2.94, 95% confidence interval (CI) 1.93-4.49, $I^2$ =24.3%], systemic lupus erythematosus (OR 2.80, 95% CI 1.30-6.02, $I^2$ = 57.0%), and small vessel vasculitis (OR 2.45, 95% CI 1.55-3.88, $I^2$ =48.5%). Increased risks were also indicated for rheumatoid arthritis (OR 1.53, 95% CI 0.80-2.25, $I^2$=88.1%). The overall odds ratio for all four connective tissue diseases was 2.45 (95% CI 1.77-3.40, $I^2$=82.9%). Heterogeneity was small for systemic sclerosis, moderate for systemic lupus erythematosus, and small vessel vasculitis, and high for rheumatoid arthritis as indicated by the $I^2$ values. Funnel plots strongly indicated publication bias and the reviewed studies had several limitations like inappropriate control groups, low response rates, qualitative exposure information, lack of exposure response data, low diagnostic specificity, and limited confounder adjustment.

***Conclusion:*** This review provides some evidence to the hypothesis that quartz exposure is associated with the occurrence of connective tissue diseases: systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and small vessel vasculitis. More high quality studies are needed in order to confirm or refute a causal relation between quartz exposure and the risk of connective tissue diseases.

**37. A Variant of the Hepatocyte Growth Factor Receptor MET Is Associated with Impaired HGF Signaling: Possible Role in Scleroderma Lung Disease**

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*[*]T. Akter and Y. Shirai contributed equally to this work*

**Objective:** MET is a transmembrane protein and receptor for hepatocyte growth factor **(HGF)**. Previously, we showed that HGF down-regulates connective tissue growth factor (CTGF, CCN2) and collagen in lung fibroblasts isolated from Caucasian but not from some African American SSc-ILD patients. In one such cell line with impaired HGF signaling, we have identified a D1398G variant of the HGF receptor. **This study was undertaken to investigate effects of the D1398G mutation on lung fibroblasts (LF) and alveolar epithelial cells (AEC).**

**Materials and Methods:** D1398G MET mutant was created using the QuickChange Site-Directed Mutagenesis XL kit from Stratagene; MET wild type and D1398G adenoviruses were generated by AdEasy Vector System, Quantum Biotechnology. MET expression in lung tissue was studied by immunofluorescence; expression and phosphorylation of proteins in cultured cells were studied by immunoblotting; Ras and caspase activity were assessed by spectrophotometric methods; C-terminal fragment of MET was measured by indirect ELISA.

**Results.** When compared with MET wild type, upon exposure to HGF the D1398G variant of MET was associated with reduced phosphorylation on tyrosine residues and also with reduced activation of Ras and MAPK in **LF and AEC.** In both SSc LF and TGFβ-treated normal LF transfected with MET wild type, HGF decreased collagen, CCN2 and smooth muscle α-actin (SMA). However, HGF had no such effects on collagen, CCN2, and SMA in LF transfected with MET D1398G. MET, which is expressed in lung tissue mainly in AEC, can be cleaved by caspase-3 to yield a terminal 10-amino-acid-fragment that protects AEC from apoptosis. Cells transfected with the variant D1398G MET were unable to either generate M10 or protect AEC from apoptosis. This appears to be due to the fact that the D1398G mutation disrupts the Caspase-3 cleavage motif DEVD.

**Conclusions:** D1398G mutation in the HGF receptor MET is associated with impaired HGF signaling in both LF and AEC. Clinical correlation is required to determine if ILD in SSc patients bearing such a mutation is more severe, as suggested by these observations, than in SSc patients possessing wild type MET.

## 38. Reliability and Validity of the Total Joint Count and Swollen Joint Count in Early Diffuse Systemic Sclerosis

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**Background:** Clinical trials in diffuse cutaneous Systemic Sclerosis (dcSSc) sometimes include the tender joint count (TJC) and swollen joint count (SJC) as secondary outcome measures; however, these outcomes have not yet been validated in dcSSc. Our objectives were to assess inter and intrarater reliability of the TJC and SJC and to compare physician joint examinations with musculoskeletal ultrasound (MSK-US) as a gold standard.

**Methods:** On a single day 7 patients in the Prospective Registry of Early Systemic Sclerosis (PRESS) cohort underwent 2 separate TJC/SJC by 10 rheumatologists and had MSK-US of the bilateral hands and wrists (22 joints). We computed inter and intrarater reliability for TJC/SJC and compared the TJC/SJC to the MSK-US to determine sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

**Results:** The mean TJC was 4.2 (2.0). The interobserver reliability for the TJC was 0.97, and the intraobserver reliability for the TJC was 0.99, showing almost perfect agreement. The mean SJC was 1.3 (0.8). The interobserver reliability for the SJC was 0.24, showing fair agreement, and the intraobserver reliability for the SJC was 0.71, showing substantial agreement.

9.7% (15/154) of joints showed synovitis or synovial thickening on MSK-US. Sensitivity, specificity, PPV and NPV are shown below.

**Conclusion**: We noted excellent inter and intrarater reliability for the TJC and acceptable intrarater reliability for the SJC in patients with early dcSSc. TJC/ SJC showed low sensitivity, but high specificity when compared with MSK-US. In this group with low prevalence of MSK-US abnormalities, the PPV of the TJC/SJC was low, and the NPV was high. There was poor agreement between the TJC/SJC and MSK-US in early dcSSc.

| | Sensitivity – mean(SD) | Specificity – mean(SD) | PPV – mean(SD) | NPV – mean(SD) |
|---|---|---|---|---|
| SJC1 | 0.020 (0.045) | 0.956 (0.028) | 0.039 (0.076) | 0.894 (0.009) |
| SJC2 | 0.014 (0.045) | 0.947 (0.048) | 0.017 (0.047) | 0.869 (0.008) |
| TJC1 | 0.093 (0.034) | 0.778 (0.033) | 0.046 (0.014) | 0.881 (0.013) |
| TJC2 | 0.035 (0.037) | 0.900 (0.063) | 0.046 (0.048) | 0.866 (0.005) |

## 39. Inter and Intrarater Reliability of the Modified Rodnan Skin Score in Early Diffuse Systemic Sclerosis

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**Background:** The Modified Rodnan Skin Score (MRSS) is a semiquantitative assessment of skin thickness and a commonly used outcome measure in Systemic Sclerosis (SSc) clinical trials. The MRSS has been shown to have acceptable inter and intrarater reliability. Our objective was to determine the inter and intrarater reliability of the MRSS in patients with early diffuse cutaneous (dc)SSc in the Prospective Registry of Early Systemic Sclerosis (PRESS) Cohort.

**Methods:** Seven patients with early dcSSc, defined as less than 2 years from the first non-Raynaud's symptom of SSc, from the PRESS Cohort were examined by 10 rheumatologists twice in one day. Prior to the exercise, the rheumatologists received training in the MRSS examination. For the continuous variable MRSS we computed the inter- and intrarater reliability by fitting a linear mixed model to the examiners' ratings with random effects for patient, rater and patient by rater.

**Results:** The mean age of the patients was 41.6 (19.8) years and the mean disease duration from the first non-Raynaud's symptom was 2.7 (0.8) years. Three patients were female and 4 male.

The interrater reliability for the MRSS was 0.81, and the intrarater reliability for the MRSS was 0.94. The interobserver mean for the MRSS was 14.67 and the within-patient standard deviation (SD) was 4.04. The intraobserver mean for the MRSS was 15.04 and the within-patient SD was 2.30.

**Conclusion:** We found the MRSS to have an inter-rater reliability of 0.81 and intrarater reliability of 0.94, suggesting almost perfect agreement. The interobserver within-patient SD was 4.04 and the intraobserver within-patient SD was 2.30 which are comparable to previously published figures of 4.6 and 2.45, respectively.[1] Our study was limited due to a small number of patients and the potential for recall bias, although measures were taken to prevent this. This study confirms the reliability of the MRSS in the study of patients with early dcSSc.

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[1] Clements, PJ, et al. J Rheumatol 1995;22:1281-5

## 40. Interrater Reliability of Nailfold Capillaroscopy using Widefield Microscopy

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**Introduction:** The presence of nailfold capillary (NFC) abnormality is part of the 2013 ACR/EULAR Classification Criteria for Systemic Sclerosis (SSc). NFC findings include the presence of capillary dilation, hemorrhage, and neoangiogenesis, and it has been shown that these patterns have prognostic value. Patients in the Prospective Registry of Early Systemic Sclerosis (PRESS) cohort have NFC evaluation using widefield microscopy (WM) at eleven centers in the United States. Our objective in this study was to assess the interrater agreement of the PRESS investigators assessment of NFC by WM in order to ensure the quality our data collection.

**Methods**: Ten investigators from the PRESS registry assessed 26 WM photomicrographs of patients with early diffuse cutaneous (dc)SSc. The investigators have attended training on NFC, but no specific training was provided prior to this exercise. Each case was rated as normal, mild, moderate, or severe for NFC dilation, hemorrhage, and neoangiogenesis. Data were captured via surveymonkey and analyzed in two ways: using all 4 categorical variables and collapsing the variables to normal/abnormal. Krippendorf's alpha reliability estimate was used to quantify the interrater agreement.

**Results**: Intrarater reliability ranged from substantial to almost perfect agreement for NFC hemorrhage to substantial agreement for dilation of NFC loops to fair agreement for neoangiogenesis. Assessment with four variables showed improved agreement when compared to 2 variables.

**Conclusions:** Substantial reliability is seen in NFC rating by WM among PRESS investigators, which attests to quality data collection for this procedure. Classification of specific NFC abnormalities using the terms normal, mild, moderate, and severe is feasible and can be used reliably. The prognostic significance of NFC in the PRESS cohort is a subject for future study.

Table 1. Interrater Reliability - Krippendorf's alpha reliability estimate (95% CI)

| | 4 variables (normal, mild, moderate, severe) | 2 variables (normal/abnormal) |
|---|---|---|
| NFC Hemorrhage | 0.7991 (0.7528, 0.8403) | 0.8205 (0.6801, 0.9262) |
| Dilated Capillary Loops | 0.6538 (0.5930, 0.7093) | 0.4869 (0.2846, 0.6934) |
| Neoangiogenesis | 0.3738 (0.2820, 0.4644) | 0.2706 (0.0879, 0.4527) |

**41. An Elegant Method of ELISA Calibration with an Illustration of Quantitating MET Receptor Fragment, a Novel, Mechanism-Based Biomarker for Scleroderma Interstitial Lung Disease**

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**Background:** Interstitial lung disease (ILD) is a severe complication and leading cause of mortality in scleroderma (SSc). Although SSc-ILD is detected in more than 90% of patients, disease progression is very heterogeneous, demanding mechanism-based biomarkers that will accurately predict pathways activated in those patients with progressive disease. Preliminary studies have suggested bronchoalveolar lavage fluid concentration of M10, a cleavage product of the HGF receptor, MET, might be used as an early biomarker of alveolar injury in SSc-ILD. A method for measuring the concentration of M10 in biological fluids using an ELISA is therefore warranted.

**Materials and methods:** A model based calibration approach using a non-linear function fitting known concentrations of a peptide against absorption values measured using an ELISA is proposed. A calibration function is constructed from the fitted model using the inverse transformation of the non-linear model. Approximate confidence interval for the calibration estimate is also provided. A step by step procedure for applying this approach will be presented.

The proposed approach will be illustrated in the context of M10 as a potential biomarker for SSc-ILD. For this purpose, several experiments measuring known serial concentrations of synthesized M10 were performed under various experimental conditions, including incubation time, plate type, and buffer type, etc. Feasibility of ELISA as a way to measure M10 was assessed by comparison of objective properties of the model fit in addition to clinical usefulness of the final model.

**Results:** A model was identified as being ideal for M10 ELISA results that is generalizable and interpretable. Moreover, for all values of optical density at 460 nm (up to 1,000,000), a mean and 95% confidence interval could be provided. From these results the ideal plate type seems to be a microplate with high binding capacity and the buffer type is PBS, and the change in accuracy from incubation time was negligible.

**Conclusions:** This statistically rigorous approach to evaluating ELISA results has an application in wide range of fields. Moreover, the method provides a more concise estimate of M10 concentration along with a confidence limit, which could lead to more accurate diagnostic procedures in patients with SSc-ILD and other fibrotic diseases.

**42. miR-29 therapeutics inhibit ECM production and fibrosis**

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**Background:** miRNAs are small, non-coding RNAs that act as negative regulators of gene expression. miRNAs play a crucial role in tissue homeostasis and are frequently dysregulated in disease. The miR-29 family (miR-29a, b and c) are uniformly downregulated in fibrotic diseases and the therapeutic benefit of increasing miR-29 levels has been shown for heart, kidney, liver, lung and systemic sclerosis. Among the predicted and proven targets of the miR-29 family are multiple extracellular matrix proteins and profibrotic molecules, as well as regulators of endothelial-mesenchymal transition (EMT).

**Materials and methods:** A synthetic, chemically stabilized, oligonucleotide mimic of miR-29b (promiR-29; MRG-201) and a locked nucleic acid (LNA)-modified inhibitor of miR-29 (antimiR-29) were generated at miRagen Therapeutics. C57BL/6 mice were utilized, with or without a full-thickness incision to upregulate ECM production in the skin. MRG-201, antimiR-29 or PBS were administered via intradermal injection. Microarray analysis (Agilent) was performed on total RNA isolated from the treatment sites to identify genes that were differentially regulated by promiR-29 and/or antimiR-29 in vivo. MRG-201 was further tested in rats, rabbits and human fibroblasts to confirm translation to additional species, and in a bleomycin-induced lung fibrosis model in mice to confirm efficacy in preventing fibrosis in other organs and tissues.

**Results:** Microarray analysis identified 222 genes that were reciprocally regulated by promiR-29 and antimiR-29 in vivo in mouse. Notably, functional annotation identified Extracellular Matrix as a key pathway that was modulated by miR-29. Repression of numerous growth factors, collagens, chaperones and processing enzymes involved in the collagen fibrillogenesis pathway was observed with MRG-201 treatment. Repression of these miR-29 target genes was confirmed in rat and rabbit in vivo and in human fibroblasts in vitro. Additionally, systemic MRG-201 treatment was shown to repress collagen expression and abrogate the development of pulmonary fibrosis in the bleomycin mouse model when used either as a preventative or therapeutic treatment approach.

**Conclusions:** These results demonstrate that exogenous miR-29 treatment represses key pathways associated with fibrosis in multiple organs, and that MRG-201 prevents or treats cutaneous and lung fibrosis. These findings indicate that MRG-201 may be effective in the treatment of cutaneous scleroderma and SSc-ILD.

## 43. Impaired glycocalyx in sublingual microvessels of patients with systemic sclerosis

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**Background:** Our group has previously described the sublingual frenulum abnormalities in systemic sclerosis (SSc, scleroderma). Video-microscopy of the sublingual mucosa permits direct observation of the microcirculation with the possibility of identifying novel clinical biomarkers and patho-physiological insight into SSc. Our goal was to use this technique to measure vessel perfusion, vessel density, and a validated marker of glycocalyx in a cohort of SSc patients and age- and sex-matched healthy controls.

**Methods:** Ten patients fulfilling the 2013 ACR/EULAR classification criteria for SSc (49 ± 11 yrs; 8 F, 2 M) and ten age and sex matched healthy controls (46 ±10yrs; 8F, 2M) were studied under a 4-hour fasted state. Video-microscope images of the sublingual mucosa were obtained using a Glycocheck device and acquisition and analysis of images were automated by the Glycocalyx Measurement Software. The primary variable of interest was the penetration of red blood cells (RBC) into the microvessel wall barrier region (perfused barrier region, PBR). A diminished glycocalyx allows greater RBC penetration and a higher PBR score. We also determined the number of well perfused vessels/mm$^2$, total number of vessels/mm$^2$ and the longitudinal vessel fraction that is filled with red blood cells (RBC%).

**Results:** The SSc cohort included 4 diffuse cutaneous and 6 limited cutaneous SSc patients. All were antinuclear antibody (ANA) positive and 9 were SSc-specific antibody positive. Mean disease duration was 7.25 years (1-25 years). Glycocheck scores demonstrated that sublingual microcirculation PBR was 10% lower in healthy controls vs. SSc (1.9±0.1 vs. 2.1±0.2, $p<0.05$), indicating a diminished glycocalyx in SSc patients. RBC filling was higher in the capillaries in healthy controls vs. SSc (75.6±3.4% vs. 66.6±5.4%, $p<0.05$), indicating lower RBC perfusion of the sublingual tissue in the SSc cohort. There were no differences between the groups in the total number of vessels and the number of well perfused vessels (Both $p>0.05$).

**Conclusions:** This preliminary study indicates that the microvascular glycocalyx is diminished and RBC perfusion is lower in SSc. Confirmation of these findings requires a larger cohort to be studied before the functional significance and clinical utility can be determined.

**44. Role of inflammation and microbiota in a progressive Topoisomerase I peptide-loaded dendritic cell induced model of systemic sclerosis associated with CXCL4 production**

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**Background and objective:** Dendritic cells (DCs) are implicated in pathogenesis of autoimmune disorders and DNA topoisomerase I (TopoI) is a candidate autoantigen for systemic sclerosis (SSc). Increasing evidence supports the concept that fibrosis in SSc is a pathological consequence of an aberrant inflammatory response that fails to resolve. Recent studies also highlight the impact of microflora in controlling severity of autoimmune disorders. The aim of this study is to first induce a progressive disease with an inflammatory response that precedes the development of fibrosis by immunization with TopoI peptide-loaded DCs and next, utilize this model to manipulate gut flora in order to modulate fibrosis.

**Methods:** TOPOIA and TOPOIB peptides were selected from different regions of TopoI. Mice were repeatedly immunized with either TOPOIA or TOPOIB -loaded LPS matured DCs to induce experimental SSc. In some experiments, breeding pairs and mice were given antibiotics, streptomycin or vancomycin, in their drinking water.Fibrosis in lung and skin was quantified by measurement of hydroxyproline content and trichrome (Masson) stain. Pulmonary inflammation was quantified in lung tissues, bronchoalveolar lavage (BALF) and lung explant cultures. Anti-TopoI specific antibodies were measured in sera by ELISA.

**Results:** Temporal analysis showed that immunization with TOPOIA DCs results in progressive development of disease with perivascular and peribronchial inflammation that precedes the peak of pulmonary fibrosis. TOPOIA DCs immunized mice also develop robust skin fibrosis. Pulmonary inflammation in TOPOIA DC group correlates with transient CXCL4 production, a biomarker of early diffuse cutaneous human SSc (dcSSc) and increased cellular infiltration in BALF. In contrast, immunization with TOPOIB DCs, but not TOPOIA DCs, induced an IgG2a anti-TopoI autoantibody response along with mild skin fibrosis. At a late time point (6 weeks after the last immunization), inflammation waned but fibrosis persisted and, both TOPOIB DCs and TOPOIA DCs groups developed diffuse skin fibrosis as well as IgG2a anti-TopoI autoantibody response. Manipulation of gut flora with either streptomycin or vancomycin resulted in no development of pulmonary fibrosis.

**Conclusion:** TopoI peptide-loaded DC induced model of experimental SSc closely mimics the systemic fibrosis, inflammation, transient CXCL4 production, vasculopathy, and autoantibody responses characteristic of human dcSSc. Manipulation of gut flora in this model resulted in no pulmonary fibrosis, thus opening a potential avenue to treat/manage this incurable disease. Therefore, this model may serve as a tool to further understand disease pathogenesis and test new therapeutic approaches.

**45. Genome-Wide DNA Methylation Analysis in Blood and Dermal Fibroblasts from Twin Pairs Discordant for Systemic Sclerosis Reveals Distinct Signatures in Limited and Diffuse Disease Subsets**

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**Background:**The reasons underlying the wide variation in disease heterogeneity and severity in systemic sclerosis (SSc) remain unknown. The low concordance rate in monozygotic twins suggests an important role for epigenetic factors in SSc susceptibility. This analysis was conducted to characterize DNA methylation patterns in SSc.

**Methods:** Genome-wide methylation was assessed on approximately 480,000 CpG sites using genomic DNA isolated from 1) whole blood from 20 twin pairs discordant for limited cutaneous SSc (lcSSc) and 10 twin pairs discordant for diffuse cutaneous SSc (dcSSc), and 2) skin fibroblasts cultured from dermal punch biopsies of 7 twin pairs discordant for dcSSc and 5 twin pairs discordant for lcSSc. An efficiency analysis was performed with caGEDA to determine best normalization and feature selection methods and to identify differentially methylated probes between unaffected and affected twins. Ingenuity Pathway Analysis was used for pathway analysis.

**Results:** In blood, a total of 206 and 409 CpGs were differentially methylated in the twin pairs discordant for lcSSc and dcSSc, respectively. In fibroblasts, a total of 110 and 220 CpGs were differentially methylated in lcSSc and dcSSc, respectively. Dramatic differences were observed both between tissues and disease subsets. While in blood there was a higher ratio of hypomethylated to hypermethylated probes in lcSSc (1.2:1), conversely in fibroblasts this ratio was observed in dcSSc. Only 1.3% of differentially methylated CpGs were common in the blood between lcSSc and dcSSc, while 6.5% were common in fibroblasts. In each disease subset, less than 2% of differentially methylated CpGs were common to blood and fibroblasts. Despite the enrichment of different pathways and biological functions in each disease subset driven by differential hyper- or hypomethylation of different genes, most of these pathways can be placed into broader categories implicating an overall involvement of developmental and cancer functions.

**Conclusions:** The distinct methylation patterns observed in blood and fibroblasts between lcSSc and dcSSc corroborate a similar observation reported in skin fibroblasts and suggest that subset-specific epigenetic signatures may be, at least in part, responsible for the clinical heterogeneity of the disease. These data also support a role for DNA methylation differences in mediating susceptibility to SSc.

**46. The HLA-B35 allele modulates ER stress, inflammation and proliferation in PBMCs from Limited Cutaneous Systemic Sclerosis Patients**

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**Background:** The HLA-B35 allele has been shown to be strongly associated with Scleroderma in Italian PAH patients, Choctaw Indians and Brazilian patients. We recently reported that HLA-B35 induces endothelial cell dysfunction via activation of ER stress/UPR and upregulation of the inflammatory response. Because, we also observed a correlation between activation of ER stress/UPR and upregulation of IL-6 in PBMCs from lcSSc-PAH patients, the goal of this study was to assess whether the presence of HLA-B35 contributes to the elevated ER stress in lcSSc PBMCs.

**Methods:** PBMCs were purified from healthy controls (n=49 HC) and lcSSc patients, (n=44 with PAH, and n=53 without PAH). PBMCs from each group were stratified for the presence of HLA-B35. Global changes in gene expression in response to HLA-B35, HLA-B8 or empty lentivirus were investigated by array analysis in HC PBMCs. Total RNA was extracted and qPCR was performed to measure gene expression.

**Results:** ER stress markers, in particular the chaperones BiP and DNAJB1 were significantly elevated in PBMC samples carrying the HLA-B35 allele. Similarly, IL-6 expression was significantly higher in HLA-B35 lcSSc (PAH and NoPAH) PBMCs and positively correlated with ER stress markers. We observed increased HMGB1 levels in lcSSc PBMCs when compared to HCs, which were further increased by the presence of HLA-B35. Microarray analyses were used to further understand the role of HLA-B35. Among genes downregulated by B35 lentivirus compared to B8, we observed genes related to complement (C1QB, C1QC), cell cycle (CDNK1A) and apoptotic (Bax, Gadd45) pathways. Genes with increased expression levels were related to proliferation (FYN). In particular, complement genes, C1QC and C1QB, which were elevated in lcSSc compared to HCs, showed decreased levels in B35 subjects. We also observed low levels of cyclin inhibitors (p21, p57) and pro-apoptotic genes (Bax, Gadd45) in lcSSc B35 subjects. On the other hand, high levels of FYN1, a tyrosine kinase known to be involved in proliferation of immune cells, were elevated in lcSSc B35. These changes in gene expression, which correlated with the presence of HLA-B35, were also observed in HCs.

**Conclusion**: These studies showed that the presence of HLA-B35 correlates with increased expression of ER stress, inflammation and proliferation related genes in lcSSc patient PBMCs, as well as healthy individuals, suggesting that carriers of the HLA-B35 allele may have slightly elevated constitutive level of ER stress and inflammation, which could make them more sensitive to additional stress triggers.

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**47. Does the clinical context improve the reliability of rheumatologists grading digital ulcers in systemic sclerosis?**

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**Purpose:** Digital ulcers (DUs) are often a primary end point in SSc clinical trials, although the reliability of rheumatologists grading DUs is poor to moderate at best. Previous DU reliability studies have been based upon visual inspection DU alone, potentially missing 'real-world' clinical contextual information. Our aim was to investigate whether this clinical information improves reliability of DU grading.

**Method:** 80 photographs of a range of SSc digital lesions were collected with the following clinical information: lesion duration (patient reported), pain on a visual analogue score (100 most severe) and its temporal relationship (i.e. less, the same or worse than a week ago), and discharge (patient reported and clinician observed). A custom web-based interface was constructed to facilitate the study. Rheumatologists were invited to participate through SSc networks and received all images with or without context. Lesions were graded on an ordinal scale of severity: 'no ulcer', 'inactive ulcer' or 'active ulcer'. Reliability was in general assessed using a weighted kappa coefficient. Ordinal logistic regression was used to explore the association between the context and grading.

**Results:** 51 rheumatologists completed the study (25 with and 26 without context) from 15 countries, performing 4590 image gradings (including 510 repeats). The intra and inter-rater reliability both without and with the context is presented below. Pain VAS (OR = 1.02, 95% CI 1.01 to 1.03, P = <0.001) and patient-reported discharge (OR 2.67, 95% CI 2.02 to 3.53, P = <0.001) were associated with increased lesion severity, and duration with reduced severity (OR 0.81, 95% CI 0.76 to 0.86, P = <0.001).

**Conclusions:**

1. The overall intra and inter-rater reliability did not significantly improve with the clinical context.

2. There was a trend that some clinicians may use the clinical context to help classify lesions as 'no ulcer'.

3. Patient discharge and pain were associated with increased lesion severity and duration with reduced severity.

| | | INTRA-RATER RELIABILITY | | INTER-RATER RELIABILITY | |
|---|---|---|---|---|---|
| | | **Without context** | **With context** | **Without context** | **With context** |
| **Overall** | | 0.64 | 0.71 | 0.32 | 0.36 |
| **Dichotomised** | **No ulcers vs. inactive/active ulcers** | 0.71 | 0.82 | 0.22 | 0.26 |
| | **No ulcers/inactive ulcers vs. active ulcers** | 0.74 | 0.72 | 0.32 | 0.35 |

**48. Thermographic abnormalities predict future digital ulcers in patients with systemic sclerosis**

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**Background:** Digital ulcers (DUs) are common in patients with systemic sclerosis (SSc) and associated with significant pain and disability. Several authors have recently proposed that capillaroscopic change (assessing microvascular structure) is a predictor of future DUs. Our aim was to investigate whether functional digital vascular disease, as assessed by thermography, is also predictive of future DUs.

**Materials and methods:** This was a retrospective case note review of patients with SSc undergoing thermography at a tertiary SSc centre between 01/01/2005 and 31/12/2009 (by an observer unaware of the thermography findings). Abnormal thermography was defined as a dorsal-distal difference of ≤1°C (fingertip cooler) at 30°C. Baseline demographics and disease characteristics were documented, together with DUs episodes and severity (intravenous prostanoid therapy, surgery and/or amputation) for three years post thermography.

**Results:** 69 patients with both normal and abnormal thermography were included in the analysis. Patient characteristics were (normal vs. abnormal): age (51.5 vs. 57.7 years), sex (female: 82.6% vs. 81.2%) or disease subtype (dcSSc: 24.4% vs. 30.4%). Patients in the 'abnormal thermography' group were more likely to be on treatment for RP (37.7% vs. 59.4%), whereas, more 'normal thermography' patients were current smokers (26.1% vs. 18.8%) at baseline. Abnormal thermography patients were more likely to die or be lost to follow-up (11.6% vs.30.4%).

Patients with abnormal (vs. normal) thermography were more likely to develop an episode of DU: clinician observed and/or patient reported (31.9% vs. 15.9%) (P=0.03) or clinician observed (23.2% vs. 14.5%) (P=0.191) and multiple DU episodes: clinician observed and/or patient reported (15.9% vs. 5.8%) (P=0.056) or clinician observed (14.5% vs. 4.4%) (P=0.041). Although DU severity was based upon small numbers, 'abnormal' patients required more prostanoid therapy (7.3% vs. 2.9%) (P=0.245) and surgery (10.1% vs. 1.5%) (P=0.029), but not amputation (0% vs. 1.5%) (P=0.316).

**Conclusions:** 1. SSc patients with abnormal thermography were more likely to develop future DUs, including multiple episodes.

2. There is a suggestion that DU severity was greater in the abnormal thermography group.

3. Future research is warranted to explore the additional predictive benefit of combining structural (capillaroscopy) and functional (i.e. thermography) microvascular techniques to predict DUs in SSc.

**49. Dimethyl fumarate prevents and reverses PAH in hypoxic mouse model and exerts anti-fibrotic effects through proteasomal degradation of Sp1, TAZ and β-catenin.**

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**Introduction:** Pulmonary arterial hypertension (PAH) is a serious, progressive and fatal complication of systemic sclerosis (SSc). There is evidence for an important role of oxidative stress in the development and progression of PAH. DMF (Tecfidera) is an anti-oxidative agent, approved by the FDA for treatment of multiple sclerosis. In this study we evaluated DMF as a potential therapy for PAH.

**Methods:** Dimethyl fumarate (DMF) was administered daily in a chronic hypoxia mouse model of PAH in preventive and therapeutic modes. The readouts of the experiment were: right heart hypertrophy, Doppler echocardiography, right heart systolic blood pressure, whole lung gene expression and lung immunohistochemistry. *In vitro* studies were performed on human primary arterial endothelial cells (HPAECs) and primary human lung fibroblasts from SSc-PAH patients and healthy controls. HPAECs were exposed to 2.5%$O_2$, lipopolysaccharide (LPS), NRF2 siRNA in combination with DMF treatment. Lung fibroblasts were treated with TGFβ, NRF2 siRNA, MG132 with or without DMF co-treatment.

**Results:** DMF treatment of a PAH mouse model both prevented and reversed hemodynamic changes, right heart ventricular hypertrophy and vascular muscularization in lung. DMF also attenuated–lung damage caused by oxidative stress. Gene expression levels of pro-inflammatory cytokines IL-6, OSM and CCL2, pro-fibrotic mediators TGFβ and CTGF and immune cell infiltration were significantly reduced in lungs of treated mice. In cultured HPAECs DMF normalized hypoxia induced upregulation of NOX4, IL-6, OSM, TGFβ and PDGF genes by targeting the NFκB, STAT3 and JNK signaling pathways. DMF also suppressed TGFβ dependent gene expression including CTGF, ET-1, PAI1, COL1A1 and COL1A2 in human lung fibroblasts. *In vitro* studies both in endothelial cells and fibroblasts showed that the presence of NRF2 is dispensable for the anti-inflammatory and anti-fibrogenic properties of DMF. Mechanistically, DMF treatment of fibroblasts had no significant effect on canonical TGFβ signaling through Smads but instead led to a proteasomal degradation of a key collagen transcriptional activator Sp1 and pro-fibrotic mediators: TAZ and β-catenin.

**Conclusion:** DMF has anti-oxidative, anti-inflammatory and anti-fibrotic potential both *in vitro* and *in vivo* and may provide a new treatment for SSc-PAH.

**50. Analysis of Immune Cell Dysfunction in Systemic Sclerosis Patients**

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**Background:** Systemic Sclerosis (SSc) is an autoimmune connective tissue disorder associated with tissue fibrosis leading to extensive morbidity and mortality due to this, often irreversible, fibrosis. We hypothesized that immune dysfunction can contribute to disease pathogenesis in these patients.

**Materials and Methods:** We designed a novel fifteen color flow cytometry panel to characterize co-inhibitory receptor expression on five separate immune cell types from PBMCs of SSc patients and healthy controls. PBMCs were stimulated in vitro and supernatants were analyzed by ELISA for cytokine production.

**Results:** We found no change in the relative frequencies of cells in the adaptive compartment of SSc patients; however, we found a general increase in the expression of the co-inhibitory receptors PD-1, LAG-3, and Tim-3. Interestingly PD-1 is decreased on the memory subset of CD8+ T cells. An increase in the expression of these receptors has been previously associated with chronic T cell activation, supporting the presence of immune dysfunction. Upon in vitro stimulation, PBMCs from SSc patients produce less interferon gamma and interleukin 10, supporting the notion that these cells in patients are dysfunctional and possibly exhausted in the context of chronic immune activation in this autoimmune setting.

**Conclusions:** Our data reveals the possibility that immune cell dysfunction plays a role in the complex pathogenesis of SSc.

**51. Investigating the role of IL-31 in promoting itching and dermal fibroblast activation in scleroderma**

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**Background:** In systemic sclerosis (SSc) pruritus can be a dominant symptom resistant to therapy in a subgroup of patients. IL-31 is a Th2 cell derived cytokine leading to severe itching in other conditions including atopic dermatitis and T cell lymphoma. We measured IL-31 levels in SSc blister fluid (BF) and plasma, sought correlation with clinical severity and itching, and studied the effect of recombinant IL-31 on fibroblasts.

**Materials and Methods:** IL-31 was assayed by ELISA of BF sampled from the involved forearm skin of SSc patients and healthy controls (SSc n=28, Controls n=15), and matched plasma. IL-31 receptor mRNA was assayed by qPCR of SSc and control fibroblasts and blister roof tissue lysates. Recombinant IL-31 (50 ng/ml) was added to normal dermal fibroblasts and the induced responses determined by protein assays, scratch migration and gel contraction.

**Results:** IL-31 was greatly increased in SSc BF compared to controls (mean 99.4 pg/ml vs 2.3 pg/ml SSc vs controls, $P<0.0003$) and in plasma (1370 vs 196 pg/ml, $P<0.01$). Raised BF IL-31 was a characteristic of a subgroup of SSc from both limited and diffuse clinical subsets. BF but not plasma IL-31 correlated with clinical itch score (Spearman analysis; $P=0.0369$; Spearman $r=0.7050$). Both normal and disease fibroblast extracts contained IL-31 receptor mRNA (qPCR relative copy number 6.4 SSc, 1.4 control) also seen in epidermal tissue extracts (18.4 SSc, 8.2 in control). Treatment with IL-31 led to induction of type I collagen protein but not CTGF, enhanced migration (residual wound area in IL-31 treated $0.2mm^2$ versus $0.9mm^2$ controls, $P<0.001$, inhibited by Wortmanin and U0126) as well as gel contraction by normal fibroblasts.

**Conclusions:** This is the first analysis of IL-31 in SSc, showing increased expression in the disease microenvironment affecting a subgroup of patients. Also IL-31 receptor was present in SSc fibroblasts and tissues at increased levels, and recombinant IL-31 protein induced normal fibroblasts in an SSc-like pattern. We propose that IL-31 could link T cell autoimmune responses to downstream fibroblasts activation in a subgroup of SSc patients. Blocking IL-31 therapeutically may benefit SSc in such patients, by reducing fibroblast activation and pruritus.

**52. Epstein-Barr virus induces activation of inflammatory markers via TLR8 transduction pathway in scleroderma infected Monocytes**

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**Background/purpose:** Monocytes from patients with systemic sclerosis (Scleroderma, SSc), are characterized by the increased expression of IFN-regulatory genes, implicating dysregulation of the innate immune response in activation of these cells. However, what triggers and sustains monocyte activation in SSc remains unclear. Since Epstein-Barr virus (EBV) mRNA and proteins were found in fibroblasts and endothelial cells in SSc skin, we sought to determine whether EBV might also infect monocytes and contribute to their activation in SSc.

**Methods:** Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) depleted of CD19+ cell fraction, using CD14/CD16 negative depletion (CD14-) (Human Monocyte Enrichment Kit without CD16 Depletion**,** EasySep, StemCell). Circulating monocytes from diffuse cutaneous SSc (dcSSc) (n=8) and healthy donors (HDs) (n=6), were examined for the presence of EBV lytic proteins using Immunofluorescence (IF). EBV-p2089 recombinant virus was used to infect dcSSc and HDs monocytes. Gene expression of IFNs, TLRs (TLR7/8/9) and innate immune mediators (IRF5/7) was evaluated in EBV-p2089 infected dcSSc and HD monocytes using real-time PCR, and proteins examined by IF staining and Western Blot. Flow cytometry was performed on dcSSc and HD PBMCs labeled with phycoerythrin (PE), allophycocyanin (APC), fluorescein isothiocynate (FITC) and PE-cyanine7 (PE-Cy7) conjugated mouse monoclonal antibodies (mAb) against human CD14, CD16, CD163, CD206, CD169/siglec1, CD4, CD8, CD20, CD19, CD23. THP1 monocytes were stimulated with TLR7 (R837) and TLR8 synthetic ligands (cpd14b, R848).

**Results:** We found that EBV lytic proteins (Zebra, BFRF1 and gp-350/220, were expressed in skin macrophages and in circulating monocytes from SSc patients, while no expression of lytic EBV was detected in monocytes/macrophages from HDs. Infection of SSc monocytes by EBV-p2089 strongly induced TLR8 expression, while no induction of TLR7 and TLR9 was observed in the infected cells. EBV also significantly induced markers of activated monocytes, such as IRF7, IRF5, Siglec1 and IL-6. Further supporting the importance of TLR8 activation in SSc, expression of TLR8 was significantly increased in freshly isolated monocytes from dcSSc patients compared to HDs. Furthermore, distinct monocyte subsets (CD14+/CD16++) and activation markers were identified in dcSSc PBMCs compared to HDs by FACS analysis. Activation of TLR8 by synthetic ligands mimicked EBV effects on TLR8, inducing IRF7 and inflammatory cytokines, whereas the TLR7 agonist did not induce monocyte inflammation markers on THP1 cells.

**Conclusion:** These data suggest that SSc monocytes are carrying an EBV lytic infection. Activation of TLR8 by EBV RNA might represent a novel mechanism in mediating monocyte inflammation in SSc by which EBV triggers the innate immune response in infected cells.

## 53. Duplex ultrasonography of digital arteries in patients with systemic sclerosis

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**Background:** Systemic Sclerosis (SSc) is a connective tissue disease characterized by continuous fibrosis and vasculopathy. The progressive remodeling of vessels often leads to a permanently decreased acral perfusion and the development of digital ulcers. Capillaroscopy is able to detect early microangiopathic disturbances [1] and is used in the diagnostic and risk stratification in patients with SSc. Duplex ultrasonography is an important tool in general vascular diagnostics. It detects macroangiopathic changes in digital arteries of SSc patients, which do not occur in subjects with primary Raynaud phenomenon [2]. The role of duplex ultrasonography (DUS) in the risk stratification of future digital ulcers remains unclear.

**Patients and methods:** 89 patients (77 with SSc, control group of 12 subjects with MCTD, UCTD, SLE, primary Raynaud, Sjögren's syndrome and Granulomatosis with polyangiitis; 70.8% female, mean age 55.3 years [19-81 y.]; mean duration of disease 7 years [0-33 y.]) were examined. We performed DUS on 32 arteries of hands and fingers after a five-minute water bath with 102 °F. According to Schmidt et al. [2], the patients were divided in three groups: *group 1* (not exceeding one narrowed or obliterated vessel), *group 2* (2-15 pathologic vessels) and *group 3* (16-32 pathologic vessels). Chi square test and Mann Whitney test were performed for statistical analysis.

**Results:** The distribution of DUS groups showed a significant difference between SSc and controls (p=0.0002): Inconspicuous findings (*group 1*) in duplex ultrasonography were found in 41.7% of controls but only in 5.2% of patients with SSc. DUS findings with mostly pathologic vessels (*group 3*) were found in 22.1% of SSc patients but not in any of the other subjects.

There is a significant association in SSc between the result of duplex ultrasonography and former digital ulcers (p=0.0226) as well as capillaroscopic findings: patients with an "active" or "late" pattern showed a significantly higher percentage of pathologic vessels in sonography (p=0.0001).

**Conclusions:** The duplex ultrasonography of digital arteries detects vasculopathy in patients with SSc and is associated with microangiopathic changes and former digital ulcers. Therefore, it might be a useful tool in the risk stratification of future vascular complications like the development of new digital ulcers.

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## 54. Adipose loss of co-repressor NCoR Attenuates Bleomycin-Induced Skin Fibrosis by enhancing PPAR-gamma signaling

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**Background:** The adipogenesis master regulator PPAR-gamma (PPARg) is regulated by repressors such as NCoR. Systemic sclerosis (SSc) is associated with impaired PPARg expression and function and altered adipokine homeostasis. Loss of intradermal adipose tissue is prominent in the skin in SSc, as well as in mouse models of skin fibrosis.

**Materials and methods:** To test the hypothesis that decreased adipose PPARg has a pathogenic role in dermal fibrosis and to ask whether rescuing its function might be beneficial, we characterized skin fibrosis in mice with adipocyte-specific NCoR ablation using the bleomycin model.

**Results**: NCoR null mice on a high-fat diet showed PPARg activation, enhanced insulin sensitivity and alterations in serum adipokines. Moreover, NCoR null mice were resistant to bleomycin-induced changes in adipocyte size and function and loss of intradermal adipose tissue. Importantly, NCoR null mice had attenuated skin fibrosis, which was reversed by pharmacological inhibitors of PPARg. Taken together, these findings suggest that enhanced dermal adipogenesis mediated by tissue-specific PPARg activation modulates skin fibrosis.

**Conclusions:** These results strongly suggest that intradermal adipose plays an active role in skin fibrosis. Targeting adipogenesis might therefore represent an innovative approach to control skin fibrosis in SSc.

## 55. Analysis of blood- and skin-derived DNA Topoisomerase I-specific T cells annotates a potential inflammatory role for the skin microenvironment in scleroderma

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**Background:** Pathogenically relevant immune responses can be profoundly shaped by the immune microenvironment. In systemic sclerosis (SSc), a complex autoimmune disease characterized by excessive tissue fibrosis, the relationship between the skin (as a microenvironment) and the peripheral blood (representing systemic immunity) has yet to be elucidated. Focusing on the cross-talk between T cells and dermal fibroblasts, we sought to explore and compare the effects of T cells derived from the skin and the blood of SSc patients on the gene expression of dermal fibroblasts.

**Materials and Methods:** DNA Topoisomerase I (Topo I) -specific T-cell lines (TCLs) were generated from paired lesional skin biopsies and peripheral blood samples from 5 SSc patients with diffuse SSc. T-cell lines were analysed by flow cytometry and supernatants analysed by Enzyme linked immunosorbent assay (ELISA). Primary human dermal fibroblasts (n=5) were stimulated with supernatants from the pooled skin- and blood-derived TCLs, and gene expression studies were conducted using Nanostring technology. Confirmatory quantitative PCR was performed on genes of interest and ELISA was performed to validate gene expression at the protein level.

**Results:** Skin-derived Topo I-specific TCLs expressed significantly higher amounts of IL-17A and IL-13 as compared to blood-derived Topo I-specific TCLs. Expression of IL-1$\alpha$, IL-1β, IL-11, IL23A, IL-6, IL-8, CCL2, CXCL2, CXCL10 and CSF3 was significantly ($p<0.05$) upregulated in the human dermal fibroblasts stimulated with the supernatants from skin-derived T-cell lines as compared to those stimulated with supernatants from the blood-derived TCLs. Production of IL-6, IL-8, CCL2, CXCL2, CXCL10, CSF3, IL-1β and IL-11 at the protein level was significantly higher in fibroblasts stimulated with the supernatants from the skin-derived TCLs. Gene ontology studies revealed IL-17A as a top upstream regulator of these genes.

**Conclusions:** We present, for the first time, a study interrogating paired skin- and blood-derived Topo I-specific T-cell responses in SSc. Our findings demonstrate a prominent Th17 mediated pro-inflammatory gene signature in the skin microenvironment in scleroderma.

## 56. The Wnt signalling pathway in Systemic sclerosis

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**Background:** In recent years, the morphogen pathways, specifically the Wnt pathway has been implicated in the development of fibrosis in Systemic Sclerosis (SSc). Animal studies have shown that dysregulation frizzled related protein (*frzb*), frizzled class receptor 4 (*fzd4*) and secreted frizzled related protein 1(*sfrp1*) have been implicated in the pathogenesis of SSc. The objective of this study was to examine differential gene and miRNA expression specific to the Wnt pathway.

**Materials and Methods:** Skin biopsies were taken from 8 black South African patients with early (<6 years) dcSSc, one from the lateral forearm (involved) and one from the back (uninvolved). Data was generated using the Wnt pathway RT2 Profiler qPCR Arrays, as well as mRNA- and sRNA-sequencing. Data was analysed using HTqPCR and DESeq2.

**Results:** The expression data from the qPCR array suggests that the most significantly expressed genes are involved in signal transduction within the Wnt pathway. Comparison of qPCR data and mRNA-sequencing data demonstrate that *FZRB*, *FZD4* and *SFRP1* are all significantly upregulated in both the involved and uninvolved SSc skin compared to controls. There was no significant difference in gene expression between Involved and Uninvolved skin samples. The most significantly differentially expressed miRNAs were miR-335, miR-204-5p, miR-451a, miR-15a-5p, miR-15b-5p, miR-375, miR-543, miR-324-5p, miR-18b-5p and miR20b-5p.

**Conclusions:** The results of this study suggest that the dysregulation of Wnt pathway signal transduction is involved in the development of fibrosis in SSc. Involved and Uninvolved skin exhibit similar magnitudes of expression. Differentially expressed miRNAs are predicted to target genes within the Wnt pathway and there is potential for these miRNAs to be investigated as biomarkers of SSc.

**57. Differential pattern of dermal microRNA expression in limited and diffuse variants of systemic sclerosis**

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**Background:** Systemic sclerosis (SSc) is characterised by progressive fibrosis of skin and internal organs due to vascular and autoimmune insults resulting in excessive extra-cellular matrix deposition. It has two distinct clinical phenotypes, limited and diffuse, with distinct patterns of end-organ involvement and prognosis. Genomic studies have failed to show a clear genotype-phenotype correlation. Most microRNA (miRNA) profiling studies have examined either whole skin from SSc patients, purified fibroblasts or serum, with varying results. **Aim**: To evaluate miRNAs in dermal fibroblasts isolated *in situ* from patients with the two disease phenotypes compared with normal controls.

**Methods and methods**: Skin biopsies (4 limited, 4 diffuse and 4 normal) were analysed. Laser capture microdissection of papillary dermis (avoiding lymphocytes, vessels, hair follicles) was performed on 5-6 um sections to isolate tissue containing fibroblasts. microRNAs were profiled using Taqman Low-Density Array (TLDA) Human MicroRNA A + B Cards Set v3.0 (Applied Biosystems). For all samples, miRNAs were analysed using a global normalisation approach. Differentially expressed candidate miRs were then verified by individual PCR on an increased sample number (n=6) for each disease sub-type.

**Results**: A total of 754 human miRNAs were assayed, of which 194 miRNAs were detected in at least one of the 3 selected cell types. Differential expression of various miRNAs was observed in both diffuse and limited SSc papillary dermis compared to normal controls (e.g. miR 10a was down regulated and miR 203 was upregulated in SSc skin). There were also differences between the disease phenotypes eg miR 523 and miR574 were elevated selectively in limited patients whilst miR 126 was selectively elevated in diffuse patients. Pathway analysis was done to identify potential roles for these miRNAs in various pro-fibrotic pathways likely to be relevant to the pathogenesis of SSc.

**Conclusions**: microRNAs are differentially expressed in papillary dermis fibroblasts from patients with SSc. Any observed differences compared with other studies could be explained by analysis of miRNAs from papillary dermal fibroblasts isolated *in situ* rather than from whole skin or purified fibroblasts. Studies of the function and therapeutic modulation of these miRNAs to arrest disease progression are warranted.

## 58. Genetic links between NKX2-5 and Scleroderma

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**Background**: Scleroderma (SSc) is a complex multisystem rheumatic disease characterised by autoimmunity and inflammation, vasculopathy and fibrosis of skin and internal organs. When lungs are affected, patients suffer from pulmonary fibrosis (PF), pulmonary hypertension (PH) and pulmonary arterial hypertension (PAH), with SSc-PAH to be the leading cause of death among SSc patients. The hallmark of SSc-PAH is vascular remodelling, a process during which contractile quiescent vascular smooth muscle cells (VSMCs) de-differentiate to a diseased-associated synthetic phenotype leading to structural re-arrangements of blood vessels. NKX2-5 is a transcription factor that is expressed in VSMCs and has been associated with their phenotypic modulation and vascular remodelling. However, its role in human adult vessels and the way the gene is regulated remain unknown. Our aim is to explore and unravel the role of *NKX2-5* in SSc at the genetic level through a case-control genetic association study and specifically look at particular association with SSc-PH.

**Methods**: Six tagging SNPs spanning a 13.2Kb genomic region surrounding *NKX2-5* gene were genotyped in three different SSc cohorts: 1) Royal Free cohort (UK) (n=899), 2) Manchester cohort (UK) (n=600), 3) Granada cohort (Spain) (n=1736). Two different groups of healthy control samples were used: 1) UK origin (n=901), 2) Spanish origin (n=1753). The patients were grouped based on the auto-antibodies present (ATA, ACA, ARA) and further categorised into sub-phenotypes according to major organ involvement: PF, PH, PAH, and renal crisis (RC). The SNPs were selected with the *Tagger* software and genotyped using the Taqman SNP genotyping assays (Life Technologies) and the Type-it HRM genotyping kit (QIAGEN). *PLINK* and *HAPLOVIEW* were used for the genetic association analysis, the sub-phenotype and haplotype analyses, and the meta-analysis. Permutations were used to correct for multiple testing.

**Results**: Although no significant association was found in the Royal Free cohort, SNP rs3095870 was significantly associated with SSc in the Manchester (p=0.0002) and Spanish (p=0.03) cohorts. SNPs rs3132139 and rs3131917 were also associated with SSc in the Spanish cohort (p=0.005, p=0.004 respectively). SNPs rs3095870, rs703752 and rs3132139 were associated with PH in the Royal Free cohort (p=0.028, p=0.008, p=0.022) and the association of rs3132139 was replicated in the Spanish cohort (p=0.019). In addition, SNP rs3131917 is associated with ATA in the Royal Free cohort (p=0.008). A meta-analysis of all three cohorts revealed a true association of rs3131917 (p=0.007) and rs3095870 (p=0.03) with SSc overall.

**Conclusion**: *NKX2-5* contributes to the genetic background of SSc. In particular, various polymorphisms within the *NKX2-5* locus are associated with SSc overall and with disease-associated sub-phenotypes such as PH and ATA. Each polymorphism may have different functional effects on *NKX2-5* regulation that require further investigation. Taken together, these data suggest that the *NKX2-5* locus is associated with SSc.

## 59. Evaluation of NETosis and immunophenotyping profile in systemic sclerosis (SSc) patients

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***Background:*** Several alterations in adaptive and innate immunity have been described in SSc patients. Neutrophil extracellular traps (NETs) are released by dying neutrophils and may favor autoimmunity and may represent a link between adaptive and innate immunity but their role in SSc is unclear.

**Matherial and Methods:** Forty-seven healthy controls (HCs) and 50 SSc patients, including 17 non fibrotic SSc subjects (8 with earlySSc according to LeRoy and Medsger and 9 with definiteSSc according to EULAR/ACR 2013 criteria) and 33 subjects with skin involvement (18 lcSSc and 15 dcSSc) were included. Immunophenotyping profiling of 22 cell subsets within CD4, CD8 and B lymphocytes or DCs and the determination of NETosis after neutrophil purification and stimulation with 10 nM PMA was performed. Plasma levels of IL-6, IL-10, IL-15, CXCL10, BAFF, BCMA, TACI, CD40L were also determined. Student's t-test, ANOVA with post-hoc corrections, univariate general linear models with or without correction for age were used for statistics; Bonferroni corrections are applied. Predictive models were generated via the Random Forest method.

**Results:** SSc subjects showed increased NETosis (AUC under the distribution of NET sizes) compared to HCs, 10.56±7.24 vs 7.11±3.13, p=0.004; this effect was exclusively due to an increased NETosis in non-fibrotic SSc (15.15±10.01, p=0.02); no differences were observed between HC and fibrotic SSc. NETosis correlated with DLco (r=-0.4, p=0.009) and was reduced in presence of ILD (7.84±3.41 vs 12.51±8,57, p=0.013). Compared to HCs, SSc patients had fewer total lymphocytes* and CD4+ cells**, DCs* and ativated pDCs**, B-cells,** IgM memory B-cells*; increased naïve B-cells were observed in SSc*. Increased levels of IL-6*, CXCL10*, BAFF*** and decreased levels of IL-10*** were found in SSc. CD40L levels inversely correlated both with FVC or DLco*** and IL-6 levels inversely correlated with FVC*. CD21low B-cells were increased in ILD patients***. A Random Forest model comprising the % of activated pDCs, naive T-cells, Th17+, IgM-memory and CD21low B-cells was capable of discriminating SSc from HCs with high accuracy after bootstrap validation (Accuracy=84.3%).

*p<0.001; **p<0.01; ***p<0.05

**Conclusions:** SSc patients show a characteristic immunophenotype profile; NETosis may be an early event in the disease history when fibrosis has not yet developed.

## 60. Isolation and Initial Characterization of Dermal Vascular Smooth Muscle Cells in Systemic Sclerosis

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**Background:** Nearly all patients with Systemic sclerosis (SSc) suffer from vascular dysfunction as illustrated by the uniform presence of Raynaud's phenomena. The role of vascular smooth muscle cells (VSMCs) in the development of vascular dysfunction is still unknown. In this study, we isolated VSMCs from skin biopsy, and we examined their functional phenotype.

**Methods:** We obtained 4 mm punch-skin-biopsy from 3 patients with diffuse cutaneous SSc and 3 matched healthy controls. Skin specimens were treated with a mixture of proteases, then after digestion we plated cells in culture media and harvested the primary cell culture after 10 days. VSMCs were isolated by magnetic microbeads using cell surface markers. First, we depleted total cells from CD31+ cells, followed by positive selection for CD146 + cells. To confirm the identity of this cell population (CD31-CD146+), we performed immunoflorocytometry staining for smooth muscle myosin heavy chain 11 (MYH11), Desmin and NG2. We investigated cell proliferation by using Bromodeoxyuridine (BrdU) assay, and cell viability in normal culture conditions as well as low serum conditions using MTT assay.

**Results:** Out of the total cells obtained from primary cell culture, 15% were CD31- CD146 + (VSMCs). This cell population stained for smooth muscle MYH11, in addition to Desmin and NG2, while the CD31+ and the fibroblast cell populations did not. This staining pattern differentiates VSMCs from pericytes. Next, we evaluated cell proliferation using BrdU, and we demonstrated uptake of BrdU by 19% of the control-VSMCs compared to 34% of SSc-VSMCs (P= 0.0031). The MTT assay showed increase cell proliferation of SSc-VSMCs compared to control-VSMCs (0.44 and 0.25, respectively. P= 7.38E-08). Under serum starvation conditions, SSc-VSMCs exhibited more proliferation capacity than control-VSMCs (0.30 and 0.21, respectively. P= 5.73E-13).

**Conclusion:** This is the first report of the successful isolation and initial characterization of SSc-VSMCs. We believe that increased proliferation of SSc-VSMCs in association with resistance to apoptosis may greatly impact the vascular lesion in SSc. Further studies are warranted to fully understand the trigger and maintenance of the abnormal SSc-VSMCs phenotype.

## 61. Cell-Free Circulating DNA in Systemic Sclerosis: Increased Levels and Global Cytosine Hypomethylation

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**Background:** Systemic sclerosis (SSc) is an autoimmune disease characterized by endothelial dysfunction, vascular injury, and activation of fibroblasts leading to organ fibrosis. The precise etiology of SSc is not clear. We sought to study circulating cell-free DNA (cfDNA) and characterize it in an initial effort to determine if cfDNA is a useful biomarker in SSc.

**Methods:** We measured levels of cfDNA in the serum of 20 patients with SSc (13 limited cutaneous SSc, 7 diffuse cutaneous SSc) compared to 20 age-, sex-, and ethnicity-matched controls. The average age was 57.8 and 52.3 years for patients with SSc and controls, respectively (P= 0.10). The majority of subjects in this study were female (n= 17), and Caucasians (n= 19). Modified Rodanan's skin score (mRSS) was 6.5 and 20.2 for patients with limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), respectively. Moreover, we measured global methylation level of Cytosine in cfDNA by directly quantifying levels of 5-methylcytosine using 5-methylcytosine specific capture antibody.

**Results:** We demonstrate an increase in cfDNA level in patients with SSc (262.1 ng/ml) compared to healthy controls (65.9 ng/ml) (P= 0.027). The average level of cfDNA in lcSSc was (134.03 ng/ml) (P = 0.004), and (463.76 ng/ml) in dcSSc (P= 0.022). We did not find a correlation between level of cfDNA and mRSS in each subset, clinical manifestations, or type of organ involvement. We used same amount of cfDNA from each sample to measure 5-methylcytosine, and we demonstrate that cytosine methylation was significantly lower in patients with SSc (29.1%) compared to controls (54.3%) (P = 0.047). There was no significant difference in cytosine methylation between the two subsets of SSc.

**Conclusion:** We demonstrate increased level of cfDNA in sera of patients with SSc, especially in patients with dcSSc in association with global hypomethylaiton of cytosine. We did not find a correlation between level of cfDNA and mRSS and/or clinical features of SSc. The origin, functional effect and suitability of cfDNA as a biomarker in SSc need further investigation.

## 62. Effect of the mechanically-stressed microenvironment on macrophages in systemic sclerosis

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**Background:** Systemic Sclerosis (scleroderma) is a connective tissue disease of unknown etiology, characterised by inflammation, autoimmunity, vasculopathy and tissue fibrosis. Macrophages are commonly localised in scleroderma fibrotic tissue, and although they may be exposed to mechanical stress in this context, the relationship between mechanical stress and macrophage phenotype is undefined. We have shown previously a role for MRTF-A, a member of the Myocardin-Related Transcription Factor family, in scleroderma. In scleroderma fibrotic skin nuclear MRTF-A expression is increased in fibroblasts as well as infiltrating inflammatory cells. Also, MRTF-A knockout in mice reduced the basal stiffness of skin and internal organs. Furthermore, inhibition of MRTF-A signalling dampened the pro-fibrotic phenotype of scleroderma fibroblasts, resulting in decreased contraction and expression of collagen type I and CTGF. We hypothesise that macrophages also respond to the stiff microenvironment in scleroderma, in a process mediated by MRTF-A.

**Materials and Methods:** Macrophage cultures were established from peripheral blood mononuclear cells (PBMC) cultured in RPMI/M-CSF with LPS (10ng/ml) or IL-10 (10ng/ml) for M1-like or M2-like polarising conditions, on soft (4kPa) and stiff (50kPa) collagen-coated tissue culture plates to mimic healthy and fibrotic skin with increased mechanical stress (n=4 /group). MRTF-A expression was assessed by qPCR and the conditioned media were profiled by Luminex array for inflammatory cytokines and growth factors.

**Results:** We showed by qPCR analysis that scleroderma PBMC-derived macrophages express MRTF-A. Stiff matrix induced cytokine and growth factor expression including MCP-3 in both M1- and M2-polarised macrophages ($p<0.05$), and a trend towards increased PDGF-AA and IL-13 expression in M2 macrophages only.

**Conclusions:** MRTF-A signalling may link mechanical stress to macrophage activation in scleroderma, where the stiff matrix in disease tissues may promote macrophage secretion of cytokines and growth factors that exacerbate the damaging pathologic processes.

## 63. Impact of Acute Tetrahydrobiopterin Administration on Peripheral Vascular Function in Systemic Sclerosis

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**Background:** Systemic sclerosis (SSc) is an autoimmune connective tissue disease that includes marked vasculopathy, which may be related to impaired endothelial nitric oxide (NO) signaling. Tetrahydrobiopterin ($BH_4$) is an essential cofactor that modulates endothelial nitric oxide synthase (eNOS) activity, and may therefore be an important factor in the regulation of NO bioavailability. The current study tested the hypotheses that (1) Vascular function would be reduced in SSc patients compared to healthy controls, and that (2) Acute $BH_4$ administration would restore vascular function towards that of controls.

**Materials and Methods:** SSc patients were administered either $BH_4$ (10mg/kg) or placebo 5 hours prior to arrival at the laboratory (double-blind, randomized, crossover design), while control subjects were studied in the placebo condition. Vascular function was assessed via passive limb movement (PLM), which provokes a hyperemic response that is largely NO-dependent. PLM was performed with patients in the supine position by a trained investigator who moved the patient's knee joint 180˚-90˚ continuously at a rate of 1 Hz for 2 minutes. Femoral artery diameter and blood velocity were measured continuously via ultrasound Doppler, from which femoral artery blood flow was calculated. PLM responses were quantified as peak change in blood flow and blood flow area-under-the-curve (AUC) for the first minute.

**Results:** Six SSc patients (63 ± 13 years, BMI 24.1 ± 2.9 kg/m$^2$) and six healthy controls (68 ± 4 years, BMI 25.8 ± 2.4 kg/m$^2$) participated. Average SSc disease duration was 7 ± 4 years and all patients had Raynaud's phenomenon. In the placebo condition, peak change in blood flow was reduced in SSc compared to controls (203 ± 23 vs. 380 ± 53 ml/min, p=0.011), and the reduction in AUC approached significance (42 ± 15 vs. 135 ± 33 ml, SSc vs. controls, p=0.054). Contrary to our hypothesis, $BH_4$ administration in SSc patients did not alter peak change in blood flow, but tended to improve AUC (42 ± 15 vs. 66 ± 17 ml, placebo vs. $BH_4$, p=0.185).

**Conclusions:** These findings demonstrate an impairment in NO-mediated peripheral vascular function in SSc patients that cannot be fully restored following acute $BH_4$ supplementation.

## 64. Single Sample Predictor (SSP) for Molecular Classification of Skin Biopsies of Patients with Systemic Sclerosis

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**Background:** High-throughput gene expression analyses in skin biopsies from systemic sclerosis (SSc) patients has allowed the identification of four, discrete SSc 'intrinsic' subsets that coincide with, but also further subdivide the clinically defined subsets. Distinct deregulated molecular pathways underlie each subset, suggesting that clinical course and response to treatment may vary amongst patients classified in different intrinsic subsets. The goal of the present study was to develop a nanoString based biomarker gene set and associated algorithm for routine clinical use and clinical trial classification.

**Materials and methods:** We developed a 443 gene-set and an algorithm (the Single Sample Predictor (SSP) algorithm) to permit intrinsic subset classification of individual SSc patient biopsies. This algorithm was trained using a 165 microarray dataset comprised of 34 SSc patients, one morphea patient and 11 healthy controls. We tested the algorithm on merged datasets from previously published studies of Milano et al. and Pendergrass et al. of 164 microarray hybridizations representing 47 SSc patients, 4 morphea patients and 15 healthy controls. We further translated this 443 gene-set to the nanoString platform and analyzed skin biopsies from three different clinical centers to determine the accuracy of the nanoString intrinsic subset assignments by comparison with their gold standard DNA microarray based assignments. Data was normalized on nSolver; cluster and classification analyses were performed.

**Results:** The SSP performs with 95% accuracy within the training dataset and 85% accuracy across testing datasets when predicting the four intrinsic subsets. The 443 gene nanoString assay effectively assigns patients into the intrinsic subsets with 100% accuracy compared with the DNA microarray based assignments for the inflammatory, normal-like and limited subsets, and 75% accuracy for the fibroproliferative group.

**Conclusions:** Intrinsic subset classification of SSc patients can be performed routinely in the context of clinical trials and has been shown to be a useful classification measure to predict disease course and treatment response. This SSP facilitates assignment of individual patients, and the nanoString assay provides a faster, more economical and convenient approach, together enhancing our ability to provide molecular response data in clinical trials and help target appropriate therapies to specific patients.

## 65. X-ray diffraction of spontaneously draining calcinosis in patients with scleroderma

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**Background:** Calcinosis is caused by deposition of calcified materials in the soft tissues (1). Hydroxyapatite (HA) is reported to be its major constituent (2). Mechanical stress and local tissue hypoxia are believed to be important in its pathogenesis (3). Our aim was to analyze spontaneously draining material from calcinosis sites in scleroderma (SSc) patients using x-ray diffraction.

**Materials and Methods:** In this IRB-approved study, we enrolled SSc patients meeting the American College of Rheumatology criteria for definite SSc (4). Pertinent clinical data was collected. Xray diffraction data were used to determine solid phase present (e.g., HA *versus* other Ca phosphate phase) and to approximate the % amorphous and % crystalline components using a Bruker HiStar multi-wire area detector.

**Results:** Ten female subjects (see Table 1) with advanced SSc were enrolled with mean disease duration 16 years; 6 had diffuse SSc. Calcinosis occurred later in the disease course and 7 had extensive calcinosis affecting multiple sites. Draining calcinosis was collected from multiple sites, most commonly the hand. X-ray diffraction confirmed HA of varying percentages in all but one specimen. Solid samples generally contained higher amounts of HA.

**Conclusion:** By using x-ray diffraction, our study corroborates previous published reports (2, 3) that HA crystal deposition is the main constitute of SSc-related calcinosis. Solid samples contained higher amounts of HA crystals; fluid samples observed by optical microscopy to contain solids, including HA in suspension. Further research is needed to characterize the amorphous materials associated with HA deposition in SSc patients with calcinosis.

*Table 1: SSc clinical characteristics and crystal analysis:*

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**Table 1: SSc clinical characteristics and crystal analysis:**

| Patient # | SSc type | SSc/ calcinosis onset (in years) | Auto-antibodies | sites | State | Crystalline component |
|---|---|---|---|---|---|---|
| **1** | D* | 15/9 | ANA/Nucleolar/ Scl70 | elbow thigh<br>groin | liquid*<br>liquid<br>liquid | HA** (25%)<br>HA (20%)<br>HA (20%) |
| **2** | D | 19/9 | ANA/Scl70 | belly | liquid | HA (3%) |
| **3** | L** | 15/3 | ANA/ACA | groin | liquid | HA (2%) |
| **4** | L | 19/5 | ANA/ACA/RNP | finger | liquid | HA (8%) |
| **5** | L | 16/3 | ANA | Elbow over 3 days | solid<br>liquid<br>solid | HA (40%)<br>HA (40%)<br>HA (4%) |
| **6** | D | 14/3 | Scl70/RNApol3 | finger | solid | HA (15%) |
| **7** | D | 11/9 | Scl70 | finger over 3 days | solid<br>liquid<br>liquid | HA (50%)<br>HA (7%)<br>HA (10%) |
| **8** | D | 9/4 | Scl70/ANA | shoulder | Solid mass | HA (3%) |
| **9** | L | 29/4 | ANA/RNApol3/ nucleolar | Belly<br>finger | Solid***<br>solid | calcite (<1%)<br>HA (2%) |
| **10** | D | 10/2 | ANA | finger | solid | HA (43%) |

*Diffuse **Limited

## 66. Kinetics of MMF response shows SSc patients lose their inflammatory signature and rebound upon treatment cessation

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**Background:** Gene expression analysis of skin from SSc patients has identified four 'intrinsic' subsets (normal-like, limited, inflammatory, and fibroproliferative). Previously, we demonstrated that patients classified in the inflammatory subset were most likely to demonstrate improvement in skin scores during mycophenolate mofetil (MMF) treatment. Here, we examine gene expression changes and intrinsic subset assignment in biopsies from patients who completed 24 – 36 months of MMF therapy.

**Materials and Methods:** Seventy-two patients and 22 healthy controls were enrolled. Clinical data and 358 independent biopsies were analyzed. Standard of care clinical assessments were performed. Skin score was determined, and skin biopsies were obtained at baseline, 6-, 12-, 24-, and 36-months and subjected to DNA microarray analysis. Clinical response was defined as decreased mRSS≥5, the minimal clinically important difference. Twenty patients ("completers") had at least 24 months of follow-up with longitudinal skin biopsies. We calculated a normalized enrichment score (NES) for the inflammatory signature on a per-biopsy basis for completers using single-sample gene set enrichment analysis (GSEA).

**Results:** We recapitulate the four intrinsic subsets. Completers classified in the inflammatory subset at baseline show two major response patterns determined by MMF therapy status between 24 and 36 month biopsies. Three patients showed an increase in their inflammatory signature at 6 months and then a decrease at 12 and 24 months, paralleling decreases in T cell, macrophage and activated dendritic cell (DC) signatures. MMF cessation at 24 months resulted in an increase in their inflammatory signature, typically with an increase in mRSS. In contrast, three patients that continued MMF therapy showed a decreased inflammatory signature through month 36. This included decreases in T cell, macrophage and activated DC signatures with concomitant decreasing or stable mRSS during treatment.

**Conclusions:** A subset of patients in the inflammatory subset treated with MMF lose their inflammatory gene expression signature. After cessation of MMF therapy, we observed a return of their inflammatory signature and a worsening of skin disease, suggesting these patients rebound when therapy is stopped. A subset of patients remaining on MMF therapy showed a stable to decreasing inflammatory signature that coincided with stable skin disease.

## 67. Exercise blood flow is improved following acute tetrahydrobiopterin administration in patients with systemic sclerosis

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**Background:** Systemic sclerosis (SSc) is characterized by microvascular and peripheral arterial vasculopathy, which is associated with an impaired ability of the endothelium to produce nitric oxide (NO) and induce vasodilation. Tetrahydrobiopterin ($BH_4$) is an essential cofactor for endothelial NO synthase and is critical for maintaining a healthy vascular endothelium. The purpose of this study was to determine if acute $BH_4$ administration would improve exercise-mediated forearm blood flow in patients with SSc.

**Methods:** Using a double-blind, randomized, crossover design, seven patients with SSc (ages 62±5 yrs) were administered oral $BH_4$ (10mg/kg) or placebo five hours prior to study sessions. All patients reported to the laboratory at least 5 hours fasted. Patients performed static intermittent handgrip exercise at 1 Hz for 3 min at intensities corresponding to 15, 30, and 45% of maximal voluntary contraction (MVC). Forearm blood flow (ultrasound Doppler), arterial blood pressure (brachial sphygmomanometry), and heart rate (HR) were determined at baseline and the final minute of each workload. Forearm vascular conductance was calculated as brachial artery (BA) blood flow / mean arterial blood pressure (MAP).

**Results:** At baseline, there were no differences in HR, MAP, BA diameter, or forearm blood flow following $BH_4$ administration, as compared to placebo (all $p>0.05$). During handgrip exercise, HR, MAP, BA diameter, and forearm blood flow increased in an intensity-dependent manner. Although HR, MAP, and BA diameter did not differ between treatments during any workload, forearm blood flow was ~18% higher during the highest exercise intensity (45% MVC) following $BH_4$ administration, as compared to placebo (260±21 vs. 220±17 ml/min, respectively, $p<0.05$). Likewise, forearm vascular conductance was ~15% higher (2.5±0.2 vs. 2.3±0.1 U, respectively, $p<0.05$) during the 45% workload following acute $BH_4$ administration, as compared to placebo. Of the seven patients studied, one was a non-responder, while the other six had an average increase in forearm blood flow (21%) and vascular conductance of (17%), respectively.

**Conclusions:** Acute $BH_4$ administration in patients with SSc increases forearm blood flow and vascular conductance during handgrip exercise. These hemodynamic changes during exercise may indicate improved NO-mediated vasodilation in the microvasculature and/or resistance arteries in response to acute $BH_4$ administration.

**68. Genome Wide Analysis in Scleroderma Renal Crisis: Defining Genetic Risk in Patients with RNA Polymerase III Auto-Antibodies**

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**Background:** Scleroderma renal crisis (SRC) is a severe complication of systemic sclerosis (SSc). Most SSc cases demonstrate a disease-specific antinuclear antibody including anti-RNA polymerase III (ARA), anti-fibrillarin (AFA), anti-topisomerase-1 (ATA) or anticentromere (ACA). ARA defines a distinct sub-phenotype characterised by diffuse skin disease and risk of complications including SRC and pulmonary arterial hypertension. We used the strong association between ARA and SRC and the predominant occurrence of SRC early in disease to develop an extreme phenotype strategy for defining genetic factors in susceptibility to renal crisis.

**Materials and methods:** 50 patients with confirmed SRC and ARA+ and another 50 SSc ARA+ that had never developed SRC were identified from our larger SSc cohort. These cases, all with Northern European ancestry, were genotyped across approximately one million SNPs and a logistic regression was performed comparing patients with or without SRC to determine the genetic signature difference between these two groups of patients.

**Results:** 30% of ARA+ SSc developed SRC compared with 1-7% in other antibody groups ($p < 0.001$). In ARA+ cases almost all SRC occurred within 18 months of disease onset. Thus we defined a group with SRC and another at very low risk. Our genetic analysis compared ARA+ patients with SRC history (Group A) to the control group, who had been followed for > 60 months without SRC (Group B). We performed GWAS analysis on these two groups. 641,489 SNPs were analysed. The logistic regression analysis identified a number of SRC associated SNPs within genes and gene regions. Top associations were found in the complement region (P= 1.66x10-5), and in other genes including EPHA5 (P= 1.87x10-5), GRIA3 (P= 2.16x10-5), HECW2 (P= 2.71x10-5) and CTNND2 (P= 2.92x10-5).

**Conclusions:** We present a novel study using extreme phenotypes of ARA+ SSc to identify genetic association of SRC in cases that are serologically and clinically otherwise homogeneous. We identified genes including Caterin cadherin-associated protein delta 2 (CTNND2), known to regulate adhesion molecules relevant to fibrosis. Genes identified from this analysis may have general relevance to SSc vasculopathy or other forms of hypertensive thromobotic microangiopathy. Additional functional and genetic replication studies are needed.

**69. Monocyte chemoattractant protein-1 (MCP-1, CCL2) is a Potential Local Marker of Renal Involvement in Scleroderma: Measurement in Serum, Urine and Renal Tissue**

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**Background:** Renal disease in scleroderma (SSc), including scleroderma renal crisis (SRC), remains a major clinical challenge. Previous studies showed up to 50% of SSc patients have renal involvement. We sought to gain insight into the pathogenesis of SSc kidney disease by examining markers of disease in serum, urine and renal biopsy specimens.

**Materials and methods:** We collected urine and serum from 80 SSc patients, with or without renal involvement, for comparison with patients with kidney disease of other causes (n=10) and healthy controls (n=12). We performed multiplex analysis of candidate markers of disease activity or severity in SSc and renal injury: MCP-1, MCP-3, IL-6, IL-18, TNFalpha, and VEGF. In a further experiment we examined biopsies of patients with SRC using immunostaining for MCP-1.

**Results:** 40 SSC patients were in the subgroup with renal involvement ("SSc-CKD"). Serum MCP-1 was increased in SSc compared with controls, with SSc-CKD significantly lower than SSc without renal involvement. Mean serum MCP-1 was 132 pg/ml (95% CI 105-162) for SSc with normal renal function compared with 65 pg/ml in SSc-CKD (49-81, $p<0.001$ for this comparison). Conversely, urine MCP-1:creatinine ratio was higher in SSc-CKD (mean 64, 32-111) than in SSc with normal renal function (mean 23, 18-28, $p=0.046$). 20 SRC cases confirmed on histology were stained with IgG antibodies for MCP-1. Expression was highest in the tubules, interstitium and vasculature. The number of typical "onion skin" arterial lesions seen was positively correlated with the level of MCP-1 expression in the vasculature overall ($p=0.048$).

**Conclusions:** This is the first study to measure MCP-1 in the urine of SSc patients. Elevated urine MCP-1 in SSc with renal involvement was corroborated by immunohistochemistry demonstrating marked expression of the chemokine in the kidneys of affected patients. The identification of urine MCP-1 as a marker for local expression in the kidney may help define organ-specific effects of this chemokine, which has previously been reported to be increased in serum in association with pulmonary complications. Our findings support further investigation of urine concentrations of MCP-1 as a marker or mediator of renal disease in SSc.

## 70. STAT3 dependent expression of full length stem cell factor transcript maintains systemic sclerosis lung fibroblast phenotypes via autocrine c-Kit signalling

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**Background:** Systemic sclerosis (SSc) is an autoimmune disease characterised by progressive fibrosis of skin and internal organs. Development of an activated population of fibroblasts and myofibroblasts in lesional tissue is likely to be central to pathogenesis. In this study, we identify stem cell factor (SCF) as a potential driving factor in this process. SCF is a growth factor which acts via c-Kit receptor to promote proliferation and migration of hematopoietic progenitor cells, melanocytes and mast cells. Here we show additional autocrine effects of SCF on SSc lung fibroblasts in maintaining their proliferation and migration activities.

**Methods:** A novel patterned collagen chip method was used to study the orientation and migration of SSc and control lung fibroblasts. Migration was also assessed using the scratch wound assay. Proliferation was assessed using the WST-1 assay and by direct cell counting. Protein levels and gene expression of SCF and c-Kit was assessed by western blotting and qPCR. Known inhibitors of signaling pathways were analysed for their capacity to suppress basal and induced SCF and c-Kit mRNA.

**Results:** Phosphorylated c-Kit was amongst the maximally induced phosphorylated proteins in migrating lung fibroblasts. C-kit mRNA and protein was present at low and similar levels in both SSc and control fibroblasts. FACS analysis and MACS microbead sorting did not reveal a c-Kit positive subpopulation. Full length SCF mRNA was increased in SSc lung fibroblasts and tissue biopsies compared to controls, whereas the membrane-bound variant did not differ between SSc and control samples. Soluble SCF and c-Kit were not raised in plasma or tissue fluid of patients arguing against a major systemic role in this disease. Adding SCF enhanced migration and proliferation whereas neutralizing anti-c-Kit and anti-SCF antibodies blocked basal responses. Inhibition of STAT3 greatly reduced both full length SCF and c-Kit mRNA in these cells, whereas antagonism of TGFβ signal did not affect SCF levels.

**Conclusion:** Taken together these data show an autocrine function for SCF/c-Kit in scleroderma fibroblasts acting to maintain proliferation and migratory potential, which could be targeted by therapeutic antibodies or by STAT3 inhibitory drugs.

## 71. Biomarkers of microaspiration and gastro-oesophageal dismotility measurements in scleroderma-associated interstitial lung disease

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**Background:** Microaspiration of gastric contents may have a pathogenetic role in lung fibrosis. Patients affected by scleroderma-associated interstitial lung disease [SSc-ILD] are at high risk of microaspiration due to the very high frequency of gastro-oesophageal reflux [GORD]. This is the first prospective study (NCT02136394) to investigate the relationship between ILD in the context of SSc and direct markers of microaspiration into the lungs.

**Materials and methods:** We present a cross-sectional analysis of the first 27 enrolled patients (median age 59 [min/max 35/79], median FVC=74% [38/128%], median DLCO= 39% [21/72%], female 70%, diffuse SSc 33%). Pepsin concentration in exhaled breath condensate [EBC], saliva, and bronchoalveolar lavage [BAL] was selected as a biomarker of microaspiration. Collected clinical data included oesophageal manometry/24hr impedance (carried out off PPI), respiratory (K-BILD and Leicester cough questionnaires) and GORD symptom questionnaires (*UCLA SCTC GIT 2.0 Questionnaire, Reflux Disease Questionnaire RDQ),* as well as full lung function test data.

**Results:** Proximal reflux was detected in 35% of patients, median DeMeester score was 14.2 [min/max 0.8/156]. Saliva and BAL pepsin showed, respectively, a median concentration of 2.34 ng/ml [2.34/12.4] and 4.3ng/ml [2.86/11.03]. Pepsin was below the detection limit in all the EBC samples. Saliva pepsin levels were significantly correlated with several 24h impedance measurements (e.g. total reflux episodes, r=0.5, p=0.01;cough index association, r=0.6, p=0.007). Pepsin was detected in the saliva of 45% (CI 95% 18-49%) of the subjects with normal DeMeester score (the global index of acid exposure). Among the small group of six patients with BAL results, all BAL samples showed detectable levels of pepsin, with three out of the six having undetectable pepsin levels in the saliva. Lung function test parameters were mildly but significantly correlated with BAL pepsin levels, but not with saliva pepsin and GORD questionnaires.

**Conclusions:** Pepsin concentration in BAL and saliva are potential biomarkers of GORD and microaspiration and may increase the sensitivity of current GORD diagnostic tests. To confirm the association between pepsin levels and clinically significant GORD in ILD-SSc we need to recruit a larger number of patients. We are also planning to prospectively evaluate the role of microaspiration/GORD markers as predictors of ILD-SSc deterioration.

**72. Extra- and intracellular activities of COMP control collagen homeostasis in skin.**

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The hallmark of scleroderma is fibrosis due to deposition of excessive amounts of extracellular matrix (ECM) components by activated fibroblasts. This deregulation is controlled by extrinsic and intrinsic mechanisms.

Other groups and ours have shown that Cartilage oligomeric matrix protein (COMP) is among the proteins which are overproduced in scleroderma, and it is part of the 4-gene signature used as biomarker. In addition to scleroderma, we have further shown highly elevated COMP levels in other fibrotic skin conditions such as the peritumoral stroma and lipodermatosclerosis associated with chronic wounds. COMP is a non-collagenous protein associated with major collagen fibrils and previously thought to be exclusively deposited in load-bearing tissues such as cartilage. We showed that COMP associates with collagen I fibrils and interacts with FACIT collagens XII and XIV in the skin.

To dissect the function of COMP in healthy and fibrotic skin, we compared the histology, biomechanical properties and response to bleomycin of wild type and COMP-null mice (kindly provided by A. Oldberg, Lund) and compared activation of and ECM production by fibroblasts isolated from the dermis of both genotypes.

Absence of COMP resulted in abnormally packed dermal collagen coincident with elevated skin elasticity, reflecting the well-characterized role of COMP in the organization of collagen networks. Injection of bleomycin resulted in attenuated fibrotic responses in COMP-null mice. We show that this is due to intracellular retention of collagen within the ER of fibroblasts and strongly reduced secretion in vitro. This finding was confirmed by electron microscopy showing grossly dilated ER structures in vivo.

From these results we conclude that in addition to the extracellular function of COMP in connective tissues, namely to bridge and thereby stabilize different ECM structures, among them collagens, within the extracellular matrix, COMP exerts a crucial intracellular function by ensuring efficient secretion of collagens. The underlying mechanism is not fully elucidated but may involve the intracellular assembly of collagen I/XII fibrils in the ER. This intracellular activity is a novel mechanism regulating collagen levels in connective tissues, which strongly impact on tissue mechanics and function.

**73. Combined pulmonary fibrosis and emphysema (CPFE) in systemic sclerosis: a syndrome associated with heavy morbidity and mortality.**

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**Background:** The syndrome of combined pulmonary fibrosis and emphysema (CPFE) has been occasionally reported in connective tissue disease. Little is known about its clinical characteristics and prognosis of in systemic sclerosis (SSc).

**Methods**: In this retrospective multi-center case-control study, we identified 36 patients with SSc who developed CPFE, and compared them with 72 SSc controls with isolated interstitial lung disease (ILD).

**Results:** CPFE prevalence was 3.6% of SSc patients, and 8.4% of SSc patients with ILD. CPFE-SSc patients were more likely to be male (75% vs 18%, $p<0.0001$), smokers (83% vs 33%, $p<0.0001$), and to have limited cutaneous SSc (53% vs 24% $p<0.01$) than ILD-SSc controls. No specific autoantibody was significantly associated with CPFE. At diagnosis, CPFE-SSc patients exhibited a marked decrease in carbon monoxide diffusing capacity (DLCO 39±13% vs 51±12% of predicted value, $p<10^{-4}$) when compared to controls, whereas lung volumes (total lung capacity and forced vital capacity) were comparable. Upon follow-up, CPFE-SSc patients more frequently developed precapillary pulmonary hypertension (PH) (44% vs 11%, $p<10^{-4}$), experienced more frequent unscheduled hospitalizations (50% vs 25%, $p<0.01$) and had decreased survival ($p<0.02$ by Kaplan-Meier survival analysis) as compared to ILD-SSc controls.

**Conclusion:** The CPFE syndrome is a distinct pulmonary complication of SSc. Due to its heavy morbidity and mortality burden, it should be identified early in the disease course.

## 74. Outcome of systemic sclerosis patients in the intensive care unit

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**Objective:** Patients with systemic sclerosis (SSc) are prone to disease-specific or treatment-related life-threatening complications that may warrant intensive care unit (ICU) admission. We herein assessed the characteristics and current outcome of SSc patients admitted to ICU.

**Methods:** We performed a single-center retrospective study over 6 years (November 2006-December 2012). All patients with SSc admitted to the ICU were enrolled. Short-term (in-ICU and in-hospital) and long-term (6-month and 1-year) mortality rates were studied, and the prognostic factors were analyzed.

**Results:** Forty one patients with a median age of 50 [interquartile 40-65] years were included. Twenty nine (72.5%) patients displayed diffuse cutaneous SSc. The time from diagnosis to ICU admission was 78 [interquartile 34-128] months. Twenty eight (71.7%) patients previously had pulmonary fibrosis, and 12 (31.5%) had pulmonary hypertension. The main reason for ICU admission was acute respiratory failure in 27 (65.8%) patients. Non-invasive ventilation was first attempted in 13 patients (31.7%) and was successful in eight of them, whereas others required endotracheal intubation within 24 hours. Altogether, 13 (31.7%) patients required endotracheal intubation and mechanical ventilation. The overall in-ICU, in-hospital, 6-month and 1-year mortality rates were 31.8%, 39.0%, 46.4% and 61.0%, respectively. Invasive mechanical ventilation was the worst prognostic factor, associated with an in-hospital mortality rate of 84.6%.

**Conclusion:** This study provides reliable prognostic data in SSc patients who required ICU admission. The devastating outcome of invasive mechanical ventilation in SSc patients pleads for a reappraisal of indications for ICU admission and early identification of patients likely to benefit from non-invasive ventilation.

## 75. Gene profiling in peripheral blood mononuclear cells reveals distinct scleroderma subsets

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**Background:** The heterogeneity of clinical features in patients with systemic sclerosis (SSc) is associated with the extent of skin involvement, severity of organ complications as well as the autoantibody profile. We have previously shown that based on clinical characteristics, SSc overlap patients are clearly different from patients with the diffuse form (dcSSc) and the limited form of SSc (lcSSc). To confirm the clinical diversity between these three main subsets, we have here used expression profiling in peripheral blood mononuclear cells to differentiate patients from healthy controls and to identify specific signatures.

**Materials and Methods:** Four different centers, the University of Cologne (Germany), the Royal Free Hospital in London (UK), the University of Paris (France) and the University of Lund (Sweden) collected peripheral blood cells of 150 SSc patients and 40 healthy controls. Total RNA was extracted, using PAXGene Blood RNA kit (PreAnalytiX GmbH). Gene expression analysis was performed, using Affymetrix Human Genome U219 Chips together with Beckman Biomek FX TPE System and for data analysis Partek Genomics Suite software has been used.

**Results:** Of the 150 SSc patients, 41 patients had dcSSc, 46 patients suffered from lcSSc and 63 patients had SSc overlap syndromes.

The analysis revealed 42 differentially expressed genes (FDR <0.05, FC >1.8) between all SSc patients and healthy controls.

The comparison of all three main subsets has shown overlapping genes, of which most were associated with the “interferon”- and “role of pattern recognition receptors in recognition of bacteria and viruses”- signaling pathways, but also specific genes. Most of the overlapping genes were associated with antimicrobial/inflammatory responses and cell signaling, which have been previously described for other dermatological diseases, connective tissue diseases and inflammatory/infectious diseases.

The selection of those genes, which were specific for dcSSc, lcSSc and overlap patients showed, that SSc overlap patients had the highest amount of differentially expressed genes (n=137), followed by dcSSc (n=19). Patients with SSc overlap syndromes had clearly more up-regulated genes (n = 137, Top 10: HLA-C ↑, CLEC2B ↑, BCL2A1 ↑, IFIT5 ↑, DDX60 ↑, CHMP5 ↑, CLEC12A ↑, SAMD9L ↑, MS4A3 ↑, AIM2 ↑) than dcSSc and lcSSc.

**Conclusions:** These data clearly support the clinical hypothesis that lcSSc, dcSSc and SSc overlap syndromes represent separate subsets within the spectrum of scleroderma. A key finding in our study is a PBMC specific gene expression signature for overlap SSc, with much more upregulated genes and much more specific genes for this subset than in the other groups.

**76. Evidence of Chronic Neutrophil Activation and Neutrophil Extracellular Trap (NET) formation in Systemic Sclerosis (SSc).** Nathalie Burg[1], Jamie Lynn Harden[1], Jessica Gordon[2], and Barry Coller[1].
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**Background**: Platelet activation has been associated with SSc but whether it contributes to SSc pathogenesis is unclear. One possible link is that chronic platelet activation in SSc potentiates or induces NET formation, leading to auto-antigen expression and endothelial damage.

**Methods: MPO and MPO-DNA quantification:** Plasma MPO was measured by ELISA. Plasma MPO-DNA complexes were measured by capturing the complexes with anti-MPO antibody and detecting them with and HRP labeled antibody to DNA.

**Comparison of neutrophil MPO and platelet-neutrophil aggregates in SSc and controls**: Whole blood was stained for MPO, Histone 1(H1), CD11b, and αIIbβ3 for analysis by flow cytometry and in some cases simultaneous light and fluorescent microscopy (Imagestream).

**Neutrophil isolation**: Neutrophils (PMN) were isolated from EDTA-treated whole blood using negative immunodepletion or Polymorphoprep. Immunofluorescent staining was performed after cytospin.

**Assessment of 3-chlorotyrosine protein adducts in plasma**. Plasma proteins were immunoprecipitated with an antibody to 3-chlorotyrosine and analyzed by SDS-PAGE; Coomassie blue stained bands were evaluated by mass spectroscopy.

**Detection of MPO and neutrophil elastase (NE) in skin biopsies of SSc patients and healthy controls**: Punch biopsy samples were analyzed by immunohistochemistry and immunofluorescence.

**Results**: Plasma MPO and MPO-DNA levels were elevated in SSc patients compared to controls (325±225 ng/ml vs 73±56 ng/ml; p=0.003; n=8) and (1.7±0.5 vs. 1.0±0.4; p=0.01, n=6), respectively. Flow cytometry showed that neutrophils from SSc patients had lower MPO compared to controls (geometric mean 6403±1797 vs. 14894±5525, n=10, p=0.01). Rare NETs and large platelet-leukocyte aggregates were detected ex-vivo on Imagestream in 2/3 patients and 0/3 controls. Polymorphoprep isolated PMNs showed increased NETosis compared to controls. 2/3 biopsies from SSc patients showed markedly enhanced staining of MPO and NE in subcutaneous adipose tissues compared to 2 controls. SSc plasmas demonstrated higher levels of proteins immunoprecipitated with antibodies to chlorotyrosine than control plasmas (n=8); mass spectroscopy identified fibronectin, apolipoprotein A1, apolipoprotein B100, fibrinogen, and α2 macroglobulin.

**Conclusions:** Our data indicate that both plasma MPO and MPO-DNA levels are elevated in SSc, supporting enhanced intravascular NET formation. The increased levels of chlorotyrosine on plasma proteins in SSc, a specific marker of MPO enzymatic activity, may contribute to SSc pathogenesis.

## 77. Prevalence and Risk Factors for Left Ventricular Diastolic Dysfunction in Scleroderma

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**Background/Purpose:** Left ventricular diastolic dysfunction (LVDD) is more common in systemic sclerosis (SSc) compared to the general population (1). Focal myocardial ischemia and fibrosis are thought important in its pathogenesis (2). LVDD is associated with increased mortality and little is known about risk factors. Advanced SSc lung complications may be more commonly associated with LVDD (3).

**Methods:** We collected clinical information from retrospective chart review of SSc (4) outpatients seen consecutively in our clinics. LVDD was confirmed by the last echocardiogram (tissue doppler) report. Interstitial lung disease (ILD) confirmed by high resolution chest CT (HRCT) and pulmonary hypertension (PH) diagnosed by right heart catheterization. Univariate and multivariate regression analyses were conducted to determine risk factors associated with LVDD.

**Results:** Table 1 shows 300 charts reviewed and 133(44%) had LVDD. Univariate analysis found patient's advanced age, disease duration (from onset of Raynaud's phenomenon), Anti-centromere antibody, presence of SSc lung complications, systemic hypertension, smoking, valvular heart disease, chronic kidney and thyroid diseases were commonly associated with LVDD. However, using multivariable logistic regression analysis, advanced age was the most significant factor associated with LVDD, followed by systemic hypertension, and then SSc lung complications.

**Conclusion:** The prevalence of LVDD was 44%. Advanced age, systemic hypertension, and SSc pulmonary complications (ILD or PH) were independent risk factors for LVDD. LVDD should be considered in any SSc patient with dyspnea; further research to find better treatment options for LVDD is needed to improve outcomes in this patient population.

**Table 1. Clinical characteristics in SSc patients with and without LVDD**

| Characteristic | Absent LVDD (N=167) | Present LVDD (N=133) | p-Value |
|---|---|---|---|
| **Age, yrs (Mean±SD)** | 53 ± 13 | 66 ± 10 | <0.001* |
| **Female, n(%)** | 141( 84) | 116(87) | 0.5 |
| **BMI: Normal, n(%)**<br>**Underweight**<br>**Overweight**<br>**Obese** | 75(45)<br>14(8)<br>52(31)<br>28(18) | 55(41)<br>9(7)<br>42(33)<br>27(20) | 0.8 |
| **Race: Caucasian,n(%)**<br>**Hispanic**<br>**AA**<br>**Asian**<br>**Native American** | 105(63)<br>19(11)<br>22(13)<br>10(6)<br>11(7) | 94(71)<br>8(6)<br>12(9)<br>8(6)<br>11(8) | 0 .3 |
| **Type of Scleroderma:**<br>**Diffuse,n(%)**<br>**Limited**<br>**Overlap** | <br>63(38)<br>54(32)<br>50(30) | <br>42(32)<br>61(46)<br>30(22) | 0.05 |
| **Disease Duration, (Mean±SD)yrs** | 12 ± 8 | 18 ± 12 | <0.001* |
| **Duration Of Raynads, (Mean±SD)yrs** | 12 ± 9 | 18 ± 13 | <0.001* |
| **mRSS (Mean±SD)** | 12 ± 10 | 9 ± 9 | 0.05 |
| **NailfoldCapillaroscopy: N/(%)**<br>**Normal**<br>**Abnormal** | <br>61(37)<br>86(51) | <br>51(38)<br>57(43) | 0.3 |
| **Autoantibodies:**<br>**ANA, n(%)**<br>**Anti-nucleolar**<br>**Anti-centromere**<br>**Anti-Scl70**<br>**Anti-RNA pol 3**<br>**Anti-RNP antibody**<br>**Anti-PMScl** | <br>147(86)<br>28(17)<br>38(22)<br>48(29)<br>13(8)<br>21(13)<br>5(2) | <br>118(84)<br>15(11)<br>44(33)<br>29(22)<br>10(8)<br>14(11)<br>1(1) | <br>0.9<br>0.16<br>0.04*<br>0.2<br>0.9<br>0.6<br>0.2 |
| **Pulmonary fibrosis**<br>**Absent n(%)**<br>**Present n(%)** | <br>81 (49)<br>86 (51) | <br>48 (36)<br>85 (64) | 0.03 |
| **Pulmonary Hypertension n(%)** | 18 (11) | 36 (27) | <0.001 |
| **Systemic Hypertension n(%)** | 39(23) | 64(48) | 0.001 |
| **Valvular heart disease** | 27(16) | 38(29) | 0.01 |

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**Table 2: Multivariate Regression Analysis for factors associated with LVDD**

| | b* | p-value | Crude OR (95% CI) | Adjusted OR (95% CI) | P -value |
|---|---|---|---|---|---|
| Age | 0.308113 | **0.000003*** | 1.60 (1.50-1.70) | 1.39 (1.26 – 1.52) | <0.001 |
| Disease Duration | 0.146509 | 0.192798 | | | |
| Duration of Raynaud (yrs) | -0.052186 | 0.635159 | | | |
| Systemic Hypertension | 0.150522 | **0.00853*** | .30 (1.16-1.46) | 1.16 (1.04-1.30) | <0.01 |
| Smoking history | 0.088763 | 0.099324 | | | |
| Thyroid disease | -0.038341 | 0.501586 | | | |
| Valvular heart disease | 0.052880 | 0.339044 | | | |
| Renal disease | 0.053526 | 0.325202 | | | |
| Pulmonary fibrosis | 0.120043 | **0.03971*** | 1.13 (1.01-1.27) | 1.13 (1.01-1.26) | < 0.04 |
| Pulmonary hypertension | 0.133191 | **0.01769*** | 1.23 (1.10 – 1.38) | 1.16 (1.02 – 1.27) | <0.02 |
| Anti-Centromere | 0.086025 | 0.162729 | | | |

**78. Adiponectin is an endogenous modulator of matrix remodeling linking intradermal adipose and skin fibrosis**

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**Background**: Systemic sclerosis (SSc) skin fibrosis is associated with attenuated dermal adipose tissue and adipogenic gene expression. Levels of the adipokine adiponectin (APN) and its receptors, are both reduced in SSc, and inversely correlated with the modified Rodnan skin score. We investigated the role of APN in the pathogenesis of skin fibrosis in mice with genetic APN gain- and loss-of-function, and determined the effects, mechanism and therapeutic potential of APN-derived synthetic peptides on the fibrotic process in vitro and in vivo.

**Methods**: Genetic and pharmacological manipulation of APN signaling was evaluated in mouse models of skin fibrosis. Novel APN-derived peptides targeting APN receptors were designed and synthesized, and their effects on fibrotic responses were examined in human and mouse fibroblasts, and in a mouse model of skin fibrosis.

**Results**: Mice lacking APN developed exaggerated cutaneous fibrosis and loss of intradermal adipose upon bleomycin challenge. In contrast, ΔGly-APN mutant mice, which produce ~2-fold elevated levels of APN, were protected from skin fibrosis and showed preferential expansion of intradermal white adipose tissue. To directly evaluate the role of APN signaling in skin fibrosis, recombinant APN, as well as synthetic APN-derived peptides were used. In skin fibroblasts, APN attenuated collagen synthesis, myofibroblast transformation and other fibrotic responses mediated via the energy-sensing enzyme AMP kinase; anti-fibrotic responses were associated with reduced focal adhesion kinase (FAK) activation and focal adhesion disassembly. Treatment of mice with APN-derived short peptides induced potent AMPK activation in target organs in the absence of toxicity. Significantly, peptide treatment prevented, as well as reversed, bleomycin-induced cutaneous fibrosis.

**Conclusions**: We identified an important homeostatic role for APN in negative regulation of skin collagen deposition and myofibroblast accumulation, highlighting a novel link between metabolism and skin fibrosis. The anti-fibrotic effects involve AMPK activation and focal adhesion disassembly. Rescuing APN signaling in SSc with synthetic peptides represents a novel therapeutic approach.

**79. The pr79. ofibrotic functions of TAZ and YAP are inhibited by dimethyl fumarate in systemic sclerosis fibroblasts**

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**Background:** Systemic sclerosis is a devastating multi-organ fibrotic disease with few treatment options. Fumaric acid esters, including dimethyl fumarate (DMF, Tecfidera) represent a novel class of molecules that drive the antioxidative response by activating the Nrf2 signaling pathway. DMF has shown therapeutic effects in models of multiple sclerosis, cardioprotection, neuroprotection, neo-angiogenesis and renal fibrosis. We sought to determine if DMF has a direct effect on profibrotic gene expression in scleroderma dermal fibroblasts.

**Methods:** Adult SSc and normal fibroblasts obtained from dermal biopsies were serum starved overnight followed by 1 hour pretreatment with DMF and 1, 3, and 6 hour treatments with TGFβ or S1P. RNA and protein levels of CTGF, ET-1 and IL-6 were determined by qPCR and Western blot. Nuclear and cytoplasmic extracts were assayed for levels of phospho-SMAD1, phospho-SMAD2/3, TAZ and YAP. TAZ and YAP staining was performed on DMF treated normal and SSc fibroblasts cultured on polyacrylamide gels of varying stiffness (600 Pa vs 6 kPa). C57BL/6 mice received subcutaneous osmotic pump delivery of bleomycin continuously from day 0 to 7 with daily DMF or vehicle IP injections from day 0 to 21.

**Results:** DMF pretreatment led to complete blockade of TGFβ and S1P induced CTGF, ET-1 and IL-6 gene expression and CTGF protein in cultured fibroblasts. This correlated with a rapid loss of TAZ and YAP protein from the nucleus and phosphorylation of YAP on serine 127, with no change in SMAD phosphorylation. Immunostaining demonstrated nuclear localized TAZ and YAP in normal and SSc fibroblasts in culture, and this staining was reduced by either DMF treatment or lowering substrate stiffness. siRNA mediated depletion of TAZ/YAP mimics the effects of DMF on CTGF, ET-1 and IL-6 expression. Subcutaneous bleomycin delivery induced an increase in expression of CTGF, ET-1 and IL-6 in the skin which was prevented by concurrent DMF injections.

**Conclusions:** Dimethyl fumarate reduces nuclear levels of TAZ and YAP in scleroderma dermal fibroblasts which is sufficient to fully inhibit the acute profibrotic response to TGFB and S1P. DMF prevents dermal fibrosis in a mouse model, further supporting its potential for the treatment of SSc dermal fibrosis.

**80. Predictors of cardiovascular complications and mortality in Systemic Sclerosis (SSc) patients with myocardial fibrosis and microvascular cardiac damage**

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**Background:** In previous studies we showed that prevalence of myocardial fibrosis in SSc patients is 45% and is associated to diffuse disease (dcSSc) and lower left ventricle ejection fraction (LVEF); microvascular damage was also very frequent (79%). Our aim was to identify baseline characteristics associated to the development of cardiovascular outcomes (heart failure, coronary artery disease, arrhythmias, vasculopathy, and death) in SSc patients with previously documented myocardial fibrosis and microvascular damage.

**Patients and Methods:** We included 62 SSc patients who participated in the study of prevalence of myocardial fibrosis (2008-2010) and in our local SSc cohort. We performed baseline clinical evaluation, cardiac MRI, coronary angiotomography, transthoracic echocardiogram, and yearly clinical and cardiovascular evaluation that included Medsger's severity scale items, electrocardiogram, echocardiogram, chest X ray or HRCT and spirometry; we registered presence and severity of internal organ involvement and cardiovascular outcomes. Ordinal variables were analyzed using Chi square test and Fisher test when appropriate, numeric variables were compared using Student's t test or Mann Whitney U when appropriate, logistic regression was used to perform multivariable analysis.

**Results:** We obtained follow-up information from 61 patients (29 dcSSc, 32 lcSSc), mean follow up was 43.5 months. Univariate analysis showed that elevated basal ultrasensitive CRP was associated to higher overall mortality at the end of follow-up (p=0.003, OR=22, 95% CI 2.3-209), and microvascular damage at baseline was associated to recurrent digital tip ischemic ulcers (p=0.05). Multivariable analysis showed that: myocardial fibrosis, particularly in the middle LV segments was associated to the development of heart failure (p=0.04, OR 8.9, 95%CI 1.07-76); lower LVEF was associated to the development of coronary artery disease (p=0.02, OR 0.66, 95%CI 0.47-0.9); finally, insertion point fibrosis (p=0.01, OR 11.1, 95%CI 2.5-55.5) and elevated ultrasensitive CRP (p=0.04, OR 5.2, 95%CI 1.82-25.6) were associated to recurrent digital tip ulcers.

**Conclusions:** This study shows that elevated ultrasensitive CRP, the presence of myocardial fibrosis and microvascular damage are predictors of cardiovascular outcomes in SSc patients. Patients with myocardial fibrosis experience progressive decline in LVEF when compared to those without fibrosis. Future studies should focus on therapeutic strategies for this group of patients.

**81. Differential production of Th17-related cytokines by several TLR ligand-stimulated monocyte derived dendritic cells from systemic sclerosis (SSc) patients.**

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**Background:** Systemic sclerosis (SSc) patients exhibit alterations in innate and acquired immunity that lead to increased production of pro-inflammatory cytokines and may be associated to disease progression and phenotype. Pathogenic mechanisms of these alterations are not fully understood. Aim: To determine the effect of TLR3, TLR4 and TLR9 stimulation of monocyte derived dendritic cells (Mo-DCs) in the production of Th17-related molecules in SSc patients.

**Patients and Methods:** We included 16 SSc patients (4 early limited cutaneous lcSSc, 4 late lcSSc, 4, early diffuse cutaneous dcSSc and 4 late dcSSc patients) and 5 non-related healthy controls. We isolated peripheral blood mononuclear cells (PBMCs) from 55 ml of venous blood. Mo-DCs were differentiated from ex-vivo purified CD14+ monocytes, stimulated with TLR agonists (LPS/TLR4, poly:IC/TLR3, CpG/TLR9) and co-cultured with PBMCs. Cytokine levels in supernatants were measured after 96 h, by Luminex. Differences were evaluated by Mann-Whitney U test.

**Results:** All SSc patients were females (mean age 48+/-14.4 years). We found significantly higher levels ($p<0.05$) of IL-6, IL-10, IL-17F, IL-22, IL-23, IL-31, IL-1b and IFN-$\gamma$ in LPS/TLR4-stimulated Mo-DCs-PBMC co-culture supernatants from lcSSc patients when compared to those from dcSSc patients. In contrast, Mo-DCs-PBMC co-cultures from dcSSc patients produced higher levels of IL-33 with and without TLR agonist stimulation than lcSSc patients' co-cultures. When SSc patients were categorized as early and late SSc we found that LPS/TLR4-stimulated Mo-DCs-PBMC co-cultures from early SSc patients produced higher amounts of IL-6, IL-10, IL-17F, IL-22, IFN-$\gamma$ and TNF-$\alpha$ when compared to those from late SSc patients. Interestingly, IL-33 secretion was increased with or without stimulation with TLR agonists ($p<0.05$) in Mo-DCs-PBMC co-cultures from late SSc in comparison with those from early SSc patients.

**Conclusions:** Mo-DCs stimulation with TLR4 ligand potently induces cytokines involved with the pro-inflammatory Th17 phenotype. TLR4-stimulated Mo-DCs from lcSSc patients produce higher amounts of Th17 (IL-6, IL-22 and IL-23) and Th1 cytokines. Early SSc patients exhibit enhanced Th17 responses. Mo-DCs from dcSSc and late SSc patients exhibit enhanced production of IL-33. Our findings suggest that functional variations in the production of Th17 cytokines by Mo-DCs are associated with different SSc clinical subsets and phases.

## 82. Unraveling the genetics of Scleroderma in African-Americans

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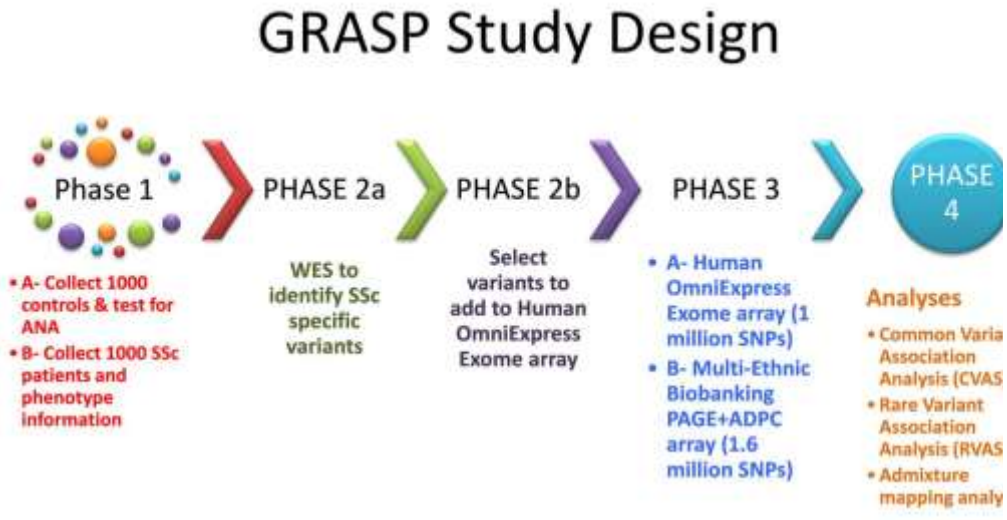
***Background:*** African-Americans (AA) have a higher incidence and prevalence of systemic sclerosis (SSc; scleroderma) than European-Americans (EA). AA develop SSc at an earlier age, with a more severe phenotype, interstitial lung disease and an increased risk for mortality as compared to EA. Family studies and twin studies point towards a significant genetic effect in SSc pathogenesis. Majority of the AA derive their ancestry from Western Africa and Africans have a SSc phenotype that is very similar to AA and different from EA. Thus, the AA SSc population is an ideal target population to understand SSc pathogenetics. We hypothesize that the higher prevalence and severe phenotype of SSc in AA is due to the genetic variants derived from their African ancestry. We are utilizing this fact and adopting a comprehensive approach of testing common and low-frequency/rare variants and applying admixture mapping to identify SSc associated loci.

***Materials and methods:*** Samples are being collected under a multicenter consortium, GRASP (Genome Research in African-American Scleroderma Patients) across US and controls will be tested for ANA by indirect immunofluorescence (Phase-1)(Fig.1). Whole exome sequencing (WES) was performed on 400 SSc and 400 controls to identify rare variants unique to SSc (Phase-2a). 20,000 variants will be selected for replication and validation in 1000 SSc and 1000 controls and rare variant association performed (Phase-2b/4). Genotyping for common variants will be performed on the 1000 SSc and 1000 controls using multiple arrays (Phase-3). Admixture mapping analysis and common variant association analysis will be used to identify SSc susceptibility loci (Phase-4).

***Results:*** DNA from 850 AA SSc patients has been collected so far under the GRASP consortium. 1062 control serum was tested for ANA by immunofluorescence and 1039 (97.8%) were found to be ANA negative at 1:80 titer and 1000 ANA-negative controls have been selected. Individual level WES on 400 AA SSc patients and 400 controls has been completed. Rare/low-frequency variant analysis and array genotyping is ongoing.

***Conclusions:*** This is the first study of its kind and scope to comprehensively test common and low frequency/rare variants associated with SSc susceptibility in AA patients. Identifying genetic loci associated with AA SSc will help identify pathways involved in SSc susceptibility that could potentially be targeted therapeutically.



**83. African American race associated with body image dissatisfaction among patients with systemic sclerosis**

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**Background:** Studies have shown a high degree of body image dissatisfaction (BID) among patients with systemic sclerosis (SSc). We aimed to identify demographic and phenotypic characteristics that correlate with BID.

**Materials and methods:** Ninety eight patients with SSc were recruited from Georgetown University Medical Center 2003-2004. Anonymous surveys collected demographic information (age, race, gender, duration/type of SSc) and assessed degree of BID on a scale of 0-3 in relation to phenotypic features of SSc (hand contractures, finger ulcers, pigmentation changes, lip wrinkling/thinning, telangiectasias). A composite total distress score was derived. Parametric and nonparametric T tests were used to compare groups.

**Results:** Of 98 patients, 86 were female and 12 male. The majority of patients were 30-60 years old. The sample was 62% Caucasian, 27% African American, and the rest identified as "other". Twenty seven percent had limited SSc, 48% diffuse and 25% "other".

African American patients had greater total BID (p=0.002), specifically with respect to digital ulcers, pruritus, and pigmentation changes, than Caucasian participants. Patients with diffuse SSc had greater BID than those with limited disease (p=0.002).

**Conclusions:**

Our results suggest that African American patients with SSc and those with diffuse subtype suffer a higher degree of BID. Given that cognitive behavioral therapy has shown benefit in the treatment of BID, screening for and addressing this issue in SSc patients is prudent.

| Variable | AA % with None/Mild/Mod/Severe Distress | AA Mean Distress(SD) | Caucasian % with None/Mild/Mod/Severe Distress | Caucasian Mean Distress(SD) | P Value |
|---|---|---|---|---|---|
| Degree of Skin Thickening | 24/44/8/24 | 1.32(1.1) | 34/33/16/16 | 1.15(1.1) | 0.494 |
| Contractures of Hands | 28/12/20/40 | 1.72(1.3) | 33/26/11/30 | 1.38(1.2) | 0.287 |
| Ulcers on Fingers | 35/15/8/42 | 1.58(1.4) | 61/13/10/16 | 0.82(1.2) | **0.011** |
| Having to Itch | 12/28/32/28 | 1.76(1.0) | 48/21/18/13 | 0.97(1.1) | **0.002** |
| Dark Body Pigment | 16/32/36/16 | 1.52(1.0) | 61/23/8/8 | 0.64(1.0) | **<0.001** |
| White Body Pigment | 42/23/27/8 | 1.00(1.0) | 70/15/8/7 | 0.51(0.9) | **0.015** |
| Facial Changes | 16/40/12/32 | 1.60(1.1) | 43/25/23/10 | 1.00(1.0) | 0.021 |
| Darker Facial Pigment | 28/20/16/36 | 1.60(1.3) | 72/16/10/2 | 0.41(0.7) | **<0.001** |
| Facial White Spots | 54/15/15/15 | 0.92(1.2) | 90/7/3/0 | 0.13(0.4) | **<0.001** |
| Wrinkles Above Lip | 64/28/4/4 | 0.48(0.8) | 52/11/20/16 | 1.00(1.2) | 0.098 |
| Thinning of Upper Lip | 54/27/12/8 | 0.73(1.0) | 52/20/11/16 | 0.92(1.1) | 0.632 |
| Telangiectasias | 54/15/15/15 | 0.92(1.2) | 48/33/10/10 | 0.82(1.0) | 0.94 |
| **Total Distress** | | **14.77 (7.6)** | | **9.7 (6.6)** | **0.002** |

**Table 2. Disease Features and BID**

## 84. Identification of novel drivers of fibrosis in SSc

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**Background:** Fibrosis is a major pathological feature of many chronic diseases such as systemic sclerosis (SSc) characterised by activation of fibroblasts, accumulation of extracellular matrix (ECM) and persistent inflammation which can lead to impaired organ function. Fibrotic disorders share common features although it is unclear which of these occur as a result of shared mechanisms and pathways and which are mediated by unique organ-specific mechanisms. The aims of this project are to (1) identify common and unique genes whose expression is altered in fibrotic organs (lung, skin and kidney), (2) validate their expression in SSc cells and tissues and (3) explore their function behaviour in normal and SSc fibroblasts.

**Materials and methods**: An extensive literature search from 1988 to 2015 (*PubMed*) was carried out to identify common/unique genes involved in fibrosis of diverse aetiologies. In addition, a thorough *in silico* data-mining exercise was conducted using all published microarray data (*GEO database*). A short-list of genes was compiled based on several criteria including common/specific targets for lung, kidney and skin fibrosis, drugability and availability of reagents. Validation of expression levels of the short-listed genes was performed in human primary fibroblasts (n=3) by qPCR (mRNA), Western blotting (protein) and immunohistochemistry. Fibroblasts were derived from normal, SSc lung and skin tissue and from normal kidney. Transforming growth factor-β (TGF-β) was used to induce fibrotic genes in fibroblasts from healthy controls.

**Results: A list of the 100 most altered (up- or down-regulated) genes was compiled from which** 12 genes were short-listed. Pathway analysis of all 100 altered genes highlighted the hyaluronic acid (HA) pathway from which 2 genes were in 12-gene short-list, Hyaluronan synthase 2 (*HAS2) and* cell migration-inducing protein (*CEMIP*). *HAS2*, responsible for hyaluronan polymerization, was the most significantly up-regulated gene. Significantly elevated *HAS2* levels were observed in cells and tissues from fibrotic lung, skin and in TGF-β-treated fibroblasts from all 3 organs. *CEMIP*, a HA binding protein, was significantly down-regulated in fibroblasts from SSc lung and skin.

**Conclusions:** Taken together, these data reveal that *HAS2* is significantly up-regulated in SSc and fibrotic conditions and *CEMIP* is significantly down-regulated in SSc fibroblasts compared to controls. These data suggest that *HAS2* and *CEMIP* are potential targets for anti-fibrotic therapies.

## 85. Endothelial to Mesenchymal Transition (EndoMT) Contributes to Endothelial Dysfunction in Systemic Sclerosis associated Pulmonary Artery Hypertension (SSc-PAH)

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**Background:** Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by vascular damage, inflammation and tissue injury. Pulmonary arterial hypertension (PAH) occurs in up to 10% of SSc patients (SSc-PAH), resulting in pulmonary endothelium dysfunction and vascular remodeling. Recently endothelial-to-mesenchymal transition (EndoMT) has been implicated in a number of fibrotic diseases as a mechanism of vascular dysfunction, vessel remodeling and expansion of pro-fibrotic mesenchymal cells. Here we explore the presence EndoMT in clinical and pre-clinical SSc-PAH tissues, and establish an inducible *in vitro* model of EndoMT.

**Materials and methods:** Using lung tissue from SSc-PAH and healthy control (HC) donors, and from the murine hypoxia/SU5416 pre-clinical PAH model, co-expression of vWF/αSMA quantified by immunofluorescence. EndoMT was induced in human PAECs *in vitro* by exposure to TNFα, IL-1β and TGFβ. Western blotting and immunofluorescence was used to quantify: CD31, vWF, occludin, VE-cadherin, αSMA, calponin and collagen type 1 expression. Levels of pro-inflammatory secretion was quantified by MSD arrays on conditioned PAEC, EndoMT and HC and SSc-PAH fibroblast media. The capacity of EndoMT monolayers and mixed cultures of 1:10 EndoMT:PAECs to form cellular barriers was assessed using electric cell-substrate impedance sensing (ECIS).

**Results:** EndoMT was observed in 6% of SSc-PAH and 5% of murine hypoxia/SU5416 pulmonary arteries. PAECs treated with TNFα, IL-1β and TGFβ exhibited significant changes in morphology, loss of endothelial and elevated mesenchymal expression, and gain of migratory capacity by day 6. EndoMT cells also secreted significantly ($P<0.05$) higher pro-inflammatory cytokines levels. EndoMT cells alone or mixed cultures (1:10) with PAECs, exhibited a significant ($P<0.01$) reduction in cell-cell junction strength and barrier function (Rb value) and a greater capacity for trans-cellular trafficking (Cm value) as determined by ECIS.

**Conclusion:** Co-localisation of vWF/αSMA detected in pulmonary arteries of SSc-PAH patients and pre-clinical models of PAH, is indicative of EndoMT. We demonstrate EndoMT leads to a loss of normal PAEC morphology and enhanced secretion of pro-inflammatory chemokines. Furthermore, the presence of EndoMT cells contributed to enhanced permeability of PAEC barriers. Collectively our data suggests that EndoMT may contribute to the loss of normal endothelium function and the development of SSc-PAH.

**86. Bromodomain inhibitor JQ1 modulates collagen processing and ameliorates bleomycin induced dermal fibrosis in mice**

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**Introduction:** Systemic sclerosis (SSc) is a complex pro-inflammatory scarring disease, characterised by elevated deposition of extracellular matrix (ECM) proteins, in particular collagen type I. The disease is heterogeneous affecting both the skin and visceral organs including kidney, lung and heart. The SSc fibroblast is a key cell which promotes a pro-inflammatory and fibrotic microenvironment that can lead to the loss of normal tissue architecture and organ function. The mechanisms that contribute to the formation and persistence of the SSc dermal fibroblast remain unclear. We have previously shown the epigenetic bromodomain and extra-terminal domain-containing proteins (Brd) which bind to acetylated histone residues, play a significant role in pulmonary fibrosis. Here we seek to explore the contribution of Brd proteins in the development of dermal fibrosis using a specific inhibitor of Brd proteins (Brd 2, 3, 4 and T), JQ1.

**Methods:** We investigated the effect of JQ1 on SSc and healthy control (HC; both n≥3) dermal fibroblasts in the Scar-in-a-Jar *in vitro* model, by western blot and immuno-florescence microscopy. To determine the effect of JQ1 in a pre-clinical model of skin fibrosis, female C57BL/6 mice were given three weekly subcutaneous injections of 100µl sterile saline (n≥6) or 0.1U/ml bleomycin (n≥6) for 14 days and treated with 12mg/kg/day JQ1 (n≥6) or vehicle (n≥6). After 14 days skin and sera were taken for further investigation.

**Results:** JQ1 has a significant inhibitory effect on collagen deposition and, the secretion of IL-6 and MCP-1 in a dose dependant manner ($P<0.05$) on normal dermal fibroblasts. Consistent with this JQ1 attenuated SSc collagen deposition and processing ($P<0.05$). JQ1 markedly attenuated dermal scarring *in vivo* ($P<0.05$). We observed a significant reduction in fibrogenic markers including αSMA, connective tissue growth factor and collagen expression in the skin ($P<0.05$). Furthermore secretion of the inflammatory marker, IL-6 was significantly attenuated ($P<0.05$).

**Conclusion:** We assessed the functional effects of the Brd inhibitor, JQ1, on SSc dermal fibroblasts and the development of dermal fibrosis in a pre-clinical model. We demonstrate that JQ1 markedly attenuated the excessive deposition and processing of collagen type I by SSc fibroblasts. In keeping with Brd proteins playing a pivitol role in the development and progression of dermal fibrosis, JQ1 significantly inhibited ECM deposition *in vivo*. Our data suggests a key role for Brd proteins in the persistence of the SSc dermal fibroblast phenotype.

**87. Characterisation of a modified murine model of systemic sclerosis: subcutaneous injection of a bleomycin-containing methylcellulose-based thermo-reversible gel solution**

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**Introduction:** Systemic sclerosis (SSc) is a multisystem autoimmune disease that is characterized by inflammation, vasculopathy and fibrosis of the skin and internal organs. The murine skin fibrosis model, induced by daily subcutaneous (s.c.) injections of bleomycin (BLM) in a susceptible mouse strain, is the most widely used. A recent paper described weekly s.c. injections of BLM-containing thermo-reversible methylcellulose (MC) gels as a novel method to induce skin fibrosis in mice. We explored the potential of this method by comparing 'single' and 'double' injection protocols in a longitudinal study.

**Material and Methods:** Female C57/BL6j mice were injected s.c. by "single injection (SI)" on day 0 with 0.1 mL of saline/ MC or BLM / MC gel into the lower left and right dorsolateral skin. Half of the mice received a second injection, "double injection (DI)", with either 0.1 mL of saline / MC or BLM / MC after 14 days.

**Results:** Significant skin fibrosis, based on elevated HYP levels, was seen in the "SI" group already at day 7 reaching a plateau at day 14 that remained unchanged until day 28. A second BLM injection at day 14 led to higher skin HYP concentrations at day 21 and 28 compared to the "SI" approach. Histopathological examination showed slight-to-marked severity of fibrosis in "SI" and "DI" mice. In both groups the severity of fibrosis increased with time, reaching a plateau on days 21 and 28. The skin lesions were of higher severity in the "DI" compared to the "SI" mice. Lymphoplasmacellular infiltrates were seen in all mice receiving BLM / MC compared to saline / MC. Genomic analysis revealed significant gene expression changes at every time point in BLM / MC- treated mice compared to saline / MC-treated mice. Gene pathway analysis indicated processes such as ECM remodeling, coagulation, inflammation and cell adhesion.

**Conclusions:** Subcutaneous injection of BLM-containing thermo-reversible MC gel induced clear signs of skin fibrosis with mild inflammatory responses. This method offers significant advantages in terms of animal welfare and reduced animal handling time compared to the frequently used daily BLM injection method.

## 88. The role of the JAK/STAT pathway in regulating type I collagen expression in human Lung fibroblasts

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**Background:** Type I collagen, the most abundant collagen in the body, is composed of two α1 and one α2 subunits wrapped together in a triple helix. Collagen Iα2 (COL1α2) is the limiting factor and largely determines functional collagen levels. Excessive collagen production is a hallmark of all fibrotic diseases such as scleroderma and thus understanding how collagen production has important implications for treatment. An enhancer element upstream of the gene drives high level COL1α2 expression. STAT3 is an important transcription factor, activated through phosphorylation from the JAK kinase family (JAK1-3, TYK2). STAT3 is phosphorylated in activated fibroblasts and is reported to regulate collagen, but the precise mechanism is not fully understood. PIM1 is a downstream STAT3 target, which is required for maintaining STAT3 phosphorylation through a feedback loop.

**Methods:** We used chromatin immunorprecipitation (ChIP) to investigate the binding of STAT3 and RNA polymerase (POL II) to the COL1α2 enhancer in human lung fibroblasts. IL6 was used at a concentration range of 10-25ng/mL with the soluble IL6 receptor α supplied at a 2.5:1 mass ratio. We used the following inhibitors: Ruxolitinib (JAK1 and JAK2, 2-10 μM), WP1066 (JAK2, 1-5 μM), SD1029 (global JAK inhibitor, 1-5μM) and SMI4a (PIM1, 5-20 μM). Collagen type I expression and STAT3 phosphorylation were characterised with western blotting and qPCR.

**Results:** STAT3 and POL II binding to the COL1α2 enhancer was detected by ChIP. Depletion of phospho-STAT3 with SD1029, abolished STAT3 and POL II binding and reduced scleroderma COL1α2 mRNA back to normal levels. IL6 paradoxically increased STAT3 phosphorylation and collagen protein levels but not COL1α2 transcription. Ruxolitinib treatment increased STAT3 phosphorylation and WP1099 treatment had no effect. SD1029 and SMI4a reduced STAT3 phosphorylation.

**Conclusions:** STAT3 is essential for COL1α2 enhancer recruitment but is not a limiting factor in agreement with previous findings. JAK3 or TYK2 rather than JAK1-2, is the primary STAT3 kinase in human lung fibroblasts, and JAK1-2 inhibition may actually activate STAT3 through feedback activation of JAK3. Inhibition of PIM1, potently suppressed STAT3 phosphorylation. Our results show that JAK3 or TYK2 and PIM1, but not JAK1-2 are suitable anti-JAK/STAT targets in lung fibroblasts.

## 89. Safety and Efficacy of Subcutaneous Tocilizumab in Adults with Systemic Sclerosis: Week 48 Data From the FaSScinate Trial

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**Background:** SSc is a debilitating disease with limited treatment options. Data indicate a key role for interleukin-6 (IL-6) in the pathogenesis of SSc.

**Methods:** In this first double-blind, placebo-controlled, phase 2, proof-of-concept study, the effect of inhibiting IL-6 in SSc was explored. Pts ≥18 y with active SSc (1980 ACR criteria, ≤5 y disease duration, mRSS 15-40, and elevated acute-phase reactants) were randomized 1:1 to TCZ 162 mg or PBO SC wkly for 48 wks. Primary end point (EP) was mean change in mRSS from baseline at wk 24.

**Results:** 87 pts (43 TCZ, 44 PBO) were enrolled. At wk 24, a favorable but not statistically significant effect of TCZ over PBO on mRSS was noted (adjusted mean difference, –2.7 [95% CI: –5.85, 0.45], $p$=0.09). At wk 48, a numerically larger change was noted in the TCZ vs PBO arm (secondary EP; adjusted mean difference, –3.6 [95% CI: –7.23, 0.12], $p$=0.06). Fewer TCZ vs PBO pts showed a decline in % predicted forced vital capacity (%FVC <0; 57% vs 84%) and a >10% absolute decrease in %FVC (10% vs 23%) at wk 48 (secondary EP). Analysis of serum biomarkers revealed a rapid and sustained inhibition of CCL18 levels by TCZ. Adverse events (AEs)/serious AEs occurred in 98%/33% of TCZ and 91%/34% of PBO pts by wk 48. One death occurred in the PBO arm and 3 deaths in TCZ pts by wk 48; all were unrelated to study drug except for a fatal lung infection in 1 TCZ pt.

**Conclusions:** Treatment with TCZ resulted in consistent, but not statistically significant, improvements in mRSS at wks 24 and 48 and in PROs at wk 48. A trend toward less FVC decline with TCZ than with PBO noted at wk 24 persisted at wk 48. The effect of TCZ on serum levels of CCL18 suggests a possible activity of TCZ on M2-macrophages. Observed AEs were consistent with SSc complications and the safety profile of TCZ. Overall, the data suggests a positive risk/benefit profile for TCZ in SSc and support further evaluation of TCZ in pts with SSc.

**90. The Pathogenic role of immune complexes containing scleroderma-specific autoantibodies**

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**Background:** Systemic sclerosis (SSc) is a chronic autoimmune condition characterized by tissue fibrosis, microvascular alterations and immune dysfunction with specific autoantibodies. These autoantibodies are highly specific for SSc, and provide the most reliable tool to predict disease subset and organ involvement. Despite such diagnostic and prognostic role, no evidence supporting their pathogenic potential has been raised.

The working hypothesis envisaged that immune complexes (IC) containing scleroderma specific autoantibodies might elicit a pro-inflammatory and pro-fibrotic cascade in target cells, contributing to SSc etiopathogenesis. Since scleroderma autoantibodies bind to nucleic acids, the effects induced by SSc-IC might be mediated by Toll-like Receptors (TLR).

**Materials and methods:** Fibroblasts were isolated from healthy skin biopsies. IC were purified from sera of SSc patients bearing different autoantibody specificities (antibodies against centromeric proteins, DNA-topoisomerase, RNA-polymerase and Th/To) or healthy controls using polyethylene-glycol precipitation. Cell cultures were incubated with pathologic and control IC and TLR3 [Poly(I:C)] and TLR4 (LPS) agonists. mRNA levels of TLR3, TLR9, IFN-α and IFN-β were investigated by Real-Time PCR; ICAM-1 expression was evaluated by cell-ELISA and the secretion of IL-6 and IL-8 in culture supernatants was measured by commercial ELISA kits. The involvement of signaling pathways culminating with p38MAPK, SAPK-JNK and NFκB activation was assessed.

**Results:** Stimulation of normal skin fibroblasts with SSc-IC induced a significant increase in gene expression levels of both IFN-α and IFN-β; similar results were reported in the presence of TLR agonists but not control IC. In addition, ICAM-1 expression and IL-6 and IL-8 secretion were up-regulated by Poly(I:C), LPS and scleroderma but not control IC. The expression levels of TLR3 and -to a greater extent- TLR9 were significantly increased upon stimulation with TLR3 agonist and scleroderma but not control IC. Further, SSc-IC induced the activation of p38MAPK, SAPK-JNK and NFκB.

**Conclusions:** These data provide the first demonstration of the potential pathogenicity of IC from scleroderma patients with different autoantibodies. Indeed, SSc-IC can interact with normal skin fibroblasts, inducing a pro-inflammatory phenotype mediated by p38MAPK, SAPK-JNK and NFκB. TLR3 and TLR9 mRNA upregulation upon SSc but not control IC stimulation suggests the potential involvement of these innate immunity receptors.

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# International Workshop on Scleroderma Research

**Under the aegis of:**

BOSTON UNIVERSITY CENTER FOR PROFESSIONAL EDUCATION
BOSTON UNIVERSITY SCLERODERMA RESEARCH CENTER
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