International Workshop on Scleroderma Research

Under the aegis of:

BOSTON UNIVERSITY

BOSTON UNIVERSITY SCLERODERMA RESEARCH CENTER

NIAMS Scleroderma Center
of Research Translation,
Boston University School of Medicine

UCL

Royal Free Hampstead NHS
NHS Trust

Centre for Rheumatology and
Connective Tissue Disorders
University College London
(Royal Free Hospital Campus)

Advancing Scleroderma Research and Translational Medicine

13th INTERNATIONAL WORKSHOP on SCLERODERMA RESEARCH

August 3rd – 7th, 2013
Boston, Massachusetts

www.bumc.bu.edu/sclerodermaworkshop

Organizers

Co-chairs
Professor Dame Carol Black
Professor Robert Lafyatis

Organizing Committee
Professors David Abraham, Christopher Denton, Carol Feghali-Bostwick, Armando Gabrielli, Thomas Krieg, Luc Mouthon, Marlene Rabinovitch, Kristofer Rubin, Maria Trojanowska, John Varga, Michael Whitfield.

Coordinators
Kate Brennan, Kim Fligelstone, Millie Williams, Pamela Yeomans

Acknowledgements

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for their support of the Workshop:

GOLD LEVEL SPONSORS

SCLERODERMA FOUNDATION
SUPPORT · EDUCATION · RESEARCH

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United Therapeutics
CORPORATION

Workshop Information

Conference Office (10)
10 Buick Street, G-18

Opening times:

Saturday	1pm-5pm
Sunday	8am-3pm
Mon-Wed	8am-5pm

Accommodations (10)
10 Buick Street

Workshop Sessions (9)
Dance Theatre @ Fit/Rec Center
Entrance off Buick Street, opposite 10 Buick Street Residences

Poster Session (10)
18th Floor Lounge @ 10 Buick Street
Access to the Lounge for set up will be from Saturday, August 3 at noon. Posters will remain up for the duration of the Workshop.

Meals (4)
West Campus Dining Hall
Breakfast and lunch only. Please have your conference badge with you.

Welcome Reception (7)
Burke Club Room @ Agganis Arena (2nd Floor)
Sunday, 5:45-7:15pm

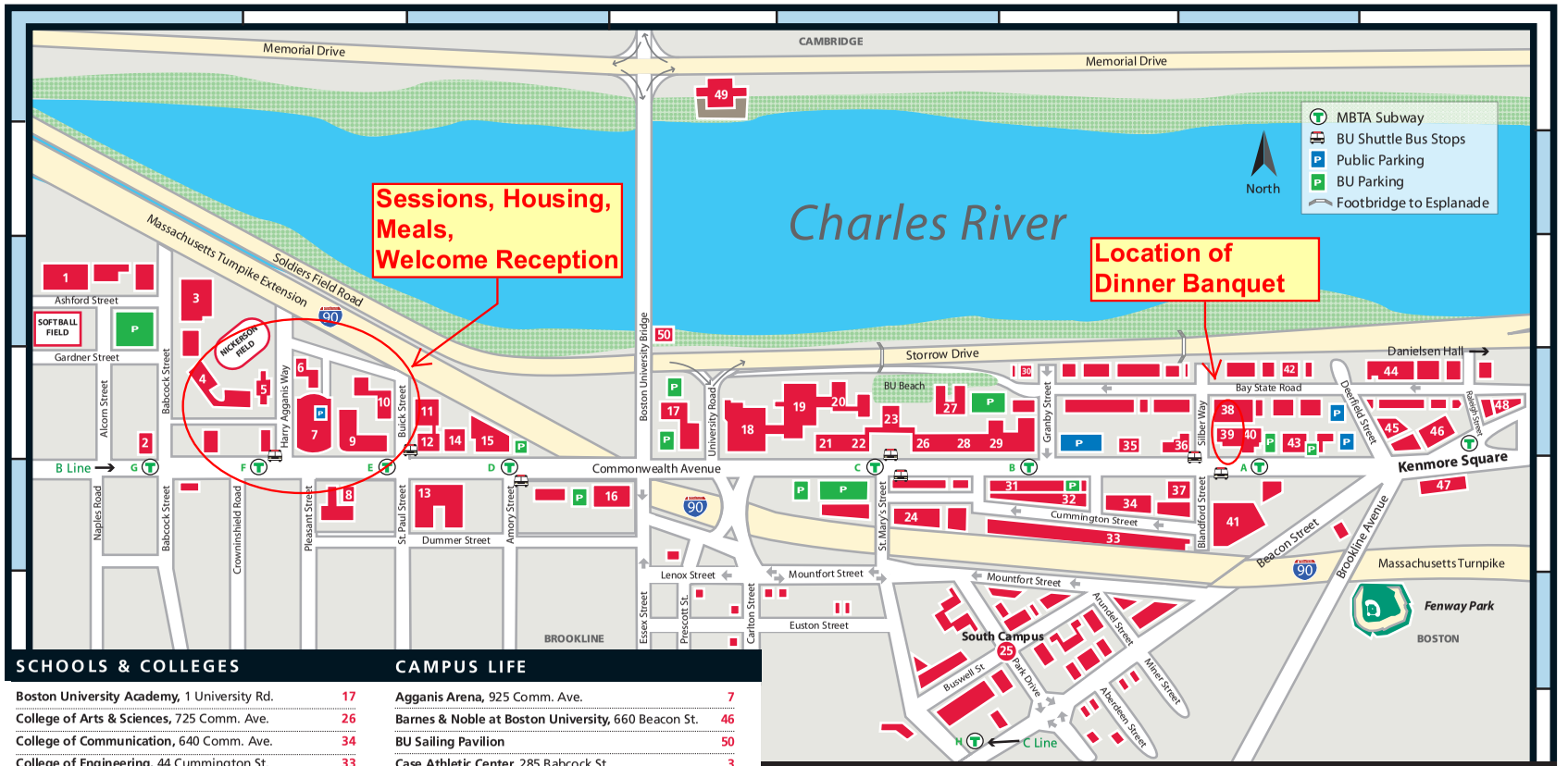
Dinner Banquet (39)
Metcalf Trustee Center Ballroom, One Silber Way, 9th Floor
Tuesday, August 3rd, 6-9pm

Getting to the Banquet:
Shuttle (Free): The Boston University Shuttle leaves from in front of 33 Harry Agganis Way at 5:30pm & 5:45pm and arrives at the corner of Blandford Street and Commonwealth in about 6 minutes.
Cross Commonwealth Ave and walk up Silber Way (Sherborn Street); the entrance to 1 Silber Way is on your right.
The return shuttles leave from the corner of Commonwealth and Silber Way (Sherborn Street) at 8:48pm & 9:18 and arrives back at 33 Harry Agganis Way in 7 minutes.

Trolley ($2.00 each way): Take the inbound Green Line trolley at the corner of Pleasant Street & Commonwealth Ave. or Buick Street and Commonwealth (descend at Blandford Street stop) and the outbound trolley to return (descend at Pleasant Street on the return journey).

Walking: From Buick & Commonwealth to Sherborn & Commonwealth takes approximately 15 minutes.

Boston University Charles River Campus



SCHOOLS & COLLEGES

Boston University Academy, 1 University Rd.	17
College of Arts & Sciences, 725 Comm. Ave.	26
College of Communication, 640 Comm. Ave.	34
College of Engineering, 44 Cummington St.	33
College of Fine Arts, 855 Comm. Ave.	15
College of General Studies, 871 Comm. Ave.	14
Graduate School of Arts & Sciences, 705 Comm. Ave.	28
Metropolitan College & Extended Education, 755 Comm. Ave. (Summer Term)	21
College of Health & Rehabilitation Sciences: Sargent College, 635 Comm. Ave.	35
School of Education, 2 Silber Way	36
School of Hospitality Administration, 928 Comm. Ave.	8
School of Law, 765 Comm. Ave.	20
School of Management, 595 Comm. Ave.	40
School of Social Work, 264 Bay State Rd.	27
School of Theology, 745 Comm. Ave.	22
University Professors Program, 745 Comm. Ave.	22

The Henry M. Goldman School of Dental Medicine, School of Medicine, and School of Public Health are located at the Boston University Medical Campus in Boston's South End.

CAMPUS LIFE

Agganis Arena, 925 Comm. Ave.	7
Barnes & Noble at Boston University, 660 Beacon St.	46
BU Sailing Pavilion	50
Case Athletic Center, 285 Babcock St.	3
Fitness & Recreation Center, 915 Comm. Ave.	9
George Sherman Union, 775 Comm. Ave.	18
Marsh Chapel, 735 Comm. Ave.	23
Mugar Memorial Library, 771 Comm. Ave.	19
Student Activities Center, 1 University Rd.	17
Track & Tennis Center, 100 Ashford St.	1
Tsai Performance Center, 685 Comm. Ave.	29

MAJOR RESIDENCES

10 Buick Street	10
33 Harry Agganis Way	6
575 Commonwealth Avenue	43
1019 Commonwealth Avenue	2
Danielsen Hall, 512 Beacon St.	(not on map)
Myles Standish Hall, 610 Beacon St.	48
Shelton Hall, 91 Bay State Rd.	44
South Campus	25
The Towers, 140 Bay State Rd.	38
Warren Towers, 700 Comm. Ave.	31
West Campus, 273–277 Babcock St.	4

BUILDINGS & SERVICES

Admissions Reception Center, 121 Bay State Rd.	42
Boston University Police, 32 Harry Agganis Way	5
Center for English Language & Orientation Programs, 890 Comm. Ave.	13
Dean of Students, 775 Comm. Ave.	18
Disability Services, 19 Deerfield St.	45
Educational Resource Center, 775 Comm Ave.	18
Financial Assistance, 881 Comm. Ave.	12
Hotel Commonwealth, 500 Comm. Ave.	47
Housing Office, 25 Buick St.	11
Information Technology, 111 Cummington St.	32
International Students & Scholars Office, 888 Comm. Ave.	13
Metcalf Science Center, 590 Comm. Ave.	41
Photonics Center, 8 St. Mary's St.	24
President's Office, 1 Silber Way	39
Registrar, 881 Comm. Ave.	12
Student Accounting Services, 881 Comm. Ave.	12
Student Health Services, 881 Comm. Ave. (West)	12
University Service Center, 881 Comm. Ave.	12
808 Commonwealth Ave.	16

SPECIAL ATTRACTIONS

BU Art Gallery at the Stone Gallery, 855 Comm. Ave.	15
Boston University Experience, 602 Comm. Ave.	37
Boston University Theatre, 264 Huntington Ave.	(not on map)
The Castle, 225 Bay State Rd.	30
DeWolfe Boathouse, 619 Memorial Dr.	49
Gotlieb Archival Research Center, 771 Comm. Ave.	19

Stops

- A Blandford Street
- B Boston University East
- C Boston University Central
- D Boston University West
- E St. Paul Street
- F Pleasant Street
- G Babcock Street
- H St. Mary's Street

Campus Information

617-353-INFO (4636) | AskUs@bu.edu | www.bu.edu/infocenter

Visit Boston University Online Maps based on Google Maps for directions and more information at www.bu.edu/maps.

The distance from Kenmore Square to West Campus residences is approximately 1.3 miles.

Advancing Scleroderma Research and Translational Medicine

SCIENTIFIC PROGRAM

SATURDAY, AUGUST 3

1-5pm	Registration / Poster Set-Up	

SUNDAY, AUGUST 4

8am-5pm	Registration	
8:15am	Welcome and Introduction	Carol Black & Robert Lafyatis
SESSION 1 **Integrative Medicine** Chairs – Michael Whitfield & Shervin Assassi		
8:25	Introduction	
8:30	The application of bioinformatics to autoimmune disease	Atul Butte
9:30	The genetic basis for tsk2	Elizabeth Blankenhorn
10:00	Discussion	
10:30	COFFEE BREAK	
SESSION 2 **Biomarkers** Chairs – Luc Mouthon & John Varga		
10:55	Introduction	
11:00	Biomarkers of interstitial lung disease	Naftali Kaminski
11:30	Alterations in angiogenesis in systemic sclerosis and clinical biomarkers	Marco Matucci-Cerinic
12:00pm	Discussion	
12:30	LUNCH	
SESSION 3 **ER Stress** Chairs – Maria Trojanowska & David Abraham		
1:55	Introduction	
2:00	Unfolded protein response and oxidative stress	Randall Kaufman
2:30	Mechanotransduction and redox regulation in lung fibrosis	Victor Thannickal
3:00	Discussion	
3:30	TEA BREAK	
SESSION 4 **Environmental & Infectious Disease Triggers** Chairs – Armando Gabrielli & Carol Black		
3:55	Introduction	
4:00	Identifying environmental factors in autoimmune diseases	Fred Miller
4:30	Microbial pathogens in skin disease	Sarah Arron
5:00	Discussion	
5:30	END of SESSION	
5:45	WELCOME RECEPTION – AGGANIS ARENA	

MONDAY, AUGUST 5

SESSION 5 **Molecular Genetics and Fibrosis** Chairs – Maureen Mayes & Michael Whitfield		
7:55am	Introduction	
8:00	Non-coding RNAs in skin disease	Howard Chang
8:30	Epigenetics and liver fibrosis	Derek Mann
9:00	Discussion	
SESSION 6 **High Throughput Proteomics** Chair – Monique Hinchcliff & Thomas Medsger		
9:15	Introduction	
9:20	Systems-level analysis of proteolytic events in increased vascular permeability and complement activation in skin inflammation	Chris Overall
9:50	Biomarker studies in lupus nephritis	Chandra Mohan
10:20	Discussion	
10:35	COFFEE BREAK	
SESSION 7 **Matrisomal Proteins and Adipose in Fibrosis** Chairs – Kristofer Rubin & Thomas Krieg		
10:55	Introduction	
11:00	Decoding the matrix	Richard Hynes
11:30	Advances in understanding adipogenesis and links to fibrosis	Stephen Farmer
12:00pm	Discussion	
12:15	LUNCH	
SESSION 8 **Angiogenesis & Pericytes** Chairs – Jeff Browning & Robert Lafyatis		
1:25	Introduction	
1:30	Pericytes	Annika Keller
2:00	Mesenchymal cells and pericytes in lymph node architecture	Theresa Lu
2:30	Pericytes in injury and fibrosis	Lucie Peduto
3:00	Discussion	
3:15	TEA BREAK	
SESSION 9 **Vascular Disease – Applications to Scleroderma** Chairs – Peter Merkel & Armando Gabrielli		
3:40	Introduction	
3:45	Pathophysiology of giant cell arteritis	Cornelia Weyand
4:00	Vascular capillary leak in systemic sclerosis	Tracy Frech
4:15	Biomarkers of vasculitis	Paul Monach
4:30	Discussion	
5:00	END of SESSION	

TUESDAY, AUGUST 6

SESSION 10 **Breakthroughs in Therapy** Chairs – Virginia Steen & Robert Simms		
7:55am	Introduction	
8:00	Autologous hematopoietic stem cell treatment of scleroderma – lessons learned over 15 years	Alan Tyndall
SESSION 11 **PAH & Vascular Remodeling** Chairs – Marlene Rabinovitch & Hap Farber		
8:40am	Introduction	
8:45	Cell therapy approaches for pulmonary arterial hypertension	Duncan Stewart
9:15	Inflammation and hypoxia in pulmonary arterial hypertension	Kurt Stenmark
9:45	Discussion	
10:15	COFFEE BREAK	
SESSION 12 **Industry Presentations** Chairs – David Abraham & Robert Lafyatis		
10:40	Introduction	
10:45	Evaluation of the anti-fibrotic properties of CCNs in pulmonary fibrosis	R Lemaire - Medimmune
11:00	TLR blockade in autoimmune disease	R Arbeit – Idera
11:15	Dasatinib in systemic sclerosis	B Ganguly – Bristol Myers Squibb
11:30	Pharmaceutical development of anti-fibrotic medicines for the 21st century: Enhancing our chances of success	D Budd – GlaxoSmithKline
11:45	Molecular diagnostics for systemic sclerosis: Towards personalized medicine	Y Nesbeth - Celdara
12:00pm	Lunch	
SESSION 13 **Selected Abstract Presentation** Chairs – Luc Mouthon & Maria Trojanowska		
1:25	Introduction	
1:30	Interleukin-33 is an upstream regulator of Inflammation and fibrosis	Irina Luzina
1:45	RNA-seq and miR-seq analysis of SSc skin across intrinsic gene expression subsets shows differential expression of non-coding RNAs regulating SSc gene expression	Zhenghui Li
2:00	Ephrin B2 is overexpressed in human scleroderma skin and mediates fibroblast to myofibroblast differentiations, and induces fibrosis in mice	David Lagares
2:15	SSc intrinsic subset classification in patients that demonstrate clinical improvement during treatment	Monique Hinchcliff
2:30	JNK-1 shows an association with systemic sclerosis	Sandra Guerra
2:45	Pulmonary hypertension in patients with U1-RNP antibodies	Vincent Sobanski

3 – 5pm ~ Poster Viewing ~ 18th Floor Lounge – 10 Buick Street

6PM ~ BANQUET ~ Metcalf Trustee Center Ballroom ~ One Silber Way ~ 9th Floor
Banquet Speaker: *Joseph Loscalzo, MD, PhD*, Chairman, Department of Medicine; Physician-in-Chief, Brigham & Women's Hospital; Hersey Professor of the Theory and Practice of Medicine, Harvard Medical School

WEDNESDAY, AUGUST 7

SESSION 14 **Innate Immunity & Inflammation in Fibrosis I** Chairs – Carol Feghali-Bostwick & John Varga		
7:55am	Introduction	
8:00	The inflammasome in interstitial lung disease	Augustine Choi
8:30	Lipid mediators in inflammation and fibrosis	Charles Serhan
SESSION 15 **Innate Immunity & Inflammation in Fibrosis II** Chairs – Carol Feghali-Bostwick & John Varga		
9:00	Sterile injury & inflammation	Richard Flavell
9:30	Inflammasomes in systemic sclerosis	Carol Artlett
10:00	Discussion	
10:30	COFFEE BREAK	
SESSION 16 **Monocytes/Macrophages in Fibrotic Disease** Chairs – Romy Christmann & Christopher Denton		
10:55	Introduction	
11:00	Macrophage heterogeneity and alternative activation	Siamon Gordon
11:30	Macrophage activation in fibrosis	Tom Wynn
12:00pm	Discussion	
12:15pm	LUNCH	
1:15	**Meeting – SCAR and SScores**	

SESSION 17 **Scleroderma Clinical Trials Consortium** Chairs – Virginia Steen & Lori Chung		
2:15	Introduction	
	Should there be a paradigm shift in clinical trials for SSc?	Moderator: L Chung
2:20	CRISS protocol	P Merkel
2:30 2:40	Case selection for stem cell transplantation in systemic sclerosis *Discussion*	C Denton L Chung
	Biomarkers and patient samples	Moderator: M Mayes
3:00	Use of biomarkers as outcome measures	R Lafyatis
3:10 3:20	Control of patient samples from clinical trials *Discussion*	R Simms M Mayes
	Use of observational cohorts in clinical trials	Moderator: V Steen
3:40	ESOS study	A Herrick
3:50	Statistical challenges of observational trials	S Johnson
4:00	Discussion	V Steen
END OF WORKSHOP		

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Top 6 Ranked Abstracts

1. INTERLEUKIN-33 IS AN UPSTREAM REGULATOR OF INFLAMMATION AND FIBROSIS

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Scleroderma lung disease (SLD) is the leading cause of death in patients with scleroderma, or systemic sclerosis (SSc). SLD is characterized by progressive autoimmune inflammation and fibrosis of the lungs and decrease in lung function. Despite substantial advancements in understanding of the mechanisms of SLD, better therapies need to be developed. It has become obvious that numerous, functionally redundant, molecular mediators are involved in propelling SLD. It follows that targeting a single mediator is unlikely to be a successful therapeutic strategy. We propose that identifying upstream regulators of diverse proinflammatory and profibrotic pathways may provide better candidate targets for future SLD therapies.

Interleukin (IL)-33 is a recently identified member of the IL-1 family. The family also includes IL-1α, IL-1β, IL-1ra, IL-18, IL-36α, -β and -γ, IL-37, and IL-38. IL-33 exists in two forms and acts dually. As a nuclear factor, full-length IL-33 (FLIL33) is bound to chromatin and appears to regulate gene expression. By contrast, extracellular IL-33, especially mature IL-33 (MIL33) resulting from proteolytic activation of FLIL33, binds to the cell surface receptor T1/ST2 and induces Th2 responses such as those in allergies and asthma. The latter T1/ST2-dependent activities of the MIL33 cytokine are under active investigation, whereas the former activities of FLIL33 nuclear factor have been underappreciated and not studied in sufficient detail or in association with a disease process.

In patients with SLD, patients with idiopathic pulmonary fibrosis (IPF), and in the bleomycin mouse model of lung injury, pulmonary expression of FLIL33 was elevated intracellularly, particularly intranuclearly, based on immunohistochemical analyses. There were minimal, if any, signs of Th2 activation such as pulmonary eosinophilia or goblet cell hyperplasia, further suggesting that FLIL33 rather than MIL33 was increased. Western blot assays of lung tissue homogenates confirmed minimal maturation of FLIL33 into MIL33. Analysis of the subcellular distribution of overexpressed IL-33 forms in normal primary fibroblast cell cultures revealed that FLIL33 was localized almost exclusively (>95%) in the cell nucleus and was not secreted from the cells, whereas MIL33 was predominantly cytoplasmic and secreted.

Recombinant adenoviral gene delivery of FLIL33 and MIL33 to mouse lungs resulted in strikingly different phenotypes. As expected based on the previous works of other groups with MIL33, gene delivery of this form induced eosinophilia, goblet cell hyperplasia, and increases in Th2 cytokines IL-4, IL-5, and IL-13. Significant elevations were also noted in the levels of IL-17, KC, and MCP-1, whereas the levels of IFN-γ were significantly decreased. In contrast, similar gene delivery of FLIL33 induced pulmonary lymphocytosis and neutrophilia, as well as a modest increase in MCP-1 without affecting the levels of other measured cytokines. Whereas the effects of MIL33 were abrogated by gene deficiency of T1/ST2, the effects of FLIL33 were not. Gene delivery of FLIL33 in cell culture and in vivo caused

elevation of the expression of CCL2, IL-6, MMP3, MMP10, MMP13, and HSP70 mRNAs and proteins, which are all known to contribute to inflammation and scarring.

Gene delivery of FLIL33 combined with bleomycin challenge in vivo had a potentiating effect on pulmonary lymphocytosis; accumulation of collagen; expression of HSP70; and the levels of total and active TGF-β, CCL6, MCP-1, and MIP-1α. These combined effects significantly exceeded the sum of the effects of FLIL33 gene delivery and bleomycin challenge alone ($p < 0.05$). Smad2/3 phosphorylation was also increased in the double-injury model. By contrast, the combined effects of FLIL33 expression and bleomycin on BAL counts of macrophages and neutrophils were additive of these two treatments alone. The Th2 cytokines IL-4, IL-5, and IL-13 were not part of this synergy, and gene deficiency of T1/ST2 did not affect the synergistic nature of the "double-hit" from FLIL33 expression and bleomycin injury. It was concluded that elevated FLIL33 expression in fibrotic lung disease such as that occurring in patients with SSc is a likely contributor to inflammatory and fibrotic lung injury, that this contribution is independent of the T1/ST2 receptor or Th2 cytokines, and that FLIL33 acts as upstream activator of several relevant pathways including cytokine regulation, production of matrix metalloproteinases, and expression of HSP70.

2. RNA-seq and miR-seq analysis of SSc skin across intrinsic gene expression subsets shows differential expression of non-coding RNAs regulating SSc gene expression

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Background: Systemic sclerosis (SSc) is an autoimmune disease with a heterogenous and complex phenotype. Previously, our lab has identified four gene expression subsets (fibroproliferative, inflammatory, limited and normal-like) among SSc patients by their gene expression in skin using DNA microarrays. We have extended these findings by using RNA-Seq in a subset of SSc skin biopsies to detect mRNA levels, splice variants, novel non-coding RNAs, and coding region SNPs in a lower background signal over microarray.

Methods: We performed RNA-Seq on four healthy controls and eight SSc patients skin biopsies. The eight SSc patients included five individuals that mapped to the inflammatory subset and three from the fibroproliferative subset. We sequenced the small and large RNA fraction extracted from each biopsy by Illumina Solexa sequencing. We obtained 90-100 million 50 bp paired-end reads for the mRNA fraction and 25 – 50 million 36 bp reads for the miRNA fraction.

Results: Our analyses reveal significant ($p<0.05$) gene expression differences between healthy controls and SSc patients, as well as between intrinsic subsets. Specifically, we found >1000 genes are significantly expressed in the inflammatory and the fiboproliferative subsets of patients. Many of the significant genes are involved in cellular proliferation or immune responses, consistent with results found by DNA microarray hybridization. We did not observe any significant differential splicing between the healthy controls and the SSc patients. We identified 228 novel long non-coding RNAs (lncRNAs) that are significantly differentially expressed in the inflammatory subset and fibroproliferative subsets. The lncRNAs differentially expressed in the inflammatory subset map to Gene Ontology terms including *inflammatory response*, *immune response*, *response to wounding*, and *defense response* and those in the fibroproliferative group map to *cell cycle*, *M phase*, and *RNA metabolism*. We also identified 54 miRs differentially expressed in the inflammatory subset of SSc patients. These include the well-characterized miR21 that has previously been reported to be differentially expressed in SSc as well as many novel miRs. We have previously shown CCL2 to be highly expressed in the inflammatory subset of SSc and inhibiting its function prevents development of disease in the sclGVHD mouse model. We now identify a novel miR with decreased expression that is predicted to target the 3'UTR of CCL2. We show that transfection of the CCL2-targeting miR, but not a negative control miR, into RAW264.7 macrophage cells expressing a heterologous Luciferase-CCL2 (3'UTR) reduced luciferase activity by 74%.

Conclusions: To summarize, we conducted the first comprehensive RNA-Seq study in SSc skin and identified differentially expressed non-coding RNAs genome-wide. Our findings show that a complex network of regulatory factors controls the disease specific gene expression subsets in SSc skin.

3. Ephrin B2 is overexpressed in human scleroderma skin and mediates fibroblast to myofibroblast differentiation, and induces fibrosis in mice.

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Ephrin B2 is a member of ephrin family belonging to the largest sub-family of membranous receptor protein-tyrosine kinases. The role of ephrin B2 in the pathophysiology of scleroderma (SSc) disease is largely unknown. In the present study we explored the potential of ephrin B2 in mediating fibroblast-myofibroblast differentiation and fibrosis associated with the pathophysiology of SSc disease.

Our immunohistochemistry, Real-Time PCR and western blot analysis show that Ephrin B2 expression was elevated in SSc skin compared to normal human skin. Further, ELISA results showed enhanced ephrin B2 production in SSc skin fibroblasts compared to fibroblasts isolated from healthy donors. In addition, the expression of ephrin B2 receptor, ephB4, is elevated in SSc skin compared to normal human skin. Interestingly, we identified that *in vitro* treatment of normal human skin fibroblasts with recombinant ephrin B2 is able to transform fibroblasts into myofibroblastic cells exhibiting all typical myofibroblastic-characteristics including increased stress fibre formation, increased cell spreading and focal adhesions, increased activation of focal adhesion kinase (FAK, a critical mediator of fibroblast to myofibroblast differentiation) and increased expression of alpha-smooth muscle actin (α-SMA) expressing myofibroblasts. In addition, treatment with the recombinant ephrin B2 is able to enhance fibroblast functions including increased rate of fibroblast migration and adhesion to fibronectin in both normal and SSc skin fibroblasts.

Mice were then injected subcutaneously with recombinant mouse ephrin B2/Fc (100μg/Kg/mouse) daily for two weeks and degree of fibrosis was determined. Mice treated with recombinant mouse ephrin B2/Fc exhibited significant skin fibrosis associated with enhanced collagen deposition, dermal thickness, hydroxyproline content, α-SMA-expressing myofibroblasts and increased expression of p-FAK, type I collagen and CTGF. We then generated fibroblast-specific ephrin B2 knockout mice (KO) mice in which Cre is under the control of a fibroblast-specific regulatory sequence from the proα2(I) collagen gene to achieve ephrin B2 inactivation specifically in the fibroblasts. Wild type mice and ephrin B2 mice were subjected to bleomycin-induced skin fibrosis. Results showed that all ephrin B2 KO mice showed significant protection from bleomycin-induced fibrosis associated with significant reduction in dermal thickness, skin fibrosis, collagen synthesis, α-SMA expression and phosphorylation of FAK.

Our study, for the first time, provides compelling evidence that ephrin B2 is a key mediator of fibroblast to myofibroblast differentiation and targeting ephrin B2 could open up new potential therapeutic avenues to counteract fibrotic and adhesive signaling associated with SSc and related diseases.

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4. SSc intrinsic subset classification in patients that demonstrate clinical improvement during treatment

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Background: Genome-wide gene expression analysis of skin biopsies from patients with systemic sclerosis (SSc) have been used to identify four 'intrinsic' subsets (normal-like, limited, inflammatory and fibroproliferative) that are distinct from previously proposed clinical subsets (limited and diffuse, lc and dcSSc). There is no known SSc biomarker to predict response to treatment though subsets of patients clinically improve during various immune modulatory treatments. We previously reported that patients whose modified Rodnan skin score (mRSS) improved during mycophenolate mofetil (MMF) treatment were classified in the inflammatory intrinsic subset while patients that did not demonstrate clinical improvement were classified in the normal-like or fibroproliferative subsets. The goals of this study are to identify the baseline clinical phenotypes of patients in each SSc intrinsic subset and to determine whether intrinsic subset classification remains stable in biopsies from patients that demonstrate clinical improvement, stability and worsening.

Materials and Methods: Patients with progressive skin disease in the opinion of the treating physician that warranted treatment, and who were referred to MH for study participation, were prospectively enrolled. Northwestern Scleroderma Program Patient Registry participants (Registry) and healthy subjects recruited from Northwestern without SSc were recruited as controls. Arm and back biopsies and demographic information were obtained from all participants. Patients underwent standardized clinical and laboratory assessments including pulmonary function test, 2-dimensional echocardiography with tissue Doppler and high-resolution computed tomography (HRCT) as clinically indicated. mRSS was determined by the same observer at each study visit (baseline, 6-, 12-, 24-, and 36-months). Serum autoantibodies were measured at Specialty Labs (Valencia, CA). Echocardiograms were interpreted according to standardized research protocols by one research echosonographer who was blinded to clinical data. HRCT scans were scored for total degree of lung disease according to published methods by one chest radiologist with experience in interstitial lung disease who was blinded to clinical data. Clinical response was defined as a reduction in mRSS≥5, the minimal clinically important difference.

Results: Data from 12 healthy control subjects and 38 patients with SSc were analyzed. DNA microarray and clinical data were analyzed for 12 SSc patients who consented to baseline biopsies (Registry) and 26 SSc patients who consented to longitudinal biopsies (Study) as part of a prospective clinical investigation. 4 out of 12 Registry patients (92% women) were taking MMF (2) or methotrexate (2) at time of biopsy. 22 of 26 Study patients were started on MMF (2g divided daily dose) at baseline visit. 38 SSc patients were classified as normal-like (11 pts), limited (2 pts), inflammatory (18 pts), and fibroproliferative (7 pts) intrinsic gene expression subsets. 11 of 12 healthy controls were classified in the normal-like group. Patients in the inflammatory (100% dcSSc) and fibroproliferative (86% dcSSc) subsets had higher mRSS (P=0.003) and longer SSc duration (P=0.029) compared to the other subsets. There were no statistically significant differences in autoantibodies

between the patients within subsets. Patients in the fibroproliferative subset had the highest LV mass [86.2g/m2 (14.6), P=0.027] and the lowest forced vital capacity % predicted [68 (14.5, P=0.07], while pts in the inflammatory subset had the lowest tricuspid annular plane systolic excursion [1.96cm (0.37), P=0.029] (all mean (SD)). 9 out of 26 (35%) patients with longitudinal biopsies changed intrinsic subset, and six (50% taking MMF) of these demonstrated ≥5 mRSS change. Mean (SD) disease duration and duration of follow-up was 53mo (35) and 27mo (10) respectively in the patients that changed intrinsic subset compared to 75 mo (86) and 12mo (7) in those whose subset remained stable. The most common pattern of change in intrinsic subset assignment was from the inflammatory to the fibroproliferative subset.

Conclusions: Intrinsic subset classification of SSc patients is independent of clinical subtype and autoantibody specificity. Patients in the inflammatory and fibroproliferative subsets are more likely to have heart and lung involvement. A subset of patients change from the inflammatory to the fibroproliferative intrinsic subset which may be due to increased disease duration, longer follow-up, treatment or a combination.

5. *JNK-1* shows a protective association with ACA but a risk association with ARA positive SSc patients

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Background: Scleroderma or systemic sclerosis (SSc) is a rare complex disease which affects approximately 1 in every 8-10,000 people in the UK. The exact aetiology of SSc is not fully understood, however the pathogenesis of SSc has shown dysregulation of the vascular and immune systems, both leading to fibrosis. To date, a number of candidate gene studies and Genome-wide association studies (GWAS) have shown the replication of a number of key loci. However, due to the heterogeneity of SSc, further genetic analysis is needed to fully understand the genetic component of this disease. Here we genotyped polymorphisms across *JNK-1* (c-Jun N-terminal Kinase 1) to ascertain a potential pathological role in SSc.

Methods: 728 SSc cases and 260 healthy controls were genotyped for nine *JNK-1* polymorphisms as part of a larger genotyping study. All patients and controls were of Caucasian decent and were categorised according to three mutually exclusive autoantibody status: anti-topoisomerase1 (ATA), anticentromere (ACA) and antiRNA-polymerase (ARA). Patients were further classified into sub-phenotypes according to major organ involvement; pulmonary arterial hypertension (defined as an elevation in the mean pulmonary artery pressure >25 mmHg with normal pulmonary capillary wedge pressure (< 15 mmHg) on right heart catheterisation) and renal crisis (defined by a rapidly progressive renal failure, new onset accelerated hypertension). All genotyping was performed by the KASP system (allele specific PCR, KBioscience, UK). All genotype data and sub-phenotype analysis was performed using PLINK.

Results: Our cohort consisted of 63 (9%) patients with renal crisis and 112 (15%) with pulmonary arterial hypertension. 155 (21%) patients were positive for ATA, 255 (35%) patients were positive for ACA and 140 (19%) patients were positive for ARA. The SSc cases and the healthy controls were genotyped and all SNPs and individuals passed quality control checks for Hardy-Weinberg equilibrium (p=0.05) and missingness (p=0.1). An overall case-control analysis was performed using PLINK, of which no association was found in any individual SNP or haplotype. However, sub-phenotype analysis showed associations between SNP rs2289805 with ACA (p= 0.012, OR=0.64) and ARA (p=0.002, OR=1.73). Hence demonstrating the dual role of SNP rs2289805 in SSc with a protective effect in ACA positive patients and a risk effect in ARA.

Conclusions: We report a novel association with SNP rs2289805 in both ACA and ARA positive SSc patients. *JNK-1* is a transcription factor which enhances collagen gene activities through the induction of TGF-β. Polymorphisms in *JNK-1* can lead to a deregulation of expression of collagen associated genes leading to increased collagen formation and fibrosis. These data suggest *JNK-1* to be a good candidate gene in SSc and warrants further investigation.

6. Pulmonary hypertension in patients with U1-RNP antibodies

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Objectives. Pulmonary hypertension (PH) is a leading cause of morbidity and mortality in patients with connective tissue diseases (CTD). Patients with anti-U1-RNP antibodies belong to a heterogeneous group, including systemic sclerosis (SSc), systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD). Management of these patients can be challenging, some studies have shown that immunosuppressive therapy might be beneficial in MCTD or SLE patients with PH. This study aimed to (i) describe the prevalence, clinical and hemodynamic characteristics of PH in U1-RNP positive patients and (ii) analyse survival and compare prognosis between the different CTDs associated with these antibodies.

Methods. We reviewed the clinical charts of 519 anti-U1-RNP antibody positive patients from the Rheumatology and/or Pulmonary Hypertension departments. Eighty patients had a first right heart catheterisation (RHC) performed between January 1998 and December 2012, leading to a diagnosis of PH according to Dana-Point classification. Four patients did not have PH (mean Pulmonary Arterial Pressure [mPAP] < 25 mmHg). In the 76 patients with PH, 10 had post-capillary PH (pulmonary capillary wedge pressure [PCWP] > 15 mmHg) and were classified as "group 2=PH due to left heart disease". 66 had pre-capillary PH (PCWP ≤ 15 mmHg): 6 had PH due to lung disease (group 3) and 60 had PAH (group 1=pulmonary arterial hypertension). 4 patients in the PAH group were excluded because of missing data. Continuous variables are presented with mean ± standard deviation or median (interquartile range). For binary variables, comparison between 3 PH groups was performed using Monte-Carlo method with Fischer test for comparison one by one. ANOVA was used to compare continuous variables with a normal distribution. Kruskal-Wallis test was used for non-parametric comparisons. Kaplan-Meier method with log-rank test was used for survival analysis. All statistical analyses were performed using XL-STAT, Addinsoft®.

Results. The prevalence of PH in U1-RNP positive patients in this cohort was 15%. The proportion of patients with SSc, SLE or MCTD was not significantly different between the 3 groups of PH. Autoantibodies (anti-dsDNA, anti-Sm, anti-Ro/SSA, anti-Scl70, anti-centromere), age and CTD duration at PH diagnosis, WHO functional class (FC) were not significantly different among groups. Patients in the group 3 had a lower DLCO (30% vs. 46%, p=0.042), lower mPAP (28 ± 4 mmHg vs. 41 ± 11 in group 1 and 43 ± 11 in the group 2, p=0.008). Patients in the group 2 had higher right atrial pressure (RAP, 15 ± 7 mmHg, p=0.003). Patients in the group 1 had higher pulmonary vascular resistances (680 ± 453 dynes.s.cm^{-5}) than in the others groups (p=0.003). Mean survival was 3.1 ± 1.2 years in the group 2 and 8.2 ± 1.6 years in the group 1 (log-rank p=0.167). No patient died in the group 3 during a median follow-up of 4.1 years (1.0-9.0). Age at PH diagnosis (p=0.006) and WHO FC III-IV (p=0.022) were associated with mortality in univariate analysis along with specific hemodynamic

variables: RAP (p=0.004), mPAP (p=0.003), cardiac index (p=0.003), PVR (p<0.0001), SvO_2 (p=0.0001), diastolic pulmonary arterial pressure-pulmonary capillary wedge pressure gradient (p=0.004) and transpulmonary gradient (p=0.002). In multivariate analysis, FVC at PH diagnosis (p=0.004), mPAP (p=0.001) and SSc (p=0.002) were significantly associated with mortality. Adjusted hazard ratio of mortality in SSc patients was 10.9 (95% CI: 2.4-50.6). Mean survival was 5.7 ± 0.8 years in SSc, 7.4 ± 1 years in MCTD and 8.2 ± 1 in SLE patients.

Conclusion. This study, from a large cohort of patients with CTDs, estimated a prevalence of PH in U1-RNP patients of 15%. The prognosis remains poor with a mean survival of 8.5 years. These data confirm that patients with U1-RNP antibodies are heterogeneous. Patients with SSc have the worse prognosis. Interestingly, survival of patients fulfilling MCTD classification criteria is intermediate between SSc and SLE. Further studies are needed to study the response to therapy – including PAH-specific and immunosuppressive agents.

Submitted Abstracts/Posters
(Alphabetical by Author)

7. PALMO-PLANTAR SPECIFIC LONG NONCODING RNA HOTAIR DRIVES MYOFIBROBLASTS SPECIFIC SIGNATURE IN SYSTEMIC SCLEROSIS.

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Background: The key cellular elements in the pathogenesis of tissue fibrosis are myofibroblasts. It is widely accepted that the number of myofibroblasts is increased in Systemic Sclerosis (SSc) and it correlates with the severity of tissue fibrosis. The mechanisms underlying their increased number in SSc are unknown. The heterogeneity in their frequency may explain the inconsistency of some in vitro studies published on SSc fibroblast biology, and may mirror the clinical heterogeneity of SSc patients both in natural history and severity of skin fibrosis.

Objectives: The purpose of this study was to unravel the specific transcriptome of myofibroblasts derived from SSc skin biopsies.

Methods: Four patients with diffuse rapidly progressive SSc, within18 months from skin involvement and before any immunosuppression, were enrolled in the study. Skin biopsy on forearm was performed and the fibroblasts subcultured for three passages. Two hundred-fifty acetone fixed alpha-Smooth Muscle Actin (alpha-SMA) positive cells were isolated by laser capture microdissection (LCM) for mRNA analysis by Affymetrix Gene array. qRT-PCR and in situ Hybridization were employed for validation of the results. Pathway analysis was conducted according to David-NIH software. Immunofluorescence (IF) followed by confocal laser scanning microscopy (CLSM) was conducted as well. Normal dermal fibroblasts were utilized to evaluate the effects of TGF-beta stimulation both at mRNA and protein level.

Results: qRT-PCR for alpha-SMA showed in average 3.7 fold increased expression in a-SMA in the LCM captured cells. Microarray analysis identified 269 genes upregulated more than 2 fold in the myofibroblasts. Of these, 24 were clearly reconducible to profibrotic activation, including alpha-SMA, Collagens I, VI and XI, Fibronectin, several Integrin genes, FGF7, CD36, IGF and Rho; 16 were ribosomial genes; 14 were mitochondrial genes involved in oxidative phosphorylation, including COX1, 2, 3 and 6 ND1 to 6, CYT-b and F-type ATP-ase; 28 genes were involved in cell-to-cell adhesion including JAM2, ERM, and MLC and 7 in antigen processing and presentation including RAB13, B2-microglobulin, cathepsin, HSPs, calnexin and calreticulin. The remaining genes were not

classifiable in any specific functional pathway. IF studies followed by CLSM confirmed the specific pattern of protein expression in myofibroblasts vs alpha-SMA negative fibroblasts from the same donor. Long noncoding RNA tiling array of the same cells indicated that alpha-SMA positive fibroblasts had a specific up-regulation of HOX-A9, also known as HOTAIR, which is usually expressed mainly in the dermis of the palmo-plantar region. qRT-PCR and in situ hybridization confirmed the increased expression of HOTAIR in SSc skin. Consistently with these results, skin biopsies from the forearms of SSc patients showed an increased level of the palmo-plantar specific Keratin-9.

Conclusions: Myofibroblast secretome displayed, besides predictable genes involved in the increased ECM production and TGF-beta pathway activation, a specific gene expression non TGF-inducible and suggestive of a distinct and stable differentiation lineage usually present only in the palmo-plantar region. This distinct differentiation status may account for the dermal and epidermal changes in SSc.

8. SUB-ANALYSIS OF ELF SCORE BIOMARKERS COMPONENTS INDICATES A SPECIFIC CORRELATION WITH DIFFERENT ORGAN INVOLVEMENT IN SYSTEMIC SCLEROSIS

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Background: A recent large multicenter study has identified an algorithm, known as Enhanced Liver Fibrosis (ELF), by combining the serum concentrations of amino-terminal propeptide of procollagen type III (PIIINP), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA). The algorithm has been shown to predict liver related outcomes in patients with chronic liver diseases and recently it has been shown to correlate with several measures of fibrosis in Systemic Sclerosis (SSc).

Objectives: The aim of this study was to compare the ability of ELF and its single components in correlating with the different clinical and instrumental variables to determine whether any of the three biomarkers could have a specific role as surrogate outcome measure in SSc.

Methods: The serum concentrations of the three biomarkers were analysed in 210 SSc patients employing the same platform used to calculate the ELF score (Siemens, Advia Centaur). All patients were investigated for clinical features, skin and internal organs involvement, HAQ-DI, disease severity and activity. Statistical analysis was performed using GraphPrism software.

Results: Correlation coefficients are shown in the table. All three components of ELF showed a significant correlation with mRSS and were higher in patients with flexion contractures ($p<0.05$). Interestingly, the concentration of HA was the only parameter correlating with age, muscle and heart involvement. The biomarker with better correlation with lung involvement was TIMP-1, which showed a significant correlation with DLCO% predicted value and lung severity and the serum levels were significantly higher in patients with lung fibrosis as assessed by HRCT scan ($p<0.0001$). All three serum markers correlated with HAQ-DI and both PIIINP and TIMP-1 with the EScSG-AI.

Conclusions: Sub-analysis of the single serum markers included in the ELF score algorithm suggests that the different biomarkers may function as surrogate outcome measure of specific organ involvement in SSc. In this regard, longitudinal studies assessing the sensitivity to change over time of the single biomarkers may pave the way to develop specific algorithms tailored to carry the maximum predictive value on specific organ involvement in SSc.

(median, range)	ELF score 8.61, 5.91-12.1	PIIINP (ng/mL) 5.91, 2.04-33.06	TIMP-1(ng/mL) 215.3, 88.5-531.2	HA(ng/mL) 33.85, 2.56-510.7
	r	r	r	r
Age	0.41****	0.07	0.11	0.48****
mRSS	0.28****	0.29****	0.33***	0.19*
ESR	0.25***	0.11	-0.22**	0.21**
DLCO %	-0.16*	-0.09	-0.20**	0.1
Skin_sev	0.31****	0.29****	0.38****	0.19**
Join/tendon_sev	0.23***	0.28****	0.13	0.10
Muscle_sev	0.27****	0.18**	0.09	0.22**
GI_sev	0.17*	0.16*	0.04	0.10
Lung_sev	0.12	0.05	0.18*	0.07
Heart_sev	0.22**	0.13	0.09	0.24***
EScSG-AI	0.21**	0.17*	0.20**	0.13
HAQ-DI	0.32****	0.25***	0.31****	0.23**

*p<0.05; **p<0.01; ***p<0.001 ****p<0.0001

9. Reduced Actin Polymerization in Lungs of IQGAP1-Knockout Mice: Implication for Scleroderma Interstitial Lung Disease

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Introduction: Scleroderma interstitial lung disease (SSc-ILD) is an irreversible and often progressive complication of SSc that may lead to respiratory failure and death. Myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA), appear to be the principal mesenchymal cell type responsible for tissue remodeling in SSc-ILD. Since IQ motif containing GTPase activating protein (IQGAP1) is consistently elevated in SSc-ILD myofibroblasts and in normal lung fibroblasts that are exposed to connective tissue growth factor (CTGF, CCN2), the current study was initiated to examine the role of IQGAP1 in regulating α-SMA expression and function.

Methods: Protein interaction studies were performed using co-immunoprecipitation, 2D-gel electrophoresis and mass spectrometry in lung fibroblasts isolated from SSc-ILD patients or controls. Actin polymerization was studied on whole mouse lung tissue using Actin Polymerization Kit from Cytoskeleton. Effects of IQGAP1 in vivo were investigated using the bleomycin-induced murine model of pulmonary fibrosis.

Results: Lung tissue isolated from bleomycin-treated mice is characterized by extensive peribronchial and interstitial infiltrates of inflammatory cells, thickening of alveolar walls and multiple focal fibrotic lesions. In contrast, significantly fewer cellular infiltrates and decreased thickness of alveolar septae are present in IQGAP1-knockout mice, as well when control mice are treated with IQGAP1-siRNA or CTGF-siRNA. Analysis of lung tissue by immunoblotting reveals an increase in both α-SMA and CTGF in bleomycin-treated mice, whereas intranasal administration of IQGAP1-siRNA and CTGF-siRNA (in combination) notably reduced the expression of α-SMA and diminished the severity of pulmonary fibrosis. Although IQGAP1 alone did not affect α-SMA expression, IQGAP1 co-localizes with α-SMA in stress fibers and regulates the functional activity of α-SMA. When IQGAP-1 knockout mice are treated with bleomycin, they exhibit reduced actin polymerization and less pulmonary fibrosis. Studies on lung fibroblasts isolated from patients with SSc-ILD indeed demonstrated that IQGAP1 forms a protein-protein interaction complex with α5β1 integrin and CTGF, and this interaction regulates α-SMA organization.

Conclusion: Based on our studies in lung fibroblasts we conclude that IQGAP1 forms a signal transduction complex with CTGF that involves an interaction with α5β1 integrin. As a result, IQGAP1 signaling leads to increased α-SMA organization generating amplified contractile forces in lung myofibroblasts. Targeting IQGAP1 therefore has the potential to be an effective novel strategy for the treatment of increased lung stiffness associated with SSc-ILD.

10. IL6/CCL2 interplay regulates trans-endothelial migration of mononuclear cells and fibrotic response in scleroderma fibroblasts.

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Background: IL6 is a key mediator recently implicated in activation of extracellular matrix (ECM) proteins in scleroderma (SSc) fibroblasts. CCL2 is a proinflammatory chemokine that is overexpressed in diffuse cutaneous systemic sclerosis (dcSSc). We explored interaction between these two major mediators and their role in the recruitment of inflammatory cells and the subsequent effect on fibroblast ECM production.

Methods: Dermal fibroblasts were cultured from skin biopsies from healthy controls (n=4) and early stage dcSSc (n=4). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of the latter group. Expression of CCL2 and IL6 were assessed using immunohistochemistry with specific markers CD3, CD68 and CD14 on PBMCs. IL6 activation of CCL2 and the ability of SSc fibroblast derived CCL2 to induce PBMCs migration through an endothelial layer was studied in vitro using Transwell migration assays in a co-culture system, and the effect of the PBMCs-fibroblast interaction on induction of ECM proteins, α-smooth muscle actin (αSMA) and Collagen type-I (Col-I) was measured using neutralising antibodies against CCL2 and/or IL6 ligand-receptor axis.

Results: Stimulation of normal and SSc dermal fibroblasts with recombinant human IL6/sIL-6R (range of concentrations used) led to a dose dependent increase of the CCL2 (mean ± SEM % basal expression) with IL6 (25ng/ml) and sIL-6R (20ng/ml) at 48 hours, with maximum induction of CCl2 (95± 7%). IL6 trans-signalling increased migration of PBMCs (n=3) up to (30 ±3.5 % $p<0.05$) in the presence of normal fibroblasts and (35±2.3 % $p<0.03$) in SSc fibroblasts. The migration of PBMCs was reduced by the addition of neutralising antibodies against CCL2, IL6R separately and combined by (20± 5.1 % $p<0.05$),(30 ± 5.4% p,0.04) and (20± 7.3.2%$p<0.05$) respectively in the presence of SSc fibroblasts compared to (5± 1.3 %), (9 ± 1.2%) and (9± 3.4%) respectively in the presence of control fibroblasts and. In the co-culture system, IL6 trans-signalling significantly increased CCL2 expression (33± 2.7% $p<0.03$ and 45±5.6% $p<0.04$) in normal and SSc fibroblasts respectively. CCL2 expression was reduced in the presence of anti-IL6R in both control and SSc fibroblasts. After 24 hours of trans-signalling mediated PBMC migration, the PBMCs increased expression of ECM proteins αSMA (53 ± 5.9%, $p<0.04$) and Col-I (70± 2.6%, $p<0.03$) in the presence control fibroblasts respectively and αSMA (37± 5.9%, $p<0.03$) and Col-I (47± 3.6%, $p<0.04$) in the presence of SSc fibroblasts respectively, in comparison to unstimulated cells. This was also significantly abrogated by CCL2 and IL6 neutralising antibodies. Markers for monocytes CD68, and T cells CD3 confirmed localisation of CCL2 to monocytes but no expression of IL-6 ligand was confirmed.

Conclusions: Our results suggest that PBMCs are recruited via fibroblast-derived CCL2. The recruited PBMCs further activate ECM synthesis from SSc fibroblasts and have a particularly strong effect on the production of αSMA. These data suggest interplay between fibroblast-mononuclear cells may mediate the fibrotic response and that the CCL2/IL6 axis represents a potential therapeutic target for fibrosis in SSc.

11. INTERLEUKIN 6 AND VASCULAR DISEASE IN PATIENTS WITH SYSTEMIC SCLEROSIS

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BACKGROUND: Interleukin 6 (IL6) is known to play an important role in the pathogenesis of Systemic Sclerosis (SSc), particularly in the early years of its development. It has been associated with inflammatory activity and fibrotic manifestations (extensive skin involvement, interstitial lung disease [ILD]) as well as with autoantibodies (anti-Scl70 and anti-RNA polymerase III antibodies) [1,2]. It has also been reported its association with ischemic digital ulcers (IDU) and pulmonary hypertension (PH) [1,3].

OBJECTIVES: To evaluate the usefulness of determining plasma IL6 in patients with SS, as a marker of vascular disease. To investigate its association with other clinical manifestations and biological markers of SS.

METHODS: Patients diagnosed with Limited SSc (LSSc), Diffuse SSc (DSSc), overlap syndrome and early SSc (ESSc) were consecutively included in this descriptive and cross-sectional study with analytic components. Serum IL6, biomarkers of disease activity and vascular risk, and specific autoantibodies were determined. Clinical history was reviewed. Clinical assessment was simultaneously performed, and the next variables were collected: modified Rodnan Skin Score (mRSS), finger flexion and extension, oral aperture measurement, nailfold capillaroscopic findings, sHAQ, and Cochin Hand Function Score (CHFS). Afterward, a vascular surgeon, blinded to clinical findings, underwent ultrasonographic measurement of common carotid intima-media thickness (IMT) and recorded the presence of atheromatous plaques. SPSS Statistics 17.0 was used for data analysis. Spearman correlation coefficient was used to assess the correlation between quantitative variables, and the non-parametric Mann-Whitney and Kruskal-Wallis test were used to compare quantitative and categorical variables. Qualitative variables were compared by means of the χ^2 statistic.

RESULTS: A total of 47 patients were evaluated (29 LSSc, 8 DSSc, 2 ESSc, 8 overlap syndromes): 28% with history of IDU, 9% with PH, 40% with ILD, 12% with heart disease and 30% with gastrointestinal disease (GId). Most of them were female (90%), their mean age being 56 years (SD=15), and their mean disease duration being 11 years (SD=9). The mean mRSS was 8 (SD=8). The mean IMT was 0.6 mm (SD=0.15), 11% of patients having carotid atheromatous plaques. Mean plasma concentrations of IL6 were 4 pg/mL (SD=4). IL6 concentrations showed a positive correlation with pulmonary artery systolic pressure (r=0.583), and were associated with the presence of avascular areas in capillaroscopy (p=0.001). No association was found with history of IDU or the presence of carotid atheromatous plaques, neither with the IMT. Regarding the rest of the variables analyzed, IL6 plasma levels showed a positive correlation with CRP (r=0.425), high-sensitivity CRP (r=0.455), fibrinogen (r=0.450), sHAQ (r = 0.449), and disease duration (r=0.434). A negative correlation was found with albumin (r=-0.629). IL6 plasma levels were also associated with the presence of GId (p=0.035), joint contractures (p=0.04) and anti Scl70 antibodies (p=0.002). However, IL6 plasma concentrations were lower in patients with anticentromere antibodies (p=0.01).

CONCLUSION: IL6 in SSc patients is a surrogate marker of inflammation and fibrosis, but can also be a marker of microvascular disease. These results should be taken into consideration when planning future clinical trials with IL6 as a therapeutic target for the treatment of this disease.

12. Molecular Basis for the Treatment of Scleroderma-Associated Interstitial Lung Disease with the Thrombin Inhibitor Dabigatran Etexilate

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Background/Aims:

Activation of the coagulation cascade leading to activation of thrombin has been extensively documented in various forms of lung injury including systemic sclerosis-associated interstitial lung disease (SSc-ILD). We previously demonstrated that the direct thrombin inhibitor dabigatran has marked anti-inflammatory and anti-fibrotic effects in vitro and in an in vivo bleomycin model of SSc-ILD. The aim of this study was to investigate the effects of dabigatran etexilate on alveolar epithelial cells (AEC) and on lung myofibroblasts, two cell types that play central roles in the pathogenesis of SSc-ILD.

Materials and Methods:

Lung injury was induced in 6-8 week old female C57BL/6 mice by a single intratracheal instillation of bleomycin. Dabigatran etexilate was given as supplemented chow beginning on day one following bleomycin instillation. Mice were euthanized 2 and 3 weeks after bleomycin instillation and lung tissue, bronchoalveolar lavage fluid (BALF) and plasma were investigated. Apoptosis was measured by propidium iodine exclusion, cell death detection ELISA, and in situ cell death detection assay. Reactive oxygen species (ROS) were measured by fluoroprobe 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester (GM-H_2DCFDA) from Molecular Probes. Caspase-3, Akt, Bcl-2, CHOP, BiP, and α-smooth muscle actin (SMA) were studied by immunoblotting and immunofluorescent staining. The level of active thrombin in BALF was routinely monitored using thrombin substrate N-Benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma) by a spectrophotometric method.

Results:

We observed that treatment with dabigatran etexilate had the dual effect of reducing AEC apoptosis and decreasing myofibroblast resistance to apoptosis. Dabigatran reduces apoptosis of AEC by blocking reactive oxygen species (ROS) and by decreasing endoplasmic reticulum (ER) stress. Dabigatran eliminated thrombin-induced hydrogen peroxide and total ROS, and reduced the expression of CHOP and BiP in AEC. Dabigatran decreased the expression of cell survival markers in lung myofibroblasts in vivo. In particular, dabigatran decreased Akt phosphorylation and reduced the expression of anti-apoptotic Bcl-2, resulting in a decrease in the total number of myofibroblasts in lung tissue from bleomycin-treated mice. Thrombin activity in BALF was significantly reduced in bleomycin-treated mice receiving dabigatran etexilate as compared with bleomycin-treated mice receiving placebo, however, it was not significantly different from saline-treated control mice receiving placebo.

Conclusions:

We conclude that dabigatran etexilate ameliorates ILD by inhibiting apoptosis of AEC and by blocking resistance of lung myofibroblasts to apoptosis. Importantly, these anti-fibrotic effects of dabigatran were achieved without complete inhibition of thrombin; therefore, down regulation of thrombin by dabigatran etexilate back to physiological levels might result in successful treatment of SSc-ILD while avoiding significant risk of hemorrhage.

13. TGF-β's Role in Disease Progression in the Tsk2/+ Mouse Model of Systemic Sclerosis.

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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 old animals with Trichrome Blue staining and by a hydroxyproline assay, but an earlier 'tight' phenotype can be felt earlier at 2 weeks of age.

The Tsk2/+ mutation first occurred in a 101/H mouse after paternal exposure to N-ethyl-N-nitrosourea (ENU) a mutagenic agent, which likely caused a point mutation. This mutation is homozygous lethal and thus the mice are bred as heterozygotes. To date, the mutation responsible for the Tsk2 phenotype has yet to be elucidated, although it has been mapped to chromosome 1 between 42.3 and 52.3 megabases (Mbs). To further narrow this interval we backcrossed Tsk2/+ mice to B6 or B6.chr1-A/J consomic mice to map the mutation by microsatellite and single nucleotide polymorphism (SNP) typing. This typing has reduced the *Tsk2* interval down to a 3Mb region. Additionally, new SNPs discovered recently by 454 sequencing have allowed us to further narrow the interval to approximately 1.6 Mb.

In collaboration with the Whitfield and Ehrlich labs we used RNA-Seq and 454 sequencing to identify all genomic differences between the Tsk2/+ mice and their wildtype littermates. Here we report one SNP of interest at Chr1:45,378,353, which is within the second exon of the gene encoding the collagen alpha-1(III) chain (*Col3a1*). It causes a cysteine to serine (C33S) amino acid change. We believe this C33S SNP is the causative mutation within our mouse. To investigate the importance of this SNP in the fibrotic phenotype of Tsk2/+ mice, we performed a complementation experiment, crossing Tsk2/+ heterozygotes to heterozygous mice carrying one *Col3al*-null allele (*Col3a1*$^-$). We hypothesized that combining the *Tsk2* mutation with a *Col3a1*$^-$ allele would not be a viable genotype, similar to the Tsk2/Tsk2 homozygotes, and thus the Tsk2/Col3a1KO mice would die *in utero*. Indeed, we found the Tsk2/Col3a1$^-$ mice were nonviable, and out of 32 offspring born in this cross, the Tsk2/Col3a1$^-$ genotype never occurred. This implies that the C33S mutation in COL3A1 is the causative mutation, because the mice couldn't survive without a functional allele of *Col3a1* and C33S is the only mutation within *Col3a1* in the Tsk2/+ mice.

Previous work in our lab has established a timeline for events preceding fibrosis including early increases in COL3A1 protein and TGF-β1-induced genes. Immunofluorescence shows a marked increase in TGF-β in the skin of male mice at 2 and 10 weeks of age. Since both TGF-β and COL3A1 are increased early in development, we theorized that the C33S mutation induces TGF-β activation, which may be the trigger that leads to fibrosis. We have begun experiments to determine if the relative increase in COL3A1 can be mitigated by inhibition of TGF-β. To explore this we will use siRNA against TGF-β receptor 2 (TGFBR2) to inhibit activation of the TGF-β pathway. COL3A1 and COL1A1 levels will be measured by western blot and RT-PCR from Tsk2/+ and wildtype fibroblasts transfected with either the TGFBR2 siRNA or a scrambled siRNA control. We believe inhibiting TGF-β will decrease COL3A1 production *in vitro* to wildtype levels. If we confirm TGF-β's role *in vitro* we will attempt to inhibit TGF-β *in vivo* to prove that disease progression in the Tsk2/+ mouse model is a TGF-β mediated disease.

14. Altered expression of P2X7 receptor in the epidermis of diffuse scleroderma patients

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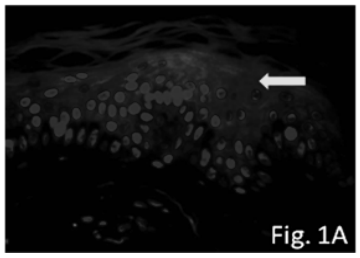
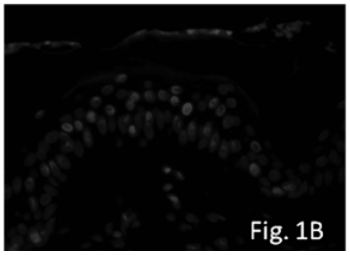
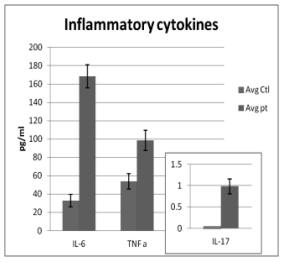
Background: SSc pathology shares features with wound healing and involves interplay between multiple cellular compartments. Previous work has identified hallmark changes in terminal differentiation in the epidermis in SSc patients. In the present study we have explored the potential role of mediators derived from the epidermis in driving disease process. P2Y1 is a trans-membrane G-protein coupled receptor expressed in the basal layer of the epidermis, and involved in controlling proliferation of keratinocytes. P2X7 is an ATP receptor cation channel, involved in terminal differentiation of keratinocytes in the stratum corneum. These receptors play an important role in inflammation and immunity. Epidermal injury induces ATP release and P2X7 signal transduction amplifies the local innate response. We hypothesised that altered ATP-P2X7 signalling in patients with SSc modulates inflammatory cytokines and angiogenic factors within the epidermism, leading to the development of autoimmunity and fibrosis.

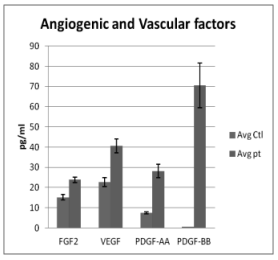
Method: We sampled the epidermis of 6 patients with diffuse cutaneous SSc, and 6 healthy controls using a dermal suction blister method. Using immunohistochemistry we stained for P2X7 (Caltag Labs, green) and P2Y1 (Abcam) and nuclei with DAPI (blue). In total 5 high power fields from each of 6 SSc and 6 control individuals were compared. The results were analysed using Chi-Squared tests. Dermal blister fluid samples (healthy control n=10, diffuse SSc n=29) were profiled by Luminex array for inflammatory cytokines, chemokines, and growth factors.

Results: The distribution of P2Y1 was uniform throughout the epidermis in 6/6 healthy controls and 6/6 patients. P2X7 was found in 6/6 healthy controls in the terminally differentiated cornified layer of the epidermis (Fig 1B). No SSc patients showed this normal terminal differentiation pattern ($p<0.001$ Chi Squared). 2/6 patients showed focally induced over expression of the P2X7 receptor throughout the epidermis (Fig 1a arrow), compared to none of the control samples. 4/6 patients showed decreased expression of P2X7 in the cornified layer of the epidermis.









Luminex array profiling of the dermal blister fluid showed a trend toward increased inflammatory cytokines (mean IL-6 in SSc 168.62 pg/ml versus 32.64 pg/ml in controls, mean IL-17 in SSc 0.98 pg/ml versus 0 pg/ml, p not significant), and angiogenic and vascular growth factors (VEGF 40.5 pg/ml in SSc, 22.7 pg/ml in controls and PDGF-BB 70.5 pg/ml in SSc versus 0 pg/ ml in controls, p not significant) (See graphs).

Conclusion: Our data show that there is altered terminal differentiation within the stratum corneum of patients with diffuse SSc. The expression of P2X7 receptors in defined foci, may lead to altered ATP-P2X7 signalling in patients with SSc. Profiling the blister fluid showed a suggestion of increased inflammatory cytokines and angiogenic and vascular factors. These factors have previously been shown to be increased within the serum of patients with SSc. This active inflammation in the epidermis provides an interesting insight into the pathogenesis of SSc and the role of the epidermis and its transdifferentiation.

15. What are the Effects of Systemic Sclerosis on Daily Function?

Paula Click Fenter, PT, DHSc, GCS; Tim Gilmore, MHS, RRT, AE-C; Samina Hayat, MD, FACP

Background/Purpose: Systemic Sclerosis (SS), a chronic multi-system disease, is an autoimmune disorder that can limit independence and function. Important are daily tasks such as walking and self care within the home. Success can be compromised is individuals are unable to manage self-care or become short of breath when doing so. The purpose of this study is to investigate the effects of SS on daily function and the impact on quality of life.

Methods: Sixteen individuals (13 African-American, 3 Caucasians) with SS were given self-report tests including the St. George Respiratory Questionnaire, Instrumental Activities of Daily Living (IADL), Barthel Index (ADLs), and Health Assessment Questionnaire Disability Index (HAQDI). Health literacy was tested prior to the former paper and pencil reports using the Rapid Estimate of Adult Literacy in Medicine (REALM). Laboratory and imaging was found in the clinical chart. (Twelve participants also performed the 6-minute walk test (6-MWT).

Results: subject demographics: average age was 59 years with SS diagnosis ranging from 1 to 30 years. Performance of the 6-MW was an average of 367 meters with normal values for 60-64 years of age being 498-603 meters for women and 558-673 meters for men. These participant's average distance is significantly lower than normal values. Half had normal oxygen saturation. One forth had pulmonary artery systolic pressure $\geq$ 30, 58% exhibited honeycombs and 59% had a positive ANA. St. George Respiratory questionnaire showed that patients reported a low number of respiratory symptoms that compromise their daily activities. Most individuals scored high on ADLs and IADS, with average HAQDI score of 1.15. According to these scores, scleroderma does not greatly impact participants' daily functional activities. More than half of the participants reported difficulty with stair climbing (8 out of 15.) The REALM score ranked an average of 7th-8th grade literacy level, meaning individuals may struggle with many patient education materials. Individuals with SS reported few respiratory symptoms but they were not able to sustain walking for a functional length of time within the normal range for their age group, indicating that functional mobility is significantly affected by this disease. Most individuals reported being able to take care of their daily functional activities and did not see themselves as having a change in their quality of life as a result of the disease except when more difficult tasks like stair climbing were involved. Being able to understand physician instructions and information about SS, as well as following the instructions of all healthcare providers is important when living with this chronic disease. When individuals have difficulty with understanding health information they may not be able to follow important instructions with medications and other treatment strategies that are important to their health.

Conclusions: Persons with SS have not generally reported to be affected greatly by the disease in self report scales, however when given specific performance tests like walking or moving the hand, measures show that changes in fact have occurred. Perhaps individuals with this disease have adapted to changes that occur gradually and do not see themselves as lacking in ability to function within their daily environment.

16. MINIMUM DIAGNOSTIC STANDARDS FOR CHILDREN WITH RAYNAUD'S PHENOMENON - RESULTS OF THE CONSENSUS MEETING IN HAMBURG (GERMANY) ON DECEMBER 9, 2012

T. Constantin 1,, C. Pain 2, N. Toplak 3, M. Moll 4, C. Iking-Konert 5, D. Piotto 6, N. Aktay 7, D. Nemcova 8, P. Hoeger 9, M. Cutolo 10, V. Smith 11, I. Foeldvari 12 and for the Juvenile Scleroderma Working Group of PReS,*

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4University Childrens Hospital Tuebingen, Tübingen,
5 Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany,
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8Children Hospital Prague, Prague, Czech Republic,
9Wilhelmstift Hospital for Sick Children, Hamburg, Germany,
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12Hamburger Zentrum fur Kinder- und Jugendrheumatologie, Hamburg, Germany

Background: Raynaud's phenomenon (RP) can be the first symptom of a connective tissue disease in children, in particular juvenile systemic scleroderma (JSSC) or systemic lupus erythematosis (SLE). However, the prevalence of RP in healthy school children has been shown to be as high as 15%[1]. There are currently no guidelines or agreed management strategies amongst Paediatric Rheumatologists on how to differentiate primary from secondary RP or how often patients require evaluation.

Objectives: To develop consensus standards for good clinical practice for children with RP.
Methods: A consensus meeting was organized in the frame of the PRES scleroderma working group. A nominal group technique was used. 75% consensus was defined as agreement.

Results: The following agreements were reached:

1. All patients with RP should be screened with an ANA test.
2. All ANA positive patients should be screened for scleroderma-specific antibodies (e.g. anti-SCL 70 and anticentromere antibodies).
3. All patients with RP should be investigated by capillaroscopy. Capillaroscopy will be classified into "normal", "aspecific changes" or "scleroderma pattern".
4. All patients who have additional symptoms pointing to a definite connective tissue disease should be evaluated according to disease specific guidelines.
5. ANA-negative and capillaroscopy-negative patients should be followed-up at least every 6 months.
6. ANA positive patients without disease-specific antibodies and with negative capillaroscopy findings should be followed-up at least every 6 months.

7. ANA and disease-specific antibody positive patients should have organ specific evaluation according to symptoms, examination and relevant to that particular disease e.g. patients who are ANA and Scl-70 positive may need organ specific evaluation for JSSC as per the Juvenile systemic sclerosis inception cohort protocol (www.juvenilescleroderma.com).
8. ANA-positive patients, who have no disease specific antibody but have positive capillaroscopy results, should be followed-up at least every 3 months.
9. ANA-negative patients with positive capillaroscopy result should be followed-up at least every 6 months.
10. The group could not reach an agreement regarding treatment, due to a lack of data for the paediatric age group. The group agreed that implementation of adult recommendations for paediatric care might be reasonable, but robust paediatric trials are needed.

Conclusions: The group made a suggestion for a standard of good clinical practice for RP in children. Our aim is that this will facilitate a large multicentre prospective follow-up study of children with RP.

17. Study CC-4047-SSC-001: A Phase 2, Proof-of-Concept, Multicenter, Randomized, Double-blind, Placebo-controlled Study to Evaluate the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics and Efficacy of Pomalidomide (CC-4047) In Subjects with Systemic Sclerosis with Interstitial Lung Disease

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Pomalidomide (CC-4047) is an immunomodulatory and antiproliferative drug with potent B cell suppressive activity currently being studied for the treatment of systemic sclerosis. This compound, which has recently been approved for the treatment of multiple myeloma, inhibits the proliferation and induces apoptosis of multiple myeloma cells, which are derived from the immunoglobulin-secreting plasma B cell lineage. Similar to its effects on tumorigenic plasma B cells, normal B cell differentiation and immunoglobulin secretion is also sensitive to pomalidomide inhibition. In the mouse bleomycin-induced skin fibrosis model, pomalidomide treatment not only prevented the development of dermal fibrosis, but also induced regression of pre-established dermal fibrosis. Pomalidomide was also effective in the tight skin mouse model of dermal fibrosis.

Due to the unique combination of immunomodulatory (pro-Th1, anti-Th2) and anti-fibrotic properties, pomalidomide is now being evaluated in a double-blind, placebo-controlled, international study for the treatment of subjects with systemic sclerosis (SSc) with interstitial lung disease (ILD). Study CC-4047-SSC-001 includes up to a 5-week Screening Period, 52-week Treatment Period, 4-week Observational Phase and up to a 5 year Long-term Follow-up Phase.

The study population (n = 88) consists of male or female subjects 18 to 80 years old. A diagnosis of limited or diffuse SSc based on American College of Rheumatology (ACR) criteria is required, with onset of the first non-Raynaud's manifestation of SSc within 7 years of Screening. Diffusion lung capacity for carbon monoxide (DLco) $\geq$ 35% and $\leq$ 80% is required at Screening along with abnormalities on high resolution computed tomography (HRCT) consistent with parenchymal changes encountered in SSc. In an effort to identify subjects at risk for progression, either forced vital capacity (FVC) readings of $\geq$ 45% and < 70% at Screening and Baseline or FVC readings $\geq$ 70% and $\leq$ 80% at Screening or Baseline with either a 5% decrease in FVC in the 24-month period prior to Baseline or an HRCT fibrosis score > 20% are required.

Subjects may continue treatment with stable doses of medications to treat SSc disease-related symptoms e.g., proton pump inhibitors, ACEI/ARB therapies, cough medication, etc. Immunosuppressives of any type, except for low-dose systemic corticosteroids ($\leq$ 10 mg prednisone or equivalent/day), are prohibited.

Key efficacy endpoints include the change from Baseline in FVC, modified Rodnan Skin Score, and UCLA SCTC GIT 2.0 scores. Safety, pharmacokinetic and pharmacodynamic parameters will also be evaluated as well.

18. Microcirculatory Responses to Ischemia in Early Diffuse Cutaneous Systemic Sclerosis Patients

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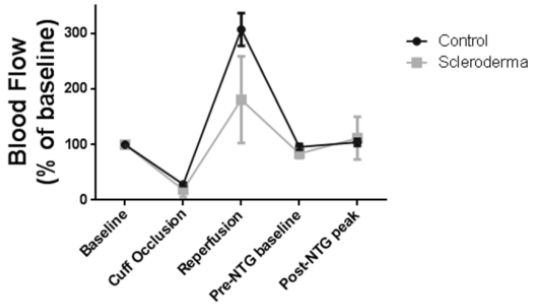
[1]UPMC and University of Pittsburgh Scleroderma Center,
[2]Vascular Medicine Institute and Critical Care Medicine

Background/Purpose: Microvascular dysfunction is a hallmark of systemic sclerosis (SSc) manifested clinically as Raynaud phenomenon and digital ulcerations (DU), although the natural history of microcirculatory abnormalities in SSc remains elusive. We sought to examine the pattern of microcirculatory flow and response to nitric oxide (NO) in a group of patients with very early diffuse SSc.

Methods: We identified patients presenting to a Scleroderma Clinic with very early diffuse SSc, defined as < 3 years since the first symptom attributable to SSc and skin thickening proximal to the elbows and knees. They were matched 1:1 to healthy controls on age, gender and race. Laser speckle contrast imaging (LSCI) of the hand was performed with the PeriCam PSI system™, which allows for continuous measurement of blood perfusion at the capillary level using a camera above the subject's hand. Measurements were taken continuously for: 5 minutes prior to (baseline) and then during arm arterial occlusion by blood pressure cuff at 300 mmHg, post-occlusion reperfusion for 5 minutes, and then for five minutes post-NTG in a temperature-controlled room. Statistical analysis was performed using SPSS.

Results: Thirteen SSc patients and 13 healthy controls participated in the study. The average age was 52, 54% were male and the mean disease duration was 2.3 (± 0.7) years. All patients were treated with either myophenolate mofetil, d-penicillamine or combination therapy. 12/13 had Raynaud symptoms, 4 had a history of DU, but none had active DU at the time of study.

Figure 1: Finger blood flow by laser speckle



SSc patients had a distinct pattern of capillary flow over the in the third metacarpal in response to ischemia when compared to controls (Figure 1). Specifically, SSc patients had both a lower peak reperfusion following ischemia (p=0.002), and a slower time to reperfusion (p=0.003). At 5 minutes after ischemia, SSc patients had a lower reperfusion nadir (pre-NTG baseline) than controls (p=0.04). After an ischemic insult, SSc patients did not mount as high a reactive hyperemic response and returned to a lower baseline flow than controls, suggesting reduced endogenous NO production. When NTG was given, SSc patients had a greater response (135%) than controls (105%; p=0.0008), suggesting greater exogenous NO sensitivity. Using these measurements to determine a receiver operating curve, the maximum reperfusion peak had an area under the curve (AUC) of 0.80 (p=0.02) to predict a ≥ 10% vasodilatory response to NTG, and the reperfusion nadir an AUC = 0.77 (p=0.03).

Conclusions: Patients with very early diffuse SSc have marked differences in microcirculatory response to ischemia compared to healthy individuals, suggestive of impaired NO production. Further studies are needed, but this noninvasive method may be clinically useful in identifying patients with Raynaud or DU who may respond to NO donors.

19. A PIONEERING MODEL OF CARE FOR PATIENTS WITH RARE DISEASE IN THE UK – CHAMPIONED BY RHEUMATOLOGISTS FROM THE ROYAL FREE & QUEEN'S HOSPITAL, UK

L Elam, E Blamey, N Quillinan, L Al Sweedan, Carol Black, Christopher Denton, K Chakravarty

BACKGROUND

Connective tissue diseases such as scleroderma are considered to be rare in the UK population, affecting approximately 1:10,000 individuals [1]. As the experience of rheumatologists in dealing with this condition is often rare, satellite clinics for scleroderma have been held at different geographical areas by the experienced specialists from the Royal Free Hospital. Prior to 2004 no such clinic for patients with scleroderma and related illnesses existed in North East London (Essex). It was unfortunate that patients had to travel a significant distance to get the appropriate specialist treatment that was required to achieve disease control. In Queens Hospital, Romford (Barking, Havering and Redbridge Trust), a super-specialist centre was developed in 2004 to specifically care for patients with scleroderma and related illnesses. Collaboration with the Royal Free Hospital, a recognised International Centre for Excellence, made this possible.

METHODOLOGY

The development of the super-specialist service at a peripheral site in North East London was conceived and initiated by one of the authors (KC) with help from colleagues in the Royal Free Hospital (CB & CD) who made regular visits at frequent intervals to support the development. Clinics were set up utilising patient profiles in a structured way with recording of clinical features, investigations and drug management to facilitate a holistic care with help from the multidisciplinary team (MDT) including specialist nurses. At the same time, parallel combined super specialist clinics were set up with respiratory medicine, dermatology and nephrology, the latter with input from a visiting Professor from Cambridge. Clinics were attended by Consultant Specialists, trainee doctors, clinical nurse specialists, staff nurses and medical students. Patient data was collected with regard to the demography and other interventions related to management. Opinions from patients were sought at regular intervals to assess quality of care.

RESULTS

Over the last 8 years (2004-2012) we have seen 80 with a mean age of 58.5 and male: female ratio 3:1. 70% of patients had ACA positive disease. Most patients had limited disease and most (85%) reported extremely high levels of service-user satisfaction, as per patient reported outcomes measures. A questionnaire was used to record satisfaction from clinical nurse specialists, trainee doctors and particularly medical students who were in attendance at the combined clinics as part of a student-selected component (SSC). Patients reported an overwhelming satisfaction about their care that was provided closer to home and was being provided by super-specialists in their local hospital. The development of such super specialist combined clinics with other specialists where necessary allowed for MDT approach to patient management with a positive impact on the quality of care delivered locally.

CONCLUSION

This 'Hub and Spoke' development of clinical care for scleroderma and related connective tissue diseases has produced a very satisfactory model, which can be replicated across a wider contingent. It was interesting to note the professional satisfaction of the multidisciplinary team including doctors, nurses and students who had the opportunity to attend these clinics and provide care that was considered by their patients to be second to none.

[1] Scleroderma Society: Understanding and Managing Scleroderma. Revised October 2008.

20. Internalization of TGF-β receptors is impaired in scleroderma

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TGF-β is a central pro-fibrotic cytokine and has been implicated as a key player in the pathogenesis of scleroderma. TGF-β initiates signaling by binding to the type I and type II TGF-β receptors. It has been shown that internalization of TGF-β receptors from the cell surface via clathrin-coated pits promotes SMAD-mediated signaling, whereas internalization through caveolae is associated with receptor degradation and thus inhibition of TGF-β signaling.

Since impaired TGF-β receptor internalization and degradation may lead to enhanced TGF-□ signaling leading to increased extracellular matrix deposition, we determined whether the internalization and/or the degradation of TGF-β receptors are impaired in scleroderma skin fibroblasts. Internalization of TGF-β receptors was performed by monitoring the uptake of ^{125}I-TGF-β1 (which mimic receptor internalization) in scleroderma and normal skin fibroblasts. To assess endogenous receptor degradation, cell surface receptors were affinity labelled using ^{125}I-TGF-β1 followed by cross-linking of the bound TGF-□□to the receptors. TGF-β receptor levels were then analyzed by SDS-PAGE and autoradiography. Interaction between caveolin-1 and TGF-β receptors was determined by immunoprecipitation of cell lysates with an anti-caveolin-1 antibody followed by Western blot with an anti-type II receptor antibody. Our results show that internalization of ^{125}I-TGF-β is markedly reduced in scleroderma skin fibroblasts when compared to normal skin fibroblasts. Furthermore, degradation of type II TGF-β receptors is markedly impaired in scleroderma skin fibroblasts. Moreover, the interaction of caveolin-1 with type II receptor was diminished in scleroderma skin fibroblasts as compared to normal skin fibroblasts.

Our findings suggest that impaired internalization of TGF-β receptors, decreased caveolin-1-type II receptor interaction as well as the resistance of type II receptor to degradation may contribute to aberrant TGF-β signaling and excessive extracellular matrix production in scleroderma skin fibroblasts.

21. Topoisomerase-1 specific T cells exhibit a proinflammatory Th17 phenotype and are quantitatively associated with interstitial lung disease in scleroderma

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Background. Anti-topoisomerase-1 (Topo-1 or Scl70) autoantibodies are present in 30-45% of scleroderma (SSc) patients and identify a more aggressive disease phenotype with worse clinical outcome and reduced survival. While autoreactive T cells may be involved in SSc pathogenesis driving tissue inflammation and damage, no study has to date reliably quantified and functionally characterized topo-1-specific T cells in SSc patients.

Methods. Peripheral blood mononuclear cells from 27 (15 Scl70-positive and 12 Scl70-negative) consecutive patients and 4 healthy donors (HD) were stimulated with topoisomerase-1 purified from baculovirus-infected insect cells in the presence of anti-CD40 blocking antibodies and autologous serum. Topo-1-responsive T cells were defined based on the expression of activation markers CD154 and CD69, and their frequency calculated as percentage of CD4+ or CD8+. The polarized functional phenotype (Th1, Th2, Th17, Th1/17) of autoreactive T cells was further determined by the surface expression of specific chemokine receptors (CXCR3, CCR6, CCR4) and validated by defining their cytokine secretion profile (IFNγ, IL4, IL17) after cell sorting. Comprehensive clinical information was obtained at the time of blood draws.

Results. Topo-1-reactive CD4+ T cells were found in all Scl70-positive subjects compared to one Scl70-negative patient and no HD [p=<0.001 and p=<0.001 respectively] with a frequency variable between 0.11% and 0.42%. Topo-1-specific T cells exhibited a predominant Th17 phenotype compared to the parent CD4+ T cell population [37±16% vs 8±5%, p=0.001]. Higher levels of Topo-1-reactive CD4+ T cells were associated with lower lung volumes [forced vital capacity (FVC) rho=-0.557, p=0.031; carbon monoxide diffusing capacity rho=-0.559, p=0.030]. Topo-1-specific CD8+ T cells were detected only in Scl70-positive patients with interstitial lung disease, and their frequency was positively associated with lower lung volumes [FVC rho=-0.552, p=0.041] and worse dyspnea [University of California San Diego Shortness of Breath questionnaire, rho=0.611 p=0.027].

Conclusions. This study suggests that Topo-1-reactive T cells can be reliably measured in the circulation of scleroderma patients and that they may contribute to disease-specific tissue injury through the secretion of pro-inflammatory and pro-fibrotic cytokines such as IL-17. Further studies will define their relevance as markers of disease activity, predictors of outcome, and disease-specific therapeutic targets.

22. Results for 6 minute walk values in healthy German children show similar results as from Britain.

Ivan Foeldvari, Günther Himmelmann

Background/Purpose

6 minute walk is a primary outcome measure in therapeutic studies for patients with pulmonary hypertension. Currently we have a two of sets of data[1, 2] regarding test results in the 6 minute walk test (6MWT) in healthy children with a large span in the norm values in the different age groups. Aim of the study was to establish norm values for healthy German children for the 6 Minute Walk Test.

Method

The team of an occupational therapist and a study nurse were visiting schools. Permission from the parents was give before the test. Always just probands from one class were invited to participate. The test were performed according the international guidelines[3]. The demographic data of the probands were collected and the parents filled out a short survey regarding the physical activity and the health condition. Children with chronic diseases, which decrease the stamina were excluded.

Results

Up till now 611probands participated from the age 5 ot 14 years. 343 of the 611 were female. The mean 6 minute walk continuously increased with age (Table 1.). It correlated in the age groups with the BMI.

Male (n=268)			Female (n=343)		
Age	Median	Mean	Age	Median	Mean
5 (n=2)	408.8	408.8	5 (n=0)	ND	ND
6 (n=21)	491.6	479.6	6 (n=18)	472.2	481.1
7 (n=42)	494.2	487.2	7(n=26)	487.9	476.9
8 (n=43)	485.0	494.8	8 (n=33)	488.2	493.8
9 (n=31)	512,0	520.1	9 (n=49)	492.0	504.5
10 (n=48)	516.8	519.7	10 (n=58)	526.3	521.3
11 (n=26)	614.9	606.5	11(n=44)	560.8	548.8
12 (n=35)	569.5	572.1	12(n=72)	551.2	552.9
13 (n=15)	569.5	558.9	13 (n=29)	556.0	557.6
14 (n=5)	499.5	491.6	14 (n=14)	521.6	540.8

Conclusion

Our results are in the range of the patients from the UK published by Lammers et al [1] and are in significantly lower range than in the Chinese population collected data by Li et al.[2]. This reflects the importance of this study to gain norm values for our patient population.

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23. Overexpressions of c-Met and CD44v6 receptors contribute to autocrine TGF-β signaling in lung fibroblasts from SSc and IPF patients

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Pulmonary fibrosis is defined by recurrent injury in alveolar epithelium where a repairing program is lost. This leads to serious and sometimes life threatening lung conditions, such as systemic sclerosis (SSc) and idiopathic pulmonary fibrosis (IPF). Fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) collagen and fibronectin, expression of c-Met receptor tyrosine kinase, elevated expression of contractile protein α-smooth muscle actin (α-SMA), synthesis of various cytokines including TGF-β1, and overexpression of the glycosaminoglycan hyaluronan (HA). Injury-derived and pathologically activated fibroblasts significantly contribute to these characteristics. Hyaluronan receptor CD44 has been shown to be critical for the recruitment of fibroblasts to the injury sites. Our recent study in inflammatory neoplastic cells indicates that many of the functions of CD44 are particularly attributed to variant 6 of CD44 (CD44v6). Moreover, splicing of CD44 variants, including CD44v6, occurs via a feed back loop, i.e. CD44v6 forms complexes with growth factors and receptor tyrosine kinases (c-Met) and then activates down stream pathways of c-Met. The question of whether the interaction between CD44v6 and TGF-β1, or cross-talk between TGF-β1 and c-Met has a significant role in regulating fibrogenic behaviors of IPF and SSc fibroblasts (*e.g.* Smad activation, α-SMA and cell proliferation) is the primary focus of this study. **We hypothesize that TGF-β/Smad signaling in SSc lung fibroblasts (SScLFbs) and IPF lung fibroblasts (IPLFbs) regulates c-Met/CD44v6 because TGF-β1 has a key role in pathogenic fibrosis.**

In support of this hypothesis, we showed that: 1) SSc and IPF lung fibroblasts exhibit increased c-Met expression compared with normal lung fibroblasts; 2) TGF-β1 autoregulates c-Met and CD44v6 in hyper proliferation of SScLFbs and IPFLFbs and TGF-β1 treatment stimulates these processes in normal lung fibroblasts (NLFbs); 3) Basal protein expression of MMP-1 is reduced in SScLFbs and IPFLFbs compared with NLFbs; 4) Basal level of HGF production is high in SScLFbs compared to NLFbs. However, HGF-treatment increases MMP-1 level in SScLFbs and IPFLFbs compared to that of NLFbs; 5) In accordance with this, c-Met induction is significantly reduced in time points beyond 72hr despite a sustained treatment of TGF-β1 in cultured SScLFbs and IPFLFbs whereas CD44v6 remains elevated during sustained treatment of TGF-β1 in these cells even after 72hr. Taken together, the results of the current study show that the overexpression of c-Met may not have unfavorable effects in SScLFb and IPFb that express higher level of HGF which may limit the adverse effects of c-Met, whereas, CD44v6 regulated collagen production may be associated with sustained CD44v6 signaling because of the effect of autocrine TGF-β1 signaling.

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24. Altered cellular responses by de-differentiated PASMCs may contribute to the development of SSc associated PAH.

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Background

Pulmonary hypertension is a significant cause of death in systemic sclerosis (SSc) and affects up to 15% of cases during the course of their disease. Pre-capillary pulmonary arterial hypertension (PAH) is a leading cause of death in SSc patients and amongst the causes of WHO group 1 PAH, SSc associated cases (PAH-SSc) have a particularly poor outcome. Pathological changes in human pulmonary arterial smooth muscle cells (PASMCs) are a key feature in the vascular remodelling that underlies the development of PAH-SSc. The de-differentiation of PASMCs from a contractile to synthetic phenotype is an important feature of vascular remodelling observed in a number of diseases including PAH-SSc. In this study we have explored cellular response of contractile and synthetic PASMCs to agonists that are implicated as pathogenic mediators in other manifestations of SSc.

Methods

Experiments were performed on commercially available PASMCs (n= 3). Contractile and synthetic PASMCs were established and markers of de-differentiation determined by Western blot and immunoflorescence (n=3). Secretions of disease relevant markers from contractile and synthetic PASMCs were determined by ELISA. The functional relevance of de-differentiation of PASMCs in response to disease relevant proteins was determined in gel-contraction, apoptosis and cell migration assays (n=3).

Results

We established a robust method to induce de-differentiation of PASMCs from contractile to synthetic smooth muscle cells. Expression of the contractile PASMC markers smoothelin and α-SMA were significantly reduced in synthetic hPASMCs ($p<0.05$). Whereas type-I collagen, CTGF, IL-6 and IL-8 were significantly elevated in synthetic compared to contractile PASMCs ($p<0.05$). Contractile PASMCs exhibited increased migration in response to PBGF-BB compared to synthetic PASMCs ($p<0.05$). BMP4 and BMP7 induced apoptosis in contractile PASMCs, whereas these effects of BMPs were blunted on synthetic hPASMCs (n=3).

Conclusion

De-differentiation of a ‘normal’ contractile to a ‘disease’ synthetic PASMC phenotype is likely to be an important feature of vascular remodelling in PAH-SSc. Here we establish a robust method to promote the de-differentiation of PASMCs from contractile to synthetic smooth muscle cells. We define a number of markers of de-differentiation, including type-I collagen, CTGF and IL-6. Further we demonstrate altered cellular responses of synthetic PASMCs to known mediators of fibrosis including PDGF-BB. Collectively these results suggest that key mediators implicated in SSc pathogenesis could promote a synthetic vascular smooth muscle cell phenotype that may contribute to the development of structural vasculopathy in the lung and potentially other relevant vascular structures in SSc.

25. Imbalance in TGFβ and BMP axis may contribute to the development of PAH in a novel murine model of SSc

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Background
Pulmonary complications in systemic sclerosis (SSc) including fibrosis and pulmonary arterial hypertension (PAH) are a major clinical problem. Survival studies have shown patients with PAH-SSc have a poor prognosis compared to those with idiopathic (iPAH) or heritable (hPAH) forms of the disease. The mechanisms that contribute to the development of PAH-SSc remain unclear. The TβRIIΔk-fib transgenic represents a novel model of PAH-SSc, which recapitulates many of the features of SSc. These mice develop fibrosis in the skin, lung and gut, and also develop a structural pulmonary vasculopathy with raised right ventricular pressure. The mechanism which contributes to the development of PAH-SSc remains unclear. However, understanding the development of PAH in this murine model may provide insight into the mechanisms and future potential therapies for the treatment in PAH-SSc.

Methods
Experiments were performed on whole lung isolates (n=6) and explant cultured fibroblasts (n=6) from the TβRIIΔk-fib murine model. Expression of components of the TGFβ superfamily and downstream signalling pathway was investigated by Western blot and immunohistochemistry on whole lung and explant cultured fibroblasts. Migration assays were performed to investigate the effects of PDGF-BB on lung fibroblasts from TβRIIΔk-fib and WT controls (n=3).

Results
Previous studies have shown that the TβRIIΔk-fib model displays increased levels of phosphorlyated Smad 2/3 which is indicative of enhanced TGFβ signalling. Consistent with an imbalance in the TGFβ/BMP axis we observed a significant reduction in BMPRII levels in the TβRIIΔk-fib model in whole lung isolates ($p<0.05$), and explant cultured fibroblasts ($p<0.05$). Furthermore explant cultured fibroblasts isolated from this model exhibited a blunted induction of phospho-Smad1 and downstream genes in response to BMP ligands ($p<0.05$). TβRIIΔk-fib lung fibroblasts also exhibited enhanced migratory response compared to wild type controls ($p<0.05$).

Conclusion
In hPAH 70% of patients possess mutations in the BMPRII gene, which leads to a reduction in functional cell surface associated receptor. Here we demonstrate the TβRIIΔk-fib transgenic murine model of PAH-SSc exhibits reduced expression of BMPRII in whole lung isolates and explant cultured fibroblasts. The model also has reduced downstream signalling independent of mutations in the BMPRII. Interestingly these cells exhibit a heightened migratory response to PDGF-BB. Collectively our data suggests loss of BMPRII expression by non-genetic means may in turn contribute to the development of PAH in the TβRIIΔk-fib mouse model of PAH-SSc by promoting an imbalance in the TGFβ/BMP axis.

26. Endothelial progenitor cells form biological exclusion barriers similar to that of mature endothelial cells- A therapeutic potential in systemic sclerosis?

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Introduction

Vascular complications associated with systemic sclerosis (SSc) including pulmonary arterial hypertension (PAH-SSc), are thought to result in part from endothelial damage and loss of barrier function. The causes of this endothelial dysfunction are unclear, but the integrity of the endothelium and barrier function is likely to be significantly diminished in SSc. Late outgrowth endothelial progenitor cells (EPCs) derived from PBMCs express both endothelial ($VEGFR2^+$, $CD31^+$) and haematopoietic ($CD133^+$) markers. It is thought that they home to sites of vascular injury and differentiate into endothelial cells restoring the barrier formed by the endothelium. In SSc patients circulating levels of EPCs are reduced. This study aimed to: (i) develop a robust method to isolate and grow healthy control (HC) and SSc EPCs from peripheral blood mononuclear cells (PBMCs) and (ii) To compare the cellular functions of EPCs to mature endothelial cells.

Methods

Peripheral blood was taken from HC donors (n=10) and SSc patients (n=10) using sodium citrate as an anti-coagulant. EPCs were cultured from isolated PBMCs, and EPC colonies grown to passage 4. EPCs and human pulmonary artery endothelial cells (hPAECs) were seeded into transwell inserts and grown to confluence. Cells were incubated with TNFα (5ng/ml), and their capacity for form biological barriers assessed using FITC-albumin (1.25mg/ml). The capacity of EPCs to support immune cell transmigration was assessed using neutrophils isolated from HCs, along with FITC-albumin 'leak'; these were quantified by cell count and fluorescent absorbance respectively over time. We further assessed the responses of EPCs to TNFα stimulation by ELISA to quantify pro-inflammatory cytokine release compared to mature endothelial cells.

Results

We demonstrate that EPCs form a functional biological exclusion barrier with similar capabilities as mature hPAECs *in vitro*. TNFα significantly enhanced the permeability of EPCs ($P<0.05$) and hPAECs ($P<0.05$) monolayers. Consistent with EPCs possessing similar cellular activities as mature endothelial cells, TNFα stimulated neutrophil transmigration in monolayers of EPCs ($P<0.05$) and hPAECs ($P<0.05$) and enhanced the secretion of IL-8 in both EPCs ($P<0.01$) and hPAECs ($P<0.05$). We sought to determine the frequency of EPC colony formation from PBMCs and found no significant difference in the capacity to form EPC colonies in HC and SSc patient PBMCs, with colonies present in 70% and 60% of cultures respectively.

Discussion

We have developed a robust method for isolating EPCs from PBMCs. We have demonstrated that endothelial progenitors can maintain an endothelial barrier consistent with that observed by mature hPAECs *in vitro*. We have established that EPCs respond to TNF in a similar manner to mature PAECs, secreting pro-inflammatory cytokines such as IL-8 and supporting neutrophil transmigration. We have further shown no significant difference in the capacity of PBMCs from SSc patients to form EPC colonies compared to healthy control donors. The biological function and importance of EPCs from SSc patients in vasculopathy, restoration and maintenance of the endothelial barrier function remains unclear.

27. COELIAC DISEASE IN SCLERODERMA - A study of associated gastrointestinal manifestations and impact of screening in Scleroderma

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BACKGROUND: Coeliac disease (CD) is an autoimmune disease with a reported prevalence 4-7% among Scleroderma (SSc) patients. This association has not been well characterized in a large cohort of patients. Our aims are to evaluate the clinical picture, nutritional complications, and clinical response after gluten free diet in scleroderma patients with CD (SSc-CD), compared with a group of SSc patients with gastrointestinal (GI) symptoms and negative coeliac antibodies.

PATIENTS AND METHODS: This is a retrospective study of well-characterised SSc cohort of 1920 patients from 2008 to 2013 with data collected through clinical database and patient records including all SSc patients diagnosed with CD by duodenal biopsy (n=12), and 96 of 236 SSc patients with GI symptoms and negative coeliac antibodies. 4 patients in the SSc-CD group had confirmed positive coeliac antibodies. Small bowel symptoms include chronic diarrhoea, bloating, constipation and discomfort.

RESULTS: The prevalence of CD with positive coeliac antibodies in our SSc cohort with GI symptoms was 4/236 (1.69%). 92.6% were female, with median (interquartile range, IQR) of age at SSc onset of 45.0 (33.0 – 55.7) years, and 49.3 (41.0 – 55.7) years at CD diagnosis. 18.5% patients had diffuse subset and 66.7% of the patients were Caucasians. 33 (30.6%) had an overlap with another systemic autoimmune disease (17 had polymyositis-dermatomyositis, 8 rheumatoid arthritis, 5 Sjögren's syndrome, 2 systemic lupus erythematosus and 1 pANCA-vasculitis). Among both groups, 30 patients (27.8%) had another organ-specific autoimmune disease including hypothyroidism or psoriasis. A higher frequency of psoriasis was identified in 25% of SSc-CD patients compared with 2.1% (p=0.009) in negative-coeliac antibodies patients. SSc-CD patients had increased frequency of myopathy (33.3% vs 10.4%, p=0.04), and anti-PM/Scl antibody (25.0% vs 3.1%, p=0.01). 88% of entire cohort had small bowel symptoms with no statistical differences between the two groups. Chronic diarrhoea was the most common symptom in 62.0% of patients. Upper endoscopy was performed in all SSc-CD patients and in 41.7% with negative-coeliac antibodies, with no statistical differences in frequency of esophagitis, gastritis, gastric antral vascular ectasia (GAVE) or macroscopic duodenitis. There were no differences in the prevalence of bacterial overgrowth or nutritional complications. Among all patients 44.4% had vitamin D deficiency, 40.7% iron deficiency and 7.4% osteoporosis. After gluten-free diet all SSc-CD patients had an improvement of small bowel symptoms. 50% of these patients achieved complete remission and the symptoms recurred in the remaining half with median (IQR) 5.0 (2.5 – 8.25) years. There was significant improvement in chronic diarrhoea (75% to 41.7%, p=0.04), abdominal distension or bloating (50% to 16.7%,p=0.04), and in weight loss (33.3% to 0%, p=0.04).

CONCLUSIONS: The clinical presentation of CD may be indistinguishable to SSc-GI disease and our study indicates that CD in this subgroup of SSc patients with GI involvement is no more common than the general population. The positive response to gluten-free diet suggests that screening for CD in selected SSc patients may be helpful.

28. The pro-fibrotic role of β-catenin in dermal fibroblasts

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Introduction & background: Increased activity of the canonical Wnt signaling pathway is associated with fibrosis in skin, kidney, and lung. β-catenin is the central transducer of this signaling pathway and acts as a transcriptional co-factor in the nucleus to regulate target gene expression. Here, we investigate the role of increased active β-catenin in dermal (skin) fibroblasts. This study is relevant to scar formation and several connective tissue disorders including scleroderma.

Results: Skin of patients with systemic sclerosis, morphea, keloid scar, and nephrogenic systemic fibrosis has an increased percentage of dermal fibroblasts expressing active β-catenin. Moreover, publicly available microarray expression data from skin fibroblasts affected by Dupuytren contracture and keloid scar demonstrate upregulation of Wnt pathway components by gene set enrichment analysis. Expression of constitutively active β-catenin in a subset of mouse dermal fibroblasts causes progressive fibrosis in vivo within three weeks. The activated β-catenin mutant skin has increased fibroblast proliferation and increased total skin collagen. The fibrotic skin demonstrates thickened collagen fibrils and increased fibrillin. We have found that in embryonic mouse dermal fibroblasts, β-catenin is required for expression of *Tgfb1* and genes encoding members of integrin and matrix signaling networks. Preliminary mRNA expression analysis of whole skin suggests that the fibrotic phenotype is due to gene expression changes within the manipulated fibroblast population.

Conclusions & future directions: β-catenin is sufficient to cause skin fibrosis, and its elevated activity in fibrotic patient skin supports further investigation of Wnt/β-catenin pathway components and transcriptional targets to better understand and treat fibrosis. The focus of our ongoing study is to identify and test the function of key pro-fibrotic genes that are regulated by β-catenin in dermal fibroblasts.

29. Role of ERG in vascular stability and a possible connection to scleroderma vasculopathy

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Several Ets family transcription factors are known to be expressed in the vascular endothelium. One such factor, FLI1, has been implicated in the development of scleroderma (also termed systemic sclerosis, SSc) vasculopathy. However, whether other endothelial Ets factors, including FLI1's closest homolog, ERG, play any role in the scleroderma vascular disease has not been studied. We used immunohistochemistry to determine the presence and localization of ERG in scleroderma skin and lung. ERG was found to be highly specifically expressed by vascular endothelial cells and predominantly localized in the cell nucleus, in both skin and lung from control subjects. We did not observe any significant difference in ERG expression between control and scleroderma skin. However, a number of scleroderma lung samples distinctively contained ERG in the cytoplasm. This altered ERG distribution pattern was seen in both SSc-PAH (pulmonary arterial hypertension) and SSc-PH (pulmonary fibrosis) lungs, suggesting that ERG may be important for maintaining the proper vascular function in the lung.

We decided to study the function of ERG in vivo by generating *Erg* conditional knockout mice using the Cre-loxP system. To specifically ablate *Erg* in the endothelial lineage, we used the *Cdh5-Cre* as well as the *Tie2-Cre* mouse lines. Although no phenotype was observed when *Cdh5-Cre* line was used due to inefficient ablation of the *Erg* gene, *Erg* deletion with the *Tie2-Cre* line lead to embryonic lethality. Characterization of the mutant embryos showed that these embryos died during midgestation with impaired angiogenesis and compromised vascular integrity. Blood vessels in the mutant embryos appeared to have reduced coverage of pericytes. The expression of genes implicated in the disease cerebral cavernous malformations (CCM) was found to be downregulated in the mutant embryos, suggesting that they are potential ERG target genes.

To further study the role of ERG in adult life, we crossed the *Erg* floxed mice to the inducible *Rosa26-CreERT2* mouse line. Tamoxifen was administered to adult mice to induce the deletion of *Erg*. A moderate increase in vascular permeability was detected in the lung of *Erg* deficient mice, suggesting that Erg is also critical for preserving the stability of blood vessels in adult.

Taken together, these data suggest that ERG is required for the maintenance of vascular integrity, and it may also play an important role in the pathogenesis of scleroderma-associated lung complications.

30. Immunomodulatory and anti-fibrotic effects of pomalidomide, a cereblon binding drug in clinical development for the treatment of systemic sclerosis

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Pomalidomide (CC-4047) is an immunomodulatory and antiproliferative drug with potent B cell suppressive activity currently being studied for the treatment of systemic sclerosis. The direct binding of pomalidomide to cereblon, part of the DCX (DDB1-CUL4–X-Box) E3 ubiquitin ligase complex, has been implicated in the mechanism of action of this drug. Pomalidomide, which has recently been approved for the treatment of multiple myeloma, inhibits the proliferation and induces apoptosis of multiple myeloma cells, which are derived from the immunoglobulin-secreting plasma B cell lineage. Similar to its effects on tumorigenic plasma B cells, normal B cell differentiation and immunoglobulin secretion is also sensitive to pomalidomide inhibition. In cultures of differentiating B cells, pomalidomide inhibits IgG production with an IC_{50} of approximately 49 nM. Pomalidomide interferes with B cell differentiation by inhibiting gene expression of important factors required for immunoglobulin production, such as BLIMP-1, XBP-1, and IgJ. In PBMC cultures from normal donors or from individuals with autoimmune disease, pomalidomide inhibits IgM and IgG production with IC_{50}s in the range of 3.8-63 nM. In contrast to its inhibitory effects on these B cell responses, pomalidomide enhances T cell- and natural killer (NK) cell responses, including the production of Th1 cytokines and cytotoxic activity. Furthermore, the responses of PBMC to TLR4 agonism are inhibited by pomalidomide, resulting in decreased production of pro-inflammatory cytokines. In the mouse bleomycin-induced skin fibrosis model, pomalidomide treatment not only prevented the development of dermal fibrosis, but also induced regression of pre-established dermal fibrosis. Pomalidomide was also effective in the tight skin mouse model of dermal fibrosis.

These immunomodulatory effects of pomalidomide are corroborated by clinical observations. In healthy volunteers administered multiple doses of pomalidomide (0.5 mg, 1 mg, or 2 mg QD), there was evidence of enhanced T cell function *ex vivo*. In a small cohort with moderate-to-severe corticosteroid-resistant cGvHD treated with pomalidomide (0.5-3 mg QD), 2 out of 8 subjects had a complete dermal response and 1 subject had a partial dermal response, i.e., improvement of erythematous skin changes. One subject had a complete response in improvement of GI symptoms. In a study of subjects with relapsed multiple myeloma who were treated with pomalidomide (1-10 mg QOD), there was a significant decrease of peripheral blood absolute $CD19^+$ B cells in 14 of 18 subjects, but an increase in peripheral blood $CD3^+$ and $CD8^+$ T cells in 15/18 subjects. Furthermore, there was a statistically significant correlation between the 4-week B cell decrease and the percentage change in serum paraprotein (tumor cell-derived immunoglobulin). Therefore, pomalidomide has been shown to enhance T cell number and function, and suppress B cell number and immunoglobulin secretion, both *in vitro* and *in vivo*. Because of its unique combination of immunomodulatory (pro-Th1, anti-Th2) and anti-fibrotic activities, pomalidomide is currently being evaluated in a double-blind, placebo-controlled study (N=88) for the treatment of subjects with systemic sclerosis with interstitial lung disease.

31. Murine Chronic Graft vs Host Disease: A Model of Scleroderma

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Scleroderma (SSc) is a rare heterogeneous disease of unknown eitology, however SSc shares many pathologies including fibrosis of the skin and lungs with chronic graft vs host disease (cGVHD), therefore cGVHD may represent a model to investigate SSc.

In this study we developed and investigated a mouse model of cGVHD as an analogue for SSc. We used a strategy of adoptively transferring bone marrow and spleen cells across minor histocompatibility antigen mismatched strains of mice to induce cGVHD. Briefly, 6-8 week old BALB/c mice (Jax) were irradiated with 650 RADs and intravenously reconstituted with red blood cell free $2x10^6$ splenocytes and $1x10^6$ bone marrow cells harvested from B10.D2 mice (Jax). The recipient mice were monitored for signs of GVHD response including skin lesions, diarrhea and weight loss. On days 30 (n=5), 60 (n=7) and 90 (n=2) post transfer, groups of transplanted mice were anesthetised and placed on a mechanical ventilator and physiological measurements taken, they were subsequently euthanized and tissue samples collected for analysis.Three mice displayed an adverse reaction to the transplant protocol with significant skin blistering & hair loss, as well as having lung inflammation and an emphysema-like phenotype. Two of these mice were euthanized on day 60 with data full collection and the third mouse euthanized on day 78 with partial data collection. Compared to control, transplanted mice had significantly decreased lung compliance on day 30 ($p=0.0002$) which continued to decrease through days 60 and 90 ($p<0.00001$). Within the transplanted group there was significant decrease in lung compliance from day 30 to 60 ($p=0.03$) and day 30 to 90 ($p=0.02$). Airway resistance between control and transplanted groups was increased on days 60 ($p=0.0005$) and day 90 ($p=0.008$). Lung lavage showed increased neutrophils in transplanted mice on days 30 ($p<0.0001$) and 60 and 90 ($p=0.252$), however histological analysis did not reveal any significant collagen deposition, inflammation or gross pathologies in the transplanted groups. Skin collagen content was calculated through measurement of the collagen deposition from the dermis extending to the subcutaneous layer. Control mice had significantly more skin collagen deposition on day 30 ($p=0.003$), however transplanted mice had more skin collagen at day 60 ($p=0.01$). There was no difference in proteinuria (mg/L) between groups at individual time points, however there was a significant difference between the transplanted and control groups over the 90 days ($p = 0.04$). Despite changes in proteinuria, no gross histological changes were observed.

In contrast to previous reports, we did not find significant lung collagen deposition in our model; however we did observe significantly decreased lung compliance and increased dermal collagen deposition which is in accordance with previous research. The SSc cGVHD model developed in this study has recapitulated key pathologies seen in scleroderma provides an opportunity to study disease mechanisms.

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32. Expanded Analysis of Pathway Gene Signatures in Systemic Sclerosis Reveals Key Mediators Driving Pathology within Each of the Intrinsic Subsets

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Background: Systemic sclerosis (SSc) is characterized by fibrosis of the skin and internal organs, vascular abnormalities, immune activation, and extracellular matrix deposition. Recent advances in genome-wide expression profiling have shed light onto the underlying gene expression driving disease pathogenesis, revealing 'intrinsic' disease subsets that can measure and clarify the heterogeneity of clinical disease. We previously performed microarray analysis of fibroblasts treated with exogenous TGFβ and IL13, which revealed gene signatures strongly correlated with distinct subsets of disease. Furthermore, the TGFβ signature was associated with increased modified Rodnan skin score (mRSS) and higher likelihood of scleroderma lung disease.

Methods: Since our initial investigations, an additional 226 skin biopsies have been analyzed. These data were merged into a single dataset consisting of 329 microarray hybridizations from 111 patients, representing 287 unique biopsies. Based upon gene clustering results we identified three pathways that may be driving pathogenesis of specific SSc intrinsic subsets in addition to the previously described TGFβ and IL13/IL4 pathways: sphingosine-1-phosphate (S1P), PDGF, and PPARγ. These gene signatures were examined alongside public microarray data for nine additional agonists (poly(I-C), IFNα, LPS, TNFα, ionomycin-PMA, dexamethasone, and our previously published TGFβ, IL4, and IL13), and compared to our SSc skin biopsy microarray database to assess the contribution of each pathway to intrinsic gene expression subset classification.

Results: Analysis of the 329-array dataset showed patient gene expression groups (fibroproliferative, inflammatory, limited, and normal-like) as previously described. A distinct fifth group, termed the proliferative-inflammatory subset, was also evident. The strongest overall correlation between any pathway and intrinsic subset was seen between PDGF and the fibroproliferative subset. Previous reports have linked this subset to TGFβ. Enrichment of TGFβ signaling was seen among the Milano et al. cohort of fibroproliferative patients, but was more variable across subsequent datasets; instead, TGFβ showed a much stronger overall correlation to the inflammatory subset. This indicates that TGFβ expression can occur across the intrinsic subsets but may be most prominent in the inflammatory group.

The inflammatory subset showed a clear and consistent association with NFκB-activating pathways. The convergence of strong TGFβ and TNFα gene expression signatures observed in this subset, along with pervasive inflammatory infiltrates, suggests a Th17-like immune response, consistent with recent reports. In contrast, the limited subset was strongly associated with IFNα, and negatively correlated to PDGF. IFNα is a potent inhibitor of both Th17 differentiation and PDGF expression. Together, these divergent signals suggest distinct immunoregulatory mechanisms underlying clinically diffuse and limited disease.

No correlation is seen between NFκB-activating pathways and the intermediate proliferative-

inflammatory subset, despite immune cell signatures evident in skin biopsy data. Instead, this subset shows modest correlation to PDGF. TGFβ and TNFα drive expression of PDGF; combined with downregulation of immune signaling, this pattern of expression is suggestive of a possible transitional state linking a TGFβ/Th17 driven inflammatory response to a more fibroproliferative response driven by PDGF.

Multiple pathways may be predictive of clinical outcomes. TNFα, S1P, PDGF, TGFβ, and IL4 were all strongly associated to mRSS ($P < 0.001$); the best overall correlation was seen for TGFβ, consistent with previous observations. IL13 showed a significant association with shorter disease duration in diffuse patients suggesting an initial spike in Th2-mediated immune responses, which diminishes over time. Other clinical covariates, including lung disease and autoantibody profiles, are currently being examined.

Conclusions: Together, these analyses suggest specific and common mechanisms of immune regulation driving pathogenesis in each of the intrinsic gene expression subsets. Tailoring therapies to target each of these mechanisms within a specific subset may improve therapeutic outcomes.

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

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***Objectives*:** Plasma S-nitrosothiols (RSNOs) act as a nitric oxide (NO) reservoir *in vivo* and circulating NO donors [1]. In systemic sclerosis (SSc) endothelial NO synthesis is impaired. This study for the first time investigated the RSNO levels in the pathogenesis of SSc and primary Raynaud's phenomenon (PRP) patients and as an index to NO status.

Introduction*:** RSNOs are organic compounds containing nitroso group attached to the sulfur ("S") atoms of the thiols of a organic group and for the reaction of NO with thiols form S-nitrosothiols; where, R denotes the organic group which may be a small molecule containing a thiol or a protein with one or more thiols (R-S-N=O). RSNOs donate NO to specific thiol-regulatory-effector sites of enzymes and signaling proteins by transnitrosation [2]. RSNOs bind to SH groups of cysteine residues of proteins and low molecular weight thiols by covalent addition of a NO moiety (NO^+) *in vivo* which regulate the function of broad spectrum of proteins in the cells by S-nitrosylation [3]. The formation of RSNOs may protect against the cellular toxicity associated with oxidative stress caused by excess NO. A breakdown product of RSNO is NO_2^-. Systemic sclerosis (SSc) is a connective tissue disorder with over production of collagen and vascular dysfunction that may also affect internal organs (dSSc). DSSc patients have increased concentrations of serum and skin nitrated proteins [4]. ***Patients and methods: 45 Patients with SSc (34 lSSc and 11 dSSc) were selected with informed consent and ethical committee approval at the Rheumatology clinics of the Royal Free Hospital. Patients with PRP (16) and 26 controls, with a similar age and gender profile, participated. RSNO and nitrite concentrations were assayed from plasma by detection of NO released by copper/iodine-iodide mediated cleavage of RSNOs to form NO with subsequent quantification by chemiluminescence [5]. ***Capillary scores*** were determined by nailfold video-capillaroscopy [6] ***Skinscores*** in patients were measured using the modified Rodnan skin score [7]. The levels of controls were correlated with those of the patients to their biological age, sex, nitrite, skin scores and disease duration. ***Results***: In the patient groups RSNOs were often below the level of detection (1nM). A striking significant reduction in the level of detectable RSNOs was observed in all patients compared to those of controls where the level was 6 ± 0.8, $n = 26$; versus all patients with SSc (1.17 ± 0.3, $n = 45$, $P = 2.584E-08$; lSSc (1.31 ± 0.37, $n = 34$, $P = 1.06E-06$), dSSc (0.73 ± 0.41, $n = 11$, $P = 0.0025$) and in PRP (2.12 ± 0.57, $n = 16$, $P = 0.002$). There were no differences in nitrite levels compared to controls. The low RSNOs in SSc were correlated to higher skin score and duration of disease. RSNO levels decreased with age (above 37 years of age) in controls. ***Conclusion*:** These findings strengthen the view that NO metabolism is profoundly disturbed in SSc and that low levels of RSNO may play a key role in its progress. The evaluation and study of the concentration of RSNOs in plasma of patients in PRP and SSc and correlation with some clinical status may help in safe treatment of NO donor drugs.

34. Matrix stiffness drives fibroblast activation and experimental skin fibrosis through enhanced FAK signaling.

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Increases in extracellular matrix (ECM) stiffness due to increased deposition and cross-linking of collagen are characteristic of chronic fibrotic disorders, including scleroderma (SSc). Whereas the ability of excess ECM proteins such as collagen to distort tissue architecture is well known, the ability of altered ECM mechanical properties to affect cellular signaling and contribute to fibrosis progression is now being increasingly appreciated. We used atomic force microscopy (AFM) to demonstrate that median dermal stiffness increased 4.9-fold in the well characterized experimental mouse model of scleroderma dermal fibrosis induced by bleomycin. We found that in contrast to the quiescent state of primary mouse skin fibroblasts grown on substrates of stiffness equivalent to that of normal dermis, both proliferation and αSMA expression of these cells increased when grown on substrates of stiffness progressively increasing to levels observed in mouse dermis following bleomycin challenges. This induction of fibroblast activation by increased ECM stiffness was FAK-dependent. ECM-induced fibroblast proliferation and αSMA expression were both significantly reduced by treatment with the selective FAK inhibitor PF-562,271. This inhibitor dramatically induced apoptosis of fibroblasts differentiated into myofibroblasts by growth on pathologically stiff substrates, but did not increase apoptosis of fibroblasts grown on normal stiffness substrates. FAK inhibition with PF-562-271 *in vivo* markedly reduced experimental skin fibrosis when administered either in a preventative or therapeutic regimen. Translational studies of skin biopsies from individuals with SSc showed co-localization of FAK activation and αSMA expression. These data suggest that FAK inhibition has the potential to interrupt a positive feedback relationship between matrix stiffness and fibroblast activation that may amplify dermal fibrosis in scleroderma. The ability of FAK inhibition to induce apoptosis of myofibroblasts in pathologically stiff matrices may give drugs targeting FAK the ability to induce the regression of already-established fibrosis.

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35. The anti-angiogenic VEGF-165b isoform is elevated in both anti-centromere and anti-topoisomerase positive systemic sclerosis patients.

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Background: Systemic sclerosis (SSc) is an autoimmune disease that has clinical manifestations including inflammation and vascular insufficiency of skin and internal organs. Many of the disease characteristics are associated with dysfunctional angiogenesis in the skin and major organs. Vascular endothelial growth factor (VEGF) is a pro-angiogenic cytokine that is commonly up regulated in many SSc patients, which does not fit with the vasculopathy seen in patients with SSc. An anti-angiogenic splice variant, $VEGF_{165}b$, has recently been demonstrated to be upregulated in the tissues and plasma of SSc patients.
We hypothesized that the detection of increased VEGF levels in SSc patients is primarily due to upregulation of $VEGF_{165}b$, and that $VEGF_{165}b$ levels might be different between anti-centromere and anti-topoisomerase positive SSc patients.

Objectives: 1) Develop and validate a reliable $VEGF_{165}b$ ELISA, and assess performance in freeze/thaw and plasma/serum reproducibility tests. 2) Measure VEGF and $VEGF_{165}b$ levels in the plasma of well-characterized patients with SSc. 3) Determine whether VEGF and/or $VEGF_{165}b$ levels distinguish clinically relevant subsets of SSc patients.

Methods: Peripheral blood samples were collected from 52 SSc patients and 26 healthy controls. The SSc patients were recruited from the Scleroderma Registry and Repository (Benaroya Research Institute, Seattle, WA). Autoantibody status was determined by initial chart review at the time of sample acquisition. The $VEGF_{165}b$ assay was developed using commercially available reagents (R&D Systems) and run at the UMN Cytokine Reference Laboratory. The assay was tested using spiked recombinant $VEGF_{165}b$ and total VEGF, samples that had undergone multiple freeze-thaw cycles, and matched plasma and serum samples. Group comparisons were performed using Mann Whitney tests, and the Spearman rank method was used for correlation analysis.

Results: $VEGF_{165}b$ is up regulated in SSc patient plasma samples. VEGF and $VEGF_{165}b$ readings were stable through multiple freeze/thaw cycles and were highly correlated in matched plasma and serum samples. Plasma $VEGF_{165}b$ levels, but not total VEGF, were significantly elevated in patients who were positive for anti-centromere autoantibodies (CENP; $p=0.0497$) or anti-topoisomerase antibodies (Topo-1; $p=0.0019$) compared to patients who lacked these autoantibodies.

Conclusion: $VEGF_{165}b$ may be significantly elevated in autoantibody-distinct subsets of SSc. Further investigations into the utility of $VEGF_{165}b$ as a biomarker and a therapeutic target are warranted.
This project was supported by funding from the Minnesota Partnership for Medical Genomics, and the Lupus Foundation of Minnesota summer fellowship. The Benaroya Institute Immune-mediated disease registry provided serum and plasma samples.

36. Bleomycin Delivery by Mini-Osmotic Pump: A Superior Model for Human ILD

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Interstitial lung disease (ILD) encompasses several chronic, progressive, irreversible respiratory disorders associated with pulmonary parenchymal fibrosis, including idiopathic pulmonary fibrosis (IPF) and scleroderma lung disease (SSc ILD). The pulmonary fibrosis in ILD is characterized by prominent subpleural fibrosis, mild inflammation, fibroblast/myofibroblast proliferation and excessive extracellular matrix (ECM) accumulation, alveolar epithelia cells (AECs) injury and hyperplasia, and loss of the master regulatory protein caveolin-1 (Cav-1) from fibroblasts. Because bleomycin causes lung fibrosis when used in cancer chemotherapy, it has been used for decades to model pulmonary fibrosis and human ILD in rodents. In most studies, bleomycin has been delivered directly into the lung by intratracheal or intraoral administration; however, bleomycin has also been delivered using subcutaneous osmotic Pumps. Here we have compared the effects in mice of bleomycin delivered by the "Pump" or the "Direct" route to determine which one is a better model for human ILD. Male CD1 mice (ten weeks old) were treated with bleomycin either by the "Direct" route (a single intraoral administration of 2 U/kg) and sacrificed on days 7 or 14, or by the "Pump" route (the pump delivers 100 U/kg bleomycin continuously over 7 days, then is removed) and sacrificed on days 10, 21, 28, or 35. Tissue sections were stained immunohistochemically for inflammatory cell markers, ECM proteins, and proteins associated with fibrosis and compared to similarly stained sections from human control subjects and SSc ILD patients. The Pump method was much better than the Direct method as a model for SSc because: 1) Masson's staining of collagen is apparent in the Pump model and in SSc ILD, but not in the Direct model; 2) Lung injury/fibrosis is limited to the subpleural portion of the lung in the Pump model and in SSc ILD, while the entire lung is affected in the Direct model; 3) Conversely, there is massive inflammation throughout the lung in the Direct model while inflammation is limited in the Pump model and in SSc ILD; and 4) Skin fibrosis is observed in the Pump model and SSc, but not in the Direct model. The Pump model is also more convenient and humane than the Direct model because there is less weight loss and mortality in the Pump model.

37. HLA-B35 induces expression of IL-6 in immune and endothelial cells via Heat Shock Protein upregulation

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Purpose: We recently demonstrated an association between Endoplasmic Reticulum (ER) stress/Unfolded Protein Response (UPR) gene activation and limited cutaneous systemic sclerosis (lcSSc) patients with Pulmonary Arterial Hypertension (PAH). In particular, we showed a positive correlation between expression of BiP/DNAJ and IL-6, as well as BiP/DNAJ and severity of PAH (PAP) in PBMC samples from patients with lcSSc, suggesting that ER stress/UPR may contribute to altered function of circulating immune cells in patients with lcSSc (Lenna et al. A&R 2013). The presence of the HLA-B35 allele has emerged as an important risk factor for the development of PAH in patients with SSc. The goal of our study was to determine whether HLA-B35 could contribute to ER stress/UPR signature and inflammation in lcSSc-PAH.

Methods: Peripheral Blood Mononuclear Cells (PBMCs) were purified from healthy controls (HC, n=36) and lcSSc patients, classified as having PAH (lcSSc-PAH, n=38) or no PAH (lcSSc-NoPAH, n=39). PBMCs from each group were stratified for presence of HLA-B35. HC PBMCs were transduced with Lentivirus expressing HLA-B35 (or control virus HLA-B8) for 48 hours. Human Dermal Microvascular Endothelial Cells (HDMECs) were transduced for 48 hours with Adenovirus expressing HLA-B35 (or control virus HLA-B8) or treated with Thapsigargin (TG) (10pM), a known ER stress inducer for 24 hours. Total RNA was extracted and the quantitative real-time polymerase chain reaction (qPCR) was performed to measure gene expression.

Results: BiP and DNAJB1 were consistently higher in HLA-B35 positive PBMCs compared with HLA-B35 negative samples, with statistically significant differences observed in each group (BiP $p<0.05$, HC B35- vs HC B35+, lcSSc-NoPAH B35+ vs lcSsc-NoPAH B35- and lcSSc-PAH B35+ vs lcSsc-PAH B35-; DNAJ $p<0.0001$, HC B35- vs HC B35+, $p< 0.05$ lcSSc-NoPAH B35+ vs lcSsc-NoPAH B35- and lcSSc-PAH B35+ vs lcSsc-PAH B35-). Likewise, IL-6 expression levels were consistently higher in HLA-B35 positive lcSSc PBMCs ($p<0.05$ lcSSc-NoPAH B35+ vs B35- and lcSSc-PAH B35+ vs B35-) but not in HCs. To further understand the relationship between HLA-B35, HSPs and inflammation, we overexpressed HLA-B35 in PBMCs and HDMECs. In both cell types we observed upregulation of BiP, DNAJ and IL-6. To determine whether HSPs were involved in HLA-B35 mediated IL-6 upregulation, we used an HSP70 inhibitor, KNK437. Treatment with the inhibitor led to a reduction of IL-6 mRNA levels in response to HLA-B35 in both endothelial cells and PBMCs.

Conclusions: Our data suggest that HLA-B35 contributes to elevated level of HSPs and IL-6 in patients with lcSSc. Furthermore our data suggest that HSP70 may be directly involved in upregulation of IL-6.

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38. Effect of A Selective Na/K-ATPase/Src Receptor Antagonist on SSc Fibrotic Signaling

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INTRODUCTION: Systemic sclerosis (SSc, scleroderma) is a chronic autoimmune disorder of unknown etiology or effective therapy. The disease is characterized by vascular obliteration, excessive extracellular matrix deposition and fibrosis of involved organs. We have shown previously sustained activation of Na/K-ATPase/Src receptor signaling in several models of chronic organ fibrosis. Furthermore, our recent data suggest that blocking the abnormal Na/K-ATPase/Src receptor function by pNaKtide, a specific receptor antagonist, reduces collagen expression and attenuates organ fibrosis.

OBJECTIVES: In this study, we examined the Na/K-ATPase/Src receptor in SSc fibroblasts and the effect of pNaKtide on SSc fibrotic phenotype.

METHODS: Primary dermal fibroblasts from diffuse cutaneous SSc patients and age, race, and gender matched healthy donors were isolated and cultured. The differences in Na/K-ATPase α1/Src receptor signaling in these fibroblasts were assessed by Western blot and immunostaining. Effect of pNaKtide on collagen expression was investigated by Western blot.

RESULTS: Expression of type I collagen in SSc fibroblasts was significantly higher than that in control fibroblasts, demonstrating that the isolated cells maintain the pathologic fibrotic phenotype *in vitro*. Significant cytosolic distribution of Na/K-ATPase α1 was noted in both control and SSc fibroblasts. Activated Src in SSc fibroblasts, as indicated by the phosphorylation at Tyrosine 418, was about 2.5-5 folds higher than that seen in control fibroblasts, suggesting a possible role for Src signaling in the pathogenesis of SSc fibrosis. Addition of pNaKtide resulted in inhibition of Src phosphorylation at Tyrosine 418 in SSc fibroblasts, but not in normal cells. Furthermore, type I collagen expression in SSc fibroblasts was significantly reduced by pNaKtide. Further investigation is under way to assess pNaKtide's effectiveness in animal models of SSc.

CONCLUSIONS: SSc fibroblasts had significantly higher activation of Src kinase compared to normal fibroblasts. Specific Na/K-ATPase/Src receptor antagonist pNaKtide inhibited Src activation and reduced the augmented expression of type I collagen in SSc fibroblasts. Antagonizing the Na/K-ATPase/Src receptor may be a novel therapeutic strategy for SSc.

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39. What are Systemic sclerosis-related calcinoses made of?

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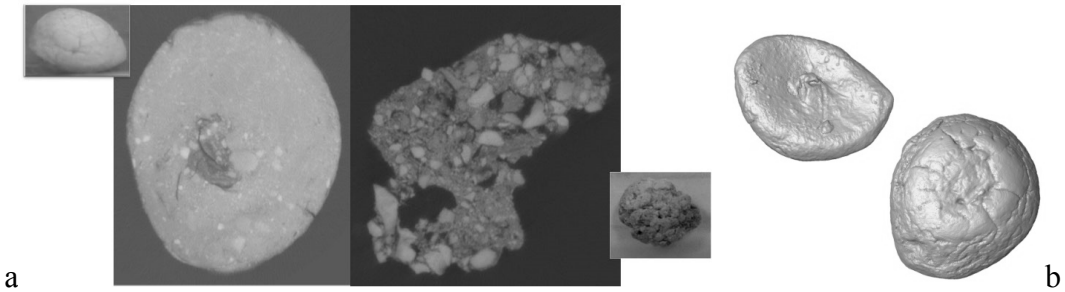
Background and aim: Approximately 25- 40% of patients with systemic sclerosis will develop calcinosis, with the knees, elbows and fingertips being commonly affected.[1] There is limited information in the literature on the composition of calcinotic lumps,[2,3] although what is available (mostly X-ray diffraction data, microscopy and thermal analyses) suggests that they consist of B carbonated apatite. The aim of this research was to unite all of these methods and more to provide a complete image of the structure and composition of calcinoses associated with systemic sclerosis.

Methods: Micro-computed tomography (XCT), thermal (TGA), powder x-ray diffraction (PXRD), elemental, electron microscopy (SEM) and infra-red (IR) analyses were carried out to determine the elemental composition and internal structure of the deposits. Four samples received from patients were analysed using a variety of these methods. The calcinotic deposits had either extruded spontaneously or were surgically removed.

Results: PXRD and elemental analyses indicate that hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the main component of the sample, but the Ca/P ratio is higher than expected at 2.1 (1.7 for hydroxyapatite), which indicates the presence of carbonate, and this is confirmed by IR and TGA studies. The internal composition of these deposits was probed by SEM and XCT, which show that the samples have very different structures, despite having similar elemental compositions. This is shown in figure 1a, where the sample on the right is visibly more porous than that on the left. The images taken were reconstructed into images that showing the surface features of the deposit using XCT, as seen in figure 1b.



a b

Figure 1. a) CT Slices from two of the samples with relevant photographs b) 3D reconstruction of the sample surface

Conclusion: Calcinotic deposits were found to consist of hydroxyapatite with a carbonated component. A greater understanding of the composition of these structures could lead to a better understanding of their formation, potential prevention and improved treatments to reduce their size safely and effectively.

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40. Conserved gene expression modules across the intrinsic subsets in scleroderma skin reveal a network of pathways interconnected with genetic polymorphisms.

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Background: Genome-wide analysis of mRNA levels is now a standard technology and a key component of translational medicine. Although significant insights have been gained by analysis of individual datasets for end target tissues, integrative analysis across multiple cohorts provides additional power and the ability to test the replication of prior findings. We analyzed three independent skin genome-wide gene expression datasets from three cohorts of SSc patients with diffuse, limited and localized disease (Milano et al. 2008, Pendergrass et al. 2012, Hinchcliff et al. 2013, Hinchcliff et al. in prep). These three studies total 329 microarray hybridizations representing 287 unique biopsies from 111 patients. These data allow for a systematic reanalysis of the scleroderma transcriptome and, in particular, allow us to identify the conserved molecular drivers of the intrinsic gene expression subsets (inflammatory, fibroproliferative, limited and normal-like). The goal of this study was to identify the subset-specific molecular 'modules' that are conserved across multiple, independent data sets and to build a network of their interrelationships with each other. We focused on the inflammatory and fibro-proliferative subsets as they capture the majority of patients with diffuse SSc.

Materials and Methods: We developed a novel data mining tool based on consensus clustering from the machine learning field. We paired this with publicly available bioinformatics tools and databases. We collected all published, statistically significant genome-wide association study (GWAS) single nucleotide polymorphisms (SNPs) from genome.gov. In addition, we used the IMP functional Bayesian network that aggregates all publicly available gene expression data into a predicted gene-gene interaction network.

Results: We find that a relatively discrete set of ~ 250 genes whose co-expression is conserved across the three cohorts underlies each intrinsic gene expression subset. We did not find modules that were consistently differentially expressed between all SSc samples and healthy controls. In order to determine how these discrete sets of conserved genes may be interrelated, we used these conserved modules as input to a global gene expression Bayesian network. In contrast to our prior studies that have suggested the subsets are independent and stable, our bioinformatic analysis suggests a theoretical path by which the subsets could be connected raising the notion that patients may change subsets during treatment or with longer disease duration. We identify three distinct modules associated with the inflammatory subset: a human leukocyte antigen (HLA) module, an interferon inducible module, and a TGFβ/ECM module. Likewise, the fibro-proliferative subset contains two distinct modules: a cell proliferation module and a TGFβ/ECM module. Notable genes in the conserved network are FBN1, duplication of which causes fibrosis in the Tsk1 mouse model, and several genes with SSc-associated polymorphisms including AIF1, GRB10, and NOTCH4. The network is enriched for co-expression interactions with SSc-associated polymorphic genes STAT4, IRF5, IRF8, and several HLA genes as well as with putative predictors of Modified Rodnan Skin Score (MRSS) including COMP,

THBS1, SIGLEC1, and IFI44, indicating that MRSS is related to all three of the core inflammatory modules, not just the profibrotic TGFβ pathway. The sharing of the TGFβ/ECM module suggests that immune suppression might push patients from the inflammatory subset to the fibro-proliferative subset without fully abrogating disease. An open label trial of Mycophenolate Mofetil (MMF) has shown that patients who improve during MMF treatment primarily map to the inflammatory subset, and some improvers lose their inflammatory signature and resemble fibroproliferative patients during treatment. Patients return to the inflammatory subset upon cessation of MMF therapy.

Conclusions: Integrative genomics provides a unique vantage point to study gene expression irregularities in SSc and to infer the drivers of those irregularities. The present study predicts that the intrinsic subsets might be connected as opposed to distinct diseases. The network approach taken here sheds light on the role of known SSc associated genetic polymorphisms as producing immune system abnormalities. This is corroborated by early data suggesting that patients can move between subsets in response to immunosuppressive therapy.

41. Aberrant Adipogenesis in the Pathogenesis of Scleroderma

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Background. A striking observation in scleroderma is the correlation between skin fibrosis and loss of subcutaneous adipose tissue (SCAT). The mechanism underlying SCAT atrophy and its significance in pathogenesis are not known. In light of emerging insights into adipogenesis and the plasticity of adipogenic progenitor cells, we investigated the mechanistic basis of adipose atrophy and its relation to fibrosis in the skin.

Methods. The kinetics of SCAT loss and its role in fibrosis was investigated in mouse models of scleroderma and genetic fate mapping was used to assess its role in fibrosis. Modulation of adipogenic differentiation was evaluated in mouse and human stem cells by real-time qPCR, immunoblotting and cytochemistry.

Results. We found that loss of SCAT preceded dermal fibrosis in the bleomycin-induced mouse model of scleroderma. Furthermore, decreased levels of adipogenic markers (*AdipoQ*, *Pparγ*, *aP2*) in lesional skin preceded the increase in fibrogenic markers (*COL1*, *Fn-EDA*, *COL5*). These observations led us to hypothesize that during fibrogenesis, mesenchymal progenitor cell differentiation was redirected away from an adipogenic and towards a fibrogenic fate. In vitro studies confirmed that profibrotic signals such as TGF-beta, PDGF and Wnt-beta-catenin preferentially promote fibrogenic differentiation of mesenchymal progenitor cells. The biological significance of preadipocyte-fibroblast transitions in fibrogenesis was directly addressed by fate-mapping studies using adipocyte-labeled transgenic reporter mice. While adipocyte-derived cells were strictly confined to SCAT, they migrated into the fibrotic dermis, and expressed fibroblast markers in transgenic mice with bleomycin-induced scleroderma. Greater than 80% of dermal myofibroblasts showed an adipocytic lineage origin.

Conclusions. Taken together, these studies suggest that adipose tissue loss may result from mesenchymal progenitor cell selection of a fibrogenic fate and may therefore represent a primary event in fibrosis. Since pharmacological manipulation of cell fate determination by mesenchymal progenitor cells is now feasible with drugs such as imatinib, we conclude that regulation of mesenchymal cell differentiation represents a novel therapeutic approach to fibrosis.

42. Molecular signatures of imatinib therapy in systemic sclerosis assign non-improvers to the inflammatory intrinsic subset

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Background: Imatinib (Gleevec®, Novartis) is a competitive tyrosine kinase inhibitor approved by the FDA for treatment of several cancers. Its potential for treatment of systemic sclerosis (SSc) has been investigated in several studies. Results have been mixed ranging from significant clinical improvements to serious adverse events. Genome-wide analysis of gene expression in response to imatinib treatment has been addressed in two studies in skin with a small number of patients. In this study, we analyzed microarray expression data in paired skin and peripheral blood mononuclear cell (PBMC) samples from 10 SSc patients in a trial of imatinib.

Methods: RNA was extracted from skin biopsies and PBMC samples of 10 SSc patients and hybridized to Affymetrix Human U133 2.0 arrays. Patients were classified as 8 improvers and 2 non-improvers based on MRSS improvement of ≥5 over 12 months. Expression values were calculated via Robust Multichip Average (RMA) and microarray batch effects adjusted via ComBat. Differentially expressed probes were identified via Significance Analysis of Microarrays (SAM) and annotated to enriched functional terms via g:Profiler. Intrinsic gene subset assignment was performed using an intrinsic gene identifier algorithm.

Results: Comparison of improvers to non-improvers at baseline in skin biopsies found differentially increased expression of 678 probes in non-improvers associated with *immune response*, *defense response*, and *cytokine production*. Analysis of the genes that changed expression after imatinib treatment identified 1,749 probes with decreased expression in improvers enriched in *cell cycle*, *DNA metabolism*, *intracellular transport* and *phosphorylation* suggesting a modulation of the fibroproliferative signature. Surprisingly, similar processes were observed down-regulated in non-improvers after imatinib treatment (447 probes, FDR<5%), but in the presence of highly expressed inflammatory signatures. We mapped each patient to their baseline intrinsic subset assignment and found that two non-improvers were from inflammatory intrinsic subset whereas most improvers were from either fibroproliferative or normal-like intrinsic subsets.

Paired PBMC samples were also analyzed for imatinib specific response signatures. 135 probes (FDR<5%) showed increased expression in non-improvers after treatment and displayed enrichment in *immune response*, *intracellular protein kinase cascade* and *response to wounding* paralleling what is observed in skin. 254 probes with increased expression in improvers at baseline (FDR<5%) were enriched in *coagulation*, *response to wounding* and *secretion*. We did observe a general down-regulation response in both skin and blood that was enriched in *intracellular protein kinase cascade*, *microtubule cytoskeleton organization* and *protein modification* suggesting a general effect of imatinib on gene expression via its kinase inhibiting activity.

Conclusions: We observe the downregulation of cell cycle-related functional terms in improvers in skin after imatinib treatment as well as the increased expression of immune system-related processes in non-responders at baseline. *Cell cycle* and *DNA metabolism* are characteristic of proliferative intrinsic subset whereas *immune response* and *defense response* are typical of inflammatory intrinsic subset. Preliminary results suggest that imatinib improvers are associated with the proliferative and normal-like subsets, whereas both non-improvers mapped to the inflammatory subset at baseline. A subset of inflammatory patients showed improvement while on imatinib therapy suggesting additional factors influence patient response.

43. A novel immune mediated model of Systemic sclerosis

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Background and objective: Systemic sclerosis (SSc) is characterized by a complex interplay of vasculopathy, inflammation, and autoimmunity resulting in fibrosis. Unavailability of small animal models that mimic this complex interplay underlines the lack of valuable targeted therapies for this devastating disease. Recent data strongly suggest that the innate immune system is important in the pathogenesis of SSc. Since dendritic cells (DCs) have been implicated in the initiation and progression of autoimmune disorders as well as development of fibrosis and, because DNA topoisomerase I (TopoI) is a specific autoantigen in SSc, we immunized mice with TopoI peptide loaded DCs to induce experimental SSc.

Method: Two peptides, TOPOIA and TOPOIB, were selected from within the TopoI protein. Mice were immunized with bone marrow DCs loaded with either TOPOIA or TOPOIB. In a combined systemic and local immunization protocol, peptide loaded DCs were injected subcutaneously and intratracheally. At experimental endpoint, lung and skin sections were examined by H&E and trichrome (Masson) staining. Lung infiltration was quantified by differential bronchoalveolar lavage cell counts. Quantification of fibrosis was done by measurement of hydroxyproline content in these tissues. Total immunoglobulin levels and anti-TopoI specific antibodies were measured in sera by ELISA. TopoI-specific CD4+ T cell cytokine responses were measured by flow cytometry based intracytoplasmic staining.

Results: We observed that repeated systemic and local immunization with TOPOIA loaded DCs induced a more severe SSc-like disease when compared to immunization with TOPOIB loaded DCs in terms of inflammation, vasculopathy, TopoI-specific antibody, T cell responses and, skin and pulmonary fibrosis. Inflammatory infiltrate in lungs consisted mainly of neutrophils and macrophages. Histological examination of lungs of immunized mice showed a pattern of combined inflammation and fibrosis, reminiscent of non-specific interstitial pneumonia seen at pathology in patients with SSc-interstitial lung disease, along with dermal, but not hypodermal, fibrosis. Furthermore, TopoI-specific T cell immune responses were biased towards a Th17 and Th17/Th1 double positive subtype.

Conclusion: This is the first demonstration that utilizing DCs loaded with the specific SSc autoantigen TopoI results in SSc-like disease in mice. A novel immune-mediated model of SSc will help to further understand the pathogenesis of disease and evaluate targeted therapies for treatment.

44. Sp1 and Smad contribute in mediating both c-Met and CD44-ligand hyaluronan synthesis in response to TGF-β1 stimulation in Scleroderma lung fibroblasts.

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Pulmonary fibrosis is characterized by recurrent injury to the alveolar epithelium that leads to distortion of the normal lung architecture. In fibrotic tissue, the repair program fails to heal the wound, and subsequent abnormal tissue repair and fibrosis occur. The ubiquitous cytokine transforming growth factor-β1 (TGF-β1) has a critical role in the development of pulmonary fibrosis. TGF-β1 expression is consistently high in affected lung fibrotic tissue and correlates with increased extracellular matrix (ECM) deposition. In the last decades, incredible progress has been made in identifying the molecular characteristics of pathways downstream of the TGF-β1 receptors (TGF-β1RI and TGF-β1RII). In particular, Smad proteins, which are TGF-β1R substrates and Smad-binding element partner molecules that translocate into the cell nucleus to act as transcription factors, have been studied extensively. The glycosaminoglycan hyaluronan (HA), the omnipresent mammalian ECM molecule, has a crucial role in the regulation of dermal fibrosis and wound healing. c-Met, a receptor for hepatocyte growth factor (HGF), is another molecule with a pivotal role in Systemic Sclerosis (SSc). In this study, northern and western blot analyses showed that TGF-β1 significantly induced expression of c-Met and HA synthase 2 (Has2) in primary SSc lung fibroblast cells, which primarily took place at the gene transcriptional level. In addition, interestingly, TGF-β1-induced c-Met and Has2 expression also depended on a functional Sp1, as silencing Sp1 blocked c-Met induction and HA production in these cells. These results provide strong evidence that TGF-β1 transcriptionally regulates c-Met and HA signalling pathways and suggests that TGF-β1 has a different mechanism in lung fibrosis diseases compared with other fibrotic pathologies. Therefore, the initial activation of TGF-β1 after alveolar epithelium injury could be beneficial by initiating the anti-fibrotic effect of HGF in this disease. However, it is conceivable to imagine that a persistent chronic exposure to hyperactive TGF-β1 signalling can also sustain HA signalling, which can eventually overpower the system in favor of profibrotic effects, the dreadful side of TGF-β1 functions.

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45. Course of disease in SSc-Overlap syndromes clearly differs from limited and diffuse cutaneous SSc - Data of the German Network for Systemic Scleroderma (DNSS)

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Abstract

Background: SSc-Overlap syndromes are a very heterogeneous and remarkable subgroup of SSc-patients, who present at least two connective tissue diseases (CTDs) at the same time, usually with a specific autoantibody status.

To determine whether patients, classified as overlap syndromes, show a disease course different from patients with limited (lcSSc) and diffuse cutaneous SSc (dcSSc).

Methods: The data of 3323 prospectively included patients, registered in the database of the German network for systemic sclerosis and followed between 2003 and 2013, were analyzed. The following statistical methods were used: Kaplan-Meier analysis, logistic regression and χ^2test.

Results: Among 3323 registered patients, 10% (325/3240) were diagnosed as SSc-overlap syndrome. Of these, 82.5% (268/325) were female with a mean age of 49.2 ±1.2 years and carried significantly more often other antibodies (71.1%; $p<0.0001$), including U1RNP-, PmScl-, Ro-, La-, as well as Jo-1- and Ku-antibodies.

These patients developed musculoskeletal involvement earlier and more often, than patients diagnosed as lcSSc and dcSSc ($p<0.0001$). The onset of lung fibrosis and heart involvement in SSc-Overlap patients was significantly earlier than in patients with limited SSc and occurred later in patients with dcSSc. Oesophagus, kidney and PAH progression was similar to lcSSc patients, whereas dcSSc patients had a significantly earlier onset. Thus, although SSc-Overlap patients have a mean mRSS similar to lcSSc patients, they develop earlier and more widespread significant organ involvement.

Conclusions: These data support the current thesis, that SSc-overlap syndromes should be regarded as a separate SSc subset, distinct from lcSSc and dcSSc, due to a different evolution of the disease, different proportional distribution of specific auto-antibodies and organ involvement.

46. Development of a novel immunoassay for the diagnosis of systemic sclerosis

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Purpose/Objective

The presence of conformational, stimulatory auto-antibodies (abs) against the PDGF receptor (PDGFR) in patients affected by systemic sclerosis (SSc) has been questioned. IgG purification procedures and technical problems in cell-based biological assays and in solid phase binding assays can disrupt agonistic conformational abs and generate artifacts. To solve these issues and to validate the presence of anti-PDGFR abs in SSc patients, we aimed at developing a simple and reproducible immunoassay to detect agonistic anti-PDGFR abs in serum samples.

Materials and Methods

i) Human monoclonal anti-PDGFR auto-abs were cloned from memory B cells of SSc patients; ii) different recombinant PDGFR conformers were tested to set up a capture ELISA able to detect serum anti-PDGFR auto-abs; iii) competitive binding assays using anti-PDGFR monoclonal auto-abs and recombinant human PDGF were performed to define the PDGFR epitopes bound by these different ligands; iv) a conformational peptide library spanning PDGFR extracellular domains was generated to validate epitope mapping data; v) peptides bound by monoclonal auto-abs were synthesized and pre-incubated with agonistic auto-abs to inhibit antibody biological activity.

Results

i) Monoclonal anti-PDGFR auto-abs generated from SSc B cell repertoire exhibited different PDGFR binding and agonistic properties; ii) a map of extracellular PDGFR functional domains was obtained: PDGFR epitopes bound by stimulatory auto-abs differed from those of non-biologically active auto-abs; iii) pre-incubation of agonistic auto-abs with peptides corresponding to their epitopes inhibited collagen stimulation in fibroblasts; iv) a competitive ELISA based on the inhibitory peptides was established to detect conformational PDGFR auto-abs in serum: this assay allowed discrimination between SSc and control sera with remarkable specificity and sensitivity.

Conclusions

The identification of the epitopes of the stimulatory, SSc-specific, anti-PDGFR auto-antibodies was crucial to develop an immunoassay discriminating between SSc and control sera. The solid phase binding assay based on these epitopes might be used as a novel tool for diagnosis of SSc and classification of the clinical subsets of disease and related conditions such as Raynaud's Phenomenon.

47. IL-6 trans-signalling mediates increased collagen via an epigenetic mechanism

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Interleukin-6 is both a pro-inflammatory and pro-fibrotic mediator. Dermal fibroblasts secrete excessive collagen I leading to extracellular matrix deposition and fibrosis ultimately leading to loss of function. IL-6 receptors are not expressed on dermal fibroblasts and so need to signal complexed with soluble IL-6R via the shared receptor gp130 which causes signal transduction leading to gene expression. We have shown T cells to be the source of sIL-6R and we now examined the downstream signalling pathways. miR are non coding RNA species that affect gene expression by binding the 3'UTR and suppressing mRNA. They are considered 'fine tuners' of gene expression by targeting many genes. A specific miR, 29a is associated with fibrotic conditions and likely targets key matrix genes. Human dermal fibroblast where treated with IL-6 and sIL-6 (trans signalling) pre treated with STAT3 inhibitor S31-201, in separate experiments miRNA29a was also measured with the use of Taqman miRNA specific miR29a primers, plus inhibitors of TGF-beta receptors to inhibit the TGF-beta pathway. SSc diseased fibroblasts from SSc patient biopsies were also quantified for miRNA29a transcripts. In separate experiments the STAT3 inhibitor was incubated with disease fibroblasts and collagen 1 and miRNA29a was measured with qPCR. The result indicated that inhibition using pharmacological approaches to STAT3 resulted in reduced collagen expression in dermal fibroblasts indicating that the STAT3 pathway plays a pivotal role. Furthermore inhibition of TGF-beta signalling reduced IL-6-mediated collagen production and abrogated IL-6-facilitated repression of miR29a. Transfection of miR29a precursors resulted in suppression of collagen transcription after 24 hours culture. SSc disease fibroblasts had reduced basal levels of miR29a and this could be reversed by blockade of the IL-6 signalling pathway. In conclusion IL-6 trans signalling mediates collagen expression via STAT3 mediated repression of the collagen targeting miR29a, thus leading to upregulation of its target. Diseased fibroblasts have suppressed miR29a and this can be reversed leading to suppression of collagen via targeting IL-6 trans signalling.

48. DECREASED INTERLEUKIN-20 LEVEL IN PATIENTS WITH SYSTEMIC SCLEROSIS ARE THEY RELATED WITH ANGIOGENESIS?

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Background: The angiogenesis-antiangiogenesis balance is disturbed in systemic sclerosis (SSc). The endothelium in SSc loses its normal characteristics, and attains vasospastic, procoagulant and proinflammatory features; endothelial apoptosis increases dramatically. Various studies revealed that Th17 cytokine group was associated with angiogenesis and also activated fibroblasts. IL-20 is a proinflammatory cytokine: it stimulates angiogenesis, arteriogenesis apoptosis and contributes actively to the pathogenesis of diseases like RA, atherosclerosis. Also, it was reported that VEGF and TGF-β which play roles in SSc are also associated with VE-cadherin.

Objectives: In this study, we aimed to evaluate the relation between angiogenesis indicators and T helper 17 cytokine group in patients with SSc which is a disease characterized by impaired angiogenesis and autoimmune response. In our study, patients with SSc are compared with patients with primary Raynaud's phenomenon (RP) and healthy controls.

Methods: Forty SSc patients (38 females, 2 males, mean age: 48.35±13.2), 18 primary RP cases (16 females, 2 males, mean age: 48.1±9.3), and 20 healthy controls (19 females, 1 male, mean age: 49.3±8.5) were included in our study. The demographic and clinical features of patients with SSc were recorded. The serum levels of vascular endothelial growth factor (VEGF), vascular endothelial (VE)-cadherin, IL-20, IL-22 and IL-23 were assessed.

Results: In SSc group, IL-20 level was significantly lower than in both primary RP group and controls (p
values <0.001). VE-cadherin level in SSc was significantly higher than in primary RP (p=0.016). The IL- 22, IL-23 and VEGF levels of SSc, primary RP and control groups were similar (p values >0.05). In SSc
patients. IL-23 correlated negatively with VEGF (r=-0.36, p=0.025), and positively with VE-cadherin (r=0.55, p<0.001). In SSc, IL-20 levels correlated with ESR (p=0.032, r=0.339), CRP (p=0.049, r=0.311) and disease duration (p=0.044, r=0.32). SSc patients with limited involvement had significantly higher VE-cadherin levels than SSc patients with diffuse involvement (4.32±6.5 vs. 1.79±0.9, p=0.044). Anti-centromere positive patients (6.59±10.5) had significantly higher levels of VE-cadherin when compared to anti-centromere negative ones (2.72±3.8) (p=0.036).

Conclusions: We observed that IL-20 which is an IL-10 group angiogenesis indicator were observed to be suppressed in SSc, suggesting abnormal angiogenesis.

Key words: Systemic sclerosis, interleukin-20, VE-cadherin, Th 17, interleukin-23

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49. The Role of the transcription factor Nkx2-5 in Scleroderma-Associated Pulmonary Arterial Hypertension

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Introduction: One of the major causes of death in scleroderma is pulmonary arterial hypertension (SSc-PAH). Although disease management has improved significantly in recent years, patients still have poor survival and limited treatment options. PAH is a severe condition which results in structural remodelling within the lung, in particular thickening of the pulmonary arteries and cardiac manifestations including right ventricle hypertrophy. The cause(s) of PAH are unknown, but activation of adventitial fibroblasts, vascular smooth muscle cells and the elevated deposition of extracellular matrix components notably collagen type I in vessels are key features of disease pathogenesis. We have previously shown that the transcription factor Nkx2.5 is as master regulator of the expression of Collagen type I by vascular smooth muscle cells by identifying an Nkx2-5 activated vessel specific response element in the Collagen Ia2 far upstream enhancer region. Nkx2-5 is a cardiac specific transcription factor essential in cardiac development. The aim of this study was firstly to look at expression of Nkx2-5 in the vessels of patients with scleroderma associated pulmonary arterial hypertension (SSc-PAH) and secondly, to assess the effect of Nkx2-5 deletion on vascular remodelling of the pulmonary vasculature.

Methods: We used pulmonary tissue from health control subjects (n=3) and SSc-PAH patients (n=3). We also bred Nkx2-5^{flox} with Col1□2CreERT mice to create inducible conditional Nkx2-5^{flox}Col1□2CreERT null mice. Upon administration of tamoxifen, Nkx2-5 expression is deleted in all cells expressing the Collagen 1□2 enhancer driven Cre recombinase such as the collagen type I synthesising VSMC in the pulmonary vasculature. The mice were placed in a hypoxic chamber at 10% oxygen for 21 days, the right ventricular systolic pressure (RVSP) was measured before the mice were sacrificed. Morphometric studies of the vasculature to assess masculinisation, vessel compliance were carried out as well as right ventricular hypertrophy.

Results: We demonstrated that Nkx2-5 is expressed by de-differentiated pulmonary arterial smooth muscle cells (PASMC) undergoing phenotypic modulation, in remodelling vessels and in the pulmonary vasculature of patients with SSc-PAH.

In the hypoxia model, we demonstrated that RVSP of the all control groups was elevated as expected to 40.18 ±2.44 mmHg and 37.6±1.83 mmHg respectively compared to that of Nkx2-5 null mouse group which was significantly lower at 29.15± 2.3 mmHg ($p=0.0076$). Significantly less vascular muscularisation ($p<0.00005$) in small, medium and large vessels of Nkx2-5 null mice was observed compared with control mice and myography revealed that Nkx2-5 null mouse vessels were less stiff and more compliant. A significant decrease in RV/LV+S ratio was observed in Nkx2-5 null mice compared with control mice ($p=0.016$) although Nkx2-5 expression was not deleted in the heart, as the Col1□2 enhancer is not activated there during the hypoxic insult.

Conclusions: Nkx2-5 is expressed in SSc-PAH and is involved in the vascular remodelling and deposition of collagen type I. Deletion of Nkx2-5 in the hypoxia induced model of PAH results in decreased RVSP, decreased vascular remodelling and right ventricular hypertrophy.

This work was funded by Arthritis Research UK and the British Heart Foundation.

50. Constitutive expression of JUN B contributes to Collagen type I deposition and dermal fibrosis in Systemic Sclerosis

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Objectives:
Collagen type I deposition in the skin of Systemic sclerosis patients, is a characteristic of the disease and of fibrotic diseases in general. The molecular mechanisms that result in the deposition of Collagen type I remain unclear. It is now well established that the Collagen1A2 far upstream enhancer is regulates Collagen IA2 over-expression in adult tissues undergoing repair and in pathology. Several transcription factors from the Activator Protein 1 (AP-) family have been shown to modulate extracellular matrix production in SSc and animkla models of fibrosis. Here, we investigate the AP-1 members, in particular c-Jun and Jun B, and their role in the activation of the Collagen IA2 enhancer and resulting collagen type I deposition in dermal fibrosis observed in Systemic Sclerosis (SSc).

Methods:
Collagen IA2 enhancer activity was determined in dermal fibroblasts from scleroderma patients (n=5) and healthy controls (n=5) by transient transfection, mutagenesis, electrophoretic mobility shift assays and chromatin precipitation assays. AP1 family member expression in scleroderma and healthy control skin was analysed, by western blotting, immunohistochemistry and nuclear localisation was visualised by immunofluorescence. Jun B expression was targeted using specific small interfering (si)RNA.

Results:
The Collagen IA2 enhancer contains a SMAD independent TGF□-induced response element that is activated by JunB expression. JunB is not expressed in normal fibroblasts but constitutively expressed in the nucleus of dermal fibroblast explanted from SSc biopsies. Occupation of JunB on the Collagen IA2 enhancer element results in the engagement of the enhancer to the proximal promoter and in overexpression of Collagen type I. Deletion of the Jun B binding site within the enhancer results in downregulation of Collagen type I activity. Inhibition of Jun B results in a lower Collagen type I expression in fibroblasts and inability for dermal fibroblast to migrate into a wound.

Conclusions:
The data show that JunB is a key regulator of collagen type I overexpression in scleroderma skin through the occupation of a TGF-□ induced responsive regulatory element in the Collagen I A2 enhancer and that this element is continuously active in scleroderma dermal fibroblasts, thus contributing to the fibrotic phenotype. The data identifies JunB as a potential anti-fibrotic target.

This work was funded by Arthritis Research UK.

51. Flow Cytometric Detection and Characterization of Fibrocytes in Fibrotic Lung Disease: Increased Number and Altered Phenotype

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Introduction: Historically the cells that overexpress collagen in fibrotic lung disease were believed to be activated resident fibroblasts. Recent evidence suggests that some fibroblast precursors originate in the bone marrow and migrate into damaged lung tissue. In particular, monocytes differentiate into cells known as fibrocytes that are defined by their expression both of the pan-leukocyte marker CD45 and the fibroblast marker collagen I (Col I). A nagging problem in this field is that it has been difficult with existing antibodies to distinguish between specific and non-specific labeling with Col I antibodies, particularly in the mouse. Our goals in the current study were: 1) Prepare anti-peptide antibodies in which specific Col I labeling could be demonstrated by peptide competition experiments; 2) Using these antibodies quantify fibrocytes in a mouse model of lung fibrosis; 3) Determine whether fibrocytes are bone-marrow derived by their expression of GFP; and 4) characterize fibrocytes in terms of their expression of several proteins associated with cell migration and ECM production.

Methods: Antibodies against the Col Iα1 C-terminal propeptide were prepared. Western blotting and flow cytometry experiments demonstrated that the antibody can be competed by specific peptide but not by non-specific peptide. To evaluate lung fibrosis, mice previously irradiated and engrafted with bone marrow from mice that constitutively express GFP in all their cells were treated with either bleomycin or saline vehicle delivered by osmotic pump. To analyze lung tissue by flow cytometry, single cell suspensions were prepared using collagenase.

Results: An issue in this field is whether Col I+ cells are Col I expressing cells or cells that have absorbed Col I to their cell surface. Because the pro-peptide is only attached to Col I in cells that are expressing the molecule, the use of the pro-peptide antibody definitively identifies fibrocytes. In addition, the generation of antibodies for which specificity could be demonstrated by peptide competition allows a clearcut identification of CD45+/Col I+ fibrocytes. Bleomycin-induced lung fibrosis leads to a large increase in the number of fibrocytes present. The identity of these fibrocytes as bone marrow-derived cells was strikingly confirmed by their expression of GFP.
Other proteins of interest (HSP47, CXCR4, tenascin C, periostin) were expressed at much higher levels in bleomycin fibrocytes than in CD45+/Col I- and CD45-/Col I+ cells. Interestingly, while CD45+/Col I+/GFP+ fibrocytes are readily detected in control lung tissue, these control fibrocytes express much lower levels of HSP47, CXCR4, tenascin C, and periostin than do bleomycin fibrocytes. We also noted a major population of CD45-/Col I+ "resident fibroblasts" in control lung tissue that were also GFP+, suggesting that resident fibroblasts are in large part bone-marrow derived cells.

Conclusion: These studies present improved methods for identifying fibrocytes and demonstrate that fibrosis is accompanied both by an increase in the number of fibrocytes present in lung tissue AND by a change in their phenotype (to high HSP47/CXCR4/tenascin C/periostin).

52. Enhanced Expression of CCR receptors in healthy AA and SSc ILD monocytes

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Background. Scleroderma-associated Interstitial Lung Disease (SSc ILD) is more prevalent and more severe in African Americans (AA) than in Caucasian (C) patients, but little is known of the factors underlying this health disparity. We reported recently that healthy AA monocytes share abnormalities with SSc ILD monocytes including low caveolin-1 levels and hypermigration towards chemokine SDF-1 due to upregulation of its receptor CXCR4. In the current study we have investigated other chemokine receptors that are involved in SSc ILD, namely CCR1, CCR2, and CCR3. The aim of this study was to determine whether these receptors are also overexpressed in SSc ILD and healthy AA monocytes and the functional consequences of their overexpression.

Methods. The study was approved by the university's IRB for Human Subject Research. Monocytes were isolated from the blood of SSc ILD patients and healthy donors by negative selection. SSc ILD patients fulfilled the ACR criteria for the classification of systemic sclerosis. Monocyte migration was assayed in Multiwell Chemotaxis Chambers using cells treated with the caveolin-1 scaffolding domain (CSD) peptide or control peptide. CCR1, CCR2, CCR3, ERK, pERK, Src, pSrc, Lyn, pLyn, and caveolin-1 levels were determined by Western blotting and immunostaining.

Results. In the current study, we observed that the expression of CCR1, CCR2, and CCR3 is enhanced in SSc ILD monocytes and healthy AA monocytes compared to healthy C monocytes. In accord with these findings, compared to healthy C monocytes, the migration of healthy AA monocytes toward the CCR2 ligand MCP-1 was enhanced two-fold ($p < 0.05$) and toward the common CCR1,2,3 ligand MCP-3 was also enhanced two-fold ($p < 0.05$). SSc ILD monocyte migration (compared to C monocyte migration) toward MCP-1 was enhanced 10-fold ($p < 0.001$) and toward MCP-3 was enhanced 5-fold ($p < 0.001$). In all cases, treatment with CSD inhibited migration > 50 %, demonstrating that the enhanced migration of SSc ILD and healthy AA monocytes is due to their relative lack of caveolin-1. To study signaling downstream from CCR1, CCR2, and CCR3; healthy C, healthy AA, and SSc ILD patient monocytes were treated with MCP-1. Baseline activation of ERK and Src was two-fold increased in AA compared to C while Lyn activation was similar in C and AA. All three kinases were greatly enhanced in SSc ILD. MCP-1 activated ERK in both C and AA monocytes, but had no effect on Src and Lyn. MCP-1 did not increase the already high level of ERK activation in SSc ILD monocytes, but did further increase the already high levels of Src and Lyn activation. In contrast, MCP-3 appears to inhibit ERK, Src, and Lyn in all three groups; suggesting that MCP-1 and MCP-3 signaling during cell migration occurs through distinct mechanism. The relevance of these observations to human disease was demonstrated in immunostaining experiments showing that CCR1, CCR2, CCR3, MCP-1, and MCP-3 are all overexpressed in SSc ILD lung tissue compared to control healthy lung tissue.

Conclusion. Our results demonstrate that low caveolin-1 levels may play a role in the predisposition of the AA population to SSc ILD via the regulation of the expression of chemokine receptors CCR1, CCR2, and CCR3 in monocytes and signaling downstream from these receptors through the activation of ERK, Src, and Lyn.

53. Influence of interleukin and angiotensin converting enzyme pathway gene polymorphisms on the severity of organ involvement in patients with systemic sclerosis.

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Background. Systemic Sclerosis is an autoimmune disease with involvement of several internal organs. Inflammatory and vascular abnormalities play an important role in its pathophysiology. Several polymorphisms of interleukins, components of the renin-angiotensin system and endothelins have been evaluated in autoimmune diseases in diverse ethnic groups with variable results.

Patients and Methods. We evaluated 23 single nucleotide polymorphisms (SNPs) related to IL-1, IL-6, IL-10, angiotensin converting enzyme (ACE), angiotensin II receptor type 1 (AGTR1) and endothelin 1 (IL-1B 511 T/C 3, IL-1F 10.3 C/T 4, IL-1RN 6/1 C/T 5, IL-1 RN4 T/C 6, IL-6 1426 G/T 3, IL-6 572 G/C, IL-10 1082 A/G, IL-10 592 A/C, IL-10 819 C/T, ACE rs4291 A/T, ACE rs4318 A/G, ACE rs4335 A/G, ACE rs4343 A/G, ACE rs4344 A/G, ACE rs4353 A/G, ACE rs4363 A/G, AGTR1 rs275651 A/T, AGTR1 rs275652, AGTR1 rs275653 A/G, AGTR1 rs5182 T/C, AGTR1 rs5183 A/G, End rs1800541 G/T, End1 rs1800541 G/T, End1 rs5369 A/G, End1 rs3087459 A/C) in a group of 159 Mexican Mestizo SSc patients without overlap syndromes (73 patients with dcSSc and 86 with lcSSc) from our SSc cohort, and 199 ethnically matched healthy controls. Severity of the organ involvement was evaluated using the Medsger severity scale; scores of 3 or 4 according to this scale were considered as “severe” involvement. Statistical analysis was performed using SPSS v.19 and Epi-Info 7.0. Chi square was used to compare genotype and allele frequencies between groups. Results were considered significant when two-tailed p values were <0.05 and they were corrected for multiple comparisons using the Bonferroni test.

Results. There were no differences in the polymorphism frequencies between SSc patients and controls or between dcSSc and lcSSc patients. Severe vascular involvement was associated to the G allele (p=0.02, OR 3.3, 95%CI 1.2-9.1) and the GT genotype (p=0.006, OR 5.2, 95%CI 1.6-16.2) of the AGTR1 rs275652 gene polymorphism, and to the G allele (p=0.008, OR 3.9, 95%CI 1.4-10.4) and the AG genotype (p=0.001, OR 6, 95%CI 1.9-18.6) of the AGTR1 rs275653 gene polymorphism. Severe interstitial lung disease was associated to the CC genotype of the IL-1 F 10.3 C/T 4 gene polymorphism (p=0.001, OR 17, 95%CI 2.1-137) and to the CC genotype of the IL-1 RN 6/1 C/T 5 gene polymorphism (p=0.002, OR 8.4, 95%CI 1.3-53). Severe joint involvement was associated to the C allele (p=0.01, OR 3.5, 95%CI 1.4-8.8) and the CC genotype (p=0.003, OR 17.5, 95%CI 1.7-177) of the IL-1F 10.3 C/T 4 gene polymorphism.

Conclusions. Severe vascular involvement in SSc is associated to SNPs at the angiotensin II type 1 receptor, while severe interstitial lung disease and severe arthritis are associated to SNPs at IL-1 F and IL-1 RN in Mexican Mestizo SSc patients, emphasizing the importance of the participation of such pathways in the pathogenesis of this disease. Functional correlation with serum levels of these components of the renin-angiotensin system and these cytokines would be of interest.

54. Antinuclear Antibody Negative Systemic Sclerosis

Salazar GA, Assassi S, Wigley F, Hummers L, Varga J, Hinchcliff M, Khanna D, Schiopu E, Phillips K, Furst DE, Steen V, Baron M, Hudson M, Taillefer SS, Pope J, Jones N, Markland J, Docherty P, Khalidi NA, Robinson D, Simms R, Silver R, French TM, Fessler B, Molitor J, Segal B, Al-Kassab F, Perry M, Yang J, Zamanian S, Reveille JD, Arnett FC and Mayes MD

Objective:
To examine the demographic and clinical characteristics of systemic sclerosis (SSc) patients without antinuclear antibodies (ANA) compared to ANA positive patients.

Methods:
SSc patients enrolled in the Scleroderma Family Registry and DNA Repository were included.
Relevant demographic and clinical data were entered directly by participating sites or by chart review.
Autoantibodies were determined at one site utilizing commercially available kits.

Results:
This study included 3249 patients, of whom 208 (6.4%) were ANA negative. The proportion of male patients was higher in the ANA negative group (OR 1.65 p=0.008).
ANA negative patients experienced significantly less vasculopathic manifestations of SSc. The percent predicted diffusion capacity of carbon monoxide (DLco) was higher in ANA negative patients (p=0.03). Seven ANA negative patients had pulmonary arterial hypertension (PAH) per right heart catheterization (RHC) versus 213 ANA positive (OR= 0.23 p=0.0004) indicating that PAH was significantly less common in the ANA negative group. They also less often had telangiectasias and digital ulcers/ pits (p=0.01 and p=0.0004, respectively).
Although, diffuse cutaneous involvement was more common, the modified Rodnan Skin Score (mRSS) was lower in the ANA negative group (2.4 points lower, p=0.018). Furthermore, they experienced more malabsorption (p=0.003). There was no difference in frequency of pulmonary fibrosis and scleroderma renal crisis. All-cause mortality was also not different between the two groups (p=0.28).
The above observations remained significant after adjusting for potential confounders (age, disease duration, gender, disease type)

Conclusion:
ANA negative patients constitute a distinct subset of SSc characterized by a higher proportion of men, more frequent lower gastrointestinal involvement, a higher proportion of diffuse cutaneous disease but less severity of skin fibrosis overall and fewer vasculopathic features of the disease.

55. Correlation between patient self-report of symptoms of Raynaud's phenomenon and objective assessment of digital microvascular perfusion using infrared thermography

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Background: Patient self-report of digital colour changes form the basis of a clinical diagnosis of Raynaud's phenomenon (RP) and help classify patients with early systemic sclerosis (SSc). There is a high reported prevalence of RP symptoms in fibromyalgia syndrome (FMS) but little evidence of a true vasculopathy in this condition. We report the findings of a large retrospective study designed to evaluate the relationship between patient self-report of digital colour changes and objective assessment of digital microvascular function using infra-red thermography (IRT) in patients with primary RP, SSc and FMS.

Methods: Retrospective review of all patients referred for thermographic evaluation between 2010 and 2012 was undertaken. Our thermographic protocol includes the completion of a patient-reported questionnaire documenting symptoms of RP. Thermographic assessment of the resting longitudinal thermal gradient of all fingers was undertaken by calculating the mean distal dorsal difference (DDD) at 23^{o}C (without prior knowledge of the clinical diagnosis). A negative DDD is suggestive of digital microvascular dysfunction. A subsequent case note review was undertaken to select out patients with a clinician diagnosis of primary RP (ANA negative), SSc (sclerodactyly in conjunction with a SSc-specific autoantibody and/or abnormal nail fold capillaroscopy) and FMS (1990 criteria).

Results: One hundred and thirty eight patients were evaluated (83 PRP, 12 SSc, 43 FMS). No pattern of digital colour change discriminated between the three groups (Table). In all groups a monophasic pattern of colour change was the most commonly reported symptom, with white being the most frequent colour change. In the SSc group, a triphasic colour change was least commonly reported. Thumbs were generally more commonly affected in SSc. Thermographic assessment revealed most pronounced peripheral microvascular dysfunction in SSc followed by primary RP and then FMS respectively.

* p=0.09 vs. FMS	**Primary RP (n=83)**	**SSc (n=12)**	**FMS (n=43)**
Age, mean (SD)	47.8 (14.8)	53.1 (13.6)	43.8 (10.8)
Females, n (%)	64 (77.1)	10 (83.3)	39 (90.7)
White, n (%)	58 (69.9)	9 (75)	29 (67.4)
Blue, n (%)	36 (43.4)	7 (58.3)	14 (32.6)
Red, n (%)	35 (42.2)	5 (41.7)	21 (48.8)
Purple, n (%)	39 (47)	3 (25)	16 (37.2)
Monophasic, n (%)	23 (27.7)	5 (41.7)	14 (32.6)
Biphasic, n (%)	21 (25.3)	4 (33.3)	11 (25.6)
Triphasic, n (%)	17 (20.5)	1 (8.3)	8 (18.6)
Numbness, n (%)	71 (85.5)	12 (100)	41 (95.3)
Symptoms in absence of cold, n (%)	45 (54.2)	8 (66.7)	25 (58.1)
Thumbs involvement, n (%)	30 (36.1)	7 (58.3)	17 (39.5)
Toes involvement, n (%)	65 (78.3)	8 (66.7)	35 (81.4)
Average DDD ^{o}C, median (IQR)	-1.05 (4.83)	-2.2 (4.5)	+0.62 (4.92)
Proportion of patients with a mean DDD <-1.0 ^{o}C, n (%)	42 (50.6)*	8 (66.7)*	15 (34.9)

Conclusion: Patient self-report of RP symptoms are similar across primary RP, SSc and FMS and do not aid disease classification. Objective assessment using IRT revealed strong trends for differences in baseline digital microvascular function across the 3 groups although relatively low patient numbers for SSc hampered data analysis. Despite a similar burden of patient-reported symptoms of RP, normal thermographic appearances were identified in the majority of patients with FMS.

56. Discordant regulation of profibrotic pathways by endothelin-1 in cultured mice lung fibroblasts in a transgenic mouse model of SSc

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Background: Pulmonary fibrosis (PF) is a major cause of morbidity in systemic sclerosis (SSc), occurring in more than one third of diffuse cutaneous SSc patients. Therapeutic options are limited and prognosis remains very poor. The fibro-proliferative process of the disease in which cytokines such as TGFβ and CTGF are implicated leads to deregulation of extra-cellular matrix production, fibroblasts activation and collagen deposition. Experimental data in the last decade have shown that endothelin-1 (ET-1) could play a role in PF but the recent clinical trial with the endothelin-receptor antagonist bosentan in idiopathic PF have proved disappointing (1). We have previously shown that the TβRIIΔk-fib transgenic mouse model develops many features of SSc including susceptibility to vasculopathy, skin and lung fibrosis (2,3). This study aimed to (i) explore the links between ET-1, TGFβ and CTGF in normal and pathologic lung fibroblasts and (ii) to investigate the effects of bosentan on fibrotic pathways.

Methods: The transgenic mouse strain TβRIIΔk-fib expresses a kinase-deficient type II TGFβ receptor driven by a fibroblast-specific promoter leading to balanced ligand-dependent upregulation of TGFβ signalling. Explanted lung fibroblasts from wild type WT (n=3) and transgenic TG mice (n=3) were cultured until confluent, starved for 24 hours in DMEM medium containing 0.1% FCS and then stimulated with recombinant TGFβ (2 ng/mL), ET-1 (100 nM) or both for 24 hours in the presence or absence of bosentan (10 μM). Expression of collagen type I, CTGF and β-tubulin were analysed by western-blot of cell lysates. Cell migration was assessed by scratching the cell layer before stimulation with PDGF-BB (50 ng/mL) in the presence of Mitomycin C. Cells were treated with Bosentan 10 μM or vehicle during 24 hours. Cell migration (n=2 per group) was assessed in triplicate.

Results: As reported previously, basal CTGF and collagen type I expression was significantly higher in TG fibroblasts than in WT fibroblasts ($p<0.05$). TGFβ and ET-1 significantly increased levels of CTGF expression in WT and in TG fibroblasts, with a synergistic effect when used in combination. Bosentan appeared to attenuate collagen type I expression induced by TGFβ, ET-1 or both in WT and TG fibroblasts ($p<0.05$). Bosentan did not show effect on CTGF expression either with or without stimulation by TGFβ, ET-1 or both. Bosentan inhibited migration of WT and TG fibroblasts in response to PDGF-BB.

Conclusion: ET-1 induced CTGF expression in cultured lung fibroblasts. Transgenic fibroblasts were more responsive to ET-1 stimulation. Interestingly, bosentan seemed to have a greater inhibitory effect on collagen expression on TG fibroblasts than in WT, but CTGF over-expression in cultured TG fibroblasts was not attenuated, perhaps reflecting a lack of clinical benefit in lung fibrosis trials. These preliminary data would be consistent with a role for CTGF as a pathogenic driver of the lung fibrosis observed in this TG mouse strain and further experiment with more potent ET-1 antagonists are planned.

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57. Pro-fibrotic effects of CTGF beyond fibroblasts: CTGF mediates EMT-like phenotypic changes in epithelial cells *in vivo* and *in vitro*

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Connective tissue growth factor (CTGF, CCN2) is a key mediator in the pathogenesis of chronic fibrotic diseases. Although increased CTGF expression by fibroblast cells is a hallmark of fibrosis, it is not completely understood how the paracrine effects of CTGF may influence the cell fate of adjacent epithelial cells. In order to investigate the effects of CTGF over-expression in fibroblasts of Col1a2-CTGF transgenic mice on epithelial cells of skin and lung, these tissues were examined for phenotypic markers of epithelial activation and differentiation and induction of signal transduction pathways.

In the skin, Col1a2-CTGF transgenic mice showed thickening of the dermis, as well as focal hyperplasia in the epidermis. Immunostaining revealed an increased number of α-SMA- ($p < 0.005$), Snai1- ($p < 0.001$), S100A4- ($P < 0.001$) and Sox9- ($P < 0.0004$) positive basal cells compared to wild-type ($n = 6$), suggesting a phenotypic switch in these cells. Accompanying these changes, p38 and Erk1/2 signalling were also activated in the epidermis and dermis; 3- to 7- fold increases in phospho-p38- and phospho-ERK1/2- positive cells were detected by immunostaining. Western blot analysis of keratinocyte cell extracts also showed p38 and ERK1/2 activation.

In the lung of Col1a2-CTGF mice, immunostaining revealed a marked increase in the number of cells co-expressing the epithelial marker, TTF-1 and mesenchymal cell markers α-SMA ($P < 0.01$) and Snai1 ($P < 0.0001$), indicative of epithelial-to-mesenchymal transition (EMT)-like changes. Real-time PCR analysis also showed elevated expression (5- to 15- fold change) of a host of fibrotic and EMT markers in Col1a2-CTGF lung, including TβRI, TGF-β1, Col1a2, fibronectin, PAI-1, TIMP-1, SOX9 and α-SMA. Complementary *in vitro* studies in alveolar epithelial cells showed that CTGF knockdown using siRNA (5 and 20 nM) suppressed TGF-β-induced Snail, Sox9, S100A4 protein levels while restoring expression of E-cadherin. Immunostaining showed that CTGF expression, both endogenously via adenoviral expression in epithelial cells or exogenously via treatment with recombinant CTGF (10 and 50 ng/ml), induced EMT-like morphological changes and expression of a-SMA in epithelial cells. Western blot analysis also showed a significant increase in α-SMA expression in recombinant CTGF treated epithelial cells ($P < 0.005$).

Overall, these data suggests that CTGF not only plays an important role in mediating a pro-fibrotic phenotype in fibroblasts, but also in the EMT-like switching of epithelial cells, which may also contribute to fibrogenesis by increasing the accumulation of pro-fibrotic cells.

58. Linking DNA damage to WNT signalling in systemic sclerosis

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Background. Canonical WNT signaling is found frequently activated in cancer and fibrotic diseases but the primary cause of this induction is not known. We report that the expression of WIF-1, the soluble WNT inhibitor, is silenced by DNA damage in mesenchimal and epithelial cells. In a highly fibrotic disease, such as systemic sclerosis, WIF-1 expression is silenced by the DNA damage checkpoint kinase, ATM. In normal cells, DNA damage induced by UV or ROS, silences WIF-1 expression. PDGFR agonistic autoantibodies, isolated from SSc patients, induce type I Collagen, DNA damage and inhibit WIF-1 *via* ROS and ATM in normal fibroblasts. In a model of skin fibrosis *in vivo* induced by bleomycin, WIF-1 expression is silenced with a concomitant increase of β-catenin and type I Collagen. ATM mediates these effects because in ATM-/- cells bleomycin is unable to silence WIF-1 and activate type I Collagen.

Object. The aim of this study was to identify the mechanism that liks DNA damage to alteration of Wnt pathway in SSc.

Methods. Skin fibroblasts from SSc patients and normal controls were transiently transfected with specific siRNAs against c-jun and ATF3. Expression of WIF-1, type I Collagene, c-Jun, and ATF3 was determined by real-time PCR, western blot, and immunocytochemistry. Chip assay was performed to analysed the levels of transcriptor factors on WIF-1 and type I Collagen.

Results. We found that the stress factor ATF3 and the AP1 family member c-Jun inhibit WIF-1 expression. Specifically, silencing ATF3 or c-Jun expression relieves WIF-1 downregulation and abolishes type I Collagen induction in SSc or normal stressed cells. ATF3 mediates bleomycin or DNA damage inhibition of WIF-1 expression, while c-Jun mediates the effects on type I Collagen expression. Our data indicate that ATM phosphorylates c-Jun and induces ATF3, which together silence WIF-1. Loss of damaged fibroblasts-secreted WIF-1 amplifies local Wnt action by inducing a proliferative burst of surrounding epithelial cells and terminal differentiation of fibroblasts.

Conclusions. These data provide an unanticipated link between DNA damage and Wnt signaling, which drives and modifies the fate of epithelial and mesenchymal cells in cancer and fibrosis.

59. Angiotensin II - induced skin fibrosis in mice is associated with vascular injury

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Systemic sclerosis (SSc) is an autoimmune inflammatory disorder of unknown etiology characterized by microvascular injury and fibrosis of the skin and internal organs. Angiotensin II (Ang II), a vasoconstrictive peptide, was found to be increased in the blood and skin of SSc patients compared with normal healthy donors (Kawaguchi Y. at all, 2003). In addition, we reported that Ang II induces skin fibrosis in mice (Stawski L. at all, 2012). The goal of this study was to further investigate mechanisms involved in skin fibrosis induced by Ang II as a model for the pathogenic mechanism of Scleroderma.

Methods: Ang II at a rate of 1000ng/kg/min (pressor dose) was administered continuously over 7 and 14 days by subcutaneous osmotic pump implanted under the skin of 8 weeks old C57Bl males. Apoptosis was evaluated at day 14 by TUNEL staining, and IHC staining of cleaved Caspase 3. Analysis of injured endothelial cells was performed at day 14 on paraffin sections (8μm) by IHC staining of vWF, Mmp12, and Pdgfrβ. Protein levels of Mmp12 and vWF in human dermal microvascular endothelial cells (HDMEC) in response to Ang II was evaluated by Western blot. The mRNA expression level of vascular injury markers (Angpt2, Mmp12 and Thbs1) and pericyte marker Adam12 was quantified at day 7 and 14 by real-time PCR.

Results: TUNEL and cleaved Caspase 3 staining showed increased number of apoptotic cells present in the epidermis, in the vessels and in the cells within the connective tissue in Ang II treated mice. None or few apoptotic cells were found in PBS treated mice. Immunostaining in the skin of Ang II treated mice showed increased number of vWF positive cells. vWF protein level was also increased in Ang II treated HDMEC cells. When compared with control mice, Ang II treated skin samples showed increased number of Mmp12-positive cells, including positive ECs. Mmp12 protein level was increased in Ang II treated HDMEC cells in a dose dependant manner. Pdgfrβ-positive cells were observed around the vessels in control mice, but were increased in all skin layers in Ang II mice. Real-time PCR analysis showed a statistically significant increase in the levels of vascular injury markers: Angpt2 at day 7, Mmp12 and Thbs1 at day 14 and pericyte marker Adam12 at day 7 in the skin of Ang II infused mice.

Conclusions: These observations together with the previously reported data strongly suggest that the Ang II model closely reproduces many of the key features of SSc, including vascular injury. Moreover, persistent defect of endothelial cells in this model may lead to development of a chronic fibrogenic environment resulting in activation of peri-endothelial cells that leads to fibrosis.
This study was supported NIH grant AR42334-14.

60. Investigating the role of MRTF-A in wound healing and Systemic Sclerosis

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Background The Myocardin related transcription factor (MRTF) family interacts with globular (G) actin in the cytoplasm. Following actin polymerization in response to Rho-GTPase signaling, MRTFs translocate into the nucleus to activate gene transcription of muscle specific genes such as alpha-smooth muscle actin (SMA) and fibrotic genes such as CTGF through an interaction with the serum response factor (SRF) on CArG box regulatory elements [$CC(A/T)_6GG$]. MRTF-A activated collagen gene transcription through Sp1/SRF dependent recruitment, far more than other members of the myocardin family(1). The loss of functional MRTFA by genetic deletion significantly disrupted type I collagen synthesis relative to controls and the phenotype could be rescued by overexpression of MRTF-A. MRTF-A is implicated in differentiation of myofibroblast-like cells by multiple cell lineages, epithelial (EMT), endothelial (EnMT), or smooth muscle cells to synthetic mesenchymal cells. We hypothesized that MRTFA is necessary for wound healing and is dysfunctional in systemic sclerosis (SSc).

Methods MRTF-A knockout(2) and wild type control mice were subjected to excisional wounds (4mm punch biopsy, basal wound area 12.6 mm^2) and bleomycin-induced fibrosis. MRTF-A signal transduction was studied in healthy control and SSc fibroblasts with and without the small molecule inhibitor CCG1423. SSc fibrosis responses were modelled by collagen gel contraction synthesis of CTGF, and type I collagen. MRTF-A signalling was assayed by Western blotting of nuclear and cytoplasmic extracts.

Results Following excisional wounding MRTF-A mice wounds failed to close normally and increased in size during days 1-7, wound area decreasing by day 11. When compared to wild type controls, MRTF-A knockout wounds were enlarged at day 7 (wild type area 6mm^2, knockout area 12.4 mm^2, $p<0.03$), and at day 11 (wild type area 0.42 mm^2, knockout area 3.4 mm^2, $p<0.01$). The histology of Day 11 skin wounds showing reduced scar formation and abnormal vessels.. Small blood vessels within the granulation tissue were dilated, and exhibited extravasation of red blood cells. Gel contraction by wild type fibroblasts was enhanced by TGFβ and blocked by CCG1423 1μM (basal conditions mean gel mass 0.176g, TGFβ treated 0.118g, TGFβ+CCG1423 0.238g, $p<0.002$). Dermal fibroblasts from MRTF-A knockout mice showed reduced basal gel contraction, and impaired response to TGFβ, (basal conditions mean gel mass 0.349g, TGFβ treated 0.259g, TGFβ+CCG1423 0.313g, $p<0.05$ basal vs wild type). SSc fibroblasts showed enhanced nuclear localisation of MRTF-A at 8 hours following exposure to TGFβ (4ng/ml) not seen in healthy control fibroblasts

Conclusions MRTF-A knockout mice fail to contract wounds adequately and show reduced scar formation, as well as abnormal vessels. Multiple pro-fibrosis pathways converge on MRTF-A including response to stiffness of the extracellular matrix, profibrosis growth factor stimulation, as well as transiton of epithelial cells, endothelial, and perivascular cells to mesenchymal phenotypes. CCG1423 and its derivatives may be potential anti-fibrotics to benefit SSc fibrosis.

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61. Prediction of immune system and pathway abnormalities from SSc-PAH gene expression.

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Background: Systemic sclerosis-associated pulmonary arterial hypertension (SSc-PAH) has significant mortality and no disease-modifying treatment. PAH is characterized by remodeling of the pulmonary vasculature. Although a number of pathways have been implicated, including aberrant metabolism, endothelial cell and smooth muscle cell proliferation, and inflammation, the underlying biology is not fully understood. Prior studies of peripheral blood mononuclear cell (PBMC) gene expression and serum factors from SSc-PAH patients demonstrate signs of inflammation and vascular injury. PBMC gene expression data allows for investigation of a mixture of innate and adaptive immune cells in the context of a patient's serum factor and cytokine milieu. We have performed a meta-analysis of publicly available SSc-PAH PBMC gene expression data and previously published lung gene expression data to gain insight into inflammatory abnormalities underlying SSc-PAH. The objectives of this study were to identify perturbed pathways and immune cell abnormalities in SSc-PAH, as compared to SSc patients without PAH and healthy controls, consistent across independent PBMC and lung microarray datasets.

Methods: Publicly available SSc-PAH datasets were downloaded from the Gene Expression Omnibus and pre-processed. Previously published gene expression data from SSc lung were also considered. Differentially expressed genes and perturbed pathways were identified in each dataset by SAM and GSEA, respectively. Single-sample gene set enrichment analysis (ssGSEA) was applied to each PBMC dataset independently using expression-derived cell type specific gene sets, and selected GO, KEGG, and Reactome pathways. Resulting enrichment scores (ES) were used to estimate cell type proportions (cell type ES) and magnitude of pathway signal (pathway ES) on a per sample basis. Correlations between cell type ES and pathway ES and cell type ES and clinical covariates were calculated. Global and pairwise cell type relationships were examined.

Results: Pathway and leading edge analysis in PBMC and lung data revealed aberrant glucose metabolism, cell proliferation, and endocytosis signatures in SSc-PAH patients. Of the 21 blood cell types tested, 6 are predicted to be in different proportions in SSc-PAH as compared to SSc without PAH and healthy controls in both PBMC datasets. Although these 6 cell types represent both innate and adaptive immune cells, only estimated innate immune cell proportions significantly correlate with hemodynamic and pulmonary function measures. The combination of cell type ES and multiple pathway ES can be used to generate hypotheses. For example, SSc-PAH patients with high monocyte ES also tend to have high sphingolipid metabolism signal and cell migration signaling which may indicate a chemotactic monocyte population in those patients that develop pulmonary vascular disease.

Conclusions: PBMC microarray data can be used to predict the aberrant cell type proportion and behavior in SSc-PAH patients. Poor hemodynamic parameters and pulmonary function measures correlate with increased innate immune cell signatures. From pathway analysis in both PBMC and lung tissues, we predict specific pathways are deregulated such as KEGG adipocytokine signaling, KEGG regulation of autophagy, and KEGG tryptophan metabolism that could potentially be targeted therapeutically.

62. Asymmetric dimethylarginine (ADMA) levels in the early detection of systemic sclerosis related pulmonary arterial hypertension (SSc-PAH)

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Background

Pulmonary Arterial Hypertension (PAH) is a major cause of mortality in Systemic Sclerosis (SSc), and alterations in nitric oxide (NO) metabolism and endothelial cell dysfunction are implicated in the pathogenesis of SSc-PAH. Assymetric dimethlyarginine (ADMA) is considered a novel biomarker of endothelial cell dysfunction. We sought to explore the clinical utility of ADMA as a screening biomarker for incident SSc-PAH.

Methods

ADMA levels were measured in 15 consecutive treatment-naive subjects with SSc-PAH, and compared with 30 SSc-controls without PAH. ADMA levels were assayed using high performance liquid chromatography with solid phase extraction. NT-proBNP levels were measured in the same subjects. Logistic regression models were used to explore the independent association of ADMA with PAH. Important correlations of ADMA were assessed using Spearman's rank correlation coefficient (rho). The optimal cut-points of ADMA and NT-proBNP that maximised desired test properties for screening were determined.

Results

The PAH group had significantly higher mean ADMA levels than the control group (0.76 ± 0.14 μM versus 0.59 ± 0.07 μM; $p < 0.0001$). ADMA levels remained significantly associated with PAH after the adjustment for specific disease characteristics, cardiovascular risk factors and vascular complications (all $p<0.01$). An ADMA level ≥ 0.694 μM for PAH verses controls, had a sensitivity of 86.7%, specificity of 90.0% and AUC of 0.86 for diagnosing PAH. An NT-proBNP level of ≥209.8ng/mL for PAH versus controls had a sensitivity of 93.3%, specificity of 100% and AUC of 0.94 for diagnosing PAH. A 'purely' biomarker based screening model that combined an NT-proBNP ≥ 209.8ng/mL and/or ADMA ≥ 0.694 ng/mL resulted in a sensitivity of 100% and specificity of 90% for the detection of SSc-PAH.

Conclusion

ADMA may be an important screening and diagnostic biomarker for SSc-PAH. A composite 'purely' biomarker-based screening algorithm, using NT-proBNP in combination with ADMA, may achieve an excellent sensitivity (and also specificity) for the non-invasive identification of SSc-PAH.

63. Circulating Th-associated Cytokine and Chemokines in Localized Scleroderma

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Background:

Localized scleroderma (LS) is a fibrotic autoimmune disease of the skin and underlying tissues which results in disfigurement and orthopedic complications, especially when the onset is in childhood. Identifying potential biomarkers involved with disease pathogenesis may lead to future therapeutic targets. T-helper (Th) cell subsets and their associated cytokines are thought to contribute to the pathogenesis of systemic sclerosis (SSc). Traditionally, a Th2 predominant response has been supported, but more recent data also implicate Th1, Th17, and various chemokine involvement. This concept in LS has only been partially investigated, with studies evaluating only two of the three Th cell lineages, and not examined in reference to disease activity. This study was designed to further evaluate the Th-cell associated plasma cytokine and chemokine profiles of patients with pediatric LS.

Materials and Methods:

Plasma samples were obtained from 69 pediatric LS patients and 71 healthy pediatric controls. Twenty-nine cytokines and chemokines were analyzed using a Th1, Th2, and Th17 Millipore luminex panel comparing LS to healthy controls, with additional analysis predetermined to be dedicated to disease activity parameters. Concurrent clinical outcome measures of disease activity in LS, the modified Localized Scleroderma Severity Index (mLoSSI) and the Physician Global Assessment of Disease Activity (PGA-A), were the main parameters compared to the cytokine/chemokine level. Mann-Whitney U test was employed to compare cytokine levels between LS and healthy groups and Spearman rank correlation was used to determine relationships between individual analytes and clinical parameters ($p < 0.05$). Type I error due to multiple testing was controlled for using the Benjamini-Hochberg method.

Results:

The following cytokines levels were significantly elevated in LS patients (n = 69) compared with healthy controls (n = 71); IP-10, MCP-1, IL-17A, IL-12p70, GM-CSF, IL-1b, IFN-a2. When LS patients were further divided into active (n=30) and inactive states (n=39), IP-10 was significantly elevated in the active group compared to inactive (median: 2087.3 vs. 880.5 pg/ml, respectively). IP-10 levels were also significantly correlated with the Physician Global Assessment of Disease Activity (PGA-A) score ($\rho = 0.450$, $p = 0.005$).

Conclusions:

SSc and LS share a similar histopathology with infiltration of lymphocytes and their associated effector cytokines in skin specimens. In the current study we have demonstrated, for the first time to our knowledge, a serological presence of IP-10, MCP-1, IL-17A, IL-12p70, GM-CSF, IL-1b, and IFN-a2 in localized scleroderma, with IP-10 being highly correlated to active disease.

Prior SSc studies have also found similar cytokine and chemokine profiles in the circulation, with recent literature supporting a potential role of IP-10 in SSc disease severity. These findings suggest a potential immunological link between these two clinically different diseases.

64. Simulating nailfold capillaroscopy sequences to evaluate algorithms for blood flow estimation

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Introduction. Nailfold capillaroscopy is a non-invasive technique that is used to diagnose systemic sclerosis (SSc)-spectrum disorders and assess progression over time by measuring properties of the nailfold capillaries such as vessel density, shape and size. Capillaroscopy has, until now, been applied mainly in the examination of capillary structure. However, red blood cell velocity can also be assessed, and if this could be quantified reliably, could provide clinicians with a direct measure of blood flow. Red blood cell velocity could be a useful outcome measure in clinical trials of vasoactive treatments. The purpose of this study was to simulate, in software, video capillaroscopy sequences with known properties in order to evaluate the accuracy of computer algorithms for estimating blood cell velocity under varying conditions. Specifically, we wished to quantify the effects of flow rate, red blood cell density and contrast.

Methods. We created a synthetic sequence by randomly generating a path that represented the centre of the vessel, then adding inner and outer walls for both the arterial and venous limbs. A given number of blood cells were scattered within the space enclosed by the vessel walls, and a blood cell velocity vector defined at every pixel for a given flow rate. From this flow field, we computed the position of every blood cell in every image of the sequence and rendered an ideal image sequence. To this ideal sequence, we added artefacts such as brightness and contrast variation, image noise and 'shake' (simulated patient movement artefacts).

We then applied an optical flow algorithm to estimate blood cell velocity in synthetic sequences with varying but known properties (e.g. number of cells, flow rate, contrast variation). By comparing every pixel's estimated velocity with its known true value, we quantified the effect of varying each parameter from its true value.

Results. Error increased approximately linearly with flow rate, the number of cells and contrast variation. Brightness variation also increased error, though at a sublinear rate, up to a maximum of approximately 0.5 pixels per frame. Tests on real sequences also show that flow can be detected and its direction captured, though a quantitative assessment is not possible without some physical, real-world data about flow conditions.

Conclusion. Estimating blood cell velocity from real sequences demands either hierarchical optical flow algorithms or hardware capable of capturing images at a high frame rate in order to overcome the effect of high flow rates. Image registration should also be used to minimize brightness and contrast variation, and 'shake' before estimating flow. Our model of image synthesis is well-suited to generating annotated training data from which optical flow parameters can be estimated via Machine Learning, rather than via prescribed mathematical theory. For the clinician with an interest in SSc, this is a first step in extending the capabilities of nailfold capillaroscopy to include the reliable measurement of microvascular flow.

65. The role of endothelin receptors ($ETR_{A/B}$) in fibrocyte differentiation.

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Introduction

Systemic sclerosis (SSc) is a complex autoimmune fibrotic disease, characterised by elevated deposition of extracellular matrix (ECM) proteins, in particular collagen type I. The disease is heterogeneous; organs commonly affected by fibrosis are the skin, kidney, lung and heart. Vascular complications include pulmonary arterial hypertension (PAH), which occurs in 12-40% of patients.

$CD14^+$ monocytes are a functionally heterogeneous cell type. They have been noted to differentiate into a number of cell phenotypes including macrophages and collagen-producing fibrocytes. In culture fibrocytes adopt a spindle shape, co-express haematopoietic - CD45RO and 25F9, along with mesenchymal markers including αSMA and collagen type I. Fibrocytes amplify the inflammatory/ immune response through distinct mechanisms, including antigen presentation, cytokine and chemokine secretion, and the production of MMPs. We and others have shown fibrocyte differentiation is enhanced by fibrogenic cytokines including PDGF. Here we seek to understand the mechanism by which SSc fibrocytes influence the local microenvironment of the tissue.

Methods

$CD14^+$ peripheral blood mononuclear cells (PBMCs) were isolated from SSc patient and healthy control blood. PBMCs were cultured in the presence of macrophage colony stimulating factor (MCSF; n>10) and/or endothelin-1 (ET-1; n>10); after 14 days of culture number of fibrocytes was assessed. The effect of pharmacological inhibitors including ETR_A and ETR_B antagonism on fibrocyte differentiation (n=6 SSc and control) was investigated. Secreted factors in culture media from SSc and control fibrocytes were assessed by ELISA (n=6), and the effects of conditioned media explored in 3D-collagen gel

Results

Both MCSF and ET-1 significantly induced fibrocyte differentiation, in combination differentiation was significantly augmented ($P<0.05$) in comparison to mono-treatment. SSc fibrocytes more readily differentiated from $CD14^+$ PBMCs than healthy control donors in response to MCSF ($P<0.05$), ET-1 ($P<0.05$) as well as MCSF with ET-1 in combination ($P<0.01$). ETR_A and ETR_B antagonists, BQ123 and BQ788 (respectively), and Bosentan (a dual ETR antagonist) inhibited MCSF induced fibrocyte differentiation in a concentration dependant manner. Furthermore SSc fibrocytes secreted significantly more connective tissue growth factor (CTGF) than control fibrocytes ($P<0.05$) cultured with MCSF. Consistent with fibrocytes acting in a paracrine manner, conditioned media from SSc fibrocytes promoted fibroblast gel contraction by control cells ($P<0.05$).

Discussion

Here we show $CD14^+$ SSc PBMCs more readily differentiate into fibrocytes and that activation via the ETR_A and ETR_B is essential for ET-1 and MCSF induced fibrocyte differentiation. Suggesting MCSF acts indirectly via ET-1 release; possibly resulting in a positive feedback loop. Our data suggests fibrocytes may contribute to the development of a pro-fibrotic environment through influencing tissue resident fibroblasts in a paracrine manner.

66. Lipoic acid plays a crucial role in scleroderma: Insights obtained from scleroderma dermal fibroblasts

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Purpose: Systemic sclerosis **(**SSc) is a connective tissue disease characterized by vasculopathy and fibrosis of the skin and organs. Increase in oxidative stress and platelet-derived growth factor receptor (PDGFR) activation promote collagen I (Col I) production, leading to fibrosis in SSc. Lipoic acid (LA) and its active metabolite dihydrolipoic acid (DHLA) are naturally occurring thiols that act as cofactors and antioxidants. The goal of this study was to examine whether LA is deficient in SSc patients and determine the effect of DHLA on the phenotype of SSc dermal fibroblasts. N-acetylcysteine (NAC) was included as a comparison.

Methods: Dermal fibroblasts were isolated from punch biopsies obtained from healthy subjects and patients with diffuse SSc. Superoxide levels were measured using dihydroethidium. Immunofluorescence was performed to probe for LA, Col I and α-smooth muscle actin (αSMA). Matrix mettaloproteinase-1 (MMP-1) and 3, lipoic acid synthetase (LIAS) were measured by ELISA.

Results: The expression of LA and LIAS in SSc dermal fibroblasts was lower than normal, however LIAS levels were significantly higher in SSc plasma. DHLA lowered cellular oxidative stress levels, and decreased PDGFR phosphorylation, Col I, and αSMA expression in SSc dermal fibroblasts. It also restored the activities of phosphatases that inactivated the PDGFR. SSc dermal fibroblasts produced lower levels of MMP-1 and 3 then did normal dermal fibroblasts, with levels increasing after DHLA incubation. DHLA showed better efficacy than NAC in most cases.

Conclusions: LA levels were lower in SSc skin fibroblasts than normals. DHLA not only acted as an antioxidant but also an antifibrotic since it had the ability to reverse the profibrotic phenotype of SSc dermal fibroblasts.

67. Frequency of calcinosis in a multi-center international cohort of patients with Systemic Sclerosis: A Scleroderma Clinical Trials Consortium Study

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Background and purpose: Calcinosis is a known complication of systemic sclerosis (SSc) but the prevalence is unclear. Prior single center studies have estimated that 25% of SSc patients suffer from calcinosis. We sought to determine the frequency of calcinosis in an international multi-center collaborative effort with the Scleroderma Clinical Trials Consortium (SCTC).

Methods: We developed an electronic survey using Qualtrics© (Qualtrics, Provo, UT) and distributed the survey to 109 SCTC institutions via e-mail. The information requested included the number of total SSc patients enrolled in the institution database with and without calcinosis, with additional questions regarding disease duration, demographics, and disease subtype. All SSc patients had to fulfill ACR criteria, LeRoy and Medsger criteria or have a diagnosis made by a scleroderma expert. The presence of calcinosis was defined as ever having physical examination and/or radiographic evidence of subcutaneous calcium deposition. Responses were collected during the month of February 2013.

Results: 19 centers provided information from their cohorts, including centers from the US, Europe, Canada, Australia, and Mexico. Data from a total of 7709 SSc patients was collected. The average size of the 19 cohorts that provided data was 811 patients (range 86 to 2034 patients). Calcinosis was present in 22% (total 1676) of patients. The group with calcinosis was 84% female, 92% Caucasian, 6% African American, and 4.4% Hispanic. The calcinosis group was more likely to have limited cutaneous disease (65% vs. 33% diffuse and 3% sine sclerosis, $p<0.001$). Patients with calcinosis were older (54.8±5.5 vs. 50.9±6.2 years, $p=0.105$) and had longer mean disease duration from the first non-Raynaud's symptom than those without (9.6±5.1 vs. 7.0±3.1 years, $p=0.109$), but these differences did not reach statistical significance.

Conclusions: We report a frequency of calcinosis of 22% in a multi-center international cohort of SSc patients. We are currently evaluating the internal organ, clinical, and serologic associations with calcinosis in this large cohort of SSc patients. Given that calcinosis is a frequent complication of SSc, future research efforts should be made to gain a better understanding of the pathophysiology of calcinosis in SSc, and to develop targeted therapies.

68. Association between nail fold capillaroscopy abnormalities and thermographic assessment of peripheral microvascular dysfunction in an unselected cohort of patients under investigation for symptoms of Raynaud's phenomenon

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Background: Nail fold capillaroscopy (NC) and infrared thermography (IRT) allow objective assessment of digital microvascular abnormalities in patients with Raynaud's phenomenon (RP) and have an important role in disease classification, particularly in systemic sclerosis (SSc). The relationship between NC appearances and assessment of digital microvascular function with IRT in an unselected population of patients with RP symptoms has not previously been explored.

Methods: A retrospective review of all patients investigated with both NC and IRT for symptoms of RP between August 2010 and November 2012 was undertaken. Thermographic assessment of the resting longitudinal thermal gradient of all fingers was undertaken by calculating the mean distal dorsal difference (DDD) at 23^{o}C (without prior knowledge of the clinical diagnosis or NC findings). NC images were reviewed (BV and MS) and categorized as normal, non-specific abnormalities and SSc-pattern abnormalities (Cutolo, Clin Exp Rheumatol. 2007). Indeterminate cases were reviewed by a 3rd assessor (JP) and consensus reached. A subsequent case note review was undertaken to categorize patients according to the following diagnoses; primary RP (no clinical features of CTD and ANA negative), SSc (sclerodactyly in conjunction with a SSc-specific autoantibody), FMS (1990 criteria) and other diagnoses.

Results: One hundred and forty one patients were identified. NC appearances, IRT analysis and diagnoses are summarized in the accompanying table. NC abnormalities were associated with a significantly lower median thermographic DDD indicating more severe peripheral microvascular function (p=0.017). There was no difference between thermographic findings of patients with non-specific and SSc-pattern NC abnormalities (possibly due to small patient numbers with SSc-pattern abnormalities). NC abnormalities were present in all patients with SSc and the majority (54.5%) had SSc-pattern changes. Patients with primary RP were significantly less likely to have NC abnormalities compared with SSc (p<0.0001). Non-specific NC abnormalities were identified in 15/46 (32.6) patients with primary RP but SSc-pattern abnormalities were not identified in any patients with primary RP. NC appearances were generally normal in FMS with only a small proportion of patients (3/18, 16.7%) exhibiting non-specific NC abnormalities.

	Normal NC appearance (n=83)	**Nonspecific NC changes** (n=48)	**SSc-pattern NC changes** (n=10)
Median average DDD across all fingers, ^{o}C, median (IQR) *p* value vs. normal NC appearance (Mann Whitney U)	+0.15 (4.16)	-1.98 (4.21) 0.017	-2.09 (3.71) 0.099
Proportion of patients with a mean DDD < -1, n (%)	35 (42.2)	25 (52.1)	7 (70)
Clinical diagnosis, n			
Primary RP	31	15	0
SSc	0	5	6
FMS	15	3	0
Other rheumatic Diseases	37	25	4

Conclusion: The presence of NC abnormalities is associated with thermographic evidence of more profound digital microvascular dysfunction in patients under investigation for symptoms of RP. The presence of NC abnormalities differs between primary RP and SSc. Despite a high reported prevalence of RP symptoms in FMS, the majority of patients have normal NC and thermographic findings.

69. Role of Class II Human Leukocyte Antigens in the Progression from Early to Definite Systemic Sclerosis.

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Background. Criteria for the diagnosis of Early Systemic Sclerosis (EaSSc) have been formalized by LeRoy and Medsger in 2001 and later validated by Koenig et al (2008), who showed that the vast majority of EaSSc patients will eventually develop definite SSc within few years from the diagnosis. Capillaroscopy findings as well as baseline sIL2-R or PIIINP levels may help to differentiate patients with EaSSc at high- or low-risk of progression (Valentini et al, 2012). The role of no genetic marker on disease progression has been evaluated so far.

Methods. One-hundred-ten consecutive SSc patients with a diagnosis of EaSSc according to LeRoy and Medsger (2001) and no other manifestation indicative of SSc (Fransen et al, 2012) were considered. All the patients underwent high-resolution HLA Class II typing by means of PCR with sequence-specific primers ad were prospectively evaluated for the appraisal of definite SSc according to the newly ACR/EULAR proposed criteria for SSc (van den Hoogen, Arthritis Rheum, under review). The time-to-event was analyzed by means of the Log-Rank test and Cox regression analysis to provide an estimate of the risk of progression according to the baseline characteristics; results for the genetic variables were corrected (Pc) by means of 1000-fold permutation testing.

Results. Ninety-eight out of 110 EaSSc subjects (89%) developed a definite SSc by the end of the 15-years follow-up period, with a median time-to-the event equal to 23 months from referral. The first definite SSc-manifestation was the presence of puffy fingers in 34 cases (30.9%), sclerodermatous skin changes in 22 (20%), esophageal involvement in 17 (15.5%), a reduction in DLco in 8 (7.2%), the presence of digital ulcers in 5 (4.5%) or a combination of them in 21 cases (19%) mostly involving puffy fingers (16 out of 21 cases) and/or oesophageal SSc alterations (16 out of 21 cases). Ten (21.8%) patients tested positive for anti-Topoisomerase I antibodies, 61 (55.4%) for anti-centromere antibodies and 10 (9.1%) for other SSc-specific antibodies; no autoanticorpal specificity was associated with disease progression. Similarly, the duration of Raynaud's phenomenon (RP), the gender or the age at referral were not associated with disease progression. Patients with an "active" capillaroscopic pattern had shorter times to definite SSc compared to those with a "slow" capillarocopic patterns (median survival=21 vs 31 months, HR=1.112, CI=1.012-1.274, $p<0.05$). Among HLA antigens, the strongest signal was associated with the absence of the HLA-DQA1*0101 allele (median survival=68 vs 21 months, HR=0.4678, CI=0.2904-0.7536, p=0.001, $Pc<0.05$); similarly the absence of the HLA-DRB1*0101 allele (median survival=71 vs 21 months, HR=0.4983, CI=0.2891-0.7734, p=0.002) or of the HLA-DQB1*0501 allele (median survival=68 vs 21 months, HR=0.4741, CI=0.2901-0.7589, p=0.001) was protective against disease progression. Results did not significantly change when corrected for RP duration or the autoanticorpal status.

Discussion. Antigens located within the HLA DRB1*0101-DQA1*0101-DQB1*0501 haplotipe may be helpful to differentiate EaSSc patients at low- or high-risk of progression toward a definite SSc.

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70. CD109 attenuates extracellular matrix production in scleroderma skin fibroblasts and decreases skin fibrosis in a mouse model of bleomycin-induced scleroderma.

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Despite intensive research efforts aimed at developing strategies to block the progression of fibrosis in SSc, there are currently no effective anti-fibrotic therapies available for clinical use. Transforming growth factor-beta (TGF-□) is a multifunctional growth factor important for tissue homeostasis and exhibits potent pro-fibrotic properties. Aberrant activation of TGF-□ signaling has been strongly implicated in SSc fibrosis. We have identified CD109, a glycosyl phosphatidylinositol (GPI)-anchored protein, as a TGF-β co-receptor and potent inhibitor of TGF-□ signaling *in vitro*.

To determine whether CD109 regulates extracellular matrix production in human SSc skin fibroblasts, we analyzed the effect of blocking CD109 expression using CD109-specific siRNA or addition of recombinant CD109 protein on the expression of collagen and fibronectin in those cells. We also determined CD109 expression in skin tissue and cultured skin fibroblasts of SSc patients and normal healthy subjects. Our results show that endogenous CD109 potently inhibits extracellular matrix synthesis in SSc skin fibroblasts, as well as normal skin fibroblasts. Interestingly, we found that CD109 protein levels are elevated in SSc skin *in vivo* and SSc skin fibroblasts and keratinocytes *in vitro* as compared to normal skin tissue and cells, likely reflecting an adaptive response to inhibit aberrant activation of TGF-□ signalling in SSc skin. To determine the role of CD109 in skin fibrosis *in vivo,* we generated transgenic mice that overexpress CD109 in the epidermis and subjected these mice and their wild-type littermates to bleomycin-induced skin fibrosis that model scleroderma. Our results indicate that CD109 transgenic mice display a resistance to bleomycin-induced skin fibrosis as evidenced by a significant decrease in dermal thickness, collagen cross-linking, collagen and fibronectin content and attenuated Smad 2/3 signalling as compared to wild-type littermates. Furthermore, our more recent data suggest that CD109 overexpression in the epidermis shifts the balance of TGF-□ signaling from the ALK5-Smad2/3 pathway to the ALK1-Smad1/5 pathway in epidermal keratinocytes and alter epidermal-dermal interactions to decrease extracellular matrix synthesis in the dermis.

Taken together, these findings suggest that CD109 is a TGF-□-Smad2/3 pathway antagonist and exhibits anti-fibrotic properties in experimental murine scleroderma. Thus CD109 represents a potential molecular target to decrease fibrotic responses and may have application for the treatment of fibrosis in human SSc.

71. Vasopressin Modulates Endothelial and Fibroblast Gene Expression

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OBJECTIVES: This study was triggered by the finding of increased expression of the vasopressin V1a receptor in SSc fibroblast (FB) and SSc microvascular endothelial cells (MVEC). Thus, we sought to assess the potential involvement of arginine vasopressin (AVP) in scleroderma (SSc). We examined the effects of AVP on collagen I (*COLI*) expression by SSc and control FB, endothelin 1 (*ET1*), endothelial nitric oxide synthase (*eNOS, NOS3*), and prostacyclin synthetase (*PGIS*) in normal and SSc MVEC. Finally we explored a cellular source for AVP production in SSc.

METHODS: The effects of AVP and the nonapeptide V1a antagonist FE999122(2) on *COL1, ET1, eNOS*, and *PGIS* expression in SSc and control FB and MVEC were examined by a quantitative realtime PCR and by western blot analysis. Moreover, AVP expression levels were examined in SSc and control MVEC, FB and peripheral blood mononuclear cells (PBMC) and their subsets by realtime PCR and WB.

RESULTS: Significant increase expression of the V1a receptors was noted in SSc FB and MVEC. Minimal detectable AVP expression was noted in SSc and control MVEC and FB. However, significant increase in the expression level was noted in SSc PBMC particularly in the CD14+ SSc monocytes subset.
AVP significantly increased SSc and normal MVEC expression of *ET1* and decreased *NOS3* and *PGIS* expression levels in a dose dependent fashion. The effects were abolished by the addition of the V1a receptor antagonist FE999122 (2). Moreover, AVP upregulated *COLI* expression in both control and SSc FB. This effect was also completely reversed FB by the addition of the V1a antagonist.

CONCLUSIONS: This study demonstrate that AVP can augment *ET1* and *COL1* synthesis by MVEC and FB respectively and significantly down regulate *NOS3* and *PGIS* in MVEC. Inhibition of AVP effects by the V1a receptor antagonist imply that the observed effects are mediated by this receptor. Moreover, the study suggests that the CD14+ adherent PBMC are a potential source for AVP production in SSc. The role of AVP in the pathogenesis of the vascular and fibrotic lesions in SSc warrant further investigations.

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72. An atypical cyclin-dependent kinase mediates fibrosis and is a novel target in scleroderma

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Background: Cyclin-dependent kinase 5 (CDK5) is expressed primarily in the central nervous system, where it plays important roles in axonal guidance, dopaminergic signaling and neuronal migration, and is involved in sensory pathways and pain sensing. Aberrant CDK5 function is implicated in neurodegenerative diseases including Alzheimer's disease. In contrast to other cyclin-dependent kinases, binding of the non-cyclin activator p35 is sufficient to induce CDK5 activity. Recently CDK5 substrates have been identified in macrophages, T cells and adipocytes, suggesting crucial extra-neuronal roles for CDK5 in inflammation and metabolism. A novel CDK5 substrate is the nuclear receptor PPAR-γ, a master regulator of adipogenesis and adipokine production, which has been shown to be impaired in scleroderma. These observations led us to hypothesize that the CDK5/p35 pathway might be responsible for impaired PPAR-γ function in SSc and thus play a role in the development and persistence of fibrosis.

Materials and methods: The expression of p35 was examined in skin biopsies from bleomycin-injected mice and explanted normal and scleroderma fibroblasts. The regulation of p35/CDK5 expression and activity was examined in human and mouse skin fibroblasts, progenitor cells and mature adipocytes by real-time qPCR, Western analysis and in vitro kinase assay. Effects of CDK5/p35 loss-of-function and gain-of-function were evaluated in vitro in normal and scleroderma skin fibroblasts. Effects of CDK5/p35 inhibitors on fibrotic responses were evaluated in vitro using monolayer cultured fibroblasts, ex vivo using human skin organ cultures and in in vivo mouse models of fibrosis induced by bleomycin or constitutively active AdTBR^{ca}.

Results: We found constitutively that levels of p35 were elevated in explanted scleroderma fibroblasts (n=6). p35 was also elevated in lesional skin from mice with bleomycin-induced scleroderma. In vitro, both p35 expression and CDK5 activity were strongly stimulated by TGF-β in human and mouse skin fibroblasts, mesenchymal progenitor cells and mature adipocytes. Ectopic p35 and CDK5 caused simultaneous suppression of adiponectin expression and stimulation of collagen synthesis in these cells, whereas RNAi-mediated knockdown of p35/CDK5 abrogated TGF-β-induced fibrotic gene expression. Pharmacological inhibitors of CDK5 prevented and reversed TGF-β-induced stimulation of collagen synthesis and myofibroblast differentiation, and ameliorated collagen overproduction in scleroderma fibroblasts in vitro, and prevented and reversed skin fibrosis in complementary inflammatory and TGF-ß-driven models of scleroderma in the mouse in vivo.

Conclusions: The CDK5/p35 axis has an important novel non-neuronal function in modulating fibrotic responses. Elevated p35 expression and CDK5 activity is an unexpected feature of scleroderma that might contribute to development of fibrosis. Pharmacological targeting CDK5/p35 might be novel treatment for fibrosis.

73. The Relationship Between Nailfold Capillaroscopic Assessment and Telangiectasia Score with Severity of Peripheral Vascular Involvement in Systemic Sclerosis

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Objectives: To determine the association of nailfold videocapillaroscopy(NVC) findings and telangiectasia score with DU history and severity of peripheral vascular involvement(PVI) in Systemic Sclerosis(SSc).

Methods: Fifty-nine SSc patients fulfilling Leroy and Medsger criteria were evaluated including Telangiectasia Score(TS)[1], Modifiye Rodnan Skin Score(MRSS), Valentine Activity Scale(VAS) and Medsger Severity Scale(SS). NVC was performed according to Sulli et al.[2], qualitative(early,active and late patterns) and semiquantitative assessments [capillary number(CN), irregularly enlarged capillaries(IEC), giant capillaries(GC), capillary ramifications(CR), microhaemorrhages(H),capillary array disorganisation(CAD) and microangiopathy evolution score(MES)].

Results: The mean age of patients was 45.6 years and 92% were females.The mean duration of Raynaud's(RS), non-Raynaud symptoms(NRS), skin involvement(SI)(year) were 6.1±6.5, 3.1±2.0, 3.0±2.0 years respectively. Of the patients 20(34%) were diffuse cutaneous involvement, 35(59%) were limited and 4(7%) were sine-scleroderma;13(22%) were anti-centromere(+) and 29(49%) were anti-Scl70(+). DU history (DU+) was present in 27(46%) and telangiectases were present in 34(58%).
NVC patterns of different groups were shown in Table-1. When we compare DU- and DU + groups, the mean score of CN was 1.4±0.7 vs 2.0±0.5*($p<0.001$), IEC was 1.4±0.7 vs 1.8±0.6**($p<0.05$), MES was 1.8±1.0 vs 2.5±1.5**($p<0.05$).
Current PVI grouped as Non-Severe(SS;0-1)(n=43) or Severe(SS;2-4)(n=16). The frequency of severe PVI was 22% in females(12/54) and 80% in males(4/5). The mean values of TS, MRSS, VAS, SS were similar between groups. When we compare Non-Severe and Severe groups, the mean score of CN was 1.5±0.7 vs 2.1±0.4*($p<0.001$), MES was 1.8±1.1 vs 2.8±1.6** ($p<0.05$) .

Conclusions: DU history and severe PVI in SSc was associated with capillary loss and microangiopathy. 'Early' NVC pattern was infrequent in patients with DU history and was not found in severe PVI. Severe PVI in males was more frequent than females. Telangiectasia scores were not found to be related to the severity of PVI. NVC may be a helpful method in the assessment of SSc patients for prognosis of PVI, warranting prospective studies.

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Table-1: DU History, Current Severity of Peripheral Vascular Involvement and NVC Pattern

		NVC Pattern			
		Early	**Active**	**Late**	**Total(n)**
DU history -		9	16	7	32
DU history +		1	14	12	27
PVI	**Non-Severe**	10	21	12	43
	Severe	0	9	7	16

74. Aberrant circulating platelets in patients with systemic sclerosis: roles in fibrosis

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Background: Systemic sclerosis (SSc) is a systemic fibroproliferative disease characterized by concomitant occurrence of microvascular injury and autoantibody production. It has long been suggested that many processes are shared by physiological wound healing and pathological fibrosis. During wound healing process, platelets are the first cells that home to the site of injury, and contribute to hemostasis and initiation of the wound healing process through release of a variety of growth factors, chemokines, cytokines, and inflammatory mediators. Circulating platelets in patients with SSc are known to have activated phenotype, but there are few data regarding their functional properties. In this study, we aimed to evaluate if SSc platelets represent aberrant function and are involved in the fibrotic process.

Methods: We used peripheral blood samples from 49 patients with SSc and 23 age- and sex-matched healthy controls. Genes up-regulated in SSc platelets were screened with RT^2 profiler™ PCR array (168 genes), and were subsequently verified by semi-quantitative and TaqMan® quantitative PCR. The protein expression levels of candidate genes were examined by immunoblots using platelet lysates combined with densitometry. We further evaluated effects of SSc platelets or factors up-regulated in SSc platelets on production of fibronectin in human dermal fibroblast cultures.

Results: By stepwise screening strategies based on comparisons of gene/protein expression profiles between SSc and control platelets, CXCL5 was identified as a platelet-derived factor up-regulated in SSc patients. Co-culture of fibroblasts with SSc platelets, but not with control platelets, enhanced fibronectin production, which was partially abolished by a CXCL5 receptor antagonist. In addition, recombinant CXCL5 promoted fibronectin production in fibroblast cultures.

Conclusions: These results suggest that circulating platelets in SSc patients are phenotypically and functionally altered and are involved in the fibrotic process through up-regulation of CXCL5.