17th International Workshop on Scleroderma Research

July 30-August 3, 2022

Seaport Hotel, Seaport District,
Boston, Massachusetts

www.SScWorkshop.org

Advancing Biomedical
Science Research
and Translation Medicine

Under the aegis of:

International Workshop on Scleroderma Research Corporation

University College London

With participation from:

University of Pittsburgh

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Workshop Information

Conference Office:

Saturday, July 30
1:00 pm to 4:00 pm
Seaport Hotel, Liberty A + B (Ground Level)

Sunday July 31 – Wednesday August 3
8:00 am to 5:00 pm
Seaport Hotel, Plaza Ballroom Foyer

Scientific Sessions and Dinner Meals:

Plaza Ballroom A + B
Breaks and daytime meals: Plaza Ballroom C

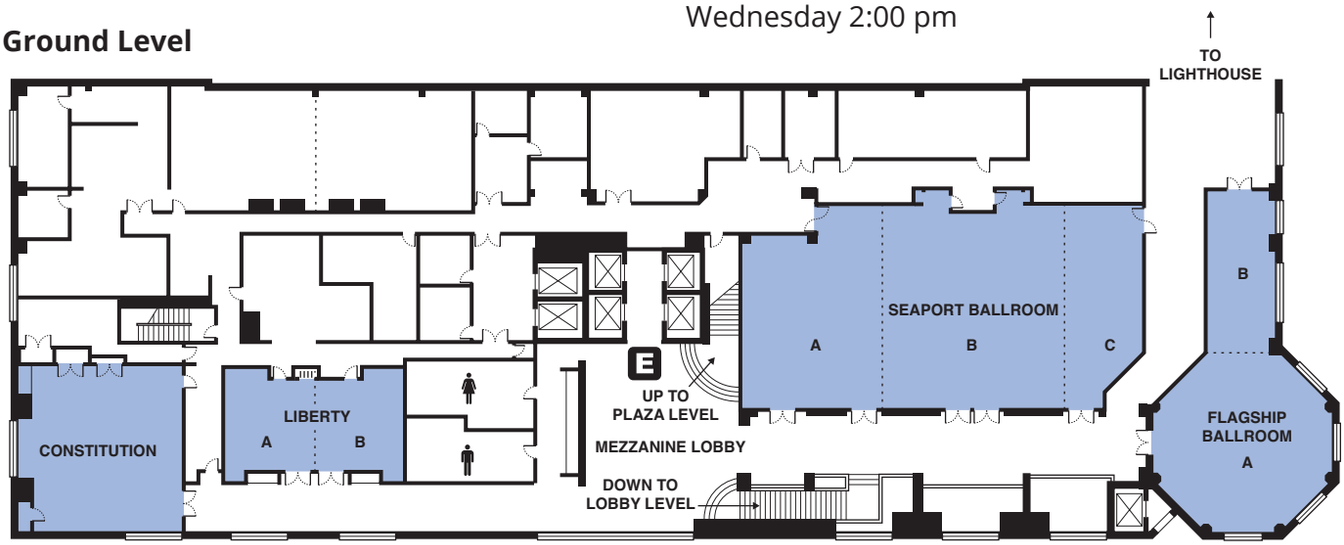
Welcome Reception:

Plaza Terrace Garden

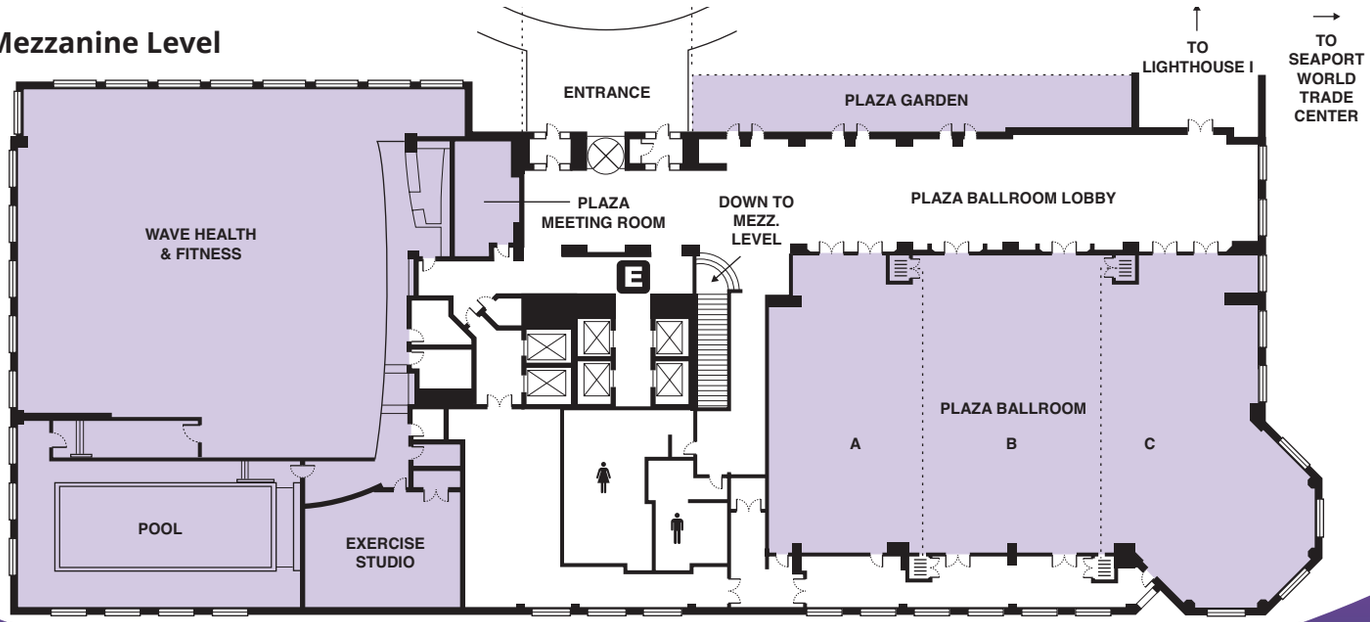
Posters:

Plaza Ballroom Foyer; Sunday AM to Wednesday 2:00 pm

Ground Level



Mezzanine Level



Acknowledgments

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Scientific Program

SATURDAY, JULY 30

1-4pm	Registration	Seaport Hotel – Liberty A + B

SUNDAY, JULY 31

8am-5pm	Registration	Plaza Ballroom Foyer
7:30-8:45	Breakfast	Plaza C
ALL SESSIONS IN PLAZA BALLROOM A + B		
8:30am	Welcome and Introduction	Carol Black & Robert Lafyatis
SESSION 1 **Special Topics – Conference Keynotes** Chairs – Christopher Denton, Joerg Distler		
8:40	Introduction	
8:45	Molecular MRI imaging in fibrosis	Peter Caravan
9:15	How studies in an ultra-rare genetic disorder led to a radical rethinking of Activin A- and BMP-mediated signaling	Aris Economides
9:45	Discussion	
10:15	COFFEE BREAK	Plaza C
SESSION 2 **New insights into SSc-ILD** Chairs – Richard Silver, Eleanor Valenzi		
10:55	Introduction	
11:00	Single cell insights into mesenchymal cell phenotype in IPF	Jonathan Kropski
11:30	Biomarkers in interstitial lung disease	Shervin Assassi
12:00	Discussion	
12:15	LUNCH	Plaza C
SESSION 3 **Altered Immunity in Fibrotic Disease** Chairs – Thomas Krieg, Valerie Horsley		
1:25	Introduction	
1:30	CD4+ T cells in lung fibrosis	Wonder Drake
2:00	Microbial metabolites and regulation of tissue macrophage function	Tracy McGaha
2:30	Discussion	
3:00	AFTERNOON TEA	Plaza C

SESSION 4 **Macrophages in Tissue Repair and Fibrosis** Chairs – Armando Gabrielli, Richard Stratton		
3:25	Introduction	
3:30	Macrophages in fibrosis	Massimo Locati
4:00	Macrophages and myofibroblast proliferation and heterogeneity during skin repair	Valerie Horsley
4:30	Discussion	
5:00	WELCOME RECEPTION	Plaza Garden Terrace
7:00	DINNER BANQUET – Plaza A + B	Joseph Zibrak, Beth Israel Deaconess Hospital, Boston

MONDAY, AUGUST 1

6:55-7:55am	Breakfast	Plaza C
SESSION 5 **Molecular Insights into Mesenchymal Cell Biology** Chairs – Maria Trojanowska, Carol Feghali-Bostwick		
7:55am	Introduction	
8:00	Transcriptional regulation of chromatin by SMAD proteins	Caroline Hill
8:30	c-JUN, CD47 and eat me signals for cell phagocytosis	Gerlinde Wernig
9:00	Discussion	
SESSION 6 **Pulmonary Hypertension: Metabolism and Therapeutics** Chairs – Paul Hassoun, Carol Black		
9:10	Introduction	
9:15	Altered metabolism in pulmonary arterial hypertension	Marlene Rabinovitch
9:45	New therapeutics for pulmonary hypertension	Vinicio de Jesus Perez
10:15	Discussion	
10:30	COFFEE BREAK	Plaza C
SESSION 7 **Genetics Insights Into Disease Pathogenesis** Chairs – Javier Martin, Maureen Mayes		
10:55	Introduction	
11:00	Functional genomics	Patrick Gaffney
11:30	GRASP genetics	Pravitt Gourh
12:00	Discussion	
12:15	LUNCH	Plaza C

SESSION 8 **Understanding the Clinical Targets in SSc - Scleroderma Clinical Trials Consortium** Chairs – Robyn Domsic, Tracy Frech		
1:15	Introduction	Tracy Frech
1:25	Assessing vascular involvement in pre-SS	Marco Matucci Cerinic
1:35	OMERACT update/overview	Susanna Proudman/ Peter Merkel
1:50	RP Patient Reported Outcomes	John Pauling
2:10	Digital Ulcer PROs	Michael Hughes
2:30	Vascular imaging techniques: Where are we with respect to clinical trials?	Graham Dinsdale
3:20	Discussion	
3:30	AFTERNOON TEA	Plaza C
SESSION 9 **Fibrosis in Other Organs** Chairs – Daniel Tschumperlin, Andreea Bujor		
3:55	Introduction	
4:00	Targeting liver fibrosis	Tatiana Kisseleva
4:30	The origin and role of cardiac myofibroblasts in fibrotic heart disease	Jeffery Molkentin
5:00	Discussion	

TUESDAY, AUGUST 2

7:30- 8:30am	Breakfast	Plaza C
SESSION 10 **New Therapeutic Approaches to SSc** Chairs – Alan Tyndall, Dinesh Khanna		
8:40am	Introduction	
8:45	Keynote - T cell targeting of myofibroblasts	Jonathan Epstein
9:15	pDC in SLE	Natalie Franchimont
9:45	Modeling novel therapeutics for SSc	Jörg Distler
10:15	Discussion	
10:30	COFFEE BREAK	Plaza C

SESSION 11 **Bioinformatics and Systems Biology** Chairs – Michael Whitfield, Robert Lafyatis		
10:55	Introduction	
11:00	Using machine learning and immunogenomic approaches to uncover biomarkers and causal latent factors of SSc severity	Jishnu Das
11:30	Bioinformatic approaches in rheumatic disease	Soumya Raychaudhuri
12:00	Discussion	
12:15	LUNCH	Plaza C

1:20PM ~ Special Lecture ~ Alan Tyndall, MB, BS, FRACP, FRCP (Ed), Professor (Emeritus), University of Basel, "Belief versus Data".

SESSION 12 **Selected Abstract Presentation** Chairs – Luc Mouthon, Thomas Medsger		
1:40	Introduction	Luc Mouthon
1:45	Erg deficiency in lymphatic endothelial cells drives pulmonary fibrosis and lymphatic dysfunction	A. Chakraborty
2:00	Epiregulin is a dendritic cell-derived EGFR ligand that maintains scleroderma skin and lung fibrosis	Ian Odell
2:15	Single-cell transcriptomic and accessible chromatin profiling of human skin endothelial cells in systemic sclerosis	Menqi Huang
2:30	Single Cell RNA-seq of scleroderma associated interstitial lung disease lung explants reveals multiple altered lymphoid subsets and population shifts	Cristina Padilla
2:45	Single-cell RNA sequencing of explanted skin from patients with systemic sclerosis unveils potent anti-inflammatory and antifibrotic effects of dimethyl-alpha-ketoglutarate	Blaz Burja
3:00	Usual interstitial pneumonia is the predominant histopathology in patients with advanced systemic sclerosis receiving a lung transplant	Eleanor Valenzi
3:15	AFTERNOON TEA	Plaza C

3 – 5pm ~ Poster Viewing ~ Plaza Ballroom Foyer

WEDNESDAY, AUGUST 3

7-8am	Breakfast	Plaza C
SESSION 13 **Translational Science in PAH** Chairs – Aris Economides, Vinicio de Jesus Perez		
8:10am	Introduction	
8:15	Pathogenesis of PAH and SSc-PAH	Paul Hassoun
8:45	Bone morphogenetic proteins in pulmonary arterial hypertension	Paul Yu
9:15	Discussion	
SESSION 14 **Applying Single Cell Technologies to Rheumatic Disease** Chairs – David Abraham, Pravitt Gourh		
9:25	Introduction	
9:30	Single cell analyses and insights in lupus nephritis	Anne Davidson
10:00	Single cell insights into Notch signaling in rheumatoid synovial fibroblast differentiation	Michael Brenner
10:30	Discussion	
10:45	COFFEE BREAK	Plaza C
SESSION 15 **Aging Research and Its Impact on Fibrosis** Chairs – John Varga, Mauricio Rojas		
11:10	Introduction	
11:15	Aging, senolytics, and mtDNA	James Kirkland
11:45	Mitochondrial dysfunction, senescence and fibrosis	Ana Mora
12:15	Discussion	
12:30	LUNCH	Plaza C
SESSION 16 **Clinical-Translational SSc** Chairs –Peter Merkel, Virginia Steen		
1:25	Introduction	
1:30	Update on combined response indices – CRISS to CRISTAL	Dinesh Khanna
2:00	Lessons from pre-scleroderma – translational studies from VEDOSS	Marco Matucci Cerinic
2:30	Reverse translation and molecular subsets defined by ANA	Chris Denton
3:00	Discussion	
END OF WORKSHOP		

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Abstracts

Scleroderma Clinical Trials Consortium Awards for Young Investigators

1. Erg deficiency in lymphatic endothelial cells drives pulmonary fibrosis and lymphatic dysfunction.

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Background:

Pulmonary lymphatics play a crucial role in lung fluid homeostasis, immune cell clearance, and tissue regeneration. Lymphatic remodeling and rarefaction have been linked with both protection as well as the pathogenesis of lung-associated injury and fibrosis, though the underlying signaling mechanism regulating these processes remains unknown. Preliminary studies from our group demonstrates that a member of the ETS transcription factor family, ERG, regulates key genes involved in lymphatic specification in lung lymphatic endothelial cells (LECs). Our central goal, therefore, is to elucidate the molecular and cellular mechanisms driving lymphatic function in lymphatic ERG deficient lungs, specifically focusing on the mechanisms driving pulmonary fibrosis.

Materials and Methods:

Mice (Prox1CreERT2 /Erg$^{fl/fl}$) with conditional knockout of Erg in the lymphatic endothelial cells (LECs) were used to study lymphatic remodeling in lung. Human lung LECs with siERG knockouts in-vitro were used to study cell specific mechanisms.

Results:

ERG silencing in human LECs reduced the expression of lymphatic identity genes (FLT4, PROX1, MMP1, and LYVE1). Lymphatic-specific knockout of Erg (Prox1CreERT2/Ergfl/fl) resulted in lymphatic vessel remodeling and upregulation of key pro-fibrotic and pro-inflammatory genes in mice (CXCL3, TNF-α, IL1B, PAI-1, MMP-12, IFN-γR1, IFN-γR2). An increase in resident parenchymal alveolar macrophages, Ly6C+ monocytes, CD103-cDC1, and eosinophils suggested tissue inflammation. Consistent with a pro-inflammatory phenotype the levels of Tgfß1 mature dimer were decreased in the Prox-1 Ergfl/fl mice lungs. Interestingly, in comparison to the controls, profibrotic stimulation of the Prox-1 Ergfl/fl mice with bleomycin resulted in complete ablation of lymphatic vessels. Both parenchyma and pleural area of the Prox-1 Ergfl/fl mice lung displayed a higher degree of fibrosis. In-vitro, treatment of fibroblasts with siERG LEC condition media increased expression of key fibroblast activation genes (Col3A, FAP) in fibroblast, suggesting that a LEC-mediated fibroblast activation might contribute to lung pathogenesis.

Conclusion:

Overall, our results show that reduced ERG expression in lymphatic endothelial cells enhances profibrotic and pro-inflammatory phenotype in lungs.

2. Epiregulin is a Dendritic Cell-Derived EGFR Ligand that Maintains Scleroderma Skin and Lung Fibrosis

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Background:

Immune cells are fundamental regulators of extracellular matrix (ECM) production by fibroblasts and have important roles in determining extent of fibrosis in response to inflammation. Although much is known about fibroblast signaling in fibrosis, the molecular signals between immune cells and

fibroblasts that drive its persistence are poorly understood. In systemic sclerosis (SSc/scleroderma) skin, EGFR ligand expression correlates with skin fibrosis severity. However, EGFR inhibition showed inconsistent results in mouse models of fibrosis. We hypothesized that an immune-mesenchymal signaling circuit underlies SSc-related fibrosis, and that targeting a specific EGFR ligand may prevent activation of pathologic fibroblasts.

Materials and Methods:
We performed scRNA-Seq on skin biopsies from patients with diffuse cutaneous SSc and healthy controls, which we compared to SSc and IPF lung fibrosis datasets. We identified enriched signaling pathways through gene ontology and receptor-ligand enrichment. Using SSc patient skin explants, we tested whether inhibition of a specific EGFR ligand could alleviate disease. Mechanistic studies were carried out in primary monocytes and dermal fibroblasts.

Results:
We identify epiregulin – EGFR signaling between dendritic cells and fibroblasts to maintain elevated ECM production and accumulation in fibrotic tissue. Mechanistic studies demonstrate that epiregulin expression marks an inducible state of the DC3 subset of dendritic cells triggered by type I interferon. DC3-derived epiregulin activates EGFR on fibroblasts, which drives a positive feedback loop through NOTCH signaling. Epiregulin was essential for persistence of fibrosis in both skin and lung, which could be abrogated by a neutralizing antibody. Notably, therapeutic administration of epiregulin antibody reversed fibrosis in patient skin and lung explants, identifying it as a novel biologic drug target.

Conclusions:
Our findings reveal epiregulin as a crucial immune signal that maintains skin and lung fibrosis in multiple diseases and is a highly promising antifibrotic target.

3. Single-cell transcriptomic and accessible chromatin profiling of human skin endothelial cells in systemic sclerosis

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Background:
Typically, vasculopathy is the earliest clinical manifestation in patients with systemic sclerosis (SSc). It is often considered to be associated with endothelial cell (EC) injury. Thus, elucidating transcriptomes and epigenomes of ECs is critical to better understand the mechanisms underlying SSc-associated vasculopathy.

Materials and Methods:
Transcriptomic and epigenetic alterations of EC populations in SSc skin were assessed by single-cell RNA sequencing (scRNA-seq; SSc n=27 and Control n=10) and single-nucleus transposase-accessible chromatin sequencing (snATAC-seq; SSc=8 and Control n=6), respectively. Immunofluorescent staining in skin and proteomics assay of patients' sera were used to confirm the altered EC phenotypes in SSc.

Results:
Transcriptomic signatures of classical vascular linages (arterial, capillary and venous ECs) and lymphatic ECs were shared by both control and SSc ECs. Comparison with control ECs, arterial ECs

in SSc skin were decreased in number and showed increased expression of genes associated with apoptosis. Two small EC subpopulations were found markedly upregulated in SSc: One subset distinctively expressed *LY6H* and *PRND* with other reported markers of tip ECs (*PGF*, *CXCR4*), and the other subset enriched with proliferative markers (*MKI67*, *TOP2A*). Interestingly, some genes that specifically expressed by tip ECs (eg. *PRND* and *PXDN*) were found among the most significantly upregulated genes in SSc skins of GENISOS and PRESS, two large cohort datasets using RNA sequencing to compare SSc skins with normal skins. Pathway analysis implied enhanced proangiogenic and proliferative activities in these two aberrant SSc-EC populations, respectively. Also, ligand-receptor analysis demonstrated altered intercellular networks of SSc EC subpopulations with neighboring cells, including perivascular cells and circulating immunocytes. Furthermore, integration of open chromatin profiles with transcriptomic analysis revealed enrichment of DNA binding sites for ETS transcription factors in both tip and SSc arterial ECs, suggesting ETS superfamily may play a critical role in driving the SSc-associated EC phenotypes.

Conclusions:
These data provide new insights into both transcriptional and chromatin alterations in driving endovascular injury and abnormal proliferation in SSc and suggest previously unrecognized EC targets for ameliorating vascular disease in SSc.

4. Single Cell RNA-seq of scleroderma associated interstitial lung disease lung explants reveals multiple altered lymphoid subsets and population shifts

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Background:
Of the serious complications that can arise, SSc associated interstitial lung disease (SSc-ILD) is the leading cause of death. Although SSc is recognized as a T cell mediated disease, the specific T cell and lymphoid subsets in SSc-ILD are not well understood. Utilizing single cell RNA-sequencing, we describe the lymphoid populations in SSc-ILD.

Materials & Methods:
Lung tissues from 13 SSc-ILD and six healthy control (HC) lungs collected were processed and analyzed by 3' 10X single cell RNA chemistry. Samples were normalized and batch corrected before unsupervised UMAP clustering and subclustered further into specific lymphoid subpopulations. Differentially expressed genes were derived to identify lymphoid subclusters. Extensive literature review, pathway analysis, and Gene Ontology were used to identify these lymphoid subpopulations. Connectome analysis visualized ligand-receptor interactions among the lymphoid subpopulations and other cell types in lung tissue.

Results:
We identified 16 lymphocyte clusters in both SSc-ILD and HC, including three populations of natural killer (NK) cells, several populations of CD8+ and CD4+ T cells, a proliferating population of lymphoid cells, and a cluster of interferon activated T cells. We observed striking shifts in lymphoid cell populations in SSc-ILD lungs. SSc-ILD lungs showed a shift in NK cells toward an activated NK cell phenotype with strongly upregulation of interferon-gamma (IFN-γ) and its associated genes. We also observed a marked increase in CD8+ tissue resident memory (TRM) cells and regulatory T cells with fewer naïve CD8+ T cells, and Th17 cells compared to HC lungs. Following connectome analysis, activated CD16+ NK cells appeared to receive signal from naïve CD8+ T cells,

proliferating NK & T cells, and regulatory T cells. CD8+ TRM appear to receive input from tissue resident NK via TGF-β, and CCL5, Th17 via CCL20, and naïve CD8+ T cells via CCL5.

Conclusion:
We described notable lymphoid cell population shifts in SSc-ILD lungs, most notably in NK and CD8+ tissue resident T cell populations. NK cells appear to be shifting toward an activated phenotype via IFN-γ signaling pathway. Several ligands associated with pro-fibrotic pathways appear to influence activated CD16+ NK cells in the SSc-ILD group.

5. Single-cell RNA sequencing of explanted skin from patients with systemic sclerosis unveils potent anti-inflammatory and antifibrotic effects of dimethyl-alpha-ketoglutarate

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Background: We have recently identified dimethyl alpha-ketoglutarate (dm-aKG) as a potential repressor of myofibroblast activation in cultured skin fibroblasts. We aimed to further analyse the clinical translation of our findings by investigating the antifibrotic capacity of dm-aKG on explanted skin biopsies from SSc patients.

Methods: Forearm skin biopsies from SSc patients (n=10) were cultured for 24h *ex vivo* ± 6 mM dm-aKG. Thereafter, skin biopsies (n=4) were dissociated using a combined mechanical-enzymatic dissociation protocol, followed by scRNA-sequencing. scRNA-seq reads were mapped to the reference genome GRCh38.p13 and the data was analysed with R/Bioconductor tools. The secretion of IL-6, procollagen-1, and C1M, from cultured skin (n=10) was measured in supernatants by ELISA. We analysed gene and protein expression in TGFβ-activated dermal fibroblasts (DF, n=10) ± dm-aKG using qPCR, Western blot and ELISA. Contractile properties of DF were assessed by gel contraction assay. Traction forces generated by DF were determined by reference-free traction force microscopy.

Results: scRNA-seq skin analysis from dissociated cultured SSc skin included 20,869 high quality single cell profiles segregating into 10 distinct skin cell populations (Fig. 1A). This analysis demonstrated decreased proportion of fibroblasts and increased proportion of keratinocytes in dm-aKG treated skin ($p<0.05$; Fig. 1B). Among skin cell types, skin fibroblasts exhibited the largest amount of differentially expressed genes upon dm-aKG treatment (44%, n=779, x-fold>0.5, FDR<0.05), suggesting that these cells are key targets of dm-aKG therapy in SSc skin. We identified inflammatory/cytokine signalling (hub genes *IL6, STAT1*) and extracellular matrix (ECM) organization (hub genes *MMP1, ITGB3*) as top downregulated biological processes in fibroblasts in dm-aKG treated SSc skin (Fig. 1C). Furthermore, dm-aKG reduced the secretion of IL-6, procollagen-1 and

C1M from cultured skin explants. In cultured DF, dm-aKG blocked the inflammatory (IL-6, pSTAT3), profibrotic (aSMA, Fibronectin, Procollagen-1, Pro-C1) and contractile activities, and significantly diminished traction forces exerted by DF on the matrix substrate.

Conclusion: Dm-aKG broadly interferes with inflammatory and ECM organizational activities of skin fibroblasts in culture and in explanted skin from SSc patients. These results confirm that dm-aKG might represent a potential new therapeutic approach for efficient targeting of skin inflammation and fibrosis in SSc.

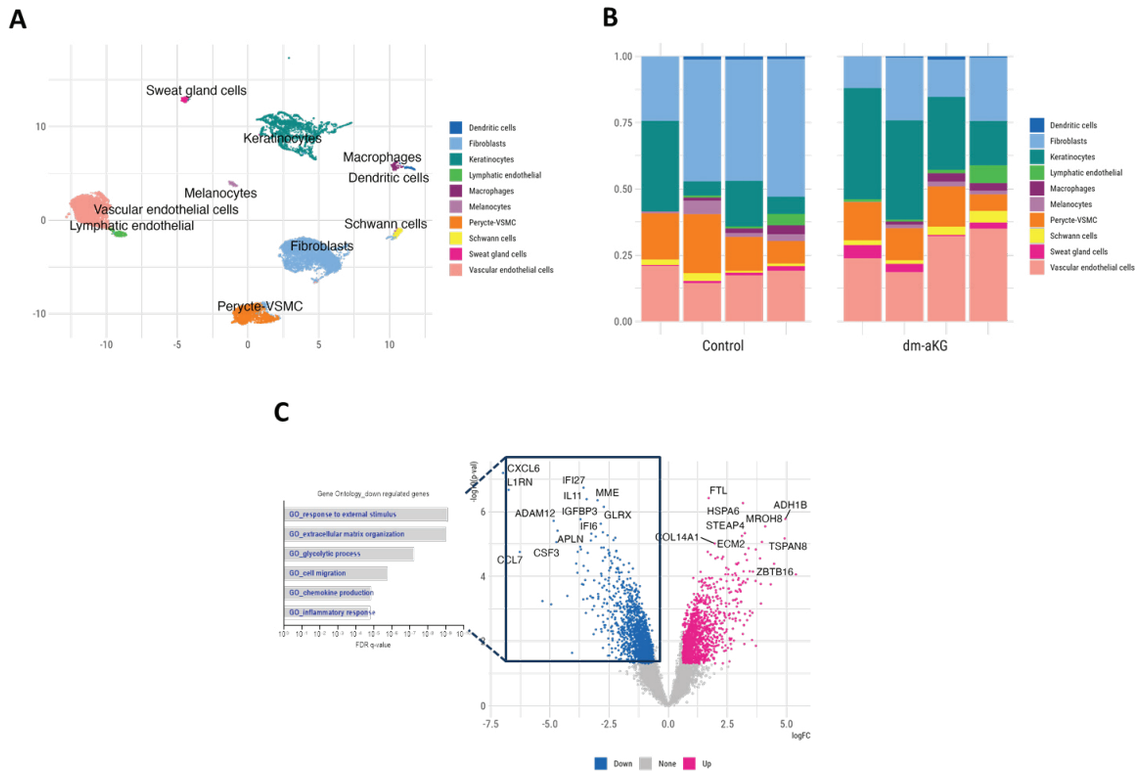

Figure 1 scRNA-seq – comparison of untreated and dm-aKG treated paired skin biopsies. (A) UMAP plot with annotated skin cells, (B) differential abundance of main skin cell types, (C) volcano plot of DE genes with top downregulated gene ontology (GO) pathways in dm-aKG treated skin fibroblasts

6. Usual Interstitial Pneumonia is the Predominant Histopathology in Patients with Advanced Systemic Sclerosis Receiving a Lung Transplant

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Background: Previous studies identifying nonspecific interstitial pneumonia (NSIP) as the predominant histopathology in SSc-ILD have primarily utilized surgical lung biopsies in early disease. These case series may only reflect the histopathology of early disease, and differ from the histopathology of advanced disease in patients with SSc complicated by respiratory failure.

Materials and methods: Patients receiving a lung transplant for a diagnosis of SSc at the University of Pittsburgh Medical Center from 2000-2021 were included (n=127) for retrospective analysis. All explanted lungs underwent histopathology review as part of routine clinical care. Histopathology

reports, demographic and clinical data were reviewed for all patients as available in the medical record.

Results: 127 patients with a diagnosis of SSc received a lung transplant during the study period, with 73 female (57.5%) and 54 male (42.5%) patients included. Usual interstitial pneumonia (UIP) was identified in 111 explants (87.4%), NSIP in 45 (35.4%) explants, organizing pneumonia in 11 explants (8.7%), and lymphocytic bronchitis in 2 explants (1.6%). Areas of both UIP and NSIP were identified in 37 explants (29.1%). Diffuse alveolar damage consistent with acute lung injury was identified in 18 (14.2%) explants. 49 (38.6%) explants had strong evidence of aspiration including food particles, cholesterol clefts, and/or airway centric giant cells and granulomas. 19 patients had pathology results available from a prior surgical lung biopsy, with a median of 4 years (range 0.25-16) between the time of biopsy and transplant. 11 patients maintained the same primary pathology on biopsy and explant (2 NSIP, 9 UIP), with 8 patients showing different pathology at the timepoints, all of whom had UIP on explant. 101 (79.5%) patients had evidence of pulmonary hypertension/vasculopathy on explant, and 116 (94.3%) of 123 patients with available data had pulmonary hypertension as defined by a mean pulmonary artery pressure $\geq$ 20mmHg or requiring pulmonary vasodilators (not prescribed for Raynauds).

Conclusions: UIP is the predominant histopathology in patients with SSc receiving a lung transplant, with many patients concurrently having both NSIP and UIP or showing a progression from NSIP to UIP over separate time points. As the largest series of SSc pulmonary explant pathology, our study confirms previous hypotheses that NSIP progresses to UIP in many patients with advanced ILD and highlights the need to include tissues from SSc patients with UIP in translational research moving forward.

Additional Abstracts Listed Alphabetically by First Author

7. Identifying a novel plasma biomarker panel for systemic sclerosis associated pulmonary arterial hypertension (SSc-PAH)

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Background: Pulmonary complications are the leading cause of mortality among Scleroderma (SSc) patients. Individuals suffering from pulmonary arterial hypertension (SSc-PAH) and pulmonary fibrosis (SSc-PF) have decreased survival rates compared to SSc patients without lung disease (SSc-NLD). Consequently, there is a need for identification of diagnostic/prognostic biomarkers and novel therapeutic targets to improve patient stratification and treatment. This study aimed to identify a panel of plasma biomarkers that can distinguish SSc patients with pulmonary complications (SSc-PAH and SSc-PF).

Materials and Methods: An enriched cohort of SSc patients with pulmonary complications (SSc-PAH, SSc-PF) or without (SSc-NLD) and a cohort of healthy controls were used in this study (n=30 per group). The inclusion criteria were SSc-PAH (mPAP$\geq$25mmHg, PAWP $\leq$15mmHg, PVR>3 wood units and no major PF; n=30), SSc-PF (> 30% disease extent on HRCT and FVC<70%; n=30). EDTA plasma and Peripheral blood mononuclear cells (PBMCs) were obtained from subjects. 184 proteins were measured using the Olink platform. Plasma protein levels were validated by ELISA in healthy controls (n=20) and patients (SSc-PAH, SSc-PF, SSc-NLD; n=20 per group). Lung tissue from healthy controls and SSc-PAH patients were stained using immunohistochemistry. Welch's 2-tailed unpaired t-tests, One-way ANOVA and the MedCalc software were used for analysis. Predictive power of the panel was tested on a validation cohort.

Results: 13 proteins were differentially expressed in SSc-PAH compared to SSc-NLD and SSc-PF ($p<0.05$). ROC analysis revealed an optimal predictive biomarker panel including RAGE, TNFRSF4, LAMP3, CXCL9, PRSS8, SOD2 and MCP-3. The panel significantly differentiated ($p<0.0001$) SSc-PAH cases from SSc-PF (AUC=1) and SSc-NLD (AUC=0.93) and distinguished all SSc cases from healthy controls (AUC=0.923, $p<0.0001$). RAGE was the most effective at discriminating SSc-PAH from SSc-PF. Validation showed significantly higher levels of RAGE in SSc-PAH (median:2145 pg/ml) compared to SSc-PF and healthy controls (median:1272pg/ml and 944pg/ml, $p<0.01$, respectively). Immunostaining showed RAGE was highly expressed in the endothelium of pulmonary arterial vessels in healthy controls but was absent in the endothelium of SSc-PAH patients.
Conclusion: This novel seven composite protein biomarker panel can potentially assist in early detection of SSc-PAH in SSc patients. RAGE is a key distinguishing marker between SSc-PAH and SSc-PF.

8. Novel metabolic markers of scleroderma associated pulmonary complications

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Background: Pulmonary complications increases mortality among Scleroderma (SSc) patients. Patients with pulmonary arterial hypertension (SSc-PAH) and pulmonary fibrosis (SSc-PF) have drastically decreased survival rates compared to SSc patients with no pulmonary abnormalities (SSc-NLD). Risk stratification and treatment of SSc patients remain challenging. This study aimed to identify plasma metabolites that discriminate between these specific SSc associated pulmonary complications.

Materials and Methods: Plasma from consented healthy controls (n=30) and patients diagnosed with either SSc-NLD (n=30), SSc-PAH (n=30) or SSc-PF (n=30) was used for untargeted metabolomic profiling. Metabolite levels were compared pairwise between groups using an unpaired t-test and correlated with each other within the groups using Pearson's correlation test. Metabolites that had a p-value of <0.0001 were considered significant. A p-value of <0.05 was used to determine the correlation significance.

Results: A total of 1001 known metabolites were detected. In SSc-PAH, 37 metabolites were significantly different compared to HC and 19 significantly altered compared to SSc-NLD. In SSc-PF, 17 were significantly different compared to HC, with no significant differences compared to SSc-NLD. 12 metabolites were significantly different between SSc-PAH and SSc-PF. 23 metabolites were exclusively altered in SSc-PAH patients compared to the other groups. Among these, were dimethylarginine (SDMA + ADMA) a marker of protein degradation and N-acetylneuraminate, associated with chronic inflammation. Pathway analysis showed SSc-PAH specific metabolites were involved in pathways, such as carnitine synthesis and fatty acid (FA) metabolism, that are involved in the regulation of endothelial and mitochondrial function. Fewer metabolites (3) were exclusively altered in SSc-PF patients. In these patients S-adenosylhomocysteine (SAH) was specifically elevated.

Conclusion: In this study, within clinically characterised groups of SSc patients with specific pulmonary complications, it was shown that SSc-PAH patients exhibited greater metabolic disruption compared to those with SSc-PF. Also, in SSc-PAH metabolic markers of increased protein turnover

and those associated with changes in endothelial and mitochondrial functions predominate. Interestingly, in SSc-PF, the elevation of SAH, a homocysteine precursor, implicates endothelial dysfunction, perhaps as a modulator of inflammation and ER stress. Validation of this metabolic marker panel is likely to be of diagnostic, prognostic and therapeutic benefit.

9. Increased expression of the ectoenzyme CD38 in peripheral blood plasmablasts and plasma cells of patients with systemic sclerosis

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Background: CD38 is a type II glycoprotein highly expressed on plasmablasts and on short- and long-lived plasma cells, but weakly expressed by lymphoid, myeloid, and non-hematopoietic cells. CD38 is a target for therapies aimed at depleting antibody-producing plasma cells. Systemic sclerosis (SSc) is an immune-mediated disease with a well documented pathogenic role of B cells. We therefore analyzed CD38 expression in different subsets of peripheral blood mononuclear cells (PBMCs) from a cohort of SSc patients.

Materials and methods: Cell surface expression of CD38 was evaluated on PBMCs from SSc patients using eight-color flow cytometry analysis performed with a FacsCanto II (BD). Healthy individuals were used as controls (HC).

Results:
Forty-six SSc patients (mean age 60, range 23-79 years; 38 females and 8 males), and thirty-two age- and sex-matched HC were studied. Twenty-eight patients had the limited cutaneous form and eighteen the diffuse cutaneous form of SSc. The mean disease duration was 7 years. Fourteen patients were on immunosuppressive therapy (13 MMF, 5 RTX).
The total percentages of T, B and NK cells were not different between SSc and HC.
SSc patients had higher levels of CD3+CD38+ T cells ($p<0.05$), higher percentage ($p<0.001$) of CD3+CD4+CD25+FOXP3+ regulatory T cells, lower percentage of CD3+CD56+ NK cells than HC ($p<0.05$).
Moreover, SSc patients had higher levels of $CD24^{high}$CD19+CD38++ regulatory B cells than HC ($p<0.01$), while the number of CD24+CD19+CD38+CD27+ memory B cells was lower ($p<0.001$).
Finally, the percentages of circulating $CD38^{high}$ CD27+ plasmablasts and $CD38^{high}$ plasma cells were both higher in the SSc group than in HC ($p<0.001$).
We did not observe any correlations between these immunophenotypes and disease subsets, duration and ongoing immunosuppressive treatment.

Conclusions:
The increased expression of CD38 in peripheral blood plasmablasts and plasma cells of SSc patients may suggest this ectoenzyme as a candidate therapeutic target, under the hypothesis that depletion of these cells may beneficially downregulate the chronic immune response in SSc patients. Validation of this data in multicenter cohorts shall be obtained prior to clinical trials with existing anti-CD38 drugs.

10. CXCL4, a chemokine upregulated in systemic sclerosis patients, abrogates TLR9 signaling and central tolerance in B cells.

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[16]These authors contributed equally: Marie Dominique Ah Kioon, Elif Çakan.

[17]These authors jointly supervised this work: Eric Meffre, Franck Barrat.

Background:

Systemic sclerosis (SSc) is an autoimmune connective tissue disease, characterized by defective central B cell tolerance. Deficiency of *MYD88*, a molecule that mediates the function of TLRs, results in a failure to silence autoreactive B cells. This suggests a role of TLRs in B cell tolerance. CXCL4, a chemokine found elevated in the sera of patients with SSc, potentiates IFN-α secretion from TLR9-induced plasmacytoid dendritic cells. The aim of this study is to investigate whether CXCL4 regulates TLR9 signaling and central tolerance in B cells.

Materials and methods:

B cells were isolated with CD19 magnetic beads from PBMCs of SSc patients and healthy donors and cultured either with medium or with TLR9 ligand (unconjugated or fluorescent) alone or with CXCL4. Pro-inflammatory cytokines gene expression and secretion were analyzed by PCR and ELISA. Delivery of fluorescent TLR9 ligand was analyzed by Amnis imagestream and confocal microscopy. To assess the effect of CXCL4 in vivo, NOD-scid-common gamma chain (γc) knockout immunodeficient mice were engrafted with $CD34^+$ human fetal hematopoietic stem cells transduced with GFP-tagged lentivirus expressing human CXCL4. B cells were purified from bone marrow and spleen from humanized mice by positive selection using CD19 magnetic beads and their new emigrant/transitional B cells were sorted. Immunoglobulins chains of these cells were cloned and transfected in fibroblasts. Polyreactivity of the antibodies released by the fibroblasts were assessed by ELISA.

Results:

Defective TLR9 signaling was observed in B cells of SSc patients. CXCL4 abrogated TLR9 response in human B cells by decreasing IL-6, TNF and IL-10 gene expression and secretion. CXCL4 increased TLR9 ligand (TLR9-L) uptake however the CXCL4 interfered with the normal trafficking of TLR9-L. Indeed, TLR9-L when given to B cells alone, was localized in lysosomes and $LAMP\text{-}2^+$ compartments

while CXCL4 prevented the delivery of TLR9-L to these vesicles. CXCL4 *in vivo* expression led to defective TLR9 responses and increased number of polyreactive B cells.

Conclusions:

Our data provide evidence for a novel mechanism by which CXCL4 impairs central B cell tolerance by altering the intracellular trafficking of TLR9 ligands, hence inhibiting TLR9 response which is required for the removal of developing autoreactive B cells.

11. Therapeutic Peptide Aur300 Targeting Cd206 Inhibits Macrophage-Dependent Inflammation, Fibrosis And Calcinosis In Scleroderma

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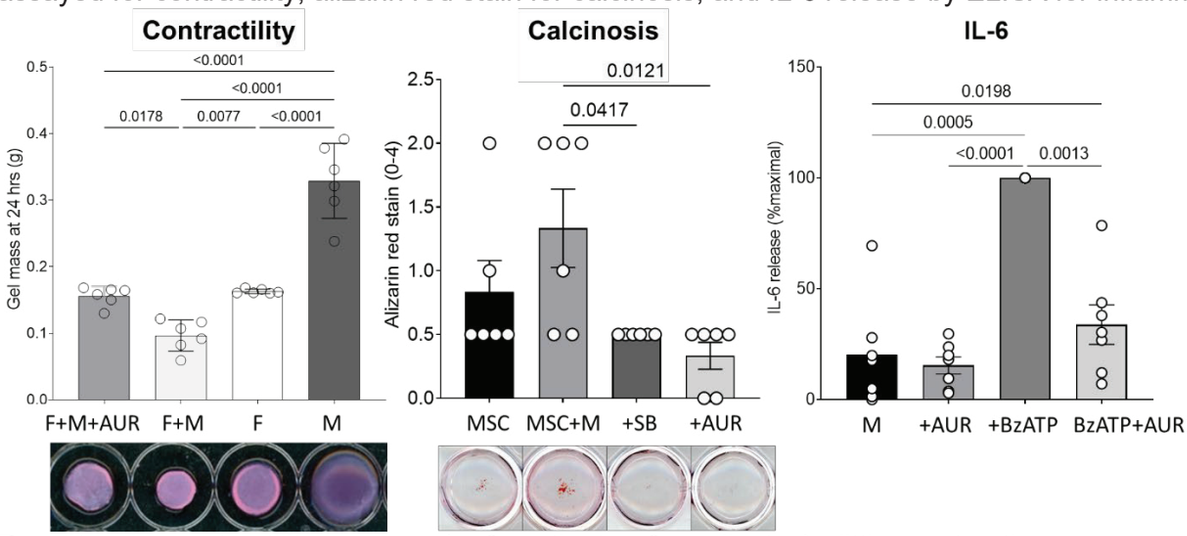
[1]UCL Centre for Rheumatology and Connective Tissue Diseases, [2]Aurinia Pharmaceuticals Inc, [3]Riptide Bioscience.

Background: Activated macrophages are believed to play a role in systemic sclerosis (SSc), where inflammatory and pro-fibrotic macrophage signatures are found in early progressive disease. Moreover, CD206-positive M2-like macrophages infiltrate around blood vessels in lesional skin, and soluble CD206 (sCD206) is elevated as a biomarker. AUR300, a therapeutic peptide, which binds macrophages via CD206 and reduces M2 dysregulation and inflammatory cytokine production, might effectively treat SSc patients with the active signature. We evaluated AUR300 in *in vitro* models of SSc macrophage-dependent inflammation, myofibroblast contractility, and calcinosis.

Materials and Methods: SSc macrophage cell lines were derived by culture of peripheral blood mononuclear cells with M-CSF(4ng/ml) for 7 days and subsequently co-cultured with SSc dermal fibroblasts in 3D collagen gels (myofibroblast model); or co-cultured in monolayer with fat derived mesenchymal stem cells (MSCs) (calcinosis model); or stimulated by 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate) (BzATP) 0.1 mM (inflammatory model). Gel weight was assayed for contractility, alizarin red stain for calcinosis, and IL-6 release by ELISA for inflammation.



Results: In contractility assays (n=3 patients, 6 replicates), little contraction was seen with macrophages alone (M), whereas SSc fibroblasts alone (F) induced moderate contraction, enhanced by co-culture with SSc macrophages (F+M) and blocked by addition of AUR300 10μM (AUR) (mean+/-SEM, M=0.329+/-0.023, F=0.163+/-0.001, M+F=0.024+/-0.010, M+F+RP=0.156+/-0.006, P<0.018, gel mass in grams). Moreover, the addition of SSc macrophages reprogrammed by 7-day culture in AUR300, effectively inhibited in this model system (not shown, P<0.02). In calcinosis assays, the addition of SSc macrophages (n=1 patient, 6 replicates) to the MSC cultures induced Alizarin red positive osteogenic foci at 21 days, blocked by AUR300 as well as by SB431542, an inhibitor of TGFβ signalling (mean+/-SEM, MSCs 0.833+/-0.247, MSCs+M 1.33+/-0.307, MSCs+M+SB 0.5+/-0, MSCs+M+AUR 0.33+/-0.105, P<0.012 for AUR300 effect). Moreover, AUR300 blocked the BzATP-dependent induction of IL-6 release by SSc macrophages (n=6 patients) (mean+/-SEM, basal M IL-6 20.2+/-9.11, +AUR 15.4+/-3.86, +BzATP 100+/-0, +BzATP+AUR 33.8+/-8.94, %maximal, P<0.0013 for effect of AUR300).

Conclusions: These data support the notion that multiple aspects of SSc could be attributable to activated macrophages. By targeting these cells, therapeutic peptide AUR300 effectively inhibits multiple disease-relevant pathways resistant to current standard DMARDs.

12. The Six-Minute Walk Test to identify systemic sclerosis patients with pulmonary hypertension.

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Background: Pulmonary arterial hypertension (PAH) in systemic sclerosis (SSc) is associated with significant morbidity and mortality and needs invasive techniques to be diagnosed. The Six-minute walk test (6-MWT) is a non-invasive exercise test to assess functional capacity. However, there is ongoing debate about the specificity of the 6-minute walking distance (6-MWD) for PAH as changes in 6-MWD can have other causes. This study aimed to identify which variable(s) of the 6-MWT is best in discriminating SSc-patients with PAH.

Methods: SSc-patients fulfilling ACR/EULAR 2013 and with PAH confirmed by right catheterization from the Leiden Combined Care in SSc (CCISS)-cohort were included. Here, 6MWT parameters and disease characteristics are annually obtained. Cases were matched for sex, age and disease duration (time since first non-Raynaud) with non-PAH SSc-controls in a 1:3 ratio. 6-MWT parameters were compared between cases and controls for the total group, and age and sex separately. Receiver operating characteristic (ROC) curves were plotted using the most discriminative values of 6-MWT for presence of PAH and mortality, taking into account age and sex as possible confounders. The DIBOSA-score was evaluated as well.

Results: We included 28 SSc PAH-patients with a 6-MWT within six months of PAH diagnosis, and were matched with 84 SSc non-PAH controls. In all patients, 6-MWD was significantly different between age groups (>65:428m, ≤65: 523m, p=0.002) and between sex (male: 531m, female: 473m, p=0.05) .

6-MWD, post-test saturation and Borg-score were significantly different between patients with and without PAH (p=0.006, p=0.002, p=0.046). Distance and post-test saturation combined yielded the most optimal AUC of 0.85 [95%CI:0.77-0.93], which slightly improved when stratifying patients for age (≤65 AUC: 0.86 [95%CI:0.76-0.97], >65 AUC: 0.87 [95%CI: 0.73-1.0]) . This combination also identified patients who died during follow-up (AUC 0.81 [95%CI:0.7-0.92]). In comparison, the DIBOSA-score yielded an AUC of 0.79 in this cohort [95%CI:0.67-0.90].

Conclusion: Our evaluation shows that 6-MWD combined with post-test saturation is most helpful in discriminating SSc-patients with PAH. Additionally, it is important to realize that both age and sex influence 6-MWD. Taken these considerations into account, 6-MWT is feasible to screen for presence of PAH in individual SSc-patients.

	No PAH ≤ 65	PAH ≤ 65	No PAH >65	PAH > 65	
Females N (%)	37 (84)	13 (81)	32 (80)	10 (83)	
Median age in years (IQR)	58 (54-62)	59 (54-63)	72 (68-75)	74 (70-76)	p=0.64
Median non-Raynaud duration in years (IQR)	7 (5-14.5)	7 (3-13.5)	4.5 (2.5-9)	5 (0.5-11)	p<0.001
Median BMI (IQR)	24.3 (21.6-27.9)	24.2 (21.3-27.1)	25.2 (21.7-28.5)	23.9 (21.8-24.7)	p=0.23
NcsSc N (%)	6 (13.6)	0	7 (17.5)	2 (16.7)	
LCssc N (%)	21 (47.7)	8 (50)	23 (57.5)	6 (50)	
Dcssc N (%)	17 (38.6)	8 (50)	10 (25)	4 (33.3)	
ACA N (%)	11 (25)	5 (31.3)	20 (50)	5 (41.7)	
ATA N (%)	17 (38.6)	3 (18.8)	6 (15)	1 (8.3)	
Presence of ILD N (%)	10 (22.7)	5 (31.3)	1 (2.5)	2 (16.7)	
Median mRSS (IQR)	2 (0-6)	6 (5-11)	3 (1-6)	4 (0-6)	p=0.009
Median 6-MWD (IQR)	548 (476-583)	470 (263-530)	463 (399-510)	286 (97-366)	p<0.001
Post-test saturation	98 (96-99)	91 (87-96)	98 (95-99)	90 (88-96)	p=0.008

13. Transcriptomic Analyses of Lung Tissues Reveals Potential Key Genes Associated with Progression of Systemic Sclerosis-Interstitial Lung Disease (SSc-ILD): A nanoString Study

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Background:

SSc-ILD is the leading cause of death in SSc affecting around 50% of the patients. Lung tissue of patients with early stage SSc-ILD is characterised by a predominant inflammatory response with inconspicuous fibrosis, but which can progress to honeycombing fibrosis. Although several biomarkers and drug targets have been proposed to monitor and halt SSc-ILD progression, none is showing satisfactory clinical benefit. A better comprehension of the molecular mechanisms underpinning SSc-ILD pathogenesis is needed to improve treatment options and progression prediction. This transcriptomic study aims to reveal the differential gene expression between healthy control (ctrl) lung sections and regions of interest (ROIs) within SSc-ILD lung tissue.

Materials and Methods:

The nanoString nCounter Human Fibrosis Panel containing 770 genes related to all stages of fibrosis was used to analyse gene expression in formalin-fixed and paraffin-embedded lung tissues with varying stages of SSc-ILD (n=18) and control lung tissue (n=6). The SSc-ILD tissues were stratified by an experienced lung pathologist into three ROIs, inflammatory (inf), prefibrotic (prefib) or fibrotic (fib). Every group comprised 6 samples. This stratification aimed to define a longitudinal simulation of

early to late phases of SSc-ILD: ctrl → inflammation → prefibrosis → fibrosis. nSolver and Rosalind software were used for data and statistical analysis.

Results:
Ctrl vs SSc-ILD comparison demonstrated 24 differentially expressed genes, of which two were with the most pronounced *p*-values. Cyclin-Dependent Kinase Inhibitor 2C (CDKN2C) was significantly overexpressed (p = 0.00052) in SSc-ILD compared to ctrl, while expression of Pellino E3 ubiquitin-protein ligase 1 (PELI1) was significantly downregulated (p = 0.0012) (fig.1). Additionally, the expression of CDKN2C and PELI1 showed an incremental increase and decrease in the four groups, respectively (fig. 3). The subgroup analysis revealed an incremental increase in pathway scores related to the severity of fibrosis (fig. 2).

Conclusions:
Expression of CDKN2C and PELI1 was associated with more severe stages of SSc-ILD on histologic assessment. We report for the first time the potential of these genes to predict progression. Further research is required for validation of these findings and includes functional studies to determine the potentiality of CDKN2C and PEL1 as putative therapeutic candidates.

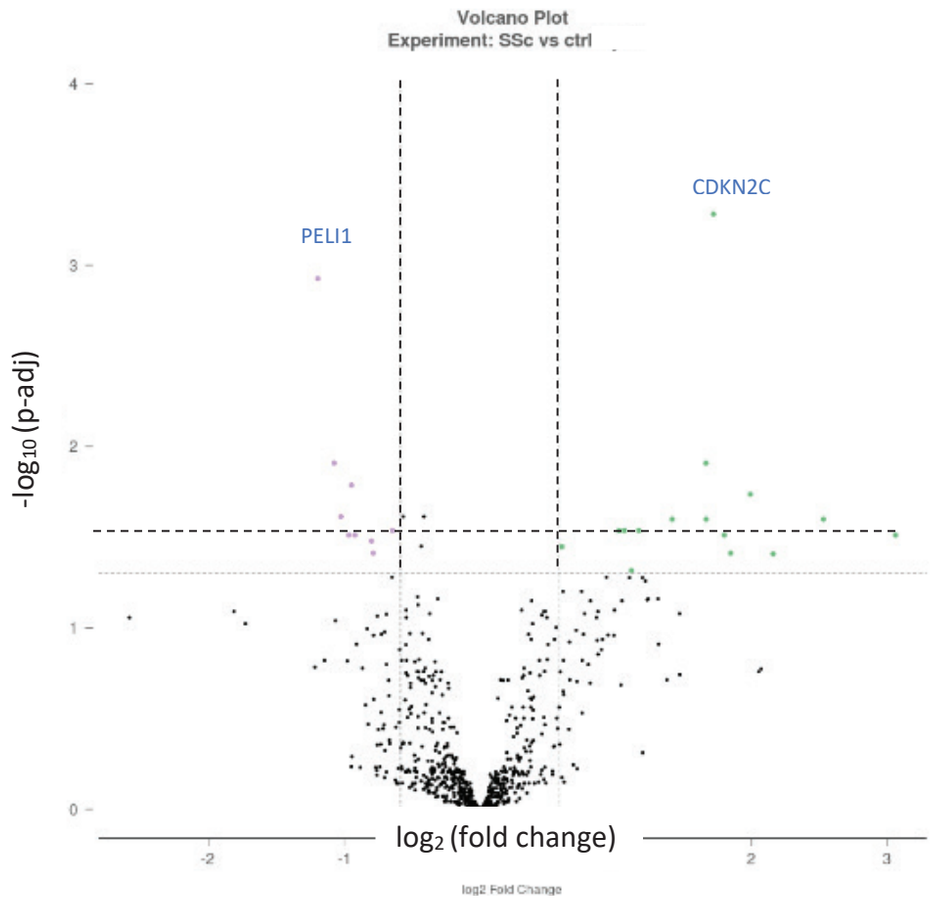

Figure 1: Volcano plot of SSc-ILD group vs ctrl (ctrl as baseline). CDKN2C and PELI1 genes are the most significant differentially expressed genes between the two groups.

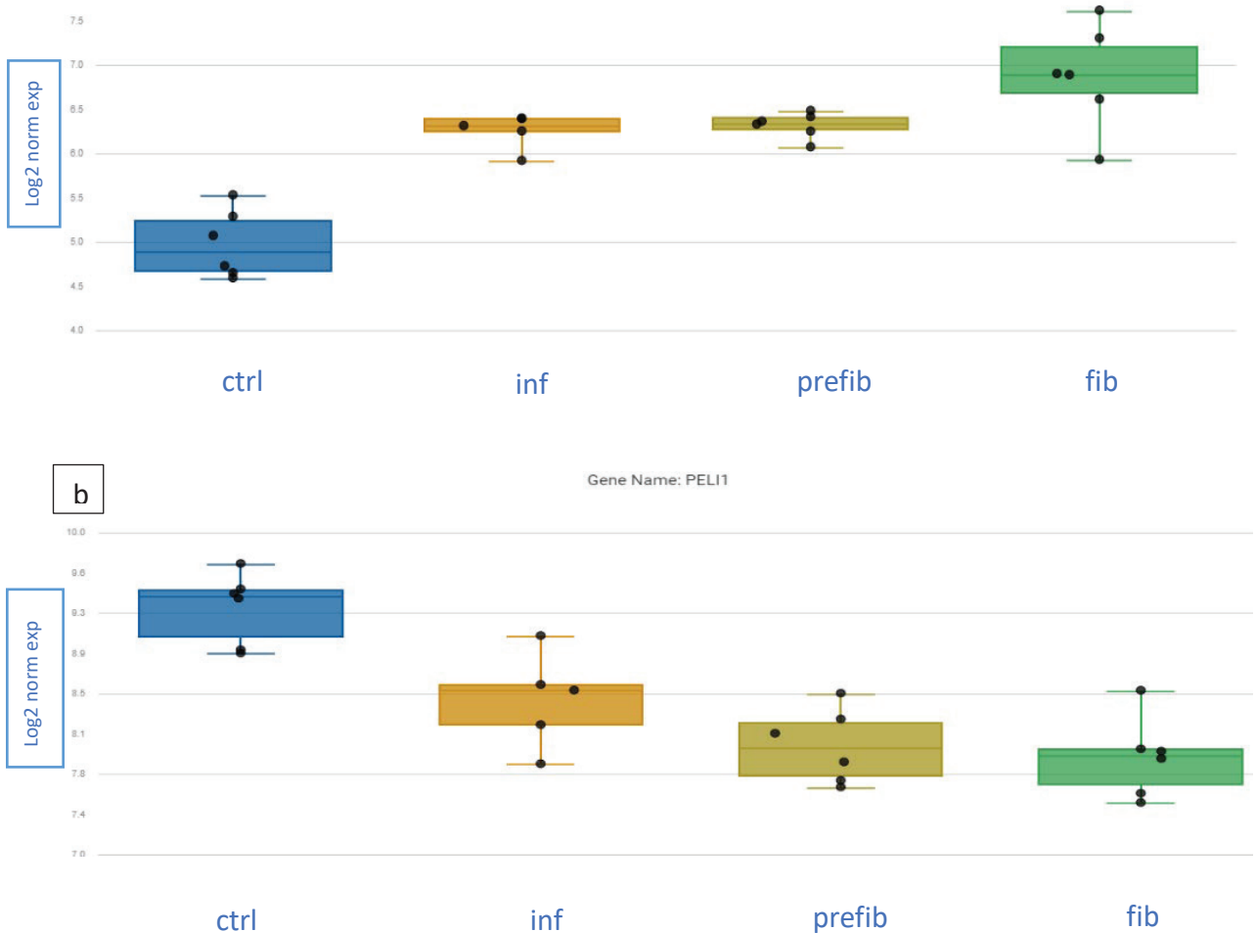

Figure 2: CDKN2C and PELI1 gene expression change between the four groups shown as (Log2) fold-change. (a) CDKN2C expression increases from the ctrl group up until the fibrotic group incrementally through inflammatory and prefibrotic groups. (b) PELI1 gene expression decreases from the ctrl group up until the fibrotic group incrementally through inflammatory and prefibrotic groups.

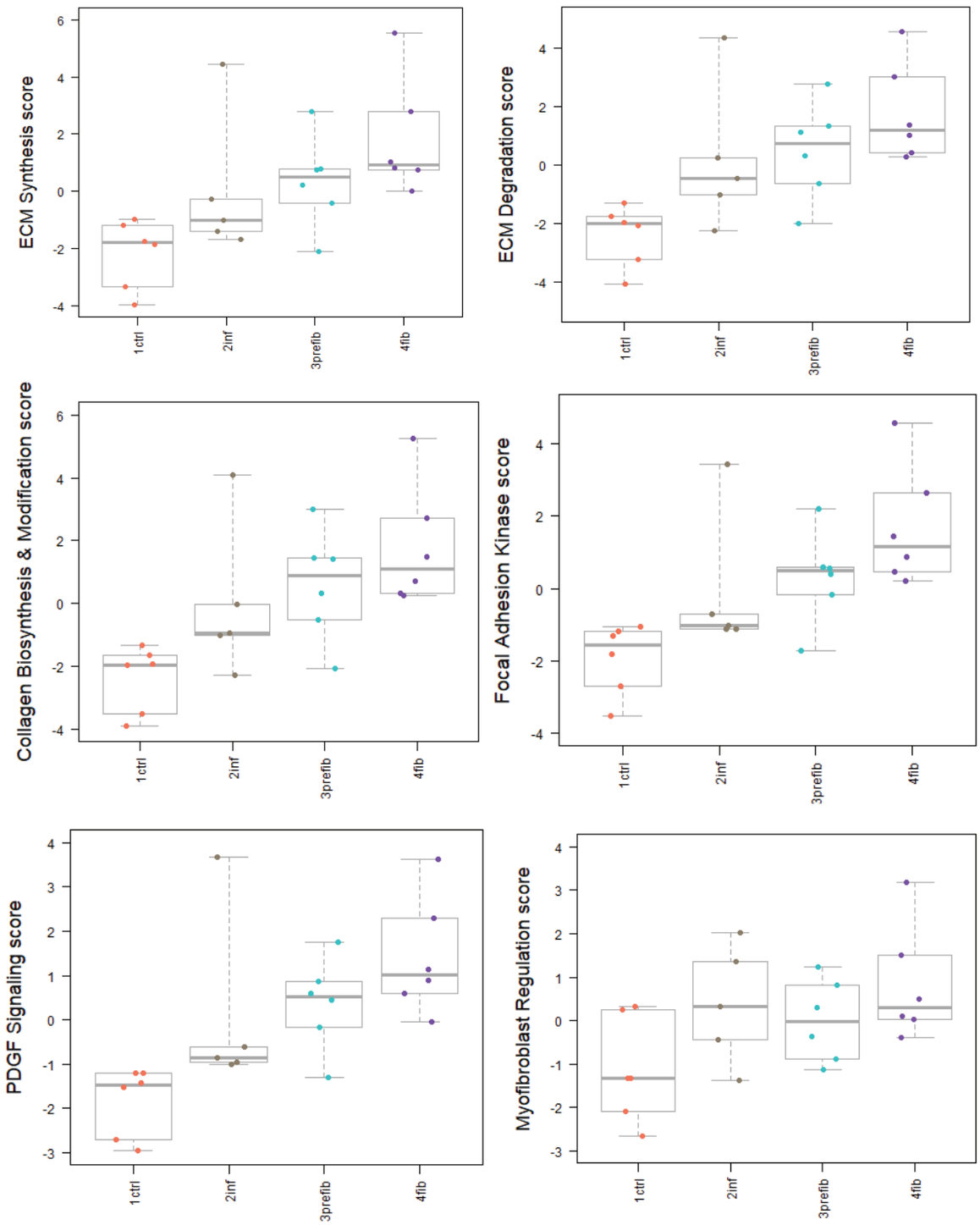

Figure 3: Pathway score analysis. Six pathways related to fibrosis and tissue remodelling are shown. Ctrl samples scored lowest while fibrotic samples scored highest. These pathways were the most differentially elevated in 51 annotated pathways within the fibrosis panel. ECM: Extracellular matrix; PDGF: Platelet-derived growth factor.

14. Effects of nintedanib on circulating biomarkers in subjects with systemic sclerosis-associated interstitial lung disease (SSc-ILD)

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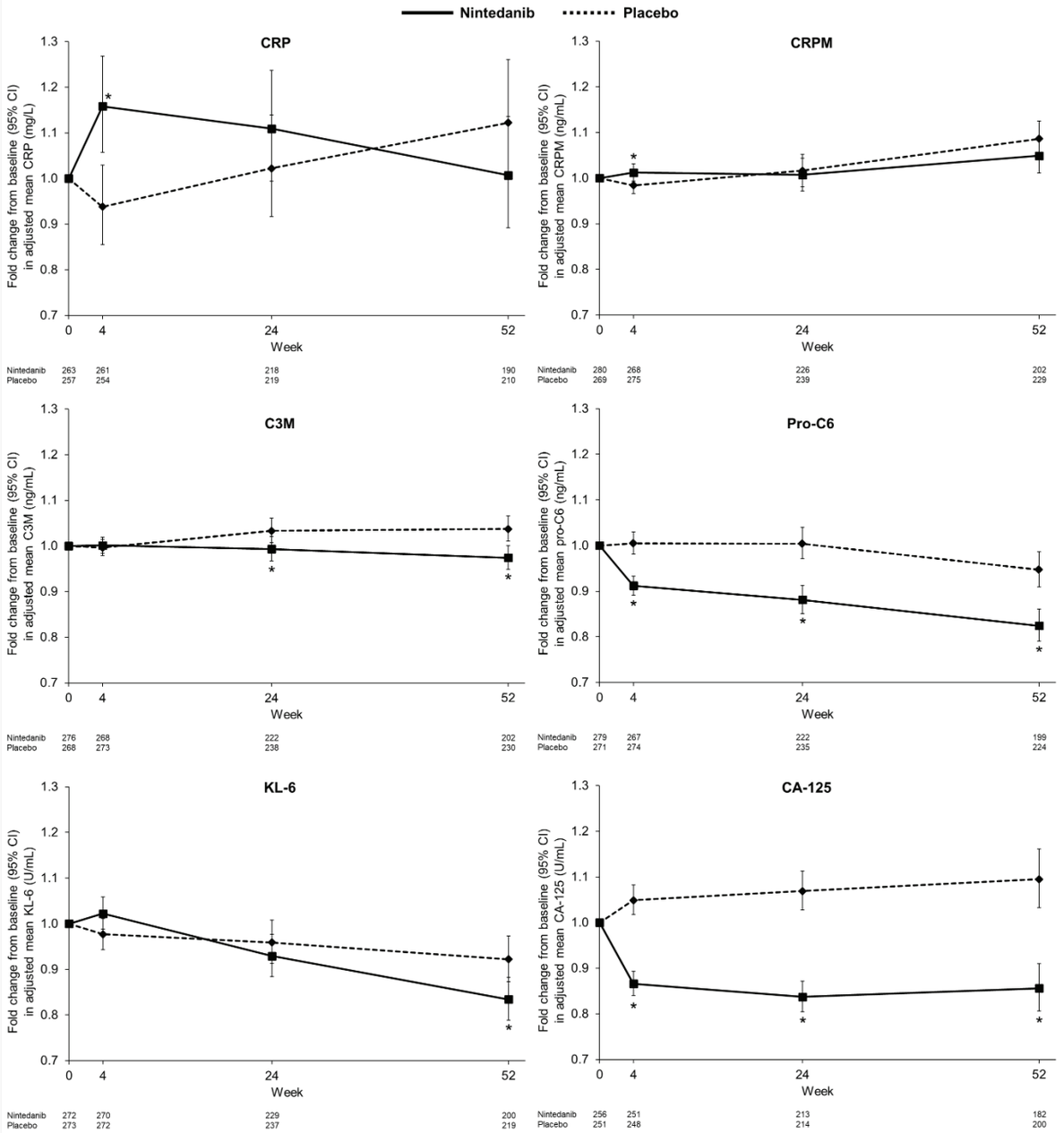
Background: In the SENSCIS trial in subjects with SSc-ILD, nintedanib reduced the rate of decline in forced vital capacity (FVC) over 52 weeks by 44% compared with placebo. We investigated the effects of nintedanib on circulating biomarkers of extracellular matrix (ECM) turnover, epithelial injury and inflammation in the SENSCIS trial.

Materials and Methods: Subjects had SSc with first non-Raynaud symptom in the prior ≤7 years, extent of fibrotic ILD on high-resolution computed tomography (HRCT) ≥10% and FVC ≥40% predicted. Patients were randomised to receive nintedanib or placebo stratified by anti-topoisomerase I antibody (ATA). Blood samples were taken at baseline and at weeks 4, 24 and 52. Fold changes in adjusted mean levels of circulating biomarkers were analyzed using a linear mixed model for repeated measures. Data were $\log_{10}$ transformed before analysis and estimates of change from baseline were back-transformed.

Results: A total of 576 subjects received trial drug (288 nintedanib, 288 placebo). A transient increase in fold change from baseline in C-reactive protein (CRP) (a marker of inflammation) was observed in subjects who received nintedanib versus placebo at week 4. After an initial increase at week 4 in the fold change from baseline in CRP degraded by MMP-1/8 (CRPM) (a marker of ECM turnover), a trend to decreasing levels was observed in subjects who received nintedanib compared with placebo at week 52. Decreases in the fold change from baseline in collagen 3 degraded by MMP-9 (C3M) and N-terminal propeptide of type VI collagen (pro-C6) (markers of ECM turnover) were observed in subjects who received nintedanib compared with placebo from week 24 and week 4, respectively. A decrease in fold change from baseline in Krebs von den Lungen-6 (KL-6) (a marker of epithelial injury) was observed in subjects who received nintedanib versus placebo at week 52. A decrease in fold change from baseline in cancer antigen 125 (CA-125) (a marker of epithelial injury) was observed in subjects who received nintedanib versus placebo from week 4 (Figure).

Conclusions: Data from the SENSCIS trial suggest that nintedanib reduced circulating levels of markers of ECM turnover and epithelial injury in subjects with SSc-ILD.

Figure. Fold changes from baseline in biomarkers over 52 weeks



*p<0.05 for difference versus placebo in fold change from baseline.
For CRP, KL-6 and CA-125: Based on linear mixed model for repeated measures for absolute change from baseline in log-transformed biomarker values, with fixed categorical effects of treatment at each visit, sex, ATA status, SSc subtype (limited cutaneous SSc, diffuse cutaneous SSc), mycophenolate use at baseline, methotrexate use at baseline, and fixed continuous effects of the baseline value of the protein biomarker at each visit, BMI and age.
For Pro-C6, C3M, CRPM: Based on linear mixed model for repeated measures for log-transformed biomarker values (including baseline timepoint), with fixed categorical effects of treatment at each visit, sex, ATA, SSc subtype (limited cutaneous SSc, diffuse cutaneous SSc), mycophenolate use at baseline, methotrexate use at baseline, and batch, and the fixed continuous effects of BMI and age.

15. Cordycepin is a potent suppressor of pro-fibrotic TGF-β induced lung and skin fibroblasts activation

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Background:
Activated fibroblasts are the main effectors in SSc. Currently, there is a lack of effective fibroblast-specific therapies. Cordycepin is an adenosine analogue identified as a major bioactive constituent in fungus *C. militaris*. Our aim was to investigate the effects of cordycepin on profibrotic activation of lung and skin fibroblasts.
Materials and methods:
Human dermal and lung fibroblasts (DF and LF, respectively) from four different healthy adult controls were cultured and treated with TGF-β (5 ng/ml) ± different doses of cordycepin (1 μM, 5 μM, 10 μM, 20 μM, 40 μM). Expression of pro-fibrotic mRNAs, such as *COL1A1, α-smooth muscle actin (α-SMA)* and *fibronectin* were analysed using qPCR. Protein levels of COL1A1, α-SMA and Smad2/3 were detected by Western blot. RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay were used to measure apoptosis in reaFl-time.
Results:
Cordycepin abrogated TGFB-induced skin and lung myofibroblast differentiation in a dose-dependent manner, as observed by decrease of *α-SMA* mRNA expression levels to 0,22 FC of stimulated cells and to 0,59 FC of unstimulated cells and protein level (27 % decrease compared to unstimulated cells). Further, cordycepin reduced the TGFB-induced production of ECM protein components, such as *COL1A1* mRNA to 0,08 FC of unstimulated cells and protein level (37 % decrease compared to unstimulated cells) as well as *fibronectin* mRNA to 0,15 FC of unstimulated cells by lung and skin fibroblasts. Cordycepin also reduced the expression of Smad2/3 on protein level for 75% compared to unstimulated cells. The utilized doses of cordycepin did not show any cytotoxic effect in DF and LF.
Conclusions:
Cordycepin inhibits TGF-β-induced expression of pro-fibrotic markers in human DF and LF, providing the rationale for its further investigation on myofibroblast function and its precise mechanism of action. Thus, cordycepin might represent a potential treatment strategy to halt skin and lung fibrosis in SSc.

16. Distinct differences in complement perturbation between scleroderma-related interstitial lung disease with and without emphysema

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Background:
Aberrant complement activation is associated with autoimmune diseases including systemic sclerosis (SSc). As a leading cause of death in SSc, interstitial lung disease (ILD) can coexist with emphysema in non-smoking patients, worsening prognosis. It has been suggested that the airway destruction observed in SSc patients may be an exaggerated inflammatory response related to increased complement activation.

Materials and methods:

We analysed 10 complement proteins in plasma samples of 16 non-smoking SSc patients with emphysema (SSc-Emp), 8 SSc no-ILD, 8 SSc-ILD patients and 8 healthy controls (HC) (Table.1) selected from 1800 SSc patients under active follow up. The extent and distribution of emphysema was evaluated on high resolution computed tomography (HRCT) and enzyme-linked immunosorbent assays (ELISA) were performed for measuring C1q, MASP-2, Factor B, Factor Bb, Factor H, C3, C3a, C4, C5, C5a and TCC (terminal complement complex). One-way ANOVA and post-hoc Tukey test were used for analysis.

Results:

HRCT confirmed that amongst the emphysema cohort, there was a spectrum of mild to severe destruction including both paraseptal and centrilobular emphysema. All 16 SSc-Emp patients had ILD and 9 of the 16 patients demonstrated perivascular emphysema not previously described in SSc The SSc-ILD group was characterised by extensive lung fibrosis on CT, mean FVC 71% predicted.

Across the four groups, C1q was significantly reduced in the SSc-ILD cohort (SSc-Emp 950±404µg/mL, SSc-ILD 355±116µg/mL, SSc-no ILD 1087±206µg/mL, HC 858±210µg/mL, p= 0.0002). In contrast, the ratio of C3a/C3 was significantly increased in the SSc-ILD group compared with the SSc-Emp group (SSc-ILD 0.12±0.07 vs SSc-Emp 0.06±0.03, p= 0.0283). The ratio of Bb/B was significantly increased in the SSc no-ILD group compared with all other groups (SSc-Emp 1.57±0.80µg/mL, SSc-ILD 1.27±0.56µg/mL, SSc-no ILD 3.86±1.12µg/mL, HC 1.89±0.75µg/mL, p< 0.0001). There was a trend towards an increased ratio of C5a/C5 in SSc-ILD and SSc-Emp compared with HC and reduced MASP2 in the SSc groups compared with HC.

Conclusions:

These data demonstrate dysregulated complement levels in three SSc subgroups. Notably, the perturbed C1q and C3a:C3 pathways in SSc-ILD are reversed in SSc-emphysema, supporting complement activation as part of the divergent tissue remodelling responses in these distinct SSc lung phenotypes.

	SSc-Emp (n=16)	**SSc-ILD (n=8)**	**SSc-no ILD (n=8)**	**HC (n=8)**
Age (mean)	53	57	65	49
LcSSc|DcSSc	10 | 5	6 | 2	7 | 1	n/a
Autoantibody profile ACA|Scl-70|ARA	2 | 7 | 2	0 | 4 | 1	4 | 1 | 0	n/a
Disease duration (years)	14	15	17	n/a
α1AT [SD] (reference range 1.2-2.6 g/l)	1.37 [+/- 0.11]	1.45 [+/- 0.28]	1.42 [+/- 0.13]	1.29 [+/- 0.19]
AP50 [SD] (reference range 50-125%)	92.8 [+/- 16.03]	95.2 [+/- 18.58]	94.6 [+/- 14.85]	103.6 [+/- 12.20]

CH100 [SD] (reference range 392-1019 IU)	780.4 [+/- 154.23]	649.5 [+/- 231.44]	537.4 [+/- 97.25]	817.4 [+/- 159.15]

Table 1. Demographics of each patient cohort including serological results for α1AT, and functional assay to rule out genetic complement pathway deficiency (AP50, CH100).

17. Transcriptional regulation of SSc dermal myofibroblasts by *FOSL2* and *FOXP1*

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Background: Systemic Sclerosis (SSc) is characterized by fibrosis, vasculopathy, and immune dysregulation. Skin fibrosis is the hallmark of SSc and is driven by the contractile action of myofibroblasts. The number of myofibroblasts in the skin correlates with the modified Rodnan skin score, the most widely used clinical measure of skin severity. Using single cell RNA sequencing, we have identified different dermal fibroblast populations and shown that SSc dermal myofibroblasts arise in two steps from *SFRP2^{hi}/DPP4* expressing progenitor population. Bioinformatic analyses of the SSc dermal fibroblast transcriptome implicated the role of transcription factors *FOSL2* and *FOXP1* in the first and second step of SSc myofibroblast differentiation respectively. Our aims are to understand the transcriptional regulation of *FOSL2* and *FOXP1* in dermal myofibroblast activity and SSc pathogenesis.

Methods: We used si-RNA to knockdown the RNA expression of *FOSL2* and *FOXP1* in primary dermal fibroblasts from SSc patients. The perturbed transcriptome, signaling pathways, and epigenetic changes were characterized using bulk RNA sequencing, Western blotting, and ATAC sequencing.

Results: We found that knocking down *FOSL2* and *FOXP1* RNA using si-RNA led to a reduction in fibrotic genes and biomarkers for SSc disease progression such as: *COL1A1*, alpha-*SMA, THBS1, PRSS23, THY1,* and *FN1.* On generating activity modules of the perturbed transcriptome, we found that the genes downregulated by si-RNA activity had a high expression in the *SFRP2^{hi}/DPP4* expressing progenitor population.

Conclusion: Our study provides a novel understanding of the transcriptional and epigenetic regulation of SSc dermal myofibroblasts by *FOSL2* and *FOXP1* and provides evidence of their role in the pathogenesis of SSc. We have identified target genes which are regulated by *FOSL2* and *FOXP1* and responsible for driving fibrosis in dermal fibroblasts.

18. Endostatin and proangiogenic markers serum levels characterize preclinical systemic sclerosis patients at risk for progression toward definite SSc

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Background: Profiling of preclinical systemic sclerosis (PreSSc) patients has been rarely performed. The knowledge of molecular mechanisms characterizing disease progression may be relevant to get

insights into SSc pathogenesis and to intercept patients at risk at an early stage. We aimed at investigating the proteomic profile of PreSSc via a discovery/validation two-step approach.

Materials and Methods: Step1: serum sample of 13 PreSSc (discovery cohort) according to 2001 LeRoy and Medsger criteria (Raynaud phenomenon plus a positive nailfold capillaroscopy and SSc specific antibodies without any other sign of definite disease) and 8 healthy controls (HCs) age, gender, ethnicity matched were collected to perform SOMAcan aptamer-based analysis. Prospective data were available up to 4±0.6 years to determine the progression to definite SSc according to the EULAR/ACR 2013 criteria. Univariate analysis was performed via prediction models in proteins with relative fluorescence units (RFU) > |1.5|-fold HCs values. Bootstrap aggregating was used to determine the accuracy of predictions (progression vs non-progression) of categorized baseline protein values. Gene Ontologies (GO terms). Step2: significant proteins from Step1 were validated via ELISAs in a validation cohort of 50 PreSSc with clinical prospective data up to 5 yrs. Time-to-event analysis for interval-censored data was used to evaluate disease progression.

Results: Step1: 286 out of 1306 analyzed proteins via SomaScan, were differentially expressed vs HCs in the discovery cohort. Ten proteins were significantly associated with disease progression; analysis of GO biological processes showed a number of differentially enriched pathways involving angiogenesis, endothelial cell chemotaxis and endothelial cell chemotaxis to fibroblast growth factor (FGF). Step2: ELISA confirmation assay showed that Endostatin (HR=10.23, CI95=2.2–47.59, p=0.003), bFGF (HR=0.84, CI95=0.709-0.996, p=0.045) and PAF-AHβ (HR=0.372, CI95=0.171-0.809, p=0.013) were significantly associated with progression after Cox-regression analysis for interval-censored data.

Conclusion: soluble factors associated with hypoxia and vasculopathy, are linked with the transition from preclinical to definite SSc, these proteins can be regarded both as markers of severity and molecules with pathogenetic significance.

19. Identifying circulating biomarkers associated with progressive interstitial lung disease in Systemic sclerosis using an enriched cohort approach

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Introduction

Systemic Sclerosis (SSc) is a rare autoimmune connective tissue disease, affecting approximately 2.5 million people worldwide and Interstitial Lung Disease (ILD) is the leading cause of mortality[1,3]. There is a clear unmet need for therapies that effectively preserve lung function and quality of life[2].
This collaboration between the centre for connective tissue diseases at UCL (UK) and Servier Pharmaceuticals (France) aims to identifying markers of ILD progression in SSc.

Study design

Patients were recruited retrospectively from the UCL database, with samples for each patient at 3 separate timepoints throughout their disease. Thirty patients were selected based on the extent and progression of their ILD lung fibrosis to provide 3 well defined groups.
SSc patients were split into 3 groups: 10 patients “no ILD” (<5% lung involvement on HRCT); 11 patients “trivial ILD” (<20% lung involvement on HRCT or indeterminate fibrosis with FVC >70% predicted); 9 patients “meaningful ILD” (patients with significant ILD progression, >20% lung involvement on HRCT).

Methods
Ninety plasma samples were available for analysis of soluble mediators by “omics” methods, namely metabolomics (Biocrates analysis kit), proteomics (mass spectrometry) and multiplex (Meso Scale Discovery electroluminescence method). Results were statistically analysed using supervised and unsupervised methods.

Results
When comparing soluble mediators in plasma samples of SSc patients, we found a significant increase in plasma levels of Fractalkine (p=0.0042), MCP-1 (p=0.0022) and PDGF-A (p=0.0323) in meaningful ILD patients compared to no ILD patients Interestingly, IL-8 plasma levels were increased in the no ILD cohort compared to meaningful ILD cohort (p=0.0276).
Metabolomic and proteomic analyses showed no significant difference when comparing patient groups over time or between collection timepoints. Plasma levels of some mediators were significantly decreased in no ILD samples when comparing metabolomic profiles between third and first collection timepoints.

Conclusion
This innovative study design has allowed us to compare well defined subgroups of SSc patients over a substantial period. We hope to understand the role of biological markers such as MCP-1, Fractralkine, PDGF-A and IL-8 in the pathogenesis of the progressive ILD phenotype, which, in turn, could facilitate early targeted immunomodulatory treatment and improve long term outcome.

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20. Oxidative Stress Imbalance in Systemic Sclerosis

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Background:
Oxidative stress can be defined as an excessive amount of reactive oxygen species (ROS) that is the net result of an imbalance between the production and degradation of ROS by antioxidants. Correct balance between ROS and antioxidant defense in the organism is important for normal functioning as some ROS are signaling molecules in cellular signaling pathways. To assess oxidative balance in patients with systemic sclerosis (SSc), we measured oxidative and antioxidant status in the circulation.

Materials and methods:
Full blood was collected from 36 SSc patients and 28 age- and sex-matched healthy controls (HC) to obtain serum. Oxidative status was assessed using the FRAS 5 system – the pro-oxidative status by the d-ROMs fast test (measuring the concentration of hydroxyperoxides - normal range 250-300 U.Carr) and serum antioxidant potential by the PAT test (measuring ferric to ferrous ion reduction - normal range 2200-2800 U.Cor). These parameters were then used to calculate the oxidative stress index (OSI), with values < 40 considered normal, 41-65 borderline and > 66 high.

Results:
Serum concentration of reactive oxidative metabolites was similar in SSc and HC (median (IQR) 340 (55) U.Carr vs. 322 (87) U.Carr, p = 0.4696), while the concentration of antioxidants was significantly higher in SSc than in HC (3263 (400) U.Cor vs. 3028 (339) U.Cor, p = 0.0115). The calculated OSI was also significantly increased in SSc patients (65 (26) vs. 50 (26), p = 0.0393).

Conclusions:
SSc patients and HC had similar serum levels of hydroxyperoxide reactive oxidative metabolites, compounds that form early in the sequence of oxidative reactions by oxidative attack on various organic substrates. However, higher serum levels of antioxidants (above the optimal range) were present in SSc patients, resulting in the elevated oxidative stress index. This highlights the oxidative stress imbalance in SSc patients and brings the role of excess antioxidants into focus for further studies.

21. Dysregulation of the master regulator TCF12 in Systemic Sclerosis myofibroblast populations

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Background:
Systemic sclerosis (SSc) is a complex disorder with both genetic and environmental factors. Defining characteristics of SSc include fibrosis, autoimmunity, and vasculopathies though onset and progression vary greatly. Interstitial lung disease (ILD) is the leading cause of mortality in the most extreme cases. Despite heterogeneity in disease phenotypes, one of the common tissue-level pathologies is an increased proportion of myofibroblasts. SSc-ILD patients have fibroblastic foci within the lungs; many fibroblasts within these foci stain with markers for myofibroblasts. Understanding the development and maintenance of myofibroblasts may aid in a deeper understanding of the disease progression. Our single-cell RNA sequencing (scRNA-seq) data allows for unbiased prediction of master regulators from specific cellular populations directly from patients.

Materials and methods:
Gene expression data associated with mesenchymal populations in our scRNA-seq dataset were fed into pySCENIC to identify regulon activity scores leading to the prediction of master transcription factors (TFs) for myofibroblasts. We identified TFs common across multiple patients; *TCF12* expression levels and regulon activity scores are both upregulated in myofibroblasts specifically. To biologically validate the role of *TCF12*, we knocked down (KD) *TCF12* with DsiRNA (IDT) on primary pulmonary fibroblasts isolated from SSc lung explants. Bulk RNA sequencing was carried out on these samples. Differentially expressed genes (DEGs) were used to create module scores in the Seurat package. This module score is applied to the scRNA-seq data sets to investigate the specific cell populations affected by that gene signature.

Results:
Bulk RNA-seq results showed that *TCF12* was repressed approximately 95% compared to controls. DEGs specific to the targeted gene were used to add a module score to the scRNA-seq data. The highest module activity scores are within the myofibroblasts and MFAP5 matrix fibroblast populations.

Conclusions:
Our *in vitro* experiments support the role of *TCF12* in myofibroblast populations directly from patients. We plan to target other predicted master regulators for myofibroblasts along with looking at various combinations of dysregulated TF expression in the future. Continued analysis of the data is

necessary to identify additional pathways of interest which may increase understanding of the pathology and hopefully provide novel therapeutic targets.

22. Perturbed lipid metabolism in fibrotic skin contributes to metabolic reprogramming in systemic sclerosis

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Background
Perturbed cellular metabolism is emerging as a driver of fibroblast activation in fibrosis. Our aims were to identify alterations in metabolic pathways in fibrotic SSc skin and unravel fibroblast-driven contributions to perturbed skin metabolism in SSc.

Methods
To screen for perturbations in metabolic pathways in SSc skin, we integrated skin transcriptomic microarray data from 76 SSc patients and 26 healthy controls (HC) from three SSc cohorts (GSE:45485, 59785, 9285/32413). Differentially expressed genes between healthy and SSc skin were identified using the limma package; gene set enrichment analysis (GSEA) was performed using the clusterProfiler package. Transcriptional changes in SSc skin were aligned with bulk RNA-seq and scRNA-seq data created from cultured human skin fibroblasts treated or not with TGFβ. Metabolomic profiling of skin fibroblasts was performed by untargeted liquid chromatography-mass spectrometry, followed by Matlab analysis.

Results
Pathway enrichment analysis of skin transcriptomes identified multiple alterations in metabolic networks in SSc skin (Fig. 1A). These alterations pointed to enhanced pyrimidine/folate metabolism and suppressed lipid metabolism pathways in SSc skin. Fatty acid metabolism (synthesis and degradation) and steroid hormone biosynthesis were the key downregulated lipid metabolism pathways in SSc skin, particularly in patients with the inflammatory intrinsic gene expression subset. These changes were similar in the affected and nonaffected SSc skin, suggesting that decrease in lipid metabolism is a generalized feature of the SSc skin. Furthermore, pathway enrichment analysis of RNA-seq and scRNA-seq data suggested that TGFβ-driven fibroblast reprogramming significantly contributes to the observed changes in lipid metabolism networks in SSc skin (Fig. 1B-D). These findings were further supported by our untargeted metabolomic analysis demonstrating large changes in fibroblast lipid metabolome following TGFβ stimulation (Fig. 1E). Notably, most of the altered metabolites in TGFβ-treated skin fibroblasts belonged to the group of glycerophospholipids.

Conclusion
Our data suggests a perturbed metabolic network in SSc with predominant suppression of lipid metabolism and identifies fibroblasts as the important contributors to observed lipid alterations in SSc skin. These results might pave the way to a deeper understanding of the interplay between the metabolic and fibrotic pathways in SSc and unravel potential metabolic targets in SSc.

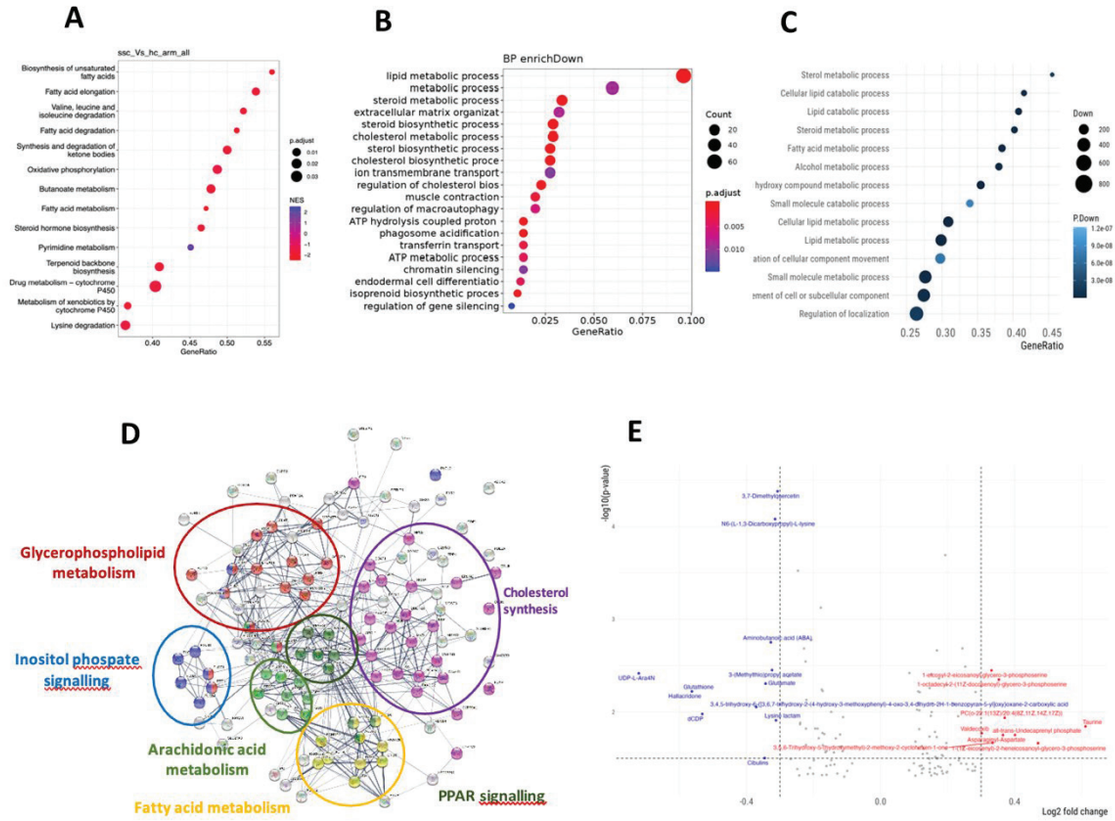

Figure 1. A) Supervised enrichment of metabolic processes in affected SSc forearm skin tissue compared to HC. B) Unsupervised analysis of downregulated GO biological process in cultured skin fibroblasts after TGFβ stimulation as analyzed by bulk RNA seq (B) and scRNAseq (C). D) STRING analysis of downregulated genes from lipid metabolism in TGFβ-stimulated skin fibroblasts analyzed by bulk RNAseq. E) volcano plot of most deregulated metabolites in TGFβ-stimulated skin fibroblasts; downregulated (red) and upregulated (blue) metabolites after TGFβ stimulation.

23. Exploring metabolism in scleroderma reveals opportunities for pharmacological intervention for therapy in fibrosis

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Background:

Recent evidence has suggested that alterations in energy metabolism play a critical role in the pathogenesis of fibrotic diseases. Studies suggested that 'metabolic reprogramming', involving the glycolytic flux and oxidative phosphorylation (OXPHOS) in cells lead to an enhanced generation of energy and biosynthesis of building blocks. The data have been associated with changes in cell proliferation, the production of extracellular matrix (ECM) and myofibroblast function. The aim of this study was to assess the molecular basis of changes in fibrotic metabolism in systemic sclerosis (Scleroderma; SSc) and highlight the most appropriate targets for anti-fibrotic therapies.

Materials and methods:
Dermal fibroblasts were isolated from five SSc patients and five healthy donors. Cells were cultured in medium with/without 2 ng/ml of TGF-β1 and with/without ALK5, pan-PIM or ATM kinase inhibitors (10 □M SB431542, 20 □M KU55933 and 5 □M KU55933, respectively). Extracellular flux analyses were performed to evaluate glycolytic and mitochondrial respiratory function. The mitochondrial network in TMRM-stained cells was visualized by confocal laser-scanning microscopy, followed by semi-automatic analysis with the MiNA macro toolset on the ImageJ platform. Protein expression levels of ECM and fibroblast components (Col-1, CTGF, Fibronectin, □-SMA), rate-limiting glycolytic enzymes, essential subunits of the five OXPHOS enzyme complexes, and GTPases and receptors involved in mitochondrial fission/fusion were assessed by western blotting.

Results:
Enhanced mitochondrial respiration coupled to ATP production was observed in SSc fibroblasts at the expense of spare respiratory capacity. Although no difference was found in glycolysis when comparing SSc with healthy control fibroblasts, levels of phophofructokinase-1 isoform PFKM were significantly lower in SSc fibroblasts (P<0.05). Our results suggest that the number of respirasomes is decreased in the SSc mitochondria; however, the organelles formed a hyperfused network, which is thought to increase mitochondrial ATP production through complementation. The increased mitochondrial fusion correlated with a change in expression levels of key regulators of mitochondrial morphology, including decreased levels of DRP1, increased levels of MIEF2 and changes in OPA1 isoform ratios. TGF-β1 treatment strongly stimulated glycolysis and mitochondrial respiration and induced the expression of fibrotic markers. The pan-PIM kinase inhibitor had no effect, whereas both ALK5 and ATM kinase inhibition abrogated TGF-β1-mediated fibroblast activation, and upregulation of glycolysis and respiration.

Conclusions:
Our data provide evidence for a novel mechanism(s) by which SSc fibroblasts exhibit altered metabolic programmes and highlight changes in respiration and dysregulated mitochondrial morphology and function, which can be selectively targeted by small molecule kinase inhibitors.

24. The effects of smoking on disease progression and expression of autoantibodies in a monocentric systemic sclerosis cohort

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Background
The correlation between smoking and systemic sclerosis (SSc) is debated [1-3] but two studies suggested a higher risk of anti-topoisomerase I (ATA) positivity in never-smokers than in ever smokers [3, 4], hypothesizing a possible aetiopathological link between smoking and ATA positivity. We conducted an explorative study to determine whether ever smokers present a distinct autoantibody profile compared to never smokers and to investigate whether smoking confers a different risk of disease progression in men and women or in patients with different autoantibodies.

Materials and methods
We included 645 patients (513 women, 132 men) of the Leiden SSc cohort between 2009 and 2020. Patients were categorized as “never-smokers” or as “ever-smokers”. The correlation between smoking habit and presence of SSc-specific antibodies was investigated. A logistic

regression model including smoking as possible predictor was built to assess the risk of organ involvement and of disease progression from baseline to last available visit.

Results
In the cohort, ATA were found in 73 (21%) never smokers and in 80 (26%) (p=0.156) ever smokers, while anti-centromere antibodies (ACA) were present in 146 (43%) never smokers and in 119 (39%) ever smokers (p=0.312). In the gender-based sub-cohorts, 67 (26%) female patients who never smoked were ATA positive, compared to 34 (14%) ever-smoking women (p<0.001), while 110 (42%) never-smokers and 129 (51%) ever-smokers were ACA positive (p=0.033). In men, 33 (44%) ever-smokers and 13 (30%) never-smokers were ATA positive (p=0.134).
In ATA-positive women, smoking was associated with progression of disease subset ("limited" to "diffuse" or "non-cutaneous" to "limited" or "diffuse") (OR 2.52, 95%CI 1.07 – 5.89, p=0.034). In ATA-positive men, a negative association was observed between ever smoking and progression of interstitial lung disease (OR 0.22, 95% CI 0.05 – 0.92, p=0.037) or presence of cardiac involvement (OR 0.10, 95% CI 0.02 – 0.50, p=0.005).

Conclusion: We observed a higher prevalence of ATA positivity among never-smoking women with SSc compared to ever-smokers, outlining the idea of a different effect of smoking on autoantibody expression between men and women. The effects of smoking on disease progression warrant further research and a project proposal is under evaluation by the EUSTAR board.

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25. Molecular and functional characterization of distinct resident and migratory fibroblast populations in systemic sclerosis skin.

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Background
Single-cell RNAseq has highlighted the molecular heterogeneity of fibroblast subpopulations in healthy (HC) and SSc skin. Using a novel cell isolation technique, we isolated "migratory" fibroblasts and "resident" fibroblasts from HC and SSc human skin. We used in vitro functional assays to delineate their fibrotic potential and compared gene expression between these two fibroblast populations. This enabled us to determine which clusters of fibroblasts, defined by scRNAseq, were present in the resident or migratory populations. We demonstrate the resident population is significantly different in SSc, and has potentially been ignored in previous studies with significant therapeutic implications.

Methods

Forearm skin punch biopsies were collected from dcSSc (n=3) and HC (n=3). Migratory fibroblasts were isolated by standard explant culture. The remaining biopsy fragments underwent collagenase digestion to yield the resident fibroblast population.
Functional characterization included 3-D collagen gel contraction, migratory scratch-wound assays. Western blot compared expression of pro-collagen I, CTGF and αSMA. Bulk RNAseq was performed on each fibroblast population, defining significant differences in gene expression as a fold change≥1.5, and adjusted p-value<0.05.
scRNAseq was performed on 12 SSc skin samples, and 3 HC, using the 10X Genomics platform.

Results

SSc fibroblasts showed a characteristic fibrotic phenotype with increased gel contraction, faster migration and overexpressed COL1, CTGF, and αSMA compared to HC. The resident SSc fibroblasts showed similarly elevated expression of COL1 and CTGF, but lower expression of αSMA compared to SSc migratory fibroblasts ($p<0.05$)(Figure 1). Functional assays confirmed differences between SSc resident and SSc migratory fibroblasts and HC fibroblasts ($p<0.05$).
There were 1483 significantly differentially expressed genes between SSc resident and migratory fibroblasts.
scRNAseq subanalysis identified 12 fibroblast clusters in SSc skin. Of these, clusters 0,1,8, and 10 showed a similar gene expression pattern to resident fibroblasts. Clusters 3, and 7 had a similar gene expression to the migratory fibroblasts(Figure 2).

Conclusion

We isolated two distinct fibroblasts populations and demonstrated both functional and transcriptional differences between these populations, more marked in SSc than in HC. Future work will focus on understanding their role in SSc pathogenesis and their contribution to disease phenotype that may facilitate a more targeted treatment approach.

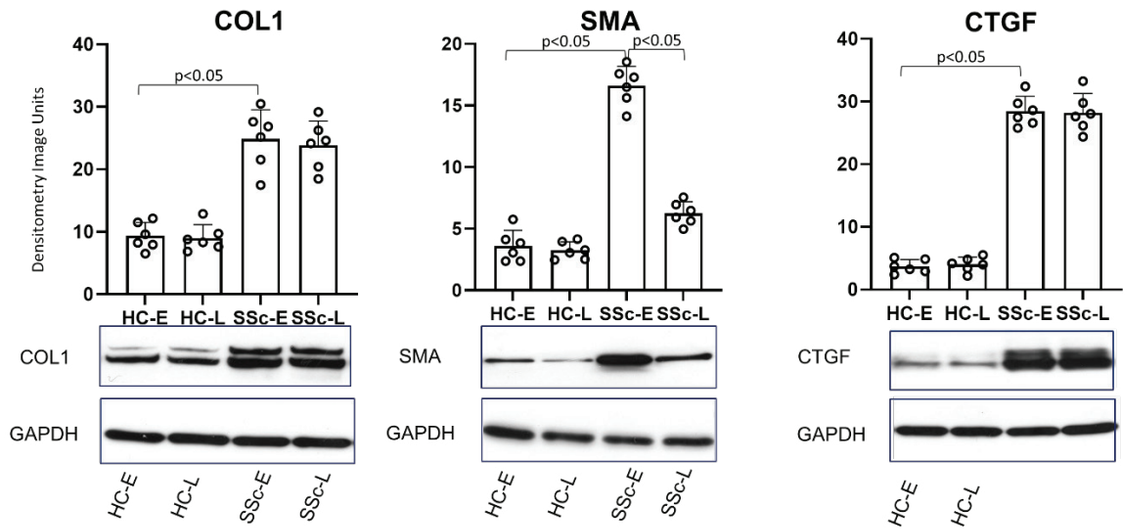

Figure 1: Western blot of showing COL1, αSMA and CTGF. Significant differences between αSMA expression in isolated fibroblast populations. E=migratory, L=resident.

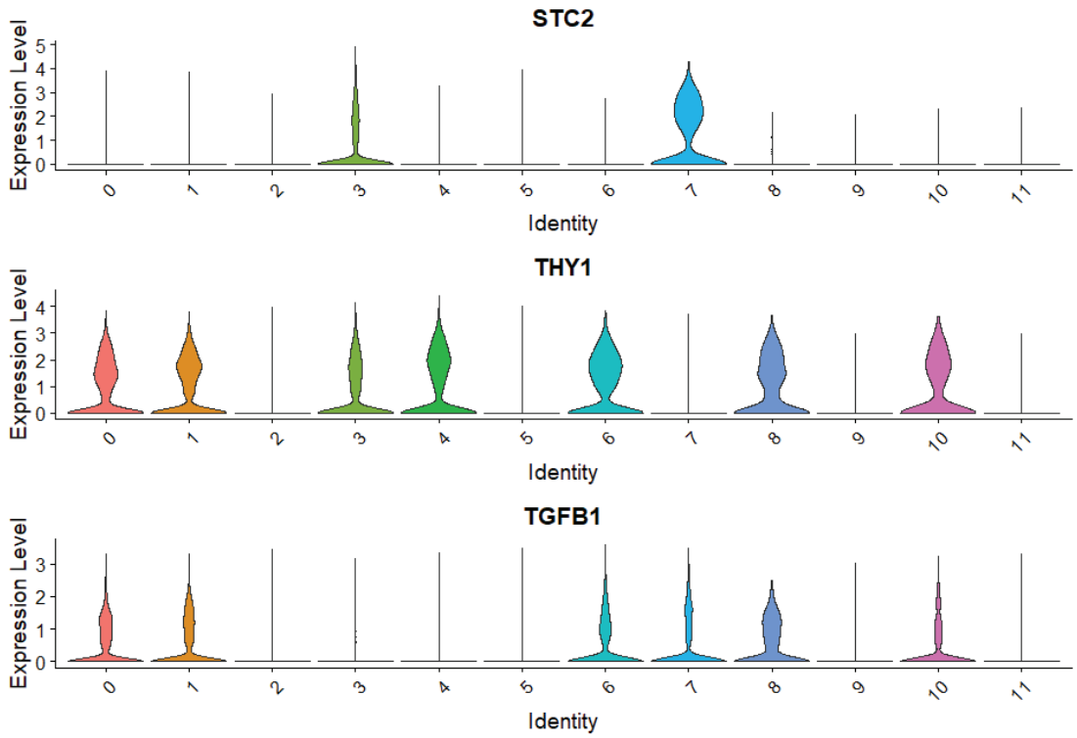

Figure 2: Differing gene expression differences between fibroblast clusters by scRNAseq. Each of these genes was significantly different between resident and migratory fibroblast populations by bulk RNAseq.

26. Developing a composite plasma biomarker for skin severity in systemic sclerosis integrating dermal blister fluid proteomics and genome-wide skin gene expression

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Background:
Recent studies have highlighted the challenge of identifying serum or plasma biomarkers which reflect skin pathogenesis in a multicompartment disease such as systemic sclerosis (SSc). Transcriptomics from skin biopsies, and blister fluid proteomics give a more specific insight into the local environment of the skin. We integrated these modalities with the aim of developing a surrogate for the modified Rodnan skin score (mRSS), utilizing the blister fluid as an anchor to identify candidate genes in the skin for skin severity that are reflected in plasma proteins.

Materials and methods
Transcriptional analysis from blood and skin, and proteomic analysis (1196 proteins) from plasma and blister fluid were performed in a cohort of SSc (n=52) and HCs (n=16). Weighted gene co-expression network analysis (WGCNA) was used to explore the association of skin gene expression, clinical traits, and blister fluid proteomics, creating modules of analytes with differing significant associations to a diagnosis of early dcSSc. Candidate hub analytes were identified as those present in both skin and blister modules, which correlated with corresponding plasma analytes.

Results

3 modules in the skin and 2 in blister fluid correlated with a diagnosis of early dcSSc, and each other. From these, 17 key hub analytes were identified, which were present in both skin and blister fluid modules (figure 1), whose expression correlated with plasma concentrations and mRSS (all with a p value <0.05). Multivariate analysis identified 4 of these analytes (COL4A1, COMP, TNC, SPON1) in the plasma, which as a composite biomarker score correlated significantly with mRSS (r=0.636, p<0.0001), and was able to differentiate disease subtype (all with a p value of <0.002, figure 2).

Conclusions

This unbiased approach has identified potential biologic candidates that are likely to be drivers of local skin pathogenesis in SSc. This technique allowed us to generate a promising composite plasma protein biomarker that could be used for assessment of skin severity, case stratification and as a potential outcome measure for clinical trials and practice. This composite score has the potential to complement clinical assessment by mRSS which has significant variability and is much less directly related to disease pathobiology.

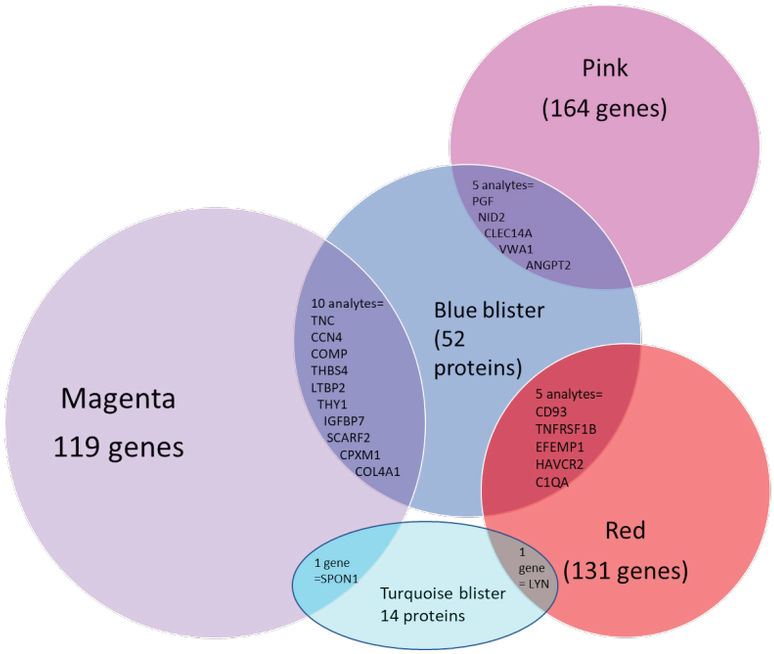
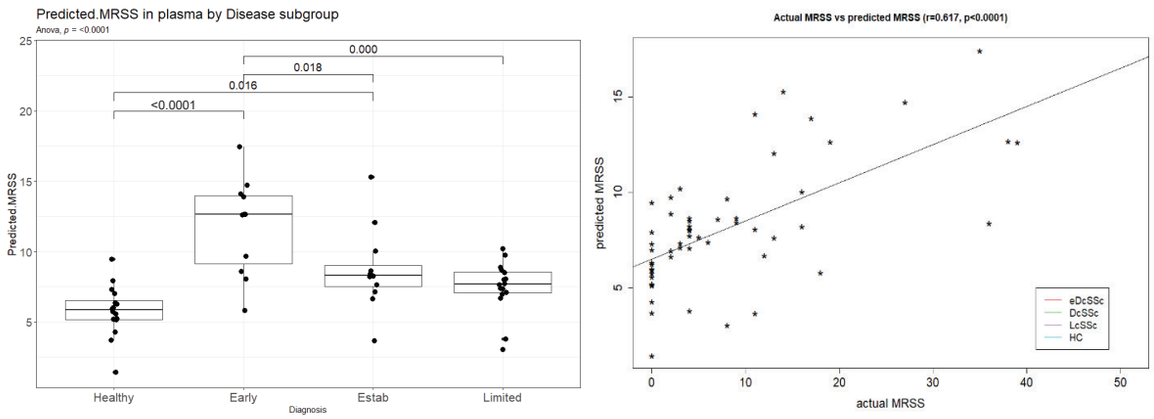


Figure 1: Venn diagram showing hub genes from blister and skin modules that have a molecular membership >0.7, gene significance of >0.6, AND present in both blister fluid proteomics, and skin transcriptomics.



27. Engagement of DNA Sensing Mechanistic Pathways in Human Dermal Microvascular Endothelial Cells.

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Background: Endothelial dysfunction is a key early event in the pathogenesis of Systemic Sclerosis (SSc) that initiates vascular disease and activation of fibroblasts. Dysregulated innate immune system characterized by Type I interferon (Type I IFN) signature, found in the blood of about 50% of SSc patients, is detected even at the early stages of the disease. Understanding the mechanisms of endothelial damage in SSc and the link to type I IFN is limited and is critical to guide therapeutic interventions at the early stages of SSc. We examined putative drivers of the type I interferon pathway and its downstream signaling in human dermal microvascular endothelial cells (HMVEC-D) to explore pathway participants and biomarker identification.
Materials and methods: HMVEC-D from six normal donors were cultured and treated with 400 ng/mL of dsDNA or 15 Multiplicity of infection (MOI) of BacMam virus for 6 & 24 hours. RNA was extracted and subjected to bulk RNA sequencing**.** Differential gene expression analysis was performed and gene co-expression analysis using patient data sets from affected skin of SSc patient cohort was carried out to identify enriched genes and intracellular signaling pathways.
Results: Stimulation with dsDNA induced the expression of 382 genes when the gene sets from 6 hours and 24-hours stimulation were intersected. Similarly, viral DNA induced differential expression of 75 genes. Overlaying the intersected gene sets over the published RNA sequencing data from affected skin of SSc patients showed enrichment of cGAS/STING genes. Further analysis identified Type I IFN pathway as one of the major DNA sensing mechanisms in endothelial cells.
Conclusions: We identified cGAS-STING pathway driven Type I IFN immunopathology in human microvascular endothelial cells upon stimulation with dsDNA or BacMam virus. This observation provides a new paradigm for understanding the contribution of type I IFN in SSc vasculopathy and offer therapeutic potential in targeting cGAS/STING pathway in the treatment of early systemic sclerosis.

28. Loss of GATA6 in lung endothelial cells leads to ER stress/UPR and contributes to PAH pathogenesis

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Background
Pulmonary arterial hypertension (PAH) is marked by increase in pulmonary vascular resistance leading to right ventricular failure and death. PAH is characterized by activation of the vascular cells- endothelial cells (ECs) and smooth muscle cells (SMCs) which influences vascular remodeling. Recent studies have shown that vascular remodeling is known to cause ER stress and contribute to proliferation and apoptosis resistance in SMCs. Studies have also shown increased ER stress markers in peripheral blood mononuclear cells. Our group has previously shown that the GATA6 levels are decreased in the pulmonary vasculature of scleroderma (SSc-PAH) and Idiopathic PAH (IPAH) patients. GATA6 is a conserved transcription factor expressed in a wide array of tissues. It is expressed in quiescent SMCs and contributes to the maintenance of its contractile phenotype.

Materials and Methods
Lung tissue samples from Healthy, SSc-PAH and IPAH patients. Human Pulmonary Arterial Endothelial Cells (HPAECs) were treated with GATA6 siRNA and cells were harvested at 24hrs for qPCR analysis. Endothelial specific GATA6 knockout (G6KO) mice were used for in-vivo experiments.

Results
Recent unpublished data from our lab shows significantly elevated expression of key ER stress/UPR markers BiP and CHOP in the lungs of SSc-PAH and IPAH patients. Nitrotyrosine expression is also significantly upregulated in these lungs. In addition, GATA6 knockdown in HPAECs show a significant increase in gene expression of ATF4 and increased gene expression of BiP, CHOP and ATF6. G6KO mice (Ghatnekar et al, 2013 Jun;182(6):2391-406) spontaneously develops PAH. A western blot and gene expression analysis from whole lung lysates of the G6KO mice show a significant elevation in BiP and CHOP. The gene expression analysis from endothelial cells (mPAECS) isolated from these mice also show increased expression of BiP and CHOP. Immunostaining of BiP and CHOP in the mPAECS show an increased nuclear localization of CHOP in G6KO mice.

Conclusions
The persistent elevation of ER stress genes and UPR can lead to further EC dysregulation and contribute to oxidative stress and inflammation which leads to the pathogenesis of PAH. This would suggest that targeting the ER stress pathway could be a potential therapeutic strategy to alleviate PAH.

29. MC_1-mediated pro-senescence therapy for the treatment of systemic sclerosis

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Background:
Systemic sclerosis (SSc) is a chronic autoimmune disease of the connective tissue with a high unmet clinical need. Patients with SSc may develop extensive fibrosis throughout the body, including in the skin, the lungs, the heart, and the gut. One of the key disease drivers are fibroblasts. Recent work from our lab has demonstrated that in another pathology driven by fibroblasts, rheumatoid arthritis, senescence induction via melanocortin 1 receptor (MC_1) activation in synovial fibroblasts resulted in a reduced aggressive phenotype and amelioration of the disease.

Materials and methods:
We investigated if MC_1-induced senescence could be exploited as a *common therapeutic strategy* for fibroblast-mediated autoimmune diseases, like SSc. Dermal fibroblasts derived from SSc patients (SDFs) were treated with BMS-470539, a selective MC_1 drug, and assessed for markers of senescence.

Results:
BMS-treated SDFs exhibited a number of hallmarks of senescence including reduced proliferation rates with increased metabolic activity, prominent beta-galactosidase staining, increased cell and nuclear size, and lysosomal expansion, thereby demonstrating this novel mechanism of senescence induction to be functional in distinct fibroblast populations.

Interestingly, no markers of DNA damage, measured by γH2AX and 53BP1 immunofluorescence, were detected, making this type of GPCR-induced senescence distinct from other previously known forms of senescence. In addition, preliminary data suggest that the pro-fibrotic phenotype of SDFs can been reversed by BMS administration, observed by modulated alpha smooth muscle actin (αSMA) expression, fibroblast migration inhibition, and reduced proliferation. Ongoing studies using the murine model of dermal fibrosis are being undertaken to translate these findings *in vivo*.

Conclusions:

In summary, our data show therapeutic potential for MC_1-induced senescence for the treatment of diseases mediated by aberrantly activated fibroblasts, evidencing the usefulness for *pathway-centred approaches* rather than organ-specific ones (e.g. joint, skin, etc) for the development of innovative therapeutic strategies exploiting shared mechanisms in autoimmune diseases.

30. Galectin-3 upregulation in SSc monocytes cells contributes to fibroblasts activation

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Background: Galectin-3 (Gal-3) is a beta-galactoside binding protein that is dysregulated in serum and skin of SSc patients and may correlate with parameters of skin, lung and heart involvement. Secretion of Gal-3 by macrophages has been implicated in fibrosis, but its specific contribution to SSc pathogenesis has not been elucidated. This study was undertaken to evaluate the profibrotic role of Gal-3 in SSc.
Materials and methods: Levels of Gal-3 were assessed by ELISA in serum, and qPCR and immunofluorescence in monocytes. Primary human dermal fibroblasts were co-cultured with Thp1 cells in the presence or absence of siRNA against Gal-3. Recombinant human Gal-3 was added to cultured dermal fibroblasts. Western blot was used to assess protein levels of collagen, and qPCR to assess profibrotic markers in transfected myeloid cells.
Results: SSc patients had higher expression of galectin-3 in monocytes, and levels of serum Gal-3 inversely correlated with parameters of lung function (FVC, FEV1 and DLCO). Coculture of fibroblasts with myeloid cells potently stimulated collagen deposition by fibroblasts, and this effect was blocked by pretreatment of myeloid cells with siRNA against Gal-3. The addition of Gal-3 to cultured fibroblasts induced upregulation of collagen deposition.
Conclusions: These findings suggest that Gal-3 overexpression by monocytes/macrophages contributes to SSc pathogenesis via profibrotic activation of fibroblasts, suggesting that Gal-3 could be a target for SSc therapy.

31. Restoring serum CXCL5 dampens inflammation and reduces skin thickness in murine systemic sclerosis via targeting neutrophil and macrophage pathways

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Background:
Myeloid cell (neutrophil and macrophage) aggregation and interferon (IFN) signalling have been shown to be upregulated in patients with systemic sclerosis (SSc). CXCL5 is a potent chemoattractant and activator of neutrophils and macrophages. In SSc patients, we found significantly lower serum CXCL5 compared to healthy individuals, and serum CXCL5 was negatively correlated with disease activity (modified Rodnan skin score). We hypothesize that the dysregulation of CXCL5 blood-tissue chemokine gradient (decreased in the circulation and increased in tissue) is a pathogenic mediator for the development of SSc. We aimed to demonstrate that restoration of serum CXCL5 reduced skin thickening in a bleomycin mouse model.

Materials and methods:
A skin fibrosis mouse model was induced using bleomycin. Subcutaneous injection of 0.5 mg/ml of bleomycin was injected to thirty-three 6- to 8-week-old DBA/2J mice at 1cm^2 defined areas on the lower back every other day for 3 weeks. This was followed by intravenous (IV) mouse CXCL5 (3 μg/kg) given twice per week for 3 consecutive weeks. Skin thickness was measured on the formalin-fixed H&E stained sections at 3 weeks post-treatment. Immune and cytokine profile changes were examined by flow cytometry and Luminex assay.

Results:
At 3 weeks post-bleomycin induction, skin thickening (324.7 ± 46.6 μm) and leukocyte infiltration (7 ± 2 per high power field (HPF)) were observed. There was low blood CXCL5, low blood and marrow neutrophils and high monocytes. Following IV CXCL5 administration, endogenous blood CXCL5 levels were restored, which persisted 7-10 days after each injection. At 3 weeks post CXCL5 treatment, there was reduction in skin thickness (253.6 ± 51.0 μm, $p < 0.01$) and leukocyte skin infiltration (2 ± 2 per HPF, $p < 0.05$). There was concomitant increase in blood and marrow neutrophils and decrease in monocytes ($p < 0.05$). This rebalance of the dysregulated immunity was supported by the decreased circulating pro-inflammatory cytokines (IL-1β, IL-2, IL-16 and IFN-γ) and chemokines (CXCL1, CXCL10, CXCL11, CCL2, CCL3, CCL4 and CCL19) as well.

Conclusions:
Restoring serum CXCL5 may represent a novel neutrophil/macrophage-targeting therapy for SSc.

32. Autoantibodies from Systemic Sclerosis patients targeting the angiotensin II type 1 and endothelin-1 type A receptor induce endothelial cell activation and pro-fibrotic responses

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3LUMC, Department of Internal Medicine (Nephrology), Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden, The Netherlands
4 University of Lübeck, Department of Rheumatology and Clinical Immunology, Lübeck, Germany.

Background: Systemic Sclerosis (SSc) is a heterogenous and potentially lethal autoimmune disease hallmarked by dysregulated immunity, vasculopathy and fibrosis. Autoantibodies directed against the angiotensin-II type -1 receptor (AT1R) and endothelin-1 type-A receptor (ETaR) are present in most patients with SSc and are associated with more severe disease

complications. AT1R and ETaR are expressed by endothelial cells (ECs), providing a potential direct link between two major pathophysiological features in SSc. Here, we aim to identify and understand autoantibody-induced endothelial cell damage in SSc.
Materials and methods: IgG of SSc patients and healthy controls (HC) was isolated and AT1R- and ETaR-mediated effects on ECs were analyzed using specific receptor antagonists. Cytokine responses induced by SSc-derived AT1R and ETaR autoantibody binding to ECs were measured by interleukin-6 (IL-6), IL-8 and transforming growth factor- β (TGF-β) ELISA. EC activation was assessed by RT-qPCR and correlated to the levels of AT1R- and ETaR antibodies measured in serum. AT1R- and ETaR autoantibody interactions with their target receptors were analyzed using a label-free whole cell xCELLigence approach.
Results: We identified that SSc-derived IgG induced upregulation of the EC activation markers monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin compared to HC IgG in an AT1R- and ETaR-mediated fashion. SSc-derived IgG induced AT1R- and ETaR-mediated expression of IL-6, IL-8 and TGF-β, which was not observed with HC IgG. Although higher levels of both autoantibodies were associated with a stronger EC responses, we did not observe EC activation in all patients with AT1R and ETaR antibodies. Of interest, xCELLigence data showed that AT1R- and ETaR autoantibodies can provide opposite signals in the ECs upon receptor binding, which can in part explain the observation that we did not observe EC activation in all patients expressing high levels of AT1R and ETaR antibodies.
Conclusions: We have shown that SSc-derived AT1R- and ETaR-targeting autoantibodies impact ECs resulting in EC activation and pro-inflammatory and pro-fibrotic cytokine release and that these antibodies can have opposite functional effects upon receptor binding. We have identified a direct pathophysiological mechanism linking AT1R- and ETaR-targeting autoantibodies to EC activation and pro-fibrotic cellular responses in SSc.

33. Rituximab and tocilizumab and their effect on lung disease progression in scleroderma. A retrospective cohort study at a single centre

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Background:
Interstitial lung disease (ILD) is one of the leading causes of mortality in systemic sclerosis (SSc). Emerging evidence suggests a beneficial effect of tocilizumab and rituximab on lung function however comparative data outside the arena of clinical trials remains limited. Deciding when and who to treat with specific treatments remains challenging.

Materials and methods:
We performed a retrospective analysis of all SSc patients attending a single specialist centre who had received rituximab, tocilizumab or both treatments. Demographic, clinical and laboratory data was collected along with lung function. Patients were excluded if lung function pre and/or post therapy was not available. Data was analysed based on anti-topoisomerase I antibody (ATA) status. Wilcoxon T test and Fishers exact test were used.

Results:
129 patients were included, of which 87 received rituximab, 32 tocilizumab and 10 both therapies. 8 of 10 (80%) patients who had received both therapies received rituximab prior to tocilizumab. 76 (58.9%) patients had diffuse SSc and 102 (79%) had ILD. Concurrent mycophenolate mofetil (MMF) was prescribed for 52 (53.6%) patients with rituximab and 17 (40.5%) patients with tocilizumab. Median pre-treatment lung function percentage forced

vital capacity (%FVC) and percentage transfer factor (%DLCO) were 67.9% and 42.5% and 85.5% and 59.8% for rituximab and tocilizumab respectively. Median change %FVC and %DLCO pre- and post-treatment were +0.35% and -0.8% for rituximab and -0.9% and +0.2% for tocilizumab. The majority of patients improved or remained stable with either treatment and fewer patients on rituximab demonstrated FVC decline compared with tocilizumab (Figure 1). The effect from rituximab was not affected by ATA status in contrast, ATA positive patients were more likely to respond to tocilizumab ($p = 0.0073$) (Median FVC change: tocilizumab; + 60ml ATA positive vs -110ml ATA negative, rituximab; 0ml ATA positive vs 0ml ATA negative, Figure 2). Disease duration and CRP had no effect on treatment response for either therapy.

Conclusions

Our retrospective cohort provides real-life data supporting the use of both rituximab and tocilizumab to stabilise ILD in SSc. Differential response based on autoantibody specificity and clinical parameters may help optimise patient selection for biological therapy in SSc-ILD.

Figure 1. Change in absolute %FVC pre and post-treatment with rituximab or tocilizumab

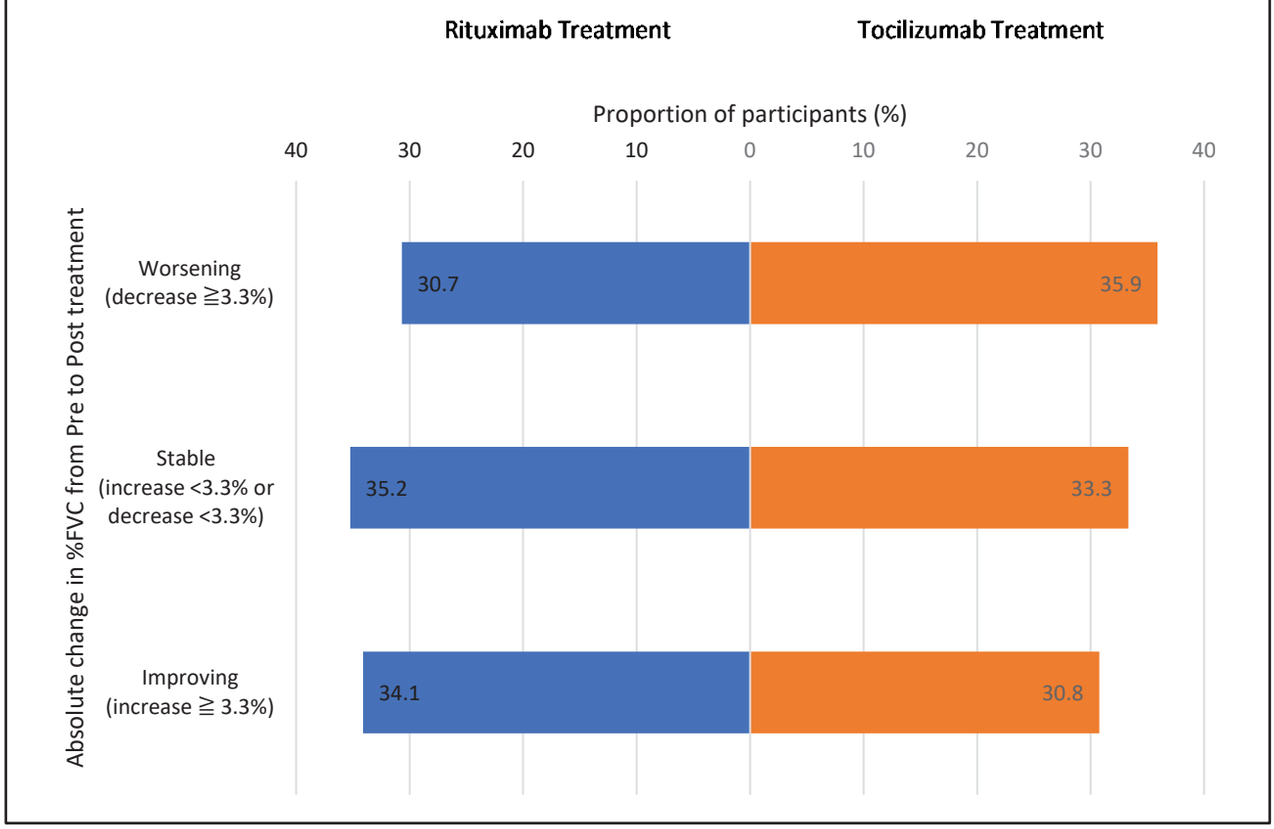
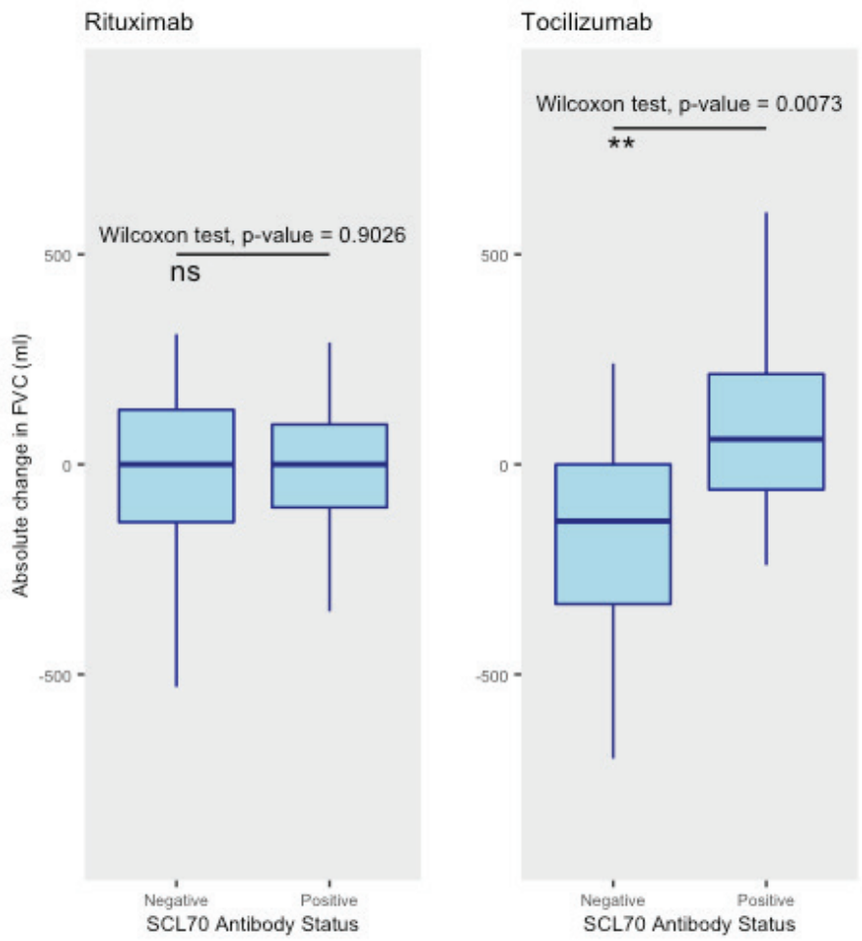


Figure 2: Absolute change in FVC (ml) for rituximab and tocilizumab treatment based on ATA antibody status



34. Investigating the role of YAP and mechanosensing to identify new drug targets in skin fibrosis

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Background:
The mechanical properties of the extracellular matrix (ECM) profoundly influence fibroblast behaviour by promoting the nuclear translocation of transcriptional coactivators such as the Yes-Associated protein (YAP), licencing the transcription of genes supporting ECM production and contractility. Canonical control of YAP is mediated via the Hippo pathway, which promotes degradation and cytoplasmic sequestration through YAP phosphorylation. A YAP signature is easily observed during wound healing and in fibrotic tissues. Recently, poorly understood, parallel, non-canonical mechanotransduction pathways, such as the Rho/Rac pathway and the NUAK kinases have emerged as the dominant regulators in mesenchymal cells.

Materials and methods:
Primary dermal fibroblasts seeded in hydrogels of defined stiffness and stimulated with/without TGFβ or serum, were used to assess mechanisms of YAP activation. YAP localization was assessed via immunofluorescence and YAP transcriptional activity was determined using a luciferase reporter gene cell line. The reporter cell line was also used to screen a focused small molecule library of potential YAP modulators. Pathways implicated in YAP function were inhibited with specific small molecules or RNA interference: Rho/Rac (Rhosin, EHT1864), SRC/cAbl (Dasatinib and PD173955), F-actin (Latrunculin B), FAK (PF573228), NUAK1/2 (HTH-01-015 and WZ4003, siRNA) and Hippo (siLATS1/2).

Results:
In dermal fibroblasts, YAP nuclear localisation is primarily controlled by mechanical stiffness and matrix attachment. YAP was strongly inhibited by F-actin depolymerization, driving YAP completely out of the nucleus in healthy, but not in scleroderma fibroblasts. Silencing LATS1/2 had no effect in attached fibroblasts, irrespective of matrix stiffness, but partially blocked the effects of actin depolymerization. NUAK1/2 inhibitors promoted YAP deactivation and reduced cytoskeletal organization while Rho/Rac inhibitors had only a modest impact. Quiescent fibroblasts expressed mostly NUAK2 and suppressed NUAK1. Fibroblasts stimulated with TGFβ, activated YAP, downregulated NUAK2 and upregulated NUAK1. Our screen identified SRC/c-Abl as an upstream YAP regulator. It was also confirmed that SRC/c-ABL lies upstream of NUAK1.

Conclusion:
The canonical Hippo pathway is deactivated upon matrix attachment in dermal fibroblasts. Instead, NUAK1 becomes the focal point of YAP control. Serum components, TGFβ and other growth factors activate NUAK1 via SRC/c-ABL, an insight that can be harnessed therapeutically.

35. Troponin I levels in systemic sclerosis patients with myocardial involvement

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345/350 words

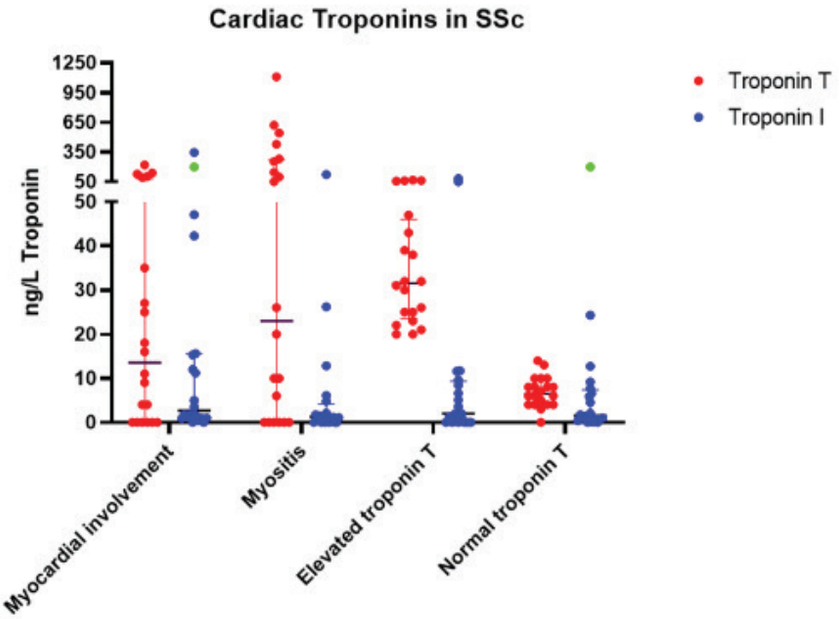
Background: As primary myocardial involvement in systemic sclerosis (SSc) is associated with worse prognosis, diagnostic tools for early recognition are essential. Troponin I has

been reported to be more specific for presence of myocardial damage than Troponin T (Hughes et al. 2015). This study aimed to evaluate the value of Troponin I to determine SSc-related myocardial involvement.

Methods: A cross-sectional observational study was performed, including four groups of 20 patients from the Leiden Comprehensive Care in SSc (CCISS) cohort: 1) SSc-patients with primary myocardial involvement, 2) SSc-patients with skeletal muscle myositis, 3) SSc-patients with elevated troponin T and CK levels without cardiac involvement, and 4) SSc-patients with normal troponin T and no organ involvement. Presence of primary myocardial involvement was determined by two experienced physicians independently (JVR and NAM) based on standardized extensive evaluation including CK, troponin T, proBNP, echocardiography, 24h rhythm registration and, preferably, MRI. Troponin I levels were measured using RayBio® Human Cardiac Troponin I enzyme-linked immunosorbent assay (ELISA) Kit, and compared between the groups using Mann-Whitney U and Kruskal-Wallis tests. Spearman's rank was performed to assess correlation between Troponin T and I.

Results: Mean age was 56.2 (SD:12.7) years, 61% was female. SSc-patients with myocardial involvement more often had diffuse cutaneous SSc (80% vs 33%, p<0.001) and a cardiovascular history (35% vs 12%, p=0.017), and less often anti-centromere antibodies (0% vs. 35%, p=0.002) compared to SSc-patients without myocardial involvement.
Median troponin I levels were not significantly higher in patients with myocardial involvement compared to patients without myocardial involvement (group 1: 2.7 ng/L [IQR:0.5 – 15.3] vs. group 2-4: 1.2 ng/L [IQR:0.1 – 6.6], p=0.117; Figure 1).
In both SSc patients with and without myocardial involvement, Troponin I levels and troponin T levels were not correlated (respectively: ρ:-0.051, p=0.864; and ρ:-0.049, p=0.729).

Conclusion: Based on our observations in SSc, troponin I is not more specific for primary myocardial involvement in SSc than troponin T. Currently, additional cases and healthy controls from our cohort are being tested to determine whether the combination of elevated troponin T and I can help to recognize SSc-patients with myocardial involvement.
Figure 1 Troponin I and troponin T in SSc patients.
• = value higher than could be measured with the used dilution.



36. Novel noncoding Y RNA drug attenuates fibrosis and inflammation in systemic sclerosis

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Background: Current therapy for scleroderma only mitigates symptoms with no available curative options. We have previously demonstrated anti-fibrotic and anti-inflammatory properties of EV-YF1, a small Y RNA abundant in extracellular vesicles. Here we describe the therapeutic bioactivity of a shorter, engineered derivative, TY4.
Materials and methods: TY4, a 24-nucleotide chemically-modified oligonucleotide bioinspired by EV-YF1, was synthesized and packaged into lipid nanoparticles. To create a model of scleroderma, C57BL/6J wild type mice (n=100 total) were injected with 100 µL bleomycin or vehicle (control) or subcutaneously every other day for 3 weeks. Animals were then randomized to receive vehicle (0.9% NaCl), TY4 scrambled (as an RNA control) or TY4. Endpoints include body weight, exercise capacity, histologic and hydroxyproline quantification of fibrosis in skin, and inflammatory markers by *qRT-PCR*.
Results: Four weeks after randomization, animals receiving TY4 demonstrated body weight recovery ($p<0.0001$) and augmented exercise capacity ($p<0.0002$) compared to vehicle and TY4 scrambled. Similarly, dermal thickness was attenuated after TY4 administration (control 195±23 µm, TY4 287 ± 45 µm, TY4 scrambled 426 ±75 µm , vehicle 419 ±116 µm $p<0.0001$) as was hydroxyproline concentration (control 0.04±0.01 µg/µl, TY4 0.02 ± 0.008 µg/µl, TY4 scrambled 0.11 ±0.05 µg/µl , vehicle 0.14 ±0.04 µg/µl $p=0.0005$). Finally, the expression of dermal inflammatory cytokines *IL-1, IL-6, IL-4*, measured by *qRT-PCR*, was diminished after TY4 administration ($p<0.0001$).
Conclusion: These results demonstrate the therapeutic bioactivity of a bioinspired new chemical entity with the potential to become a synthetic, off-the-shelf drug for scleroderma.

37. Casein kinase 2 inhibition mediates the metabolic reprogramming of pulmonary arterial smooth muscle cells in pulmonary arterial hypertension

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Background
Pulmonary arterial hypertension (PAH) is associated with many diseases including connective tissue diseases such as scleroderma (SSc). PAH involves extensive remodeling of distal pulmonary arteries. Underpinning this remodeling is the phenotypic and metabolic reprogramming of pulmonary artery smooth muscle cells (PASMCs), giving rise to hyperproliferative cells. Casein kinase 2 (CK2) is known to be involved in tumor development, including the Warburg effect characteristic of cancer cells by regulating key glycolytic enzymes. PAH-PASMCs exhibit similar metabolic changes to cancer cells, but the contribution of CK2 to these changes has not been investigated. Therefore, this study aims to investigate the role of CK2 in the metabolic and phenotypic reprogramming of PASMCs.

Materials and methods
Primary PASMCs from idiopathic PAH and healthy donors (HC) were used. PASMCs were cultured under ‘contractile’ (0.1%FBS), or ‘proliferative/synthetic’ conditions (10% FBS) and treated in the presence/absence of the CK2 inhibitor, CX-4549 (10mg/ml). PASMC phenotypic modulation was assessed using protein expression of synthetic and contractile markers. ATP production was measured using the XFp Real-time ATP Rate assay. Proliferation rates were assessed using the Presto blue cell viability assay. Mitochondrial respiration and glycolysis, after CK2 inhibition were assessed using the Seahorse XFp Mito-

Stress Test which measures oxygen consumption rate (OCR) and Glycolysis Stress Test which measures the Extracellular Acidification Rate (ECAR).

Results
Phenotypic modulation was confirmed *in vitro*, indicating the proliferative phenotype of all PAH patient PASMC cell-lines. At 0.1%FBS, HC-PASMC generate ATP mostly via mitochondrial respiration (52%) whereas PAH-PASMC mostly use glycolysis (60%). ATP production was mostly via glycolysis for both HC (61%) and PAH (63%) PAMSCs in 10%FBS. CX4945 treatment resulted in significantly reduced proliferation rates of PAH-PASMCs and inhibition of the proliferative/synthetic phenotype indicated by reductions of associated markers such as COL1. CX4945 treatment also significantly deceased mitochondrial basal and maximal respiration of PAH-PASMC by 71.3% and 49.3% respectively as well as their glycolytic capacity by 38.9% ($p<0.05$). CX4945 has no significant effect on the metabolic parameters of HC cells.

Conclusions
CK2 plays a key regulatory role in the phenotypic and metabolic reprogramming of PASMCs in PAH and can be targeted as a novel therapy.

38. Effect of nintedanib in patients with systemic sclerosis-associated interstitial lung disease (SSc-ILD) and risk factors for rapid decline in forced vital capacity: further analyses of the SENSCIS trial

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Background: In the SENSCIS trial conducted in a broad population of subjects with SSc-ILD, nintedanib reduced the rate of decline in FVC (mL/year) over 52 weeks by 44% versus placebo. Risk factors for a rapid decline in FVC in patients with SSc include early SSc, elevated inflammatory markers, significant skin involvement, and diffuse cutaneous SSc (dcSSc).
We analysed the rate of decline in FVC, and the effect of nintedanib on FVC decline, in subjects with risk factors for a rapid decline in FVC in the SENSCIS trial.

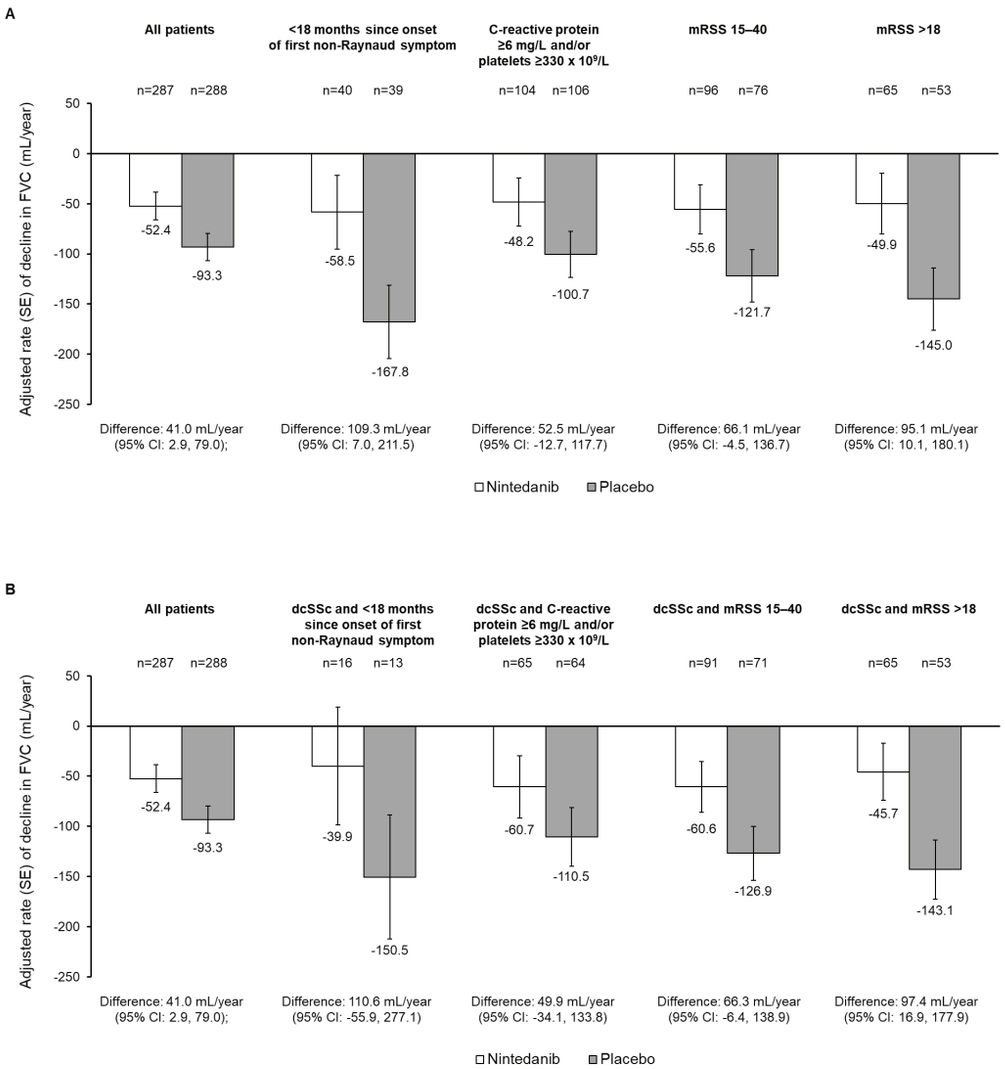
Materials and Methods: In post-hoc analyses, we analysed the rate of decline in FVC (mL/year) over 52 weeks in all subjects and in those with early SSc (<18 months since first non-Raynaud symptom), elevated inflammatory markers (C-reactive protein ≥6 mg/L and/or platelets ≥330 x 10^9/L), or significant skin fibrosis using two approaches (modified Rodnan

skin score [mRSS] 15-40 or mRSS >18) at baseline. We also analysed the rate of decline in FVC over 52 weeks in subjects with one of these risk factors and dcSSc.

Results: Of 575 subjects analysed, 79 (13.7%) had <18 months since first non-Raynaud symptom, 210 (36.5%) had elevated inflammatory markers, 172 (29.9%) had mRSS 15-40 and 118 (20.5%) had mRSS >18. Of 299 subjects with dcSSc, 29 (9.7%) had <18 months since onset of first non-Raynaud symptom, 129 (43.1%) had elevated inflammatory markers, 162 (54.2%) had mRSS 15-40 and 118 (39.5%) had mRSS >18. In the placebo group, the rate of decline in FVC over 52 weeks was numerically greater in subjects with these risk factors for rapid decline in FVC compared with all subjects (Figure). Across the subgroups, the rate of decline in FVC was numerically lower in subjects treated with nintedanib than placebo (Figure).

Conclusions: In the placebo group of the SENSCIS trial, subjects with risk factors for a rapid decline in FVC had a more rapid decline in FVC over 52 weeks than the overall trial population. By targeting fibrosis, the rate of decline in FVC in patients with risk factors for FVC decline was reduced in patients treated with nintedanib compared with placebo.

Figure. Rate of decline in FVC (mL/year) over 52 weeks in (A) all patients and in patients with risk factors for rapid decline in FVC at baseline and (B) all patients and in patients with dcSSc and risk factors for rapid decline in FVC at baseline in the SENSCIS trial.



39. Nationwide Distribution of Systemic Sclerosis (SSc) and Other Connective Tissue Disease (OCTD) in Areas of Environmental Concern

Noelle N. Kosarek[1,2], Megan E. Romano[3], Erika Moen[1], Patricia A. Pioli[4], Michael L. Whitfield[1,2]

[1]Departments of Biomedical Data Science, [2]Molecular and Systems Biology, [3]Epidemiology, [4]Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Lebanon NH 03756

Systemic sclerosis (SSc) is a rare autoimmune disease characterized by fibrosis of the skin and internal organs, vascular abnormalities, and autoantibody formation. The etiology of SSc is unknown, but has been hypothesized to result from environmental exposures in genetically predisposed individuals. Occupational, bacterial, and viral factors have been cited as potential environmental triggers of SSc. We sought to test the hypothesis that SSc and other connective tissues disease have a higher incidence at or around sites environmental exposures by analyzing the geographic distribution of these individuals. Secondarily, we wanted to ask if SSc had a random or non-random geographic distribution across the US. We selected individuals from the US Medicare database that had diagnostic codes associated with SSc and other connective tissue disease. Our study population consisted of 207,467 OCTD beneficiaries between the years 2014 and 2018. Our cohort is 68.2% female and 86.8% White. 29.8% of the beneficiaries in our cohort resided in the Southeastern US. Of the 207,467 OCTD beneficiaries, 55,696 had diagnostics codes associated with SSc. We identified statistically significant clusters of Other Connective Tissue Disease (OCTD) and SSc in Wisconsin, Michigan, Ohio, New York, Georgia, Mississippi, Nebraska, New Jersey, and Kansas using global spatial autocorrelation (Figure 1). We overlaid these clusters with superfund sites and found that a significant number of these clusters occurred in zip codes containing at least one superfund site. These locations are found in Mississippi, New York, Wisconsin, and New Jersey. We accessed contaminant information at these sites through the Environmental Protection Agency (EPA) website and compiled a list of chemicals found in sites enriched for OCTD and SSc patients. These contaminants include vinyl chloride, 1,2 dichloroethane, and other volatile organic compounds. Further concentrated studies at these locations are necessary to describe the complexity of these cases.

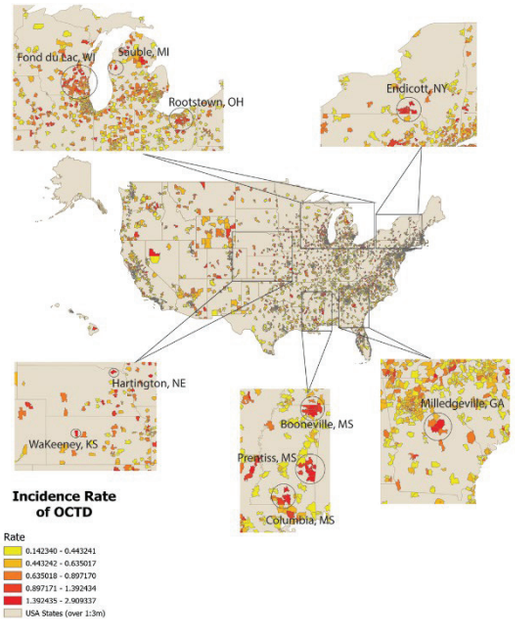


Figure 1: Distribution of the incidence rate of Medicare beneficiaries carrying a diagnosis of OCTD by zip code between the years 2014 and 2018. We noted statistically significant clusters in Wisconsin, Michigan, Ohio, New York, Georgia, Mississippi, Nebraska, and Kansas.

40. Distinct Fibroblast Populations in a Systemic Sclerosis (SSc) 3D Skin Model with Single Cell Omics.

Noelle N. Kosarek[1,2], Tamar R. Abel[1,2], Avi Smith[4], Mengqi Huang[4], Tammara A. Wood[1,2], Jonathan Garlick[4], Patricia A. Pioli[3], Michael L. Whitfield[1,2]

[1] Department of Biomedical Data Science, [2] Department of Molecular and Systems Biology, [3] Departmental of Microbiology and Immunology, Geisel school of Medicine at Dartmouth, Lebanon, NH 03756, USA [4] Department of Diagnostic Sciences, Tufts School of Dental Medicine, Tufts School of Medicine, Tufts Graduate School of Biomedical Sciences. Boston, MA 02111, USA

Systemic sclerosis (SSc) is a rare autoimmune disease characterized by fibrosis of the skin. Interactions between macrophages, fibroblasts, and keratinocytes are thought to contribute to excess collagen deposition and inflammation in SSc. Here we show that distinct fibroblast populations can be recapitulated in vitro using SSc 3D skin-like tissues. We constructed SSc and control 3D skin-like tissues using fibroblasts, monocytes, keratinocytes, and plasma from SSc patients or healthy donors. Single cell RNA-sequencing (scRNA-seq) was performed, and data was analyzed in Seurat. Fibroblasts differentiated into four distinct subpopulations in the presence of macrophages and keratinocytes. Two of these fibroblast subpopulations exhibited positive proportional differences in SSc samples compared to healthy controls. Differential pathway analysis revealed populations with high expression of gene sets associated with SSc pathogenesis. Comparison to scRNA-seq data from cells in 2D culture and from SSc and control skin biopsies shows that the 3D tissue model captures a subset of the cell populations observed in human skin. Our results suggest there is a plasticity to fibroblast cell populations that require cell-cell, cell-matrix, and circulating factors to maintain their phenotype in culture that is captured in the saSE 3D tissue model.

41. The CRISTAL Project: First steps towards the creation of an FDA-acceptable Combined Response Index for Scleroderma Trials Assessing Limited SSc

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Background:

There is a lack of relevant outcome measures to comprehensively evaluate limited cutaneous systemic sclerosis (lcSSc). The overarching objective of this international project is to create a combined response index for the assessment of lcSSc patients in clinical trials (the CRISTAL index: Combined Response Index for Scleroderma Trials Assessing Limited SSc).

Materials & Methods:

The most bothersome symptoms from the patients' perspective were identified using 4 lcSSc patient-centered focus groups. An international scoping literature review (SLR) allowed an identification of existing domains and items from the literature. A two-round international Delphi exercise was performed to enrich the list of instruments. Each expert was randomly assigned to up to three domains and experts were asked to add additional core set items (or outcome measures) to the domains they had been assigned to.

Results:

15 domains were identified as bothersome from the patients' perspective in the focus groups: 10 SSc-specific domains (Gastro-intestinal involvement, Raynaud's (and microcirculation), musculoskeletal, digital ulcers, pulmonary involvement, cardiac involvement, hand functioning, calcinosis, skin

involvement, Sicca syndrome ; and 5 general domains (Fatigue, Pain, Cognition, sleep disorders and health related quality of life).

The scoping literature review (SLR) included 3652 abstracts at screening and 270 articles for data extraction. 459 instruments among 26 domains were identified, including 164 instruments among the domains considered bothersome from the patients' perspective.

100 experts were invited to the Delphi exercise. 71 participants (71%) provided answers for at least one survey. The participants who provided answers in round 1 were invited to a second Delphi exercise to rate each item on a scale ranging from 1 to 9, for feasibility, Face validity, Content validity and Overall appropriateness for the CRISTAL Index. 59 participants (83%) provided answers to at least one survey.

The results of this Delphi Exercise will inform an upcoming nominal group technique exercise for the selection of clinician reported outcomes. The selection of patient reported outcomes will be based on cognitive debriefing with patient partners.

Conclusion:

These initial results from the CRISTAL initiative are the necessary first steps for the construction of an FDA-acceptable composite index for lcSSc.

42. Time between Raynaud's Phenomenon (RP) and non-RP in systemic sclerosis and its association with clinical characteristics and anti-topoisomerase I antibody response.

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350/350 words

Background: Autoantibodies specific for systemic sclerosis (SSc), including anti-topoisomerase antibodies (ATA), are important predictors for developing definite SSc. Progression from very early SSc to definite SSc is usually due to developing the first non-RP symptom. Very early ATA+ SSc-patients are difficult to identify, however, probably due to less time between RP and non-RP. Therefore, we used time between onset of RP and first non-RP symptom as proxy for progression to definite SSc. We hypothesized that if the ATA-response contributes to the progression of SSc, this would be reflected by higher ATA-levels in patients with less time between RP and first non-RP. Therefore, we investigated the association between clinical characteristics and ATA-response, and time between RP to first non-RP symptom in SSc-patients.

Methods: One-hundred-sixty-three ATA-IgG+ SSc-patients, fulfilling ACR/EULAR 2013 criteria, were included from Leiden CCISS-cohort. ATA-IgG/IgM/IgA levels were assessed at cohort entry. Rate of disease progression was determined as months between RP and first non-RP symptom and patients were categorized based on its terciles: 1)<=3 (n=55), 2)4–24 (n=54), 3)25–558 (n=52). Disease characteristics at cohort entry were compared between groups and correlation between progression rate and ATA-levels was analyzed using Spearman-rho. Time between RP and non-RP was compared between SSc-patients with high (>=1000) and low (<1000) ATA-IgG levels using Kruskal-Wallis-tests.

Results: At cohort entry, SSc-patients with <=3months between RP and non-RP were more often male compared to patients with 4–24months and 25–558 months (respectively:47%, 26% and 31%,p=0.035), had higher skin scores (respectively:9 [5–17], 5 [2–14] and 4 [2–7],p=0.002), and more often diffuse cutaneous SSc (respectively:53%, 52% and 23%,p=0.004). Only ATA-IgG levels were correlated with time between RP and non-RP (p=0.04; Figure 1), while ATA-IgM/IgA levels were not. Patients with ATA-IgG levels >=1000 had less time between RP and non-RP compared with patients with ATA-IgG levels <1000 (12 [1–65] vs. 7 [0–24],p=0.089).

Conclusion: SSc-patients with shorter time between RP and first non-RP symptom have higher ATA-IgG levels, potentially indicating that ATA-specific responses are associated with progression from very early SSc to definite SSc. Longitudinal studies in very early SSc are needed to confirm this.

Figure 1. Anti-topoisomerase I levels in SSc patients categorized in groups using time between Raynaud's phenomenon (RP) and first non-RP symptom

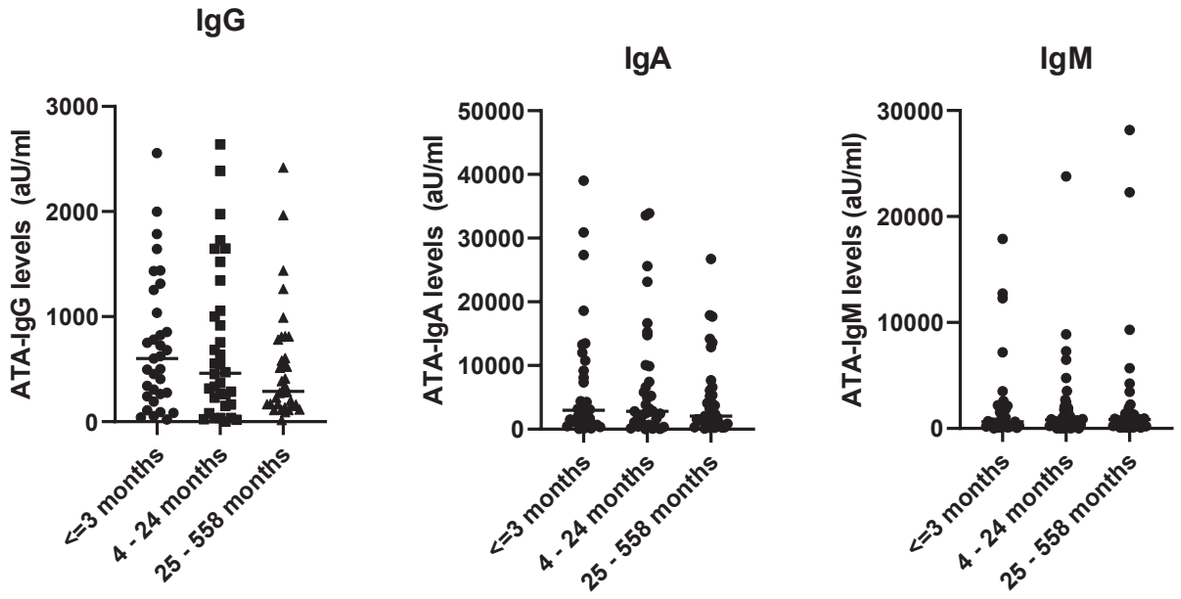

Table 1 Baseline characteristics of included ATA+ SSc patients

	Total: n=163	**Δ <= 3 months n=55**	**Δ 4 – 24 months n=54**	**Δ 25 – 558 months n=52**	**P-value**
Age, mean (SD)	52 (15)	53 (15)	52 (13)	51 (16)	0.848
Female, n (%)	106 (65%)	29 (53%)	40 (74%)	36 (69%)	0.035
Years follow-up, median (min – max)	3 (0 – 11)	3 (0 – 11)	5 (0 – 11)	3 (0 – 10)	0.105
Modified Rodnan Skin Score	6 (2 – 12)	9 (5 – 17)	5 (2 – 14)	4 (2 – 7)	0.002
Limited cutaneous SSc, n (%)	79 (48%)	22 (40%)	19 (35%)	36 (69%)	0.004
Diffuse cutaneous SSc, n (%)	70 (43%)	29 (53%)	28 (52%)	12 (23%)	
Digital ulcers, n (%)	21 (13%)	8 (15%)	7 (13%)	6 (12%)	0.879
ILD on HRCT, n (%)	103 (63%)	35 (64%)	19 (63%)	33 (64%)	0.690
ILD combined*, n (%)	41 (25%)	17 (31%)	15 (28%)	8 (15%)	0.158
ATA-IgG, median (IQR)	289 (169 – 889)	551 (253 – 1146)	470 (264 – 1056)	289 (164 – 780)	0.404
ATA-IgM, median (IQR)	829 (278 – 2156)	597 (251 – 2168)	869 (326 – 2042)	829 (278 – 1401)	0.673
ATA-IgA, median (IQR)	2072 (907 – 7900)	2656 (924 – 8637)	2851 (959 – 10071)	2072 (852 – 6086)	0.960

N: number; SD: standard deviation; SSc: systemic sclerosis; ILD: interstitial lung disease
*ILD combined: ILD present on HRCT and forced vital capacity or diffuse capacity for carbon monoxide of less than 80% of predicted

43. An association of anti-carbamylated protein antibodies and skin involvement in patients with systemic sclerosis: does it exist?

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*Both authors contributed equally
331/350 words

Background: Carbamylation is a post-translational modification of proteins that is practically irreversible and can be triggered by inflammation. Previous studies showed presence of antibodies against carbamylated proteins (anti-CarP) in systemic sclerosis (SSc) and suggested an inverse correlation between levels of anti-CarP IgG antibodies and extent of skin involvement in SSc. The mechanisms underlying this observation are unclear. This study aimed to evaluate all anti-CarP isotypes and associate these with clinical characteristics in SSc-patients.

Methods: Sera of 194 SSc-patients from the Leiden Comprehensive Care in SSc (CCISS) cohort, fulfilling ACR/EULAR 2013 criteria and with a clinical diagnosis of SSc, 83 other connective tissue diseases/Raynaud's Phenomenon, 24 rheumatoid arthritis and 98 age- and sex-matched healthy controls were tested for the presence of anti-CarP antibodies, determined by ELISA. Data on clinical characteristics were gathered at the same moment as the samples.

Results: The SSc-patients were 55 (SD:13) years old and 155 (80%) were female. Forty-four (23%) patients had anti-topoisomerase and 80 (42%) anti-centromere antibodies. The median modified Rodnan Skin Score (mRSS) was 2 (range:0–47); 39 (20%) patients had diffuse cutaneous SSc and 12 (6%) synovitis.

No differences in anti-CarP levels were observed between SSc-patients and healthy controls. 15 (8%) patients were positive for anti-CarP IgG, 16 (8%) for anti-CarP IgA, and 36 (19%) for anti-CarP IgM. There were no significant correlations between age and levels of the different anti-CarP isotypes. Anti-CarP IgA levels were significantly different between ATA and ACA positive SSc patients (ATA:616 aU/ml [359-1103]; ACA:424 aU/ml [300-673], p=0.015). A weak correlation between anti-CarP IgG levels and mRSS in the SSc-patients was found (r=0.141, p=0.049), but not for anti-CarP IgM and IgA levels. Characteristics between anti-CarP isotype positive and negative SSc-patients were comparable except for a higher mRSS score for anti-CarP IgG (Table 2).

Conclusion: All anti-CarP isotypes are found in SSc but their prevalence is comparable to healthy controls. No clear associations between skin involvement and presence of anti-CarP antibodies was found. Therefore, it is not useful as biomarker in SSc.

Table 1. Characteristics between anti-CarP isotype positive and negative SSc patients

Table 2. Characteristics between anti-CarP isotype positive and negative SSc patients

	IgG+ N=15	IgG- N=179	IgA+ N=16	IgA- N=178	IgM+ N=36	IgM- N=158
Female, n (%)	13 (87%)	142 (80%)	10 (63%)	145 (82%)	31 (86%)	124 (79%)
Age (years), mean (SD)	52 (16)	55 (13)	57 (10)	55 (14)	55 (15)	55 (13)
Disease duration (months), median (IQR)	92 (40 – 160)	43 (22 – 122)	45 (20 – 70)	46 (24 – 138)	46 (22 – 98)	45 (23 – 136)

Anti-topoisomerase antibodies, n (%)	5 (33%)	39 (22%)	7 (44%)	37 (21%)	11 (31%)	33 (22%)
Anti-centromere antibodies, n (%)	5 (33%)	75 (43%)	5 (31%)	75 (43%)	18 (50%)	62 40%)
Modified Rodnan Skin Score, median (IQR)	7 (0 – 10)*	2 (0 – 6)*	3 (0 – 6)	2 (0 – 6)	2 (0 – 6)	3 (1 – 8)
Disease subset, n (%)						
• Non cutaneous	2 (13%)	34 (19%)	3 (19%)	33 (19%)	6 (17%)	30 (19%)
• Limited cutaneous	8 (53%)	111 (62%)	9 (56%)	110 (62%)	22 (61%)	97 (61%)
• Diffuse cutaneous	5 (33%)	34 (19%)	4 (25%)	35 (20%)	8 (20%)	31 (20%)
Synovitis, n (%)	1 (7%)	11 (6%)	1 (6%)	11 (6%)	3 (8%)	9 (6%)

Anti-CarP: anti-carbamylated protein antibodies; SSc: systemic sclerosis; N: number; SD: standard deviation; IQR: interquartile range

*statistically significant different between anti-CarP isotype positive patients compared to anti-CarP isotype negative patients

44. A decade of caring for SSc patients from the Leiden CCISS Cohort: do outcomes improve?

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340/350 words

Background: Since 2009, Combined Care in Systemic Sclerosis (CCISS) is a prospective cohort of patients referred to Leiden University Medical Center for Raynaud's Phenomenon (RP), (suspected) systemic sclerosis (SSc) or connective tissue disease. This cohort is characterized by its standardized annual follow-up. Using this data, this study aimed to determine whether disease characteristics and mortality of SSc patients change over time, with cohort entry year as instrumental variable, for all patients, and male and female patients separately.

Methods: 643 SSc-patients fulfilling ACR/EULAR 2013 SSc criteria, were included, and categorized into three groups based on cohort entry year: 1) 2010–2013 (n=229,36%), 2) 2014–2017 (n=207,32%), and 3) 2018–2021 (n=207,32%). Characteristics included disease duration (defined by months since RP, first non-RP symptom and first date of diagnosis by a physician, and months RP and first non-RP symptom), interstitial lung disease (ILD), pulmonary arterial hypertension, digital ulcers (DU), diffuse cutaneous SSc, anti-topoisomerase and anticentromere antibodies, and quality of life. Characteristics were compared between cohort entry groups, using appropriate tests. Moreover, survival was evaluated between the groups for a three-year follow-up period.

Results: There were significantly more females in the 2010–2013 group compared to the 2014–2017 and 2018–2021 group (Table 1). Over time, RP, non-RP and diagnosis duration decreased (Table 1). The proportion of patients presenting with ILD and DU was highest in group 2010-2013 (Table 1). The prevalence of ILD was higher in male and ATA+ SSc patients, and decreased over the cohort-entry groups. The proportion of female SSc patients with cardiac involvement at cohort entry increased over

time, whereas the proportion of males decreased. Especially in ACA+ SSc patients, the proportion of patients presenting with DU decreased over time.
Three-year survival for the total group was similar between the cohort entry groups but the survival was worse in male compared to female SSc patients (Figure 1).

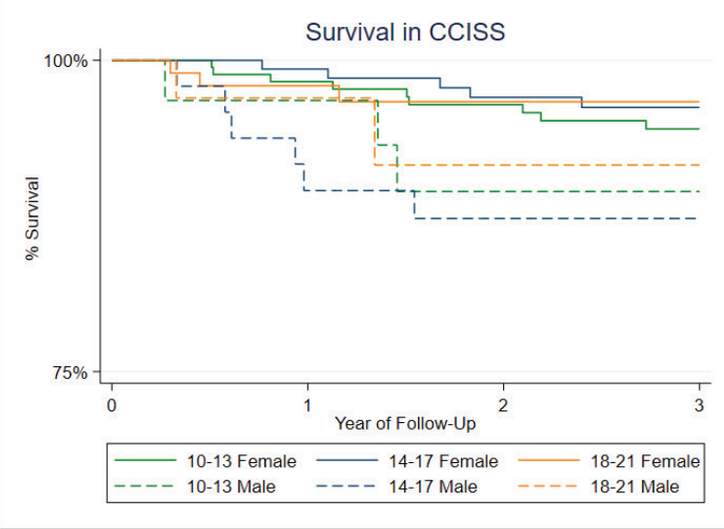
Conclusion: Despite earlier recognition and increased of drugs that have proven efficacy in SSc we do not see improvement in outcome underlining the need for better treatment/or even more timely intervention.

Table 1. Characteristics at cohort entry.

	2010 - 2013 N=229	**2014- 2017 N=207**	**2018 – 2021 N=207**	**P-value**
BASELINE				
Age, mean (SD)	53 (15)	57 (14)	55 (14)	0.003
Female	195 (85)	157 (76)	108 (74)	0.010
RP duration, months (IQR)	122 (46 – 240)	93 (20 – 202)	67 (20 – 210)	0.003
Non RP duration, months (IQR)	43 (16 – 227)	20 (5 – 112)	17 (6 – 54)	<0.001
Diagnosis duration, months (IQR)	116 (80 - 177)	65 (45 – 105)	25 (5 – 45)	<0.001
ΔRP and Non-RP, months (IQR)	24 (0 – 99)	18 (0 – 118)	22 (0 – 120)	0.337
Anti-centromere antibodies	88 (38)	88 (43)	72 (49)	0.092
Anti-topoisomerase antibodies	53 (23)	50 (24)	31 (21)	0.259
Diffuse cutaneous SSc	42 (18)	48 (23)	23 (16)	0.073
Interstitial lung disease on HRCT	99 (43)	65 (31)	43 (40)	<0.001
Pulmonary arterial hypertension	8 (4)	5 (2)	5 (3)	0.746
Digital ulcers	45 (20)	26 (13)	17 (12)	0.041
SF36 mental component score	45 (11)	46 (11)	46 (12)	0.730
SF36 physical component score	40 (11)	41 (11)	41 (12)	0.890
EQ5D	0.4 (0.2 – 0.6)	0.4 (0.1 – 0.7)	0.4 (0.1 – 0.6)	0.580
HAQ Disability index	0.8 (0.2 – 1.3)	0.6 (0.1 – 1.1)	0.8 (0.3 – 1.4)	0.460
3-year survival %	94%	94%	96%	0.941

N: number; SD: standard deviation; IQR: interquartile range; ILD: interstitial lung disease; FVC: forced vital capacity
All binary variables are presented as numbers with percentages.

Figure 1. Survival in CCISS cohort.



45. Soluble Cd206 Is Elevated In Plasma And Tissue Fluid Of Diffuse Cutaneous Systemic Sclerosis Patients Correlating With Systemic Inflammation

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Background: Although the pathogenesis of systemic sclerosis (SSc) is not fully understood, recent genome wide association studies revealed susceptibility polymorphisms in the innate immune system and network analyses showed a subnetwork of genes associated with M2 macrophage activation. CD206 is a cell surface marker of M2 macrophages, and has a role in endocytosis, particularly of mannosylated ligands. This can also exist in a soluble form, sCD206, cleaved off at the cell membrane by matrix metalloproteinases, and circulating in the periphery proportional to the level at the cell surface. sCD206 has been found to be elevated in many pro-inflammatory and pro-fibrotic conditions. Given this, it was hypothesised that sCD206 may be elevated as a biomarker in SSc and may represent part of the pathogenic mechanism.

Methods: Stored plasma samples from healthy controls (HC) and from well-characterised limited (lcSSc) and diffuse (dcSSc) SSc patients (all n=50) were assayed by ELISA for sCD206 (Cambridge Bioscience #HK381-02). Lesional tissue fluid obtained by suction blister method from n=12 HC and n=13 diffuse SSc patients, was also assayed.

Results: Plasma sCD206 was elevated in patients with dcSSc, but not lcSSc (median, range, HC 554, 120-1491, lcSSc 433, 190-1420, dcSSc 629, 216-1609, pg/ml, $P<0.05$ Mann Whitney test, HC vs dcSSc, $P<0.0004$ lcSSc vs dcSSc). When analysed by antibody subset, highest levels were seen in anti-topoisomerase (ATA) subgroup (median, range, HC 554, 120-1491, ACA 445, 190-1420, ARA 629, 311-1082, U1RNP 621, 192-1141, ATA 561, 216-1609 pg/ml, $P<0.05$ for ATA vs ACA). sCD206 was also elevated in the dermal interstitial fluid of dcSSc (median, range sCD206, HC 31, 20-51, dcSSc 41, 23-94 pg/ml, $P<0.04$). Plasma sCD206 correlated with ESR (Pearson correlation, lcSSc 0.329, $P<0.02$, dcSSc 0.364, $P<0.009$), but not disease duration or modified Rodnan Skin Score.
Discussion: sCD206 is elevated in patients with dcSSc, most significantly in those with anti-topoisomerase antibody, recognised to be a poor prognosis group. Furthermore, sCD206 correlated with ESR, a marker of systemic inflammation. These results indicate a potential role for the CD206

pathway in diffuse disease and support the use of sCD206 as a biomarker to stratify patients for therapy against pathogenic macrophages.

46. Computational Drug Repositioning Identifies EGFR and PI3K as Anti-fibrotic Targets for Systemic Sclerosis

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Background: Genome-wide analyses of gene expression in systemic sclerosis (SSc) skin biopsies have identified differential treatment responses based on the mechanism of action of the therapy. The heterogeneity of SSc suggest that subset-specific gene expression could be used to reposition existing drugs for a personalized medicine approach to treatment. The goal of this study was to identify small molecules through the Connectivity Map 2.0 (CMAP) database which can counteract the gene expression changes observed in SSc skin and test the ability of these small molecules to inhibit fibrosis in a 3D skin-like model of SSc.

Materials and Methods: CMAP data was processed with RMA, quantile normalized, and fit to a multichip linear model. Probes were collapsed as average intensity and formatted for gene fold-change as the ratio of treatment to control intensities. DNA microarray data from Milano et al (GSE9285) was analyzed by Gene Set Variation Analysis (GSVA). Pathways statistically significant and specific to the inflammatory and fibroproliferative intrinsic subsets of SSc patients were determined. Small molecules from the CMAP data which regulated pathways specific for each SSc subset were identified and tested in our 3D tissue culture system.

Results: Over 600 pathways were statistically significant in the Fibroproliferative and Inflammatory subsets of SSc patients using GSVA. Comparison to gene expression profiles in the CMAP database revealed EGFR inhibitors strongly downregulate pathways found in the inflammatory subset of SSc patients, while PI3K inhibitors were predicted to downregulate pathways in the fibroproliferative subset of SSc patients. Using self-assembled (SA) 3D skin-like tissues containing SSc or control dermal fibroblasts, we found no adverse effects on tissue growth. Tissue thickness was altered through these small molecules. In SA tissues grown from SSc patient derived fibroblasts, thickness decreased significantly after treatment with either a PI3K or EGFR inhibitor. Immunohistochemical staining shows EGFR overexpression in SSc compared to healthy control tissues.

Conclusions: We identified multiple small molecule inhibitors that downregulate the gene expression pathways shown to be highly expressed in SSc skin biopsies. Experimental validation in 3D skin-like tissues constructed from SSc or control tissues showed a marked decrease in tissue thickness suggesting reduced ECM deposition.

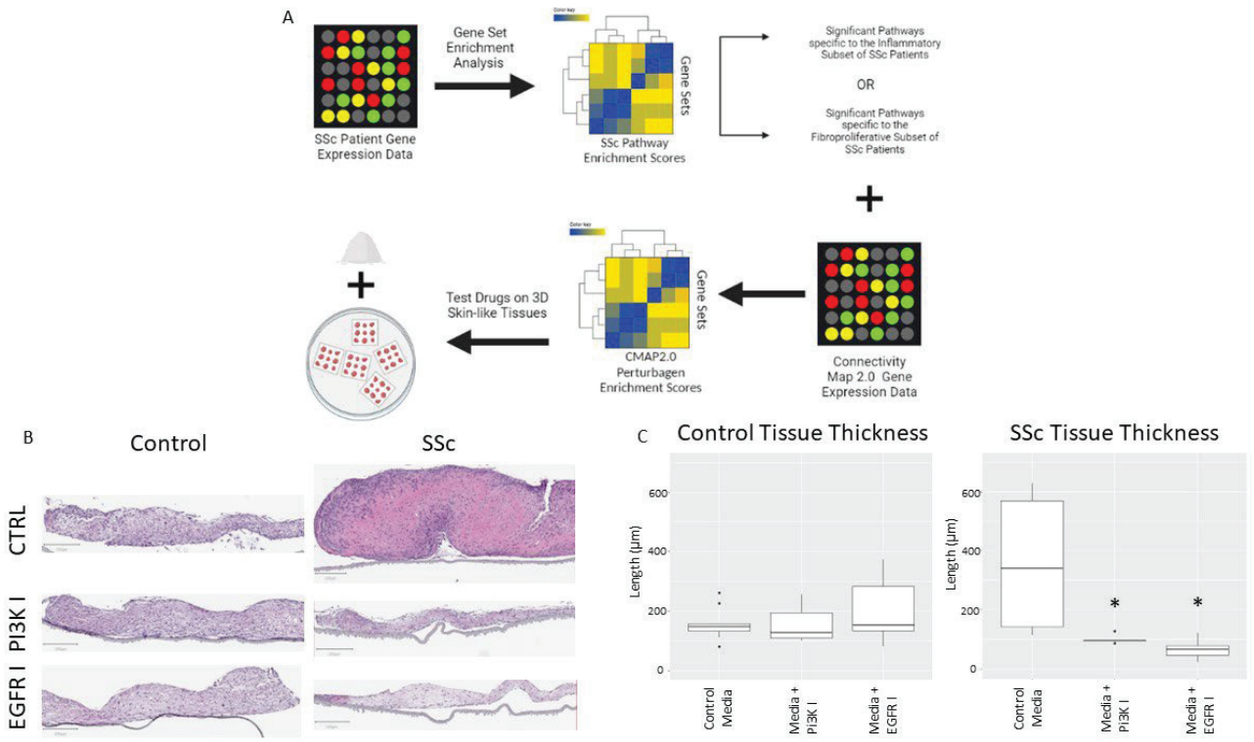

Figure 1. A) General outline of drug repositioning pipeline. GSVA is run on microarray datasets for SSc patient cohorts. Significant gene sets for each gene expression subset are then analyzed in CMAP data to find drugs which regulate these pathways. Drugs found to heavily offset this gene set dysregulation were then used in our 3D skin-like tissue culture methodology to determine drug specific effects. B) Self-assembled 3D tissues treated with either normal tissue media (CTRL), or media with added drug. C) Tissue thickness measurements of tissues harvested in B. * = $p < 0.05$

47. CD45+/ColI+ Cells in Scleroderma Interstitial Lung Disease

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Background: The role of cells of the hematopoietic lineage in fibrosis is controversial. Here we evaluate whether monocytes/macrophages only promote the activation of resident fibroblasts, or whether they can also differentiate into CD45+/Col I+ cells (fibrocytes) that exhibit many properties routinely used to describe fibroblasts.

Materials and Methods: Systemic bleomycin treatment was used to induce fibrosis in a bone marrow transplant and two transgenic mouse models. Fibrotic and control human lung tissue were also used. These tissues were analyzed by flow cytometry. Fibroblasts and fibrocytes derived from a transgenic mouse model were compared in terms of their morphology, growth, and adhesion to fibronectin. Single cell RNAseq was performed to compare numbers of CD45-/ColI+ fibroblasts and CD45+/ColI+ fibrocytes in control and fibrotic mouse lung tissue. Finally, we inhibited fibrosis in mice using a novel, water-soluble version of caveolin scaffolding domain (CSD) called WCSD.

Results: In both mouse and human tissue, we observed a large increase in fibrocyte number and Col I expression associated with fibrosis. In contrast, fibroblast number is not significantly increased. These results on increased fibrocyte number in fibrotic mouse lung tissue were strongly supported by single cell RNAseq. Many investigators claim that fibrocytes are not present among primary fibroblasts. However, we find that fibrocytes are the predominant cell type present in these cultures prior to passage. Fewer fibrocytes are present after one passage, and almost none are present after two passages. Like the classic description of fibroblasts, these fibrocytes are spindle-shaped (i.e. not the fried-egg shape of macrophages). Experiments suggest that fibrocytes are crowded out of cultures during passage because fibroblasts have a larger footprint than fibrocytes, even though fibrocytes bind more efficiently to fibronectin. Finally, we observed that in mice treated with bleomycin and WCSD, there was a large decrease in the number of fibrocytes present, but not in the number of fibroblasts, compared to bleomycin alone.

Conclusions: Fibrocytes are the major collagen-producing cell type that is increased in number in association with fibrosis. The common observation that collagen-producing spindle-shaped cells associated with fibrosis are CD45- appears to be an artifact of passage in cell culture.

48. Monocytes/macrophages promote fibrosis in systemic sclerosis

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Background:

Systemic Sclerosis (SSc) is an autoimmune connective tissue disease characterised by tissue fibrosis. While fibroblasts are the effector cells responsible for excess extracellular matrix production, immune cells are also critically involved in pathogenesis. We previously found more circulating $CD163^{+}$ non-classical monocytes in SSc compared to healthy controls (HC). While TGF-β is known as a master regulator of fibrosis, we found elevated expression of other cytokines such as IL-21 by SSc-derived macrophages. We hypothesize that monocytes/macrophages, through their secretome, promote a fibrotic phenotype in fibroblasts.

Materials and methods:

Flow cytometry. HC and SSc whole blood were subjected to red blood cell lysis. Leukocytes were counted and stained for surface markers (CD3,CD14,CD16,CD19,CD56,HLA-DR,CD163) before fixation in 1%PFA/PBS, permeabilization, TGF-β staining and analysis using LSR Fortessa and FlowJo.

Cell culture and scratch assay. Monocytes isolated from HC and SSc PBMCs by negative selection were cultured for 7 days in RPMI/10% FBS. Cells were serum-starved in RPMI/BSA overnight before media collection. Macrophage media and anti-IL-21-neutralising antibody (2.5μg/mL) were applied to scratch-wounded fibroblast monolayers (P3-6), previously maintained in DMEM/10% FBS, serum-starved and incubated with mitomycin-C (5μg/mL) prior to treatment. Linear regression was used to analyse wound closure rate and 2-tailed unpaired t-tests to compare monocyte frequencies and wound closure areas.

Results:

SSc macrophage-derived media (n=3) promoted faster migration of normal skin fibroblasts than HC-derived macrophage media (n=3, p=0.029). Flow cytometry results demonstrated increased numbers of TGF-β^+CD163$^+$ monocytes in SSc (n=8) compared to HC (n=5) blood (p=0.049). Recombinant TGF-β induced lung fibroblast migration; 24-hour post-wounding, % wound area was significantly smaller in TGF-β (10ng/mL)-treated (n=10) than BSA-control-treated (n=10, p=0.041) monolayers. Surprisingly IL-21 inhibition induced migration of skin fibroblasts cultured in HC-derived macrophage media (HC media/BSA (n=3) vs HC media/IL-21-antibody (n=3), p=0.033). However, the antibody did not affect SSc-derived macrophage media-induced migration (p=0.146).

Conclusions:

The SSc macrophage secretome promotes fibroblast migration. TGF-β may be a mediator of this. The increase in TGF-β^+ monocytes in SSc supports a predominantly M2-like phenotype in SSc. Further work is needed to elucidate other cytokines in the SSc secretome which promote a pro-fibrotic micro-environment and hence be a target for therapy.

49. T cell receptor β-chain repertoire displays alterations in African American Systemic Sclerosis patients

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National Institute of Arthritis and Musculoskeletal and Skin Diseases[1]; National Institute of Allergy and Infectious Diseases[2]; Genome Research in African American Scleroderma Patients (GRASP) Consortium[3]

Background: Systemic sclerosis (SSc) is a devastating multisystem autoimmune disorder that disproportionately affects African Americans (AA). Previous work from our lab has suggested pivotal role of *HLA-II* genes in SSc pathogenesis. *HLA-II* alleles encode for variations in antigen binding grooves of HLA proteins that present antigens to T-cell receptors (TCRs) and can contribute to autoimmunity by autoantigen presentation. TCR repertoire is HLA-restricted, antigen-specific, and highly diverse to facilitate recognition of large number of presented antigens. Alterations in TCR repertoire are suggested to predispose patients to autoimmune diseases. Thus, given the important role of *HLA-II* genes in SSc pathogenicity, we hypothesized that TCR repertoire is altered in SSc, and is distinct from healthy control TCR repertoires.

Materials and methods: Genomic DNA from 132 AA SSc patients from the GRASP consortium along with 50 AA healthy control samples were extracted and the TCR-β chain was deep sequenced using immunoSEQ assay (Adaptive Biotechnologies, USA). Data was analyzed using Immunarch to identify differences in unique clones, CDR3 length, V gene usage, J gene usage, and amino acid usage in the FG-Loop of the TCR-β between the two SSc groups. GLIPH2 is being used to identify motifs within different TCR-β clones. VDJdb will be used to predict antigen specificity.

Results: Analysis of V gene and J gene, revealed an enriched usage of *TRBV5-1* ($p=2.7x10^{-5}$), *TRBV7-3* ($p=2.1x10^{-2}$), *TRBJ2-1* ($p=9.1x10^{-4}$), *TRBJ2-2* ($p=1.5\times10^{-3}$) in SSc patients along with a decrease in usage of *TRBV6-1* ($p=8.1x10^{-10}$), *TRBV6-4* ($p=4.73x10^{-7}$), *TRBV6-5* ($p=5.2\times10^{-5}$) *TRBV25-1 (p=*$3.9x10^{-2}$), *TRBJ1-5* ($p=3.8x10^{-3}$) in comparison to the healthy controls. The analysis for TCR-CDR3 amino acid usage revealed enriched usage of hydrophobic amino acids, Leu ($p= 7.0x10^{-5}$) and Val ($p=3.3x10^{-3}$) and reduced use of negatively charged Glu ($p=9.7\times10^{-3}$) in the FG loop of SSc patients compared to healthy controls.

Conclusions: Differential V, J genes and FG-loop amino acid usage in SSc patients indicate alterations in TCR repertoire. Identifying TCR sequences unique to SSc can help locating the antigenic triggers that underlie autoimmune responses leading to SSc pathogenesis.

50. Osteoclastogenesis in patients with systemic sclerosis with and without calcinosis cutis

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Background:
Calcinosis cutis, the deposition of insoluble calcium in the skin and subcutaneous tissues, is a debilitating complication of systemic sclerosis (SSc) with no effective treatments. Although the pathogenesis of SSc-related calcinosis remains unknown, there is growing evidence of a link between calcinosis and vascular hypoxia. Osteoclasts are large multinucleated giant cells that participate in bone remodeling and can be induced by hypoxia, as shown in patients with SSc and acro-osteolysis. We hypothesize that calcinosis in SSc is a consequence of microvascular injury associated to increased osteoclasts formation that results in the local release of calcium from the osteolytic process.

Materials and methods:
We recruited 19 patients with SSc (9 with calcinosis and 10 without calcinosis) and 8 age- and gender-matched healthy controls. We collected clinical information and blood from all patients. Hand radiographs were performed in all SSc patients and scored using the validated Scleroderma Clinical Trials Consortium (SCTC) radiographic severity score for calcinosis. Osteoclasts were identified using tartrate-resistant acid phosphatase stain in cultures of peripheral blood mononuclear cells (PBMC) with RANKL, and M-CSF, and counted under light microscopy.

Results:
SSc patients were all women and Hispanic, and the majority (11, 57.89%) had limited SSc skin type. Mean age was 54±14.3 years, mean disease duration was 13.34±7.3 years from Raynaud phenomenon (RP) onset and 9.6±6.88 from first non-RP symptom, modified Rodnan Skin Score was 9.1±6.85. Patients with and without calcinosis were similar with regards to demographic and clinical characteristics, including smoking history, osteoporosis, autoantibody status, internal organ involvement, use of medications, calcium, and phosphate serum levels. Mean SCTC score in SSc patients with calcinosis was 24.4±14.98 (range 1-94.25). After 9 days in culture, PBMCs from patients with calcinosis originated a significantly higher number of osteoclasts (41.4±34.9 cells/well) than patients without calcinosis (15.3±6.9 cells/well) and healthy individuals (12.3±2.01 cells/well) (p=<0.001). The number of osteoclasts per well was strongly correlated with the severity of calcinosis (r=0.828, p=0.058).

Conclusions:
Calcinosis in patients with SSc is associated with an increased propensity of peripheral blood cells to form osteoclasts. Targeted inhibition of osteoclastogenesis may provide a specific therapeutic option for SSc-associated calcinosis.

51. Single cell transcriptome analysis reinforces the pro-inflammatory role of CD14^{+} monocytes in scleroderma patients

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Background: Systemic sclerosis (SSc) is an immune-mediated disease, which affects mostly women over 50 years old. Recently, single cell mRNA sequencing techniques (scRNA-seq) have contributed to establish the relevance of different immune cell types in SSc. However, little is known about the role of the different subsets of monocytes, which play a central role in the early inflammatory response and in the maintenance of inflammation in SSc.

Materials and methods: CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) of 8 SSc diagnosed patients and 8 sex and age matched unaffected controls. All the patients were females between 50 and 70 years old. ScRNA-seq libraries were generated using the 10x Genomics Chromium platform and the Chromium Next GEM Single Cell 5' Reagent Kits. All the sequencing reads were pre-processed using the Cell Ranger software and standard QC was performed. After QC a total of 98,543 cells remained, with an average of ~7,000 cells per sample, and 36,601 genes were detected. Dimensionality reduction and cell clustering were performed using Scanpy, establishing 5,000 high variable genes, 20 principal components and the Leiden clustering algorithm.

Results: We identified 14 different clusters, in which the cells included in 1 of these clusters were classified as non-classical monocytes, while the remaining 13 clusters were classified as classical monocytes by the CellTypist software. Then, we manually classified the classical CD14+ monocytes into clusters showing high expression of *S100* family genes and increased expression of *NFKBIA* and we were able to identify intermediate monocyte clusters. We observed an increased HLA gene expression pattern in the intermediate monocytes compared to the rest of the clusters. Moreover, differential gene expression analysis showed the upregulation of interferon family genes in SSc patients. Finally, a gene ontology enrichment analysis revealed the overrepresentation of immune response and external stimuli response pathways.

Conclusions: Our analysis of CD14+ monocytes in SSc patients showed an increased inflammatory response compared to healthy controls. These findings confirmed a clear interferon-responsive monocyte profile in SSc patients, which suggested the possible use of monocyte subtypes as biomarkers or treatment targets for the disease.

52. Alteration of protein O-GlcNAcylation in keratinocytes affects their cytokine secretion and the paracrine regulation of fibroblast function

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Background:

Keratinocytes have a crucial role in regulating fibroblast functions by secreting pro-inflammatory and pro-fibrotic cytokines via paracrine effects, and this contributes to the dysregulated fibrosis in scleroderma. O-GlcNAcylation is the addition of the GlcNAc from nucleotide uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) onto serine or threonine residues of cytosolic proteins. This is catalyzed by O-GlcNAc transferase (OGT), and removed by O-GlcNAcase (OGA). Abnormal O-GlcNAcylation of proteins is found in various chronic diseases including diabetes, cancers and Alzheimer's disease.

Materials and methods:

To determine the role of protein O-GlcNAcylation in regulating secretion of cytokines by keratinocytes and the consequent impact on fibroblasts, both primary keratinocytes and dermal fibroblasts were isolated from the wildtype C57BL/6 mouse and cultured separately *in vitro*. The keratinocytes were treated with a chemical inhibitor to either OGT (OSMI-1) or OGA (Thiamet-G) for 24 hours. At the end of treatment, the inhibitor-containing media was removed and keratinocytes were cultured in fresh media for 48 hours. The keratinocyte-conditioned media (K-CM) was then transferred from the keratinocytes to fibroblasts. After 48-hour culture in K-CM, the fibroblasts were harvested for further analysis. Alternatively, the K-CM was processed for secretome analysis by Mass Spectrometry.

Results:

1), Chemical inhibition of OGT significantly impaired keratinocyte differentiation. On the other hand, chemical inhibition of OGA enhanced keratinocyte differentiation. 2), The gene expressions of both Acta2 and Collagen I were significantly downregulated in fibroblasts cultured in OSMI-1-treated K-CM. 3), Culture in OSMI-treated K-CM also induced significant caspase-dependent apoptosis in fibroblasts. 4), Secretome analysis of K-CM revealed that the levels of profibrotic cytokines, including Thrombospondin-1 and Connective Tissue Growth Factor, were significantly lower, while the levels of CXCL1 and CD44 were significantly higher, in OSMI-1-treated K-CM.

Conclusions:

These findings demonstrate that the myofibroblast differentiation is suppressed and apoptosis is induced in fibroblasts cultured in conditioned media from OGT-inhibited keratinocytes, suggesting that inhibition of protein O-GlcNAcylation in keratinocytes alters their secretion of certain cytokines that regulate activation and turnover of fibroblasts. Protein O-GlcNAcylation may be a novel therapeutic target in treating dysregulated fibrosis in scleroderma.

53. Development of a Novel Small Molecule Anti-fibrotic and Anti-inflammatory Agent for the Treatment of Systemic Scleroderma Interstitial Lung Disease.

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Background: Evidence suggests that the apelinergic system, APJ (apelin receptor) and its ligand (apelin), in pulmonary endothelial subtypes (aerocytes and general capillary, respectively), are mediators of alveolar regeneration and that age- or disease-related attenuation of this system contributes to the interstitial lung disease (ILD) of systemic sclerosis (SSc) and idiopathic pulmonary fibrosis (IPF). In models of interstitial lung disease, loss of APJ function is associated with more severe disease while promoting APJ activation improves outcomes. Herein we describe a novel APJ agonist (APT101) and it effects in models of acute lung injury and chronic ILD.
Materials and methods: Heterologous cellular systems were used to characterize the potency, efficacy and selectivity of APT101 on APJ signaling, APJ internalization and off-target activities. Bioavailability studies and the mouse bleomycin model of lung injury and fibrosis were used to characterize the acute (prophylactic treatment) and chronic (delayed treatment) efficacy of APT101.
Results: APT101 activated APJ Gαi-mediated inhibition of cAMP generation (IC50 = 13 nM) at concentrations that have no effect on β-arrestin-mediated APJ receptor internalization (2670 nM). APT101 was bioavailable and well tolerated by the oral route in preclinical *in vivo* studies and exhibits dose proportional plasma exposures. In the bleomycin mouse model of lung injury and fibrosis the chronic administration of APT101 treatment was protective in both prophylactic (at time of injury) and treatment (7 days after injury) experimental paradigms. Lung weights, Ashcroft scores, pulmonary fibrosis and inflammatory cell infiltrates were significantly reduced in a dose-dependent manner in both studies and increases in lung hydroxyproline concentrations induced by bleomycin were blunted by 77%. Fibrinogen deposition and TIMP1 expression were also significantly suppressed.
Conclusion: These results support the further development of APT101 as a novel treatment of ILDs like SSc and IPF.

54. Non-invasive Raman spectroscopy imaging of- involved forearm skin reveals aligned collagen in dermis of systemic sclerosis patients

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Background:
Systemic sclerosis (SSc) skin disease is known to exponentially correlates systemic complications and mortality in SSc. Modified Rodnan skin score (mRSS) is used commonest in routine practice and trials but is limited by inter-rater variability.
Studies showed skin biopsy from body part of SSc patients with normal mRSS had demonstrable pathological deposition of collagen but repeat skin biopsy to track longitudinally may be unacceptable to patients. Hence, ongoing research for non-invasive tools are encouraged.

Objectives:
Our objective is to produce preliminary data in SSc compared to healthy controls'(HC) with confocal-Raman microscopy (CRM).

Methods:
Skin biopsies from SSc (n=3) and HC (n=1) were analysed under CRM with three contrast methods which are Coherent anti-Stokes Raman scattering (CARS), Second Harmonic Generation (SHG) and

Two-Photon Fluorescence (TPF) at skin depth of 150-200um. We analysed collagen and elastin fibers in the skin samples which is disease relevant end products of fibroblast activation in SSc.

Results:
The spectrometry analysis revealed denser collagen fibers in dermal layer and deposition occurs in more superficial layers of diseased skin. Peak of collagen curve were at depth of 110-130um HC vs 75-90um in SSc. Collagen fibres were more aligned in the SSc(Figure 1).
In addition to that, analysis also revealed larger number of disordered elastin fibres in the dermal layer of diseased skin.

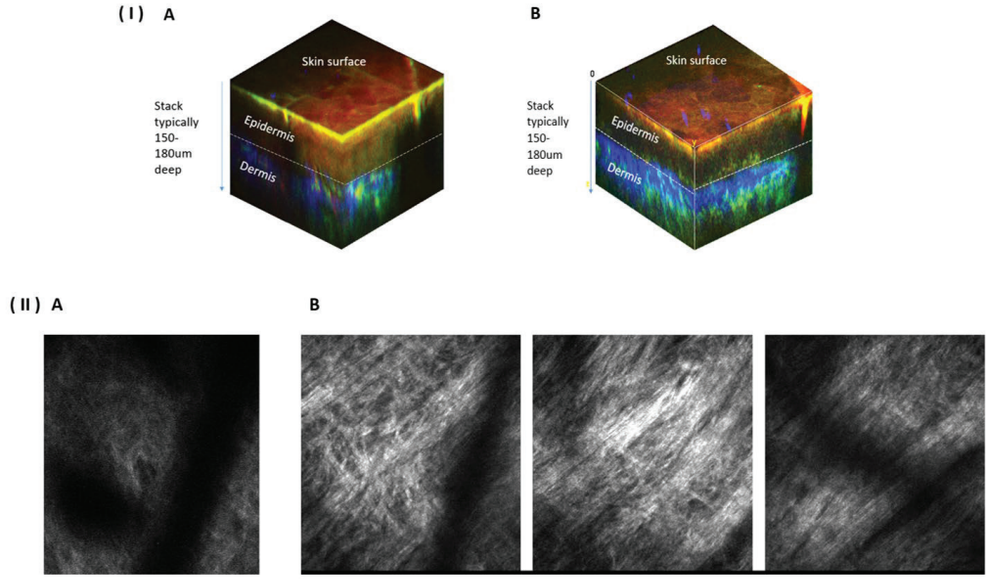

Figure 1: Confocal Raman Microscopy analysis of SSc vs HC. I : Two-Photon Fluorescence (TPF) , II : Second Harmonic Generation (SHG). A : Scleroderma , B: Healthy Control. Both contrast analysis revealed denser and more aligned collagen fibers in SSc compares to HC skin.

Conclusions:
With the demand of better tools in diagnosis of early phase of disease and therapeutic research in SSc, our work with spectrometry analysis may prove better in objective evaluation of skin changes at the molecular level. We demonstrate that the SHG is altered in the disease consistent with increased and aligned collagen in the SSc dermis that is compatible with mRSS score.
Our future work is to generate computer module in defining pathological collagen level, utilise CRM for SSc therapeutic and translational research with novel therapeutic peptides, and manufacturing of portable non-invasive handheld device that is capable to accurately diagnose early subclinical SSc as well as clinical research.

55. The anti-topoisomerase, but not the anti-centromere, B cell response in systemic sclerosis is characterized by the presence of active, Ig-secreting B cells that associate with lung fibrosis

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Background:
Systemic Sclerosis (SSc) carries the highest mortality burden among the rheumatic diseases. Disease risk and course are difficult to predict, and anti-inflammatory and B-cell depleting therapies show varying results. >95% of SSc patients harbor autoantibodies. Anti-topoisomerase antibodies (ATA) and anti-centromere antibodies (ACA) are most prevalent and associate with distinct disease phenotypes. Here, we determined phenotypic and functional characteristics of the underlying auto-reactive B cell responses to identify possible drivers and dynamic markers of disease activity.

Materials and Methods:
Peripheral blood mononuclear cells (PBMC) from ATA- and ACA-positive SSc patients were cultured with or without stimulation. ATA- and ACA-IgG and -IgA were measured in culture supernatants by ELISA. In addition, PBMC were depleted of plasmablasts by fluorescence activated cell sorting (FACS), while isolated plasmablasts were cultured separately. The presence of antigen-specific plasmablasts was confirmed by ELISPOT. Finally, the degree of spontaneous ATA secretion was correlated to the presence and degree of interstitial lung disease (ILD).

Results:
ATA- and ACA-positive SSc patients harbored circulating B cells that secreted either ATA-IgG or ACA-IgG upon stimulation, depending on their serotype. In addition, we noted spontaneous secretion of ATA-IgG and extensive secretion of ATA-IgA in ATA-positive patients. In contrast, spontaneous secretion of ACA-IgA was undetectable in ACA-positive patients. FACS and ELISPOT identified circulating plasmablasts as the source of spontaneous ATA-IgA and -IgG secretion. Spontaneous ATA secretion was remarkably higher in patients with ILD than in those without and correlated with the degree of pulmonary fibrosis.

Conclusions:
The hallmarking B cell responses underlying ACA and ATA-positive SSc display different phenotypes. ATA-positive SSc patients harbor activated ATA-IgG and -IgA B cell responses, as indicated by the spontaneous secretion of both isotypes by circulating plasmablasts. In contrast, the ACA B cell response is far less active and lacks the active IgA component, suggesting a difference in the triggers driving these B cell responses in patients. In fact, the remarkable ATA-IgA secretion points towards a mucosal trigger of this B cell response, which may be continuously active. Importantly, a link was found between the activity of the ATA B cell response and the presence and severity of ILD.

56. A Deep Neural Network Classifier to Identify Intrinsic Molecular Subsets of Systemic Sclerosis from Histological Images

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Background: Intrinsic gene analyses based on the gene expression profiles in skin have identified four intrinsic molecular subsets among patients with systemic sclerosis (SSc); these have been named the inflammatory, fibroproliferative, limited, and normal-like. SSc participants assigned to the inflammatory subset showed significant improvement in the Abatacept Systemic SclErosis Trial (ASSET). In order to provide a fast and accessible molecular subset predictor, we trained a deep neural network to efficiently identify these participants using the images of hematoxylin and eosin (H&E)-stained skin biopsies.

Materials and Methods: Skin tissue, H&E-stained images, and clinical scores were obtained from participants enrolled in the ASSET clinical trial. Image labels were molecular subset calls from a classifier using gene expression from a paired skin biopsy. A sliding window approach was used to generate small patches from whole slide images. Convolutional neural networks (CNNs) were trained on patch images, and patch-level classifications were aggregated to infer the subset of the given biopsy. Visualization for areas of the image that are important for prediction and Spearman correlations between predicted probabilities and clinical scores were conducted for validation. Final prediction for molecular subsets was determined by the combination of individual binary classifiers for the inflammatory, fibroproliferative and normal-like subsets.

Results: CNNs were trained and validated on small patches generated from 140 whole-slide images. The model was evaluated using a hold-out test set of 24 whole-slide images. The final model identified participants' molecular subset with 79.17% average accuracy when data sets are split-by-patient. Visualization of patches and predicted decisive regions provided insights regarding important parts of the histogical image contributing to the molecular subsets. Predicted molecular subset probabilities were significantly correlated with clinical inflammation and fibrosis scores performed by a blinded expert.

Conclusions: We developed an ensemble neural network classifier that classifies SSc patients into the intrinsic molecular subsets using standard H&E-stained images of skin biopsies. This is less costly than genomic assays to identify these individuals and can be applied to evaluate retrospective data where genomic data were not collected.

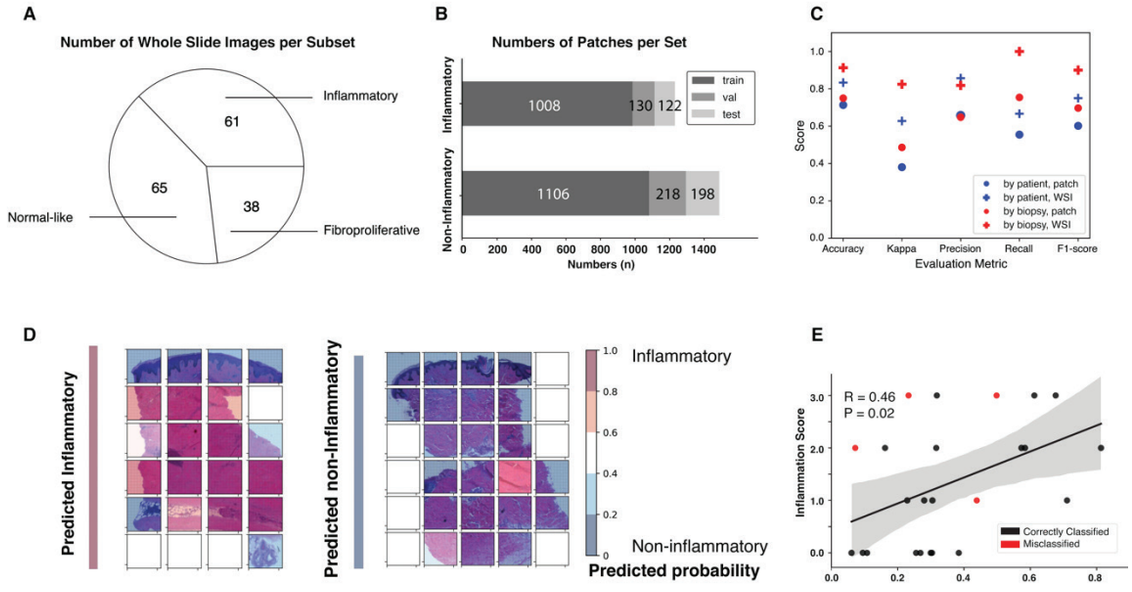

Figure 1. (A) Number of whole slide images per molecular subset. (B) Number of small patches used for inflammatory classifier model training. (C) Models' performance of inflammatory classifier on the test set for models trained on split-by-patient or split-by-biopsy datasets, patch-based and post aggregation whole-slide level. (D) Visualization of patches in a true inflammatory slide and a true non-inflammatory slide by overlapping a colored heatmap based on predicted probability. (E) Correlation of confidence scores for prediction as inflammatory subset and clinical inflammation scores.

Notes:

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