Genentech, UCB and Wyeth Pharmaceuticals, and is employed by Corrona. J.K. has received research grants and consultancy fees from Genetech and Roche. C.M. and E.V. are employees of Roche.

TABLE 1. Outcomes, Week 152

| GmTSS | TCZ8 n = 244 | TCZ4 n = 241 | Control n = 219 |
|---|---|---|---|
| Mean (S.D.) change from BL | 0.72 (2.56)*,† | 0.71 (2.14)†,‡ | 1.78 (3.64) |
| Pts with no progression (GmTSS change from BL ≤ 0), % (n) | 69 (169)*,§ | 67 (162)§,‖ | 51 (111) |
| Signs and Symptoms | | All TCZ | |
| ACR20, % (n/n) | | 80 (472/591) | |
| ACR50, % (n/n) | | 59 (346/591) | |
| ACR70, % (n/n) | | 36 (212/591) | |
| DAS28 remission, % (n/n) | | 57 (325/572) | |
| TJC and SJC = 0, % (n/n) | | 21 (137/656) | |
| HAQ < 0.5 % (n/n) | | 37 (202/552) | |

n/n: no. patients achieving end point/no. patients reaching timepoint with valid assessments. *$p < 0.0001$ vs control. †p calculated by Van Elteren test stratified by region. ‡$p = 0.0002$ vs PBO. §p calculated by logistic regression analysis adjusted for region. ‖$p = 0.0008$ vs control.

### 222. PEPTIDYLARGININE DEIMINASE FROM PORPHYROMONAS GINGIVALIS AS A POTENTIAL TARGET FOR THE TREATMENT AND PREVENTION OF RHEUMATOID ARTHRITIS

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**Background:** Porphyromonas gingivalis (P. gingivalis) is a major bacterium in the pathogenesis of periodontitis. Several epidemiological studies have shown an association between periodontitis and rheumatoid arthritis (RA). We demonstrated cross-reactivity between citrullinated human α-enolase and P. gingivalis enolase, and that the anti-citrullinated α-enolase response links with DR4 alleles and smoking, common susceptibility factors for RA and periodontitis. Recently, we reported that P. gingivalis peptidylarginine deiminase (PPAD) catalyses the citrullination of carboxy-terminal arginines to citrulline, in both bacterial and human peptides. Bacterial citrullination might therefore be aetiologically important in a subset of RA by driving the autoimmune response to citrullinated proteins. Hence, we investigated the PPAD enzyme as a potential new therapeutic target.
**Methods:** Recombinant full-length PPAD was expressed in E. coli, purified, and tested for enzymatic activity. A specific antibody to PPAD was developed by immunising rabbits with recombinant protein. The subcellular localisation of PPAD was investigated by fractionation of P. gingivalis and blotting with anti-PPAD antibody. Cross-reactivity with human PADs was examined by western blot. PPAD inhibition studies were performed using tetracycline, doxycycline, minocycline, sulfasalazine, methotrexate, and 2-chloroacetamidine. Site-directed mutagenesis was performed to investigate the contribution of a conserved cysteine residue to enzyme activity.
**Results:** Full-length PPAD was active and citrullinated α-enolase and fibrinogen peptides harbouring carboxy-terminal arginines, but not peptides with internal arginines. Blotting of subcellular fractions with a PPAD-specific antibody demonstrated that the enzyme is located on the cell surface of various P. gingivalis strains. Anti-PPAD antibody did not cross-react with human PADs. 2-chloroacetamidine proved an effective inhibitor with half-maximal inhibition (IC50) at ~25 μM. Tetracycline, doxycycline, minocycline, sulfasalazine or methotrexate did not inhibit PPAD activity. The substitution of cysteine-351 with alanine completely abolished PPAD activity.
**Conclusions:** The cell-surface localisation identifies PPAD as a putative virulence factor and suitable pharmaceutical target. Cysteine-351 as a crucial catalytic residue indicates that agents targeting cysteines can be effective inhibitors. This is confirmed by the inhibitory activity of 2-chloroacetamidine in the low μM-range. Our combined results suggest that PPAD and human PADs have different three-dimensional structures but share a similar, although not identical, catalytic mechanism. Our findings form the basis for further characterisation, such as crystallisation and the development of more potent and specific inhibitors with the potential to prevent RA by inhibiting the generation of the autoantigens which drive the disease.
**Disclosure statement:** The authors have declared no conflicts of interest.

## Scleroderma and related disorders

### 223. LONG TERM OUTCOME IN A CONTEMPORARY SYSTEMIC SCLEROSIS COHORT

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**Background:** We have previously compared outcome in two groups of systemic sclerosis (SSc) patients with disease onset a decade apart and we reported data on 5 year survival and cumulative incidence of organ disease in a contemporary SSc cohort. The present study examines longer term outcome in an additional cohort of SSc followed for 10 years.
**Methods:** We have examined patients with disease onset between years 1995 and 1999 allowing for at least 10 years of follow-up in a group that has characteristics representative for the patients we see in contemporary clinical practice.
**Results:** Of the 398 patients included in the study, 252 (63.3%) had limited cutaneous (lc) SSc and 146 (36.7%) had diffuse cutaneous (dc) SSc. The proportion of male patients was higher among the dcSSc group (17.1% v 9.9%, $p = 0.037$) while the mean age of onset was significantly higher among lcSSc patients ($50 \pm 13$ v $46 \pm 13$ years ± SD, $p = 0.003$).

During a 10 year follow-up from disease onset, 45% of the dcSSc and 21% of the lcSSc subjects developed clinically significant pulmonary fibrosis, $p < 0.001$. Among them approximately half reached the endpoint within the first 3 years (23% of dcSSc and 10% of lcSSc) and over three quarters within the first 5 years (34% and 16% respectively). There was a similar incidence of pulmonary hypertension (PH) in the two subsets with a steady rate of increase over time. At 10 years 13% of dcSSc and 15% of lcSSc subjects had developed PH (p=0.558), with the earliest cases observed within the first 2 years of disease. Comparison between subjects who developed PH in the first and second 5 years from disease onset demonstrated no difference in demographic or clinical characteristics, but 5-year survival from PH onset was better among those who developed this complication later in their disease (49% v 24%), with a strong trend towards statistical significance ($p = 0.058$). Incidence of SSc renal crisis (SRC) was significantly higher among the dcSSc patients (12% v 4% in lcSSc, $p = 0.002$). As previously observed, the rate of development of SRC was highest in the first 3 years of disease-10% in dcSSc and 3% in lcSSc. All incidences of clinically important cardiac disease developed in the first 5 years from disease onset (7% in dcSSc v 1% in lcSSc, $p < 0.001$) and remained unchanged at 10 years. As expected, 10-year survival among lcSSc subjects was significantly higher (81%) compared to that of dcSSc patients (70%, $p = 0.006$). Interestingly, although over the first 5 years the death rate was much higher in the dcSSc cohort (16% v 6% in lcSSc), over the following years it became very similar for both subsets (14% and 13% between years 5 and 10, and 18% and 17% between years 10 and 15 for dcSSc and lcSSc respectively).
**Conclusions:** Even though dcSSc patients have higher incidence for most organ complications compared to lcSSc subjects, the worse survival among them is mainly due to higher early mortality rate. Mortality rate after first 5 years of disease becomes comparable in the two disease subsets.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 224. SYSTEMIC SCLEROSIS MANAGEMENT: ARE WE DOING AS WELL AS WE COULD?

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**Background:** 2009 EULAR guidelines set clear standards for the treatment of systemic sclerosis (SSc). We wanted to assess our

management against these standards, and to look at our general management of SSc.

**Methods:** The audit was conducted within the South West Thames Regional Audit Group. All members were invited to participate by identifying SSc patients and submitting data via a proforma.

**Results:** Data on 83 patients from 9 hospitals was received. 82% were female; mean age 61yr (23-89). Commonest autoantibody status is shown in Table 1. Skin: 17 patients required immunosuppressant drugs for skin disease: MMF (8), MTX (7) (mean dose 15 mg/wk), AZA (2). Only 1 patient had an adequately documented skin score. Digital vasculopathy: 46 patients had Raynaud's phenomenon or digital ulceration requiring treatment: 28 were on 1st line therapy (Ca antagonists),17 on 2nd line therapy (iloprost or epoprostenol), 1 on 3rd line therapy (bosentan). Lung: 17 patients had CT evidence of interstitial lung disease (ILD). 6 patients were on present treatment: cyclophosphamide (2), MMF (2) steroids (2). 12/17 patients with ILD and only 42/83 total patients had annual lung function tests. Pulmonary hypertension (PAH): 18 patients were classified as having mild PAH (mean PAP 20-51 mmHg),6 as moderate (mean PAP 23-45 mmHg), 1 as severe (mean PAP 39 mmHg on treatment). WHO severity classification was only available in 13 patients (9 = I, 1 = II, 3 = III). Current treatment included diuretics (6), bosentan (3),sildenafil (2), epoprostenol (2). 3 were on home oxygen. All patients with PAH but only 54 (65%) of all patients in the audit were undergoing annual echo. BP: Only 52 (63%) had BP check each clinic visit. 58 patients had a current recorded BP and of these patients 13 were hypertensive (SBP>140 mmHg or DBP>90 mmHg). Anti-hypertensive treatment included ACE-I (25), Angiotensin II RA (19) and others (15). Renal: 16 patients had eGFR < 60 ml/min (lowest 22 ml/min). 5 patients had suffered SSc renal crisis. Digestive tract: 44 (53%) had oesophageal involvement; 14 (17%) had bowel involvement. 37 were on a PPI, 2 on pro-kinetics and 2 on rotating antibiotics. None were having NG or PEG feeding. 14 patients were current steroid users (commonest indication: joint pain (6 patients)); 12/14 had BP check every visit.

**Conclusions:** This audit shows the importance of regional networks in gathering adequate patient numbers in rare rheumatic conditions. Our SSc drug management appears to accord well with EULAR guidance. However, there is room for improvement in our monitoring of skin scores, blood pressure, lung function, echocardiography and PAH functional status.

**Disclosure statement:** The authors have declared no conflicts of interest.

TABLE 1. Auto-antibody status (% of patients)

| | |
|---|---|
| ANA | 96 |
| Centromere | 43 |
| Scl-70 | 34 |
| Ro | 6 |
| U1 RNP | 4 |
| RNA I,II, III | 1 |

## 225. CYTOKINES AND GROWTH FACTORS RELEASED INTO CONDITIONED MEDIA BY EPIDERMAL AND DERMAL EXPLANTS OF HEALTHY CONTROLS AND SYSTEMIC SCLEROSIS PATIENTS

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**Background:** Systemic sclerosis (SSc) epidermal cells show signs of injury and activation similar to changes observed during the wound healing process. Keratinocytes secrete chemo-attracting agents and growth factors influencing the phenotype and proliferation rate of fibroblasts. We hypothesized that in SSc injured epidermal cells release chemokines and cytokines capable of recruiting immune cells to the skin and promoting fibrosis. We decided to determine the array of cytokines and growth factors released by SSc epidermal and dermal cells.

**Methods:** In the preliminary study 4 mm forearm biopsies were taken from 6 healthy controls and 6 SSc patients with various stage and type of disease. Dermis and epidermis were separated using trypsin/EDTA and incubated overnight with 1 ml of serum free media. Then the media were collected and analysed using LegendPLEX/ELISA for presence of CCL20, HGF, G-CSF, GM-CSF, VEGF, PDGF-AA, PDGF-BB, MCP-1, FGF-2, IL-8, IL-1α, IL-1β and IL-1ra. The statistical analysis was performed using Wilcoxon rank-sum test.

**Results:** Predominant amongst growth factors released by the epidermal explants were chemokines MCP-1 and IL-8 (Table 1). A trend towards increased IL-8 release by SSc epidermis comparing to control epidermis was observed, similar with MCP-1. Also HGF was abundantly present in both epidermis and dermis with trend towards significant increase in the SSc dermis. Moreover, elevated levels of IL-1ra were detected in SSc dermis when matched to controls. VEGF was produced in higher amounts by SSc dermis.

**Conclusions:** Growth factors and cytokines released by SSc explants can be assessed using Luminex cytokine arrays. Chemokines IL-8 and MCP-1 predominate in the conditioned media from epidermal biopsies, whereas HGF and MCP-1 release was prominent in the dermal. The epidermis is confirmed as a possible source of chemokines in SSc. Our further work will focus on possibility to categorise SSc patients based on cytokine profiling arrays of explant skin biopsy material.

**Disclosure statement:** The authors have declared no conflicts of interest.

TABLE 1. Mean and standard error of the mean (SEM) concentration of growth factors and cytokines in the conditioned media.

| Growth factor | Control epidermis | | SSc epidermis | | Control dermis | | SSc dermis | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| CCL20 | 5.03 | 8.899 | 1.43 | 6.737 | 13.72 | 9.141 | 2.92 | 1.849 |
| HGF | 549.64 | 49.110 | 805.62 | 167.069 | 800.89 | 85.991 | 795.87 | 224.324 |
| G-CSF | 56.32 | 19.787 | 24.16 | 7.004 | 62.97 | 35.173 | 9.01 | 2.826 |
| GM-CSF | 0.00 | 0.00 | 0.00 | 0.00 | 0.20 | 0.197 | 0.00 | 0.00 |
| VEGF | 75.33 | 30.382 | 88.11 | 27.514 | 23.93 | 4.806 | 35.12 | 11.531 |
| PDGF-AA | 1.04 | 0.488 | 3.35 | 1.285 | 3.49 | 1.190 | 4.35 | 1.987 |
| PDGF-BB | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| MCP-1 | 846.14 | 372.356 | 810.40 | 452.211 | 2258.44 | 1194.114 | 422.12 | 222.845 |
| FGF-2 | 0.35 | 0.348 | 0.43 | 0.303 | 0.90 | 0.446 | 0.66 | 0.273 |
| IL-8 | 828.95 | 278.716 | 1335.83 | 504.504 | 1721.21 | 882.055 | 714.10 | 138.508 |
| IL-1α | 46.56 | 25.935 | 19.36 | 5.564 | 1.54 | 0.995 | 0.99 | 0.746 |
| IL-1β | 0.39 | 0.237 | 0.47 | 0.147 | 0.26 | 0.151 | 0.17 | 0.075 |
| IL-1ra | 110.33 | 81.088 | 40.82 | 17.946 | 2.07 | 1.792 | 7.19 | 0.075 |

## 226. LATE ONSET SYSTEMIC SCLEROSIS: A SYSTEMATIC SURVEY OF THE EULAR SCLERODERMA TRIALS AND RESEARCH GROUP DATABASE

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**Background:** The clinical course of SSc depends on subtype, organ involvement and age. Few data are reported on patients suffering from late-onset SSc.

**Methods:** We analysed data from 8554 patients prospectively followed in the EULAR Scleroderma Trials and Research (EUSTAR) group database. Late-onset SSc was defined as onset of non-RP disease features at or beyond 75 years of age. Disease characteristics, clinical features, disease course and mortality were evaluated.

**Results:** A total of 123 patients with SSc onset at or beyond 75 years of age were identified. Compared with patients <75 years they had more frequently limited than diffuse SSc and a higher prevalence of anti-centromere autoantibodies. Fewer old patients had digital ulcers. The modified Rodnan's skin score, the prevalence of lung fibrosis and renal crisis did not differ significantly between groups. Pulmonary hypertension (PH) measured by echocardiography was more prevalent in the late-onset group, as well as arterial hypertension and diastolic dysfunction. Late-onset SSc remained a positive predictor for PH in multivariate analyses. No significant difference of the two groups in skin score or diffusion capacity was observed during follow-up. Mortality due to SSc was higher in the late-onset group, but the survival time from diagnosis was longer compared with the younger patients.

**Conclusions:** Late-onset SSc shows a distinct clinical presentation and outcome. Patients with late-onset SSc suffer more frequently from the limited subtype and PH, but fewer patients have digital ulcers. PH may in part be determined by underlying cardiovascular disease.

**Disclosure statement:** The authors have declared no conflicts of interest.

### 227. INFLUENCE OF THE COLD CHALLENGE ON THE DISCRIMINATORY CAPACITY OF THE DIGITAL DISTAL-DORSAL THERMAL GRADIENT IN THE THERMOGRAPHIC ASSESSMENT OF RAYNAUD'S PHENOMENON

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**Background:** A key challenge facing clinicians evaluating patients with Raynaud's phenomenon (RP) is the early identification of those patients at risk of connective tissue disease such as systemic sclerosis (SSc), in whom RP can predate the onset of other clinical manifestations by many years. This study evaluates the influence of the cold challenge on the discriminatory capacity of the digital longitudinal thermal gradient in differentiating between primary RP and SSc.
**Methods:** A retrospective review was performed of unselected patients with primary RP ($n = 27$) and SSc ($n = 28$) in whom thermographic assessment of RP had been undertaken. The 'distal-dorsal difference' (DDD) for each digit (and a composite score of all fingers) was calculated at baseline and 10 minutes following standardised cold challenge (60s at 20°C). The discriminatory capacity of the mean DDD for individual digits, and the composite score of all fingers, before and after cold exposure was assessed.
**Results:** There was significant variation in the DDD for each digit in both groups (Table 1). The mean DDD of the thumbs was significantly higher than the digits in both PRP ($P < 0.05$) and SSc ($P < 0.005$). The fingers provided greater discriminatory capacity between patient groups than the thumbs at baseline, although the associations only achieved borderline significance owing to the large variation of the data (Table 1). The cold challenge led to signifcant increases in the magnitude of the DDD for all digits in primary RP, but not in SSc. This reduced the discriminatory capacity of the DDD after cold challenge by narrowing differences present between RP subgroups at baseline. The composite score of all fingers failed to improve the overall discriminatory capacity of the mean DDD ($P = 0.11$).
**Conclusions:** Large variation in the DDD amongst patients prevents easy discrimination between disease states although the fingers appear to provide greater discriminatory capacity than the thumbs. Relative sparing of the thumb occurs in both primary RP and SSc but this does not aid differentiation between disease states. Our findings question the value of the cold challenge in the thermographic assessment of RP and its contribution must be reappraised. Baseline assessment alone, if sufficient, may facilitate greater use of thermographic assessment of RP outside that of specialist centres.
**Disclosure statement:** The authors have declared no conflicts of interest.

TABLE 1. DDD for individual digits and a composite score of all digits, before and after cold challenge

| | Primary RP ($n = 27$) Mean (S.D.) Baseline Post CST | SSc ($n = 28$) Mean (S.D.) Baseline Post CST | Primary RP vs SSc P value Baseline Post CST |
|---|---|---|---|
| Thumb | −1.0 (2.6) −1.7 (2.0)* | −1.4 (2.5) −1.8 (3.1) | 0.51 0.95 |
| Index | −1.4 (2.7) −2.7 (3.1)* | −2.7 (2.6) −3.2 (3.1) | 0.08 0.55 |
| Middle | −1.9 (2.7) −3.1 (2.9)* | −2.9 (2.5) −3.4 (3.0) | 0.18 0.67 |
| Ring | −1.9 (2.7) −3.2 (3.1)* | −3.2 (2.4) −4.0 (2.8)* | 0.07 0.34 |
| Little | −1.9 (2.7) −3.3 (3.0)* | −3.2 (2.6) −4.0 (3.0)* | 0.06 0.39 |
| Composite of all digits | −1.62 (2.6) −2.81 (2.9)* | −2.7 (2.2) −3.28 (2.8) | 0.11 0.54 |

RP: Raynaud's phenomenon; SSc: systemic sclerosis; CST: cold stress test. *$p < 0.05$ for baseline vs post CST.

### 228. CTGF/CCN2 IS ESSENTIAL FOR SKIN SCLERODERMA

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**Background:** Connective tissue growth factor (CTGF/CCN2) is highly overexpressed in fibrotic disease including in scleroderma (systemic sclerosis/SSc. In fact, CTGF/CCN2 is a good surrogate marker for the severity of the fibrosis in this disease. However, whether CTGF/CCN2 expression functionally contributes to the fibrosis in SSc is not fully understood. The critical cell type contributing to the fibrosis in SSc is considered to be the myofibroblast. The aim of this study was to determine whether CTGF/CCN2 is required for the development of skin scleroderma and whether CTGF/CCN2 is required for myofibroblast differentiation or recruitment.
**Methods:** We subjected control mice or mice deleted for CTGF/CCN2 in fibroblasts to the bleomycin-induced model of skin SSc. The extent of fibrosis was assessed histologically using hematoxylin and eosin staining. The appearance of myofibroblasts was assessed using anti-a-smooth muscle actin antibodies. Collagen deposition was assessed using hydroxproline/proline analysis and trichrome staining. Fibroblast responses to TGFbeta were assessed using real time PCR to detect mRNA expression. Whether pericytes were induced into the fibrotic lesion was assessed using an antibody against NG2.
**Results:** Loss of CTGF/CCN2 resulted in resistance to bleomycin induced skin fibrosis; compared to controls, loss of CTGF/CCN2 resulted in resistance to bleomycin-induced skin thickness and collagen deposition (all $N = 6$, all $p < 0.01$). TGFbeta was able to induce collagen and a-SMA mRNA expression in CTGF/CCN2 knockout cells ($N = 6$; $p < 0.05$). In wild type mice, bleomycin caused the appearance of a-SMA expressing myofibroblast that were also positive for the pericyte marker NG2; whereas, in mice lacking CTGF/CCN2, a-SMA/NG2-expressing myofibroblasts were not recruited into the fibrotic lesion ($N = 6$, $p < 0.01$).
**Conclusions:** CTGF/CCN2 is required for bleomycin-induced skin scleroderma. CTGF/CCN2, as it is required for myofibroblasts/pericyte recruitment, could be a good target for antifibrotic drug intervention in SSc.
**Disclosure statement:** A.L. is a shareholder of FibroGen. All other authors have declared no conflicts of interest.

### 229. ANALYSIS OF SIGNAL TRANSDUCTION IN SYSTEMIC SCLEROSIS EPIDERMIS

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**Background:** Systemic sclerosis (SSc) is a severe disease characterised by cellular injury and activation in early stage, followed by autoimmunity and fibrosis. Previously, we found using a proteomic analysis of diffuse SSc and healthy control forearm skin biopsy material, activation of the disease epidermis. Therefore, we decided to look at the signalling pathways that might be responsible for the state of activation, and as a result lead to fibrosis.
**Methods:** Epidermal biopsies from early diffuse patients and healthy controls (both $n = 4$) were lysed for phosphorylation array analysis (Kinexus). The arrays included 880 proteins associated with diverse processes. The data were compared using permutation analysis software SAM for Excel. In addition further biopsies from both patients and controls (both $n = 6$) were studied by immunohistochemistry for proteins identified in the array analysis as altered in phosphorylation status (phospho-smad 2/3, p-38α, c-Met and c-jun).
**Results:** The array analysis indicated increase in phosphorylation status of proteins responsible for response to stress, wound healing as well as histone modifications, in the disease (Table 1). Immunohistochemistry revealed elevated phospho-p38α and phospho-c-jun in basal layer keratinocytes in SSc. Smad2/3 phosphorylation was seen throughout the SSc epidermis and in cells in the adjacent upper dermis. C-Met phosphorylation was seen in cytoplasm of SSc epidermis at all levels of differentiation.
**Conclusions:** The activated phenotype of SSc keratinocytes may be initiated by environment induced stress signaling in basal layer keratinocytes (MAPKs p38 and c-jun),or be maintained by HGF released by underlying mesenchymal cells (c-Met signaling). Cells in the upper dermis are being induced via SMAD signaling pathway and should be further characterized to distinguish epithelial-mesenchyme transition vs induction of adjacent fibroblasts. The altered differentiation in SSc epidermis may involve epigenetic histone modification.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 230. IDENTIFICATION OF POTENTIAL MARKERS OF SKIN FIBROSIS USING TARGETED DISRUPTION OF TGF BETA SIGNALING IN TRANSGENIC MICE

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**Background:** TGFβ is a profibrotic cytokine involved in tissue repair and wound healing. We have demonstrated that deletion of TβRII selectively in fibroblasts is antifibrotic. Previous studies show that mice from this strain are highly resistant to bleomycin induced experimental fibrosis. We have used this model to identify potential key mediators of fibrotic scar formation in the skin. These may include novel markers

TABLE 1.

| Biologic process | Protein | Fold increase in SSc | P value | Function |
|---|---|---|---|---|
| Stress/injury response | Jun proto-oncogene-encoded AP1 transcription factor (c-jun) | 2.37 | 0.02 | AP-1 transcription factor downstreram of C-jun N-terminal kinase stress activated MAPK |
| | Dual specificity protein kinase | 2.18 | 0.019 | Regulator of MAPKs |
| | Mitogen activated protein- serine kinase p38α (T180 Y182) | 2.13 | 0.05 | Stress-induced MAPK |
| | Mitogen-activated protein-serine kinase p38α (pan specific) | 1.53 | 0.014 | Stress-induced MAPK |
| | MAPK/ERK protein-serine kinase 2 (MKK2) | 1.73 | 0.029 | Response to extracellular growth factors |
| | Tank-binding protein-1 | 1.55 | 0.039 | type I interferon pathway/TLR3 signalling |
| | MAPK/ERK protein-serine kinase 1 (MKK1) | 1.48 | 0.039 | Response to extracellular growth factors |
| | Inhibitor of NF-kappa-B alpha (MAD3) | 1.35 | 0.019 | Regulation of NFκB pathway |
| | NF-kappa-B p65 nuclear transcription factor | 1.23 | 0.046 | Forms heterodimer with NF-κB transcription factor. Required for nuclear translocation of NF-κB |
| Regulation of tissue repair | Hepatocyte growth factor (HGF) receptor (c-Met) | 2.89 | 0.05 | Mesenchymal-epithelial signalling |
| | STAT3 | 2.18 | 0.05 | Signalling downstream of c-Met, IL-6 or other inflammatory cytokine |
| | STAT2 | 1.62 | 0.015 | |
| | Integrin-linked protein-serine kinase 1 | 2.17 | 0.04 | Coupling of signal transduciton to adhesion and motility during wound repair |
| | PDGF receptor kinase α | 1.75 | 0.017 | PDGF receptor signal transduction |
| | PDGF receptor kinase α/β | 1.63 | 0.036 | PDGF receptor signal transduction |
| | SMAD2 | 1.64 | 0.029 | TGFβ dependent signal transduction |
| Epigenetic silencing | Protein-serine phosphatase 4-regulatory subunit (PPX/A'2) | 2.57 | 0.00052 | Regulator of histone deacetylase |

that could be used to assess disease activity in systemic sclerosis (SSc).

**Methods:** We have deleted TβRII in fibroblasts of mice post-natally, using a Cre-Lox strategy that circumvents the embryonic lethality of a conventional knock-out for this gene by the administration of tamoxifen to activate Cre recombinase and hence deletion of TβRII. Gender matched littermates were studied and 4 mm dermal punch wounds performed. Wound histology was assessed for collagen deposition using Sirius red and Masson's trichrome stains. Dermal fibroblasts cultured from wildtype or mutant mice were treated with recombinant TGFβ1 (2 ng/ml) and illumina microarray gene profiling performed.

**Results:** Healing of full thickness skin wounds was severely impaired in mice after fibroblast-specific deletion of TβRII. For wildtype wounds at 14 days mean (± sd) diameter was 0.57 mm (± 0.29) compared with 1.75 mm (± 0.28) for TβRII-null-fib wounds (p = 0.001). In 7 day null wounds there was reduced expression of markers of wound healing including alpha-SMA. Illumina microarray analysis defined a TGFbeta induced gene cohort in wildtype cells and confirmed that mutant fibroblasts were essentially refractory. Key marker genes for myofibroblast phenotype were not induced and induction of several candidate profibrotic mediators including CTGF and endothelin-1 was diminished. Representative data for 5 key genes is summarised in Table 1.

**Conclusions:** Our findings confirm that an intact TGFbeta response in resident dermal fibroblasts plays a key role in normal skin wound healing. Our observations are potentially relevant to strategies that aim to attenuate fibrosis since these same approaches may have detrimental effects on connective tissue repair. This mouse strain provides a platform for better understanding human fibrotic disease such as SSc and genes that show differential expression may be candidate markers of scar formation and fibrosis.

**Disclosure statement:** The authors have declared no conflicts of interest.

## 231. HAEMOGLOBIN VIDEO IMAGING OF CONJUNCTIVAL MICROCIRCULATIONS IN SYSTEMIC SCLEROSIS

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**Background:** Haemoglobin video imaging (HVI) of the conjunctiva and episclera enables erythrocytes to be visualized at high resolution within vessel lumina. This presents the opportunity of investigating microcirculatory disease non-invasively and in real time. Systemic sclerosis (SSc) was studied as a model system due to the characteristic arterial microvasculopathy which leads to vessel occlusion and progressive tissue ischaemia.

Objective: To devise a nomenclature to describe the architecture of the conjunctival microcirculatory lobules.

To extract an initial metric by which to compare lobular architecture in patients and SSc controls.

**Methods:** HVI documents erythrocyte flow by illuminating the microcirculation with green light, absorbed by haemoglobin, and recording its reflection from the underlying sclera using a digital video camera (1360 x 1024 pixels). Software was developed to stabilise the image motion resulting from micro-saccades. Recordings from a 5 x 3.7mm region adjacent to the corneo-scleral limbus were captured at 30 frames /sec over 1 minute in 12 healthy controls and 12 patients with SSc. A nomenclature was developed for the microcirculation, in which each vessel segment between branch-points was assigned a number to denote its relative position in a circulatory lobule. For each lobule, the vessel segment distal to the final bifurcation and proximal to the first convergence was designated ''0''. Proximal (arterial) vessel segments were assigned ''-1'', ''-2'' etc and distal (venous) vessels ''+1'', ''+2'' etc. The mean vessel segment assignment (MVSA) for each segment was the mean of the assignments for all the lobules in which it participated. Software was developed to semi-automate the assignment and data output. The effect of arterial dropout on the MVSA was modelled according to whether the vessels downstream of the dropout are resorbed (MVSA unchanged) or resupplied by connection with an adjacent lobule, and, if resupplied, whether the connection is at a level equivalent (MVSA unchanged), above (MVSA reduced) or below (MVSA increased) that of the lost arterial segment.

TABLE 1. Normalised gene expression in skin fibroblasts from wildtype and mutant mice

| GENE | WILDTYPE (N = 6) | | | | | | NULL (N = 4) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BASAL | | TGFb1 | | | | BASAL | | TGFb1 | | | |
| | Mean | S.D. | Mean | S.D. | Fold Change | P Value | Mean | S.D. | Mean | S.D. | Fold Change | P Value |
| **alphaSMA** | 2260 | 1329 | **9363** | 3176 | **4.1** | 0.00009 | **3136** | 707 | **962** | 1220 | **1.1** | 0.23 |
| **Transgelin** | 1231 | 870 | **4748** | 1267 | **3.9** | 0.00005 | **1505** | 564 | **518** | 695 | **1.2** | 0.20 |
| **Endothelin-1** | 352 | 108 | **1115** | 304 | **3.2** | 0.00002 | **356** | 79 | **109** | 71 | **1.1** | 0.21 |
| **Myosin Heavy Chain** | 324 | 221 | **985** | 642 | **3.0** | 0.01021 | **408** | 93 | **126** | 108 | **1.1** | 0.18 |
| **CTGF** | 5783 | 3196 | **12198** | 5210 | **2.1** | 0.01014 | **4352** | 3006 | **1840** | 4426 | **1.1** | 0.11 |

**Results:** The mean and 95% confidence intervals were calculated for MVSAs arising from arterial segment levels in healthy controls. Of the 12 SSc patients, 9 had arterial MVSAs within the normal range. Three patients each had a series of arterial segments for which MVSAs fell below this range.
**Conclusions:** These data have provided a first metric by which to analyse remodeling in a multi-level living human microcirculation. They are consistent with arterial dropout and anastomosis between adjacent lobules at the zero order level. This pilot study indicates that HVI offers potential for non-invasive imaging of the microcirculation in SSc and other diseases characterized by microvascular pathology.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 232. ELEVATED MMP-7 SERUM LEVELS AS MARKER FOR TISSUE REMODELLING AND FIBROSIS IN SYSTEMIC SCLEROSIS AS A MODEL DISEASE

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**Background:** Fibrosis is characterized by an excessive accumulation of connective tissue due to a disturbed balance between synthesis and degradation of extracellular matrix proteins (ECM). This is regulated by matrix metalloproteinases (MMPs) and their inhibitors (TIMPs).

Matrix Metalloproteinase-7 is involved in the degradation of ECM in many physiological situations as well as in other disease processes, like tumor invasion, wound healing and lung fibrosis. It was the aim of the present study to investigate whether serum MMP-7 levels might reflect the tissue remodeling going on during the course of fibrotic diseases, using systemic sclerosis (SSc) a prototypic fibrotic disease as a model.
**Methods:** Serum samples were obtained from 123 patients with systemic sclerosis. MMP-serum levels of all SSc patients were compared with age matched healthy controls ($n = 22$) that had no rheumatic disease by using a commercial enzyme immunoassay kit (Quantikine).
**Results:** An increased median serum MMP-7 level of 5.15 ng/ml was found in scleroderma patients when compared to controls ($p < 0.0001$). Male patients showed a significant higher level of MMP-7 compared to female patients ($p = 0.016$) and patients suffering from the limited form of SSc (lSSc) showed lower MMP-7 serum levels compared to the diffuse form (dSSc), but their differences did not reach significance ($p = 0.064$). Diffuse type of scleroderma patients were more frequently male (26%), and suffered more frequently from PAH (22%), lung fibrosis (57%), kidney failure (24%) and heart involvement (14%), when compared to patients with the limited form of SSc. Median MMP-7 serum levels were significantly different between patients with or without lung fibrosis (5.98 ng/ml versus 4.17 ng/ml; $p < 0.016$). Accordingly, patients with dyspnea also had significantly higher MMP-serum levels ($p = 0.024$). SSc patients suffering from lung fibrosis without combined PH had lower mean MMP-7 serum levels compared to SSc patients suffering from lung fibrosis with secondary PH. Patients with a decreased DLCO below 60% of their predicted value had higher mean concentrations of MMP-7 (5.68 ng/ml) than patients with a DLCO level above 75% (3.80 ng/ml) ($p = 0.006$). There was no significant difference for other organ manifestations, e.g. oesophagus, kidney, musculoskeletal system, digital ulcerations and sicca-syndrome.
**Conclusions:** These results indicate the correlation between high MMP-7 levels and the more progressive course of fibrosis. Further analyses seem to be interesting and important, whether MMP-7 activity is directly involved in the pathophysiology of this disease and whether it naturally reflects tissue remodelling.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 233. ROLE OF ET-1 AND GM-CSF ON THE DIFFERENTIATION POTENTIAL OF MONOCYTES IN SYSTEMIC SCLEROSIS

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**Background:** Systemic sclerosis (SSc) is a rare but potentially life threatening connective tissue disease characterised by vasculopathy, inflammation, the production of autoantibodies and excessive fibrosis of skin and internal organs. Fibrosis is defined by an excess production of extracellular matrix (ECM) molecules predominantly of type I collagen. The main producers of collagen are myofibroblasts. These cells are characterized by the expression of $\alpha$-SMA.

Monocytes are the first cells that infiltrate inflamed or damaged tissue and they bear a high differentiation potential. Furthermore, in SSc it is suggested that monocytes are activated and play an important role in disease onset and progression. We postulate that monocytes can differentiate into myofibroblasts-like cells under the influence of soluble factors such as endothelin-1, IL-4 and GM-CSF which are elevated in SSc serum.
**Methods:** To study the effect of ET-1 as well as GM-CSF and IL-4, we isolated monocytes from whole blood of healthy controls or SSc patients by ficoll and positive selection with CD14 beads. Monocytes were cultured in the presence of the above soluble factors cytokines over 14 days. Expression of $\alpha$-SMA and type I collagen was then determined by Western blotting, qPCR and immunofluorescence.
**Results:** Monocytes from healthy individual and SSc patients responded different to certain stimuli. In particular, we found that differentiation of healthy monocytes into $\alpha$-SMA expressing cells is strictly dependent on GM-CSF. However, in patient monocytes ET-1 and IL-4 are also able to promote $\alpha$-SMA expression but to a lesser extend than GM-CSF. Furthermore, we observed that type I collagen expression requires GM-GSF treatment but can be enhanced with costimulation of ET-1.
**Conclusions:** These data suggest that monocytes can differentiate into a myofibroblast-like phenotype and that SSc patient monocytes require a less complex stimulation to do so. Understanding the role of monocytes in fibrosis may contribute to the development of novel therapies for systemic sclerosis.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 234. THE RELEVANCE OF THE EPITHELIAL-MESENCHYMAL TRANSITION FOR SYSTEMIC SCLEROSIS

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**Background:** Systemic sclerosis (SSc) is a disorder of systemic and dermal fibrosis of uncertain etiology. Recently, we found that SSc epidermis is abnormal, taking on an activated phenotype observed during wound healing and tissue repair. Epithelial-fibroblast interactions are important during wound repair and in fibrosis. Based on studies from transgenic mice over-expressing CTGF, our in vivo data suggested that CTGF expression in fibroblastic cells in the skin and lungs not only causes extensive fibrosis and myofibroblast transformation, but also changes in the differentiation program of adjacent epithelial cells, which directly contributes to fibrogenesis. Here, we focus on epithelial-mesenchymal transition (EMT) fibrosis disease, SSc.
**Methods:** Whole skin biopsy material from SSc and controls (both $n = 4$) was immediately frozen in liquid nitrogen after excision. Epidermal cell layer was dissected surgically from the frozen material. The tissue processing and phospho-kinase screening was performed by Kinexus (Vancouver, Canada). All experiments were performed with MEFs prepared from embryos from at least three different litters in a mixed 129Sv/J:c57Bl/6 background. MEF were plated on 0.1% gelatine coatedplates and grown in DMEM + 10%FBS. Human epithelial cells (A549) cultured with DMEM. Cells were stimulated with TGFbeta1 after serum starvation. Fibrosis and epithelial markers were examined using immunofluorescence staining and Western blot analysis.
**Results:** Phosphorylation arrays were performed on the epidermal tissue from SSc patients and healthy controls to identify signaling pathways activated in SSc epidermis. A number of EMT-related

proteins were found to have elevated phosphorylation states in SSc tissues versus controls, including c-Met, Wee1 protein-tyrosine kinase, STAT3, Integrin-linked protein-serine kinase 1($p < 0.05$). Further in vitro studies, A549 exposed to TGFbeta develop a fibroblast morphology and molecular markers associated with EMT (snail and CTGF). CTGF-specific siRNA dose-dependently suppresses TGFbeta-induced snail and CTGF protein expression towards basal levels in A549 cells. Col1a2-CTGF-transgenic MEFs expressed epithelial marks, E-cadherin, b-catenin. The transcription factors Sox9 and snail are master regulators of EMT. The transgenic MEFs showed significantly higher expression levels for matrix genes and proteins that include typel Collagen, Fibronectin. Increased expression of SMA and CTGF correlate with high levels of collagen expression.

**Conclusions:** In this study we show that phosphorylation array analysis revealed induction of EMT protein kinase signaling in SSc epidermis. Important growth factor, TGFbeta, promotes and triggers epithelial cells EMT. Col1a2-CTGF-transgenic MEFs from transgenic mice express both epithelial and mesenchymal markers. We propose that in SSc, epidermal cells are in a persistently activated state and are able to promote EMT which contributed fibrosis.

**Disclosure statement:** The authors have declared no conflicts of interest.

### 235. ELEVATION OF N-TERMINAL PRO-BNP IN CLINICALLY SIGNIFICANT SCLERODERMA HEART INVOLVEMENT

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**Background:** Cardiac involvement is common in Systemic Sclerosis (SSc) and is often clinically occult. It is recognised as a poor prognostic factor contributing significantly to mortality. Early detection of cardiac disease with non-invasive tools is therefore critical.

Aim of this study was to assess the role of N-TproBNP in a retrospective cohort of SSc patients with heart involvement.

**Methods:** 19 SSc patients (13 dcSSc and 6 lcSSc, 13 female) with cardiac involvement were enrolled. Cardiac involvement was defined as haemodynamically significant arrhythmias, pericardial effusion or congestive heart failure, requiring specific treatment. All patients had normal pulmonary artery systolic pressure and none had serum creatinine over 140 $\mu$mol/l. This group was compared with 19 age- and sex-matched SSc patients without evidence of cardiac involvement or pulmonary hypertension. Normal serum N-TproBNP levels were less than 20 pmol/l. Associations between N-TproBNP and left ventricular ejection fraction, Troponin-I, systolic and diastolic blood pressure, lung function and mRSS were determined by Pearson's correlation coefficient. Univariate mortality analysis was performed with Kaplan-Meier method, we compared survival curves with Log-rank test. Unpaired t-test was used to compare N-TproBNP values between subgroups with or without cardiac involvement. ROC curves were drawn to identify N-TproBNP levels with optimal sensitivity and specificity for diagnosis of SSc-related cardiac involvement. N-TproBNP levels were compared at presentation of cardiac involvement and at six month follow up using a paired t-test. Within the group with cardiac involvement, high levels of N-TproBNP levels were defined as above the median value of 219 pmol/l.

**Results:** Compared with those without cardiac involvement, N-TproBNP was significantly increased in SSc patients with cardiac involvement (mean $\pm$ SD 14.9 $\pm$ 14.5 pmol/l versus 1043 $\pm$ 2053 pmol/l respectively, $p = 0.037$; 95%CI 67,1989). ROC curves of N-TproBNP to predict the presence of heart involvement in SSc were drawn: a sensitivity of 100% was achieved at a cut-off of 28 pg/mL, with a specificity of 84% (95% CI 0.95-1). There was a progressive reduction in N-TproBNP after the acute phase of cardiac involvement (mean $\pm$ SD 301 $\pm$ 330 pmol/l) during 6 months follow-up (mean $\pm$ SD 87 $\pm$ 113 pmol/l; $p = 0.048$, 95%CI 2.8,425). Serum N-TproBNP levels correlated negatively with left ventricular ejection fraction (with logarithmic transformation of N-TproBNP values, $R2 = 0.52$, $p = 0.001$). However, there was no correlation between N-TproBNP and systolic and diastolic BP, troponin-I, mRSS, FVC and DLCO. In addition, higher levels of N-TproBNP did not predict survival within the two groups of patients with cardiac involvement.

**Conclusions:** These data suggest that N-TproBNP may be a surrogate marker for cardiac involvement in SSc. Further studies are required to evaluate the utility of N-TproBNP levels for cardiac assessment in SSc patients.

**Disclosure statement:** The authors have declared no conflicts of interest.

### 236. IGG FROM SYSTEMIC SCLEROSIS PATIENTS PROMOTE IL-1$\alpha$ RELEASE FROM HUMAN KERATINOCYTES

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**Background:** Recently we found that the epidermis in systemic sclerosis (SSc) has an abnormal wound healing phenotype, and that epidermal cells from SSc patients promote fibroblast activation and induce CTGF in fibroblasts. We propose that activation of the epidermal keratinocytes is an important early event contributing to the fibrosis seen. One possibility is that changes in the epidermis are being initiated by an autoimmune process. We measured binding of SSc patients' IgG to cultured human keratinocytes, and determined whether SSc patients' IgG promote keratinocyte activation in tissue culture.

**Methods:** Anti-keratinocyte antibody titres were determined for SSc patients sera (30 limited subset, 30 diffuse subsets) and sera from healthy control individuals ($n = 30$) using fixed normal human keratinocytes as target antigen. Binding was assessed using secondary HRP-labelled anti-human IgG. SSc and control IgG were affinity purified from plasma by protein A column. Normal human keratinocytes were cultured under serum free conditions and then exposed to SSc and healthy control IgG (both $n = 3$)(0, 10, 100 $\mu$g/ml). Keratinocyte activation was determined by measuring levels of IL-1alpha in cell layer and media before and after exposure to IgG for 24 hours. Additional time course experiments were performed to determine the kinetics of IL-1$\alpha$ release. Additional studies using immunofluorescence were performed to determine binding of SSc IgG to cultured keratinocytes, and to detect IgG in sections of SSc and control epidermis.

**Results:** Anti-keratinocyte antibody titres were in increased in both limited SSc and diffuse SSc sera compared to controls (Table 1). There was a non significant trend toward increased IL-1$\alpha$ release by SSc IgG 10ug/ml at 24 hours (untreated keratinocyte cell layer 47.6 pg/ml, media 1.8 pg/ml, control IgG treated cell layer 26.2 pg/ml, media 7.5 pg/ml, SSc IgG treated cell layer 33.7 pg/ml, media 27.9 pg/ml (p not significant)). A time course of IL-1$\alpha$ release following treatment with SSc and control IgG (10 $\mu$g/ml) was determined by treating keratinocytes in 6 well plates and removing media and lysing cell layer at various timepoints. SSc IgG lead to a rapidly evoked and biphasic induction of IL-1$\alpha$ release. Positive immunfluorescence for IgG was seen in 2 out of 6 recent onset SSc epidermal biopsies.

**Conclusions:** Cellular injury or activation by autoantibodies in systemic sclerosis (SSc) remains a controversial area, with variation in findings between centres, and a number of potential mechanisms shown. Our idea, supported by the above results, is that SSc antibodies induce innate responses in resident cells including keratinocytes. Possible mechanisms include induction of TLR signalling, or carriage of SSc autoantigens into target cells.

**Disclosure statement:** The authors have declared no conflicts of interest.

TABLE 1.

| | control sera ($n = 30$) | limited SSc sera ($n = 30$) | diffuse SSc sera ($n = 30$) |
|---|---|---|---|
| Anti-keratinocye ELISA (%) (median, interquartile range). | 6.11 (0-28.7) | 23.8 (5.2-43.4) | 18.8 (9.8-36.0) |
| p (Mann-Whitney test) | | $p < 0.026$ | $p < 0.030$ |

### 237. AN EXPLORATION OF PULMONARY VASCULAR VEGF SIGNALLING IN A TGF$\beta$-DEPENDENT MOUSE MODEL OF SYSTEMIC SCLEROSIS

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**Background:** Vascular complications of systemic sclerosis (SSc) are a major cause of mortality and morbidity. A role for altered VEGF signalling in PAH-SSc is supported by data that correlate circulating VEGF with mPAP at diagnosis. High circulating VEGF levels may be a marker of repair in response to vascular injury. We have therefore examined VEGF signalling in a TGF$\beta$-dependent mouse model of SSc with evidence of a constitutive pulmonary vasculopathy.

**Methods:** The transgenic mouse strain TβRIIΔk-fib expresses a kinase-deficient type II TGFβ receptor driven by a fibroblast-specific promoter leading to balanced ligand-dependent upregulation of TGFβ signalling. Pulmonary vasculopathy was confirmed by histological assessment of vessel architecture, isolated organ bath and in vivo haemodynamic studies performed on adult male transgenic and littermate wildtype animals ($n = 8$ in each group). Biochemical analysis of the VEGF and endothelin axes were performed assessing RNA by quantitative PCR and protein by Western blotting using cultured aortic and pulmonary artery smooth muscle cells, and by immunostaining of tissue sections. Results were compared to the same cells cultured under hypoxic conditions. A pharmacological inhibitor of VEGF signalling was then administered to investigate effects of perturbed VEGF signalling in vivo.
**Results:** Within the pulmonary arterial circulation, transgenic vessel wall thickness was increased, particularly in smaller vessels (30-60$\mu$m diameter) due to hypertrophy of the smooth muscle layer (mean wildtype vessel thickness:circumference ratio $0.66 \pm 0.02$, mean transgenic $0.88 \pm 0.04$, $p < 0.05$). Pulmonary arterial ring responses to direct and receptor-mediated contractile stimuli were reduced in the transgenic animals (in response to endothelin contraction at 10-5M wildtype 1.10 mN $\pm$ 0.02, transgenic $0.62 \pm 0.12$, $p < 0.05$) and right ventricular pressures were elevated in transgenic animals (wildtype mean 29 mmHg $\pm$ 4, transgenic mean 37 mmHg $\pm$ 3). Explanted transgenic vascular smooth muscle cells showed upregulation of TGFβ responsive genes including VEGF and VEGFR1 which were further upregulated in the pulmonary arterial circulation (PASMC) when compared to aortic smooth muscle cells (AoSMC) from the same animals, particularly when cultured in a hypoxic environment. Administration of SU5416, a VEGF receptor inhibitor, resulted in endothelial proliferation within the pulmonary circulation.
**Conclusions:** The pulmonary vascular phenotype of this transgenic mouse model appears to replicate key histological and pathophysiological features of human SSc, and supports a potential role for perturbed TGFβ and VEGF activity in the pulmonary circulation in this model. Administration of a VEGFR inhibitor in this model may more accurately replicate the pulmonary vasculopathy of human SSc.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 238. ANALYSIS OF POLYMORPHISMS IN THE STAT4, IRF5 AND BANK1 GENES IN UK PATIENTS WITH SYSTEMIC SCLEROSIS

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**Background:** Scleroderma or systemic sclerosis (SSc) is a complex genetic disease. Recent studies, have found consistent associations between polymorphisms in STAT4, IRF5 and BANK1 with SSc, both at a susceptibility level and with specific clinical aspects of the disease. The aim of the study was to investigate whether the previously reported association of polymorphisms in STAT4, IRF5 and BANK1 genes in patients with SSc, is also apparent in an extended group of UK Caucasian SSc patients.
**Methods:** Patients with SSc (436) and 245 healthy controls were screened for polymorphisms in STAT4 (rs1188934, rs7574865), IRF5 (rs2004640) and BANK1 (rs10516487) genes. All patients and controls were UK Caucasoid. Patients were grouped according autoantibody status in 3 mutually exclusive groups: anti-topoisomerase1 (ATA), anticentromere (ACA) and antiRNA-polymerase (ARA). We also divided the groups according organ involvement; lung fibrosis (LF) (assessed by HRCT and lung function test) and pulmonary arterial hypertension (PAH) (defined as an elevation in the mean pulmonary artery pressure >25 mmHg with normal pulmonary capillary wedge pressure (< 15 mmHg) on right heart catheterisation). Genotyping was performed by the KASPar system (allele specific PCR, KBiosciences, UK). Statistical analysis: 3x2 tables and Chi-square analysis was used to compare the distribution of the polymorphism between the groups of patients and controls. $P < 0.05$ was considered statistically significant.
**Results:** Of the SSc patients, 285 (65.4%) were limited cutaneous (lcSSc) and 151 (34.6%) were diffuse cutaneous (dcSSc). There were 182 (41.7%) patients with LF and 75 (17.2%) with PAH. One hundred and five patients (24%) were positive for ATA, 143 (32.8%) for ACA and 93 (21.3%) ARA. We found a significant difference ($p = 0.004$) in the genotype distribution of the IRF5 polymorphism between the control and SSc groups, while no association was observed between the SSc group as a whole and STAT4 or BANK1. There was a difference in the genotype distribution of the IRF5 polymorphism in the lcSSc group when compared with controls ($p = 0.004$). There was a significant variation in the allele distribution of the IRF5 polymorphism in patients with lung fibrosis ($p = 0.01$) and with ATA ($p = 0.01$) when compared with controls. Conversely, BANK1 was associated with PAH ($p = 0.0005$), but not with ACA. We also detected an association of both STAT4 polymorphisms with the presence of ACA ($p = 0.01$).
**Conclusions:** We confirm several of the reported associations of the STAT4, IRF5 and BANK1 polymorphisms with SSc in the UK Caucasian population. These genes have been described to have a role in immune regulation, and have been found to be associated with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, suggesting a common genetic pathway for autoimmunity. Our findings add further support for the role of genetic factors in the autoimmune component of SSc.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 239. ANALYSIS OF POLYMORPHISMS IN THE HOMEOBOX TRANSCRIPTION FACTOR NKX2-5 IN THE UK POPULATION OF SYSTEMIC SCLEROSIS PATIENTS

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**Background:** Systemic sclerosis (SSc) has a complex genetic background and is likely to involve several genes from different chromosomes. SSc is characterized by vascular alterations, autoimmunity and fibrosis. We have previously shown that the transcription factor NKX2-5, (chromosome 5q34), activates the collagen1α2 enhancer, stimulating collagen type I production in blood vessels. In addition we have shown abnormal expression of NKX2-5 in human pulmonary vessels of SSc patients with pulmonary arterial hypertension. Furthermore, NKX2-5 polymorphism has been recently shown to be associated with systemic lupus erythematosus, another autoimmune disease with vascular complications. We therefore considered NKX2-5 to be a good candidate gene for association studies in SSc. The aim of the study was to determine the genotype frequency of selected NKX2-5 polymorphisms in the SSc and control groups, and to ascertain the association of NKX2-5 polymorphisms with SSc and with its specific clinical and serological subgroups.
**Methods:** We screened 517 UK SSc patients and 378 healthy unrelated controls for 10 polymorphisms in the NKX2-5 gene region using a sequence-specific primer PCR (SSP-PCR). The polymorphisms studied were: rs6891790, rs1006225, rs6882776, rs3095870, rs12188595, rs3729937, rs17052019, rs11955407, rs3729938 and rs703752. All patients and controls were Caucasoid. Statistical analysis: logistic regression analyses were used to compare the distribution of NKX2-5 polymorphism alleles between the study groups. $p > 0.05$ was considered statistically significant.
**Results:** The SSc patients group (total 517; 341 limited cutaneous and 175 diffuse cutaneous) included 122 anti-topoisomerase1 antibody positive (ATA) individuals, 153 anticentromere antibody positive (ACA) individuals, and 222 individuals with fibrosing alveolitis.

All the genotypes tested were in Hardy Weinberg equilibrium. No significant difference was found in genotype, allele frequency or haplotypes for any of the investigated polymorphisms in NKX2-5, when SSc group as a whole and control subjects were compared. However, there was a significant association between the rare T allele of both rs3095870 and rs703752 polymorphisms in patients with ATA compared to SSc without this antibody ($p = 0.04$ odds ratio 1.35 confidence interval 95% 1.00 - 1.83, and $p = 0.03$ odds ratio 1.39 confidence interval 95% 1.02-1.88).
**Conclusions:** 10 polymorphisms in the region of the NKX2-5 gene were genotyped in groups of SSc patients and controls. The frequency of the rare T allele of the rs3095870 and rs703752 SNPs were associated with ATA subgroup of SSc patients. These variants are located in the promoter and the 3'UTR regions of NKX2-5, respectively. These areas are known to play a key role in the regulation of a gene expression and mRNA stability, suggesting a functional role of the SNPs in the NKX2-5 expression.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 240. AUTO-ANTIBODY STATUS AND DISEASE MANIFESTATIONS OF PATIENTS WITH SCLERODERMA ASSOCIATED DIGITAL ULCERS: DATA FROM THE DUO REGISTRY

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**Background:** Digital ulcers (DU) are a frequent, persistent and debilitating manifestation of systemic sclerosis (SSc). The DUO Registry is a European, prospective, multicentre, observational cohort study of patients with SSc associated DU; 271 centres (9 in the UK) are involved. This registry has potential to explore the relationship between DU disease and auto-antibody status and whether specific auto-antibody subsets or DU are more prone to occur in association with particular disease manifestations. Here, we describe the antibody profile, disease duration and organ-based manifestations of patients in the DUO Registry.
**Methods:** Patients undergo clinical assessments and receive standard medical care as determined by the patient's physician. All consenting consecutive SSc patients with ongoing DU irrespective of treatment regimen, are enrolled. Data collected since April 2008 includes demographics, auto-antibody status, disease history, and organ manifestations.
**Results:** Up to May 19, 2010, 1966 patients have been enrolled (82.3% female, mean $\pm$ SD age 54.2 $\pm$ 14.1 yrs. 51.1% of patients were classified with limited SSc and 38.0% with diffuse SSc. Almost all (95.6%) patients tested were positive for ANA; 45.6% for anti-Scl 70 antibodies, 43.5% for anti-centromere antibodies (ACA) and 11.2% for anti-RNA polymerase III. Few patients had a combination of these antibodies (anti-Scl 70 & ACA: n = 53, 2.7%; Anti-Scl 70 Ab & RNA polymerase III: n = 35, 1.8%). Compared with ACA-positive patients, anti-Scl 70 positive patients had shorter time periods from onset of Raynaud's phenomenon (RP) to first DU (anti-Scl 70: 6.0 [95% CI 5.3-6.6] yrs; ACA: 8.2 [95% CI 7.2-9.1] years) and from first non-RP manifestation to first DU (anti-Scl 70: 3.1 [95% CI 2.7-3.4] yrs; ACA: 4.2 [95% CI 3.5-4.8] yrs). Lung fibrosis was most common (62.8%) in the anti-Scl 70 subset. The frequency of kidney, heart and GI manifestations appeared to be similar across antibody subsets (Table 1).
**Conclusions:** Across the antibody subsets, patients positive for anti-Scl 70 had the highest frequency of lung fibrosis, and the most rapid disease evolution as reflected by the lag time from RP or first non-RP manifestation to first DU. Regardless of SSc type, our data suggest ongoing DU disease in patients with anti-scl70, which itself is a marker of poor outcome in SSc, is associated with the presence of organ involvement.
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### 241. LOSS OF PTEN EXPRESSION IN SKIN FIBROBLASTS CONTRIBUTES TO FIBROSIS IN SCLERODERMA

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**Background:** Skin fibrosis, the increased collagen deposition and contraction by fibroblasts resident within connective tissue, is a principal feature of scleroderma and is believed to result from a hyperactive tissue repair program. The protein phosphatase and tensin homolog (PTEN) suppresses various activities of cell including migration, contractility, survival and collagen production and thus may modulate tissue repair. We investigate whether loss of PTEN expression in skin fibroblasts is sufficient to result in fibrosis.
**Methods:** Skin fibroblasts were isolated from clincally affected areas of patients with early onset diffuse systemic sclerosis (scleroderma). Age- site- and sex- matched skin fibroblasts from healthy individuals served as controls. Western blot analysis was used to detect PTEN expression in normal and scleroderma fibroblasts. To assess whether loss of PTEN expression was sufficient for fibrogenesis in vivo, mice homozygous for a loxP-Pten allele and hemizygous for a tamoxifen-dependent cre recombinase expressed under the control of a fibroblast-specific type I collagen promoter were used, and either treated with tamoxifen (to delete Pten specifically in adult fibroblasts) or corn oil (vehicle; to generate control mice). Fibrosis in these mice was assessed histologically and by determining collagen levels using a hydroxyproline/proline assay. Western blot analysis was used to test whether adenoviral-delivered overexpression of PTEN rescued the fibrotic phenotype of scleroderma fibroblasts.
**Results:** PTEN protein expression was markedly decreased in scleroderma dermal fibroblasts ($N = 6$; $p < 0.05$). Mice deleted for Pten in fibroblasts (Pten -/-) possessed increased (a) thickness of the dermis (Pten +/+: 168 $\pm$ 57 $\mu$m, Pten-/- : 372 $\pm$ 112 $\mu$m, $p < 0.04$) and (b) collagen deposition (hydroxyproline content: Pten+/+: 3.7 $\pm$ 1.0, Pten-/-: 4.9 $\pm$ 0.8 g/100 g tissue $p < 0.03$) compared to controls (Pten +/+). Compared to control Pten+/+ dermal fibroblasts, cultured Pten-/- dermal fibroblasts produced elevated levels of the phosphorylated form of Akt as well as type I collagen ($N = 3$; $p < 0.05$); collagen overexpression in Pten-/- dermal fibroblasts was suppressed by inhibitors of PI3K/Akt signaling (LY294002 and wortmannin) ($N = 3$; $p < 0.05$). Reintroduction of PTEN into scleroderma fibroblasts rescued the overproduction of type I collagen by this cell type ($N = 3$; $p < 0.05$).
**Conclusions:** Loss of PTEN expression in fibroblasts is sufficient to create a fibrotic phenotype and hence could be a major factor in the development of fibrosis in scleroderma. Thus, PTEN agonists could be an important novel anti-fibrotic treatment for the skin fibrosis in scleroderma.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 242. SERUM LEVELS OF MMP7 AND MMP9 IN SYSTEMIC SCLEROSIS: CLINICAL ASSOCIATIONS AND POTENTIAL AS DISEASE BIOMARKERS

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**Background:** It has been shown that matrix metalloproteinase (MMP) 9 levels are increased in subjects with systemic sclerosis (SSc) compared to controls and that they correlate positively with modified Rodnan skin score (mRss). Raised MMP7 levels have been observed in patients with idiopathic pulmonary fibrosis (PF).
**Methods:** Using a commercially available ELISA we measured MMP7 and MMP9 levels in sera from patients with SSc and healthy controls, looking for correlations with clinical presentation.

TABLE 1.

| | ANA | Anti-Scl 70 | ACA | Anti-RNA Pol III | Anti-U1 RNP | Anti-U3 RNP |
|---|---|---|---|---|---|---|
| Diffuse SSc, n/N (%) | 637/1654 (38.5) | 451/683 (66.0) | 81/618 (13.1) | 34/77 (44.2) | 29/102 (28.4) | 11/38 (28.9) |
| Limited SSc, n/N (%) | 839/1654 (50.7) | 195/683 (28.6) | 500/618 (80.9) | 27/77 (35.1) | 31/102 (30.4) | 13/38 (34.2) |
| Overlap SSc/ Mixed CTD, n/N (%) | 120/1654 (7.3) | 17/683 (2.5) | 21/618 (3.4) | 10/77 (13.0) | 35/102 (34.3) | 12/38 (31.6) |
| Other, n/N (%) | 58/1654 (3.5) | 20/683 (2.9) | 16/618 (2.6) | 6/77 (7.8) | 7/102 (6.8) | 2/38 (5.3) |
| Lung fibrosis, % (95% CI) | 42.8 (40.4, 45.2) | 62.8 (59.1, 66.4) | 45.5 (34.1, 57.2) | 22.2 (19.0, 25.7) | 43.1 (33.4, 53.3) | 47.4 (31.0, 64.2) |
| Pulmonary arterial hypertension, % (95% CI) | 16.0 (14.3, 17.9) | 16.3 (13.6, 19.3) | 16.9 (9.31, 27.1) | 17.4 (14.5, 20.6) | 16.7 (10.0, 25.3) | 26.3 (13.4, 43.1) |
| Kidney, % (95% CI) | 5.0 (4.04, 6.21) | 5.7 (4.06, 7.67) | 7.8 (2.91, 16.2) | 3.7 (2.36, 5.51) | 8.8 (4.11, 16.1) | 13.2 (4.41, 28.1) |
| Gastrointestinal tract, % (95% CI) | 58.5 (56.1, 60.9) | 60.2 (56.4, 63.9) | 59.7 (47.9, 70.8) | 57.8 (53.8, 61.7) | 55.9 (45.7, 65.7) | 42.1 (26.3, 59.2) |
| Heart, % (95% CI) | 10.9 (9.47, 12.5) | 12.4 (9.99, 15.0) | 9.1 (3.73, 17.8) | 6.4 (4.64, 8.67) | 11.8 (6.23, 19.6) | 13.2 (4.41, 28.1) |

**Results:** Sera from 43 consecutive limited cutaneous (lc) SSc subjects, 44 early (<3 years from disease onset) diffuse cutaneous (dc) SSc patients and 20 age, gender and ethnicity matched healthy controls were analysed. There were no differences between lcSSc and dcSSc patients in terms of gender or ethnicity, age at disease onset or sample collection. As expected the lcSSc subjects more often had overlap syndromes compared to the dcSSc ones (30% v 11%, $p<0.001$), and had higher frequency of anti-centromere antibodies (49% v 2%, $p<0.001$), while anti-RNA polymerase III antibodies were found only in the dcSSc cohort (32% v 0%, $p < 0.001$). The mean ± SD disease duration at sample collection was significantly longer for the lcSSc cohort (132 ± 83 months) compared to 22 ± 12 for dcSSc cohort. There were no differences between the two subsets in frequency of clinically significant internal organ disease. MMP7 levels varied between 1.1 and 84.5 ng/mL (mean ± SD 8.8 ± 8.5, median 6.7 ng/mL) while MMP9 levels were measured between 54 and 5405 ng/mL (mean ± SD 651.9 ± 562, median 548.6 ng/mL). There was no correlation between MMP7 and MMP9 levels.

We found no gender or ethnicity differences in the levels of MMP7 and MMP9. Compared to healthy controls SSc patients had altered levels of both MMP7 and MMP9 - mean MMP7 level was 10.2 ng/mL for dcSSc, 8.9 ng/mL for lcSSc and 4.6 ng/mL for control groups ($p<0.001$) while mean MMP9 level was 892 ng/mL for dcSSc, 460.8 ng/mL for lcSSc and 607.2 ng/mL for controls ($p < 0.001$). The only significant association with autoantibodies we observed was for anti-topoisomerase I antibody (ATA) positive patients who had higher MMP7 levels (mean 14.3 ng/mL) compared to ATA negative subjects (8.23 ng/mL), $p = 0.049$. MMP7 levels were also higher in subjects with clinically significant (cs) PF (13.8 ng/mL) compared to those without (8.13 ng/mL, $p = 0.017$) and in subjects who developed renal crisis (16.5 ng/mL) compared to those who did not (8.8 ng/mL, $p = 0.016$). There was no association of MMP7 and MMP9 levels with pulmonary hypertension, cardiac scleroderma or severe digital vasculopathy. We also found no correlation between mRss and MMP7 or MMP9 levels.
**Conclusions:** Both MMP7 and MMP9 levels were higher in dcSSc subjects compared to lcSSc subjects or controls. Higher MMP7 levels were also associated with ATA positivity, development of csPF and SRC. Both MMP7 and MMP9 may have potential as biomarkers in SSc and further study is warranted.
**Disclosure statement:** The authors have declared no conflicts of interest.

### 243. ELEVATED IL-6 LEVELS ASSOCIATE WITH POOR CLINICAL OUTCOME AND MAY PROMOTE FIBROSIS IN EARLY dcSSc

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**Background:** We previously showed that thrombocytosis may identify a subgroup of diffuse SSc with increased IL-6 and high modified Rodnan skin score (mRSS). We assessed the predictive value of high IL-6 in these patients and its role in driving fibrosis in SSc.
**Methods:** IL-6 levels were assessed with immunohistochemistry and Western blot analysis. Skin biopsies from patients with early dcSSc and thrombocytosis($n = 10$,mean platelet:472x109/L,disease duration, Mean ± SEM:35 ± 9.5 months),established dcSSc($n = 8$,mean platelet:293x109/L, disease duration:128 ± 22) and controls($n = 12$) were used. IL-6 levels were measured in the supernatant from cultured SSc fibroblasts and the effect of anti-IL6 receptor antibody on collagen production was examined in normal fibroblasts stimulated with IL-6.

To investigate the link between IL-6 levels and clinical outcome serum IL-6(in pg/ml) from 39 patients with dcSSc (74% female) was quantified by ELISA. The dcSSc cases were categorised into high IL-6(≥10 pg/ml) and low IL-6 cohorts. Association between IL-6 levels and mRSS at 36months from disease onset was determined by Pearson's correlation. Mean difference of mRSS between the two cohorts of IL-6 levels over the 36-month period was compared by Student t-test. Difference in survival between cohorts was examined using Kaplan-Meier methods.
**Results:** There was greater dermal IL-6 expression in patients with early dcSSc than other subgroups. Strong immunostaining for IL-6 was associated with dermal fibroblasts, vascular structures and mononuclear inflammatory infiltrate in 80% of patients with early dcSSc. Moderate vascular expression for IL-6 was observed in 33% of healthy controls. Similar expression pattern was observed in 25% patients with established dcSSc with weak staining for IL-6 in dermal fibroblasts and inflammatory infiltrates.

IL-6 was elevated in the supernatant from cultured SSc fibroblasts ($n = 3$) by 17-fold (Densitometric Image Unit, DIU $p < 0.05$). In addition, collagen production was upregulated by 7-fold in SSc fibroblasts and similar induction by recombinant IL-6(20 ng/ml) was observed in normal fibroblasts (17.94 + 3.41 vs 2.18 + 1.85 DIU control, $p < 0.05$). This was partially abrogated with neutralising antibody against IL-6 receptor (4.26 + 2.07 DIU, $p < 0.05$).

Serum IL-6 level at presentation positively correlated with mRSS at 36 months follow-up in a subgroup of dcSSc cases ($r = 0.81$, $p < 0.01$, $n = 16$). There was a significant difference in mean mRSS between the two groups with differential IL-6 expression (10.9, 95% Confidence interval, 3.8, 18.2, $p < 0.01$). Kaplan-Meier analysis showed that the 5-year survival was 93% and 81% in the group with low and high IL-6 levels respectively ($p = 0.02$, log rank test).
**Conclusions:** This data confirms upregulation of IL-6 in some cases of early dcSSc and support the potential role of IL-6 as a surrogate marker for clinical outcome in SSc. Our study also suggests that IL-6 may a logical target for therapy in this subset of patients.
**Disclosure statement:** The authors have declared no conflicts of interest.

## Spondylarthropathies (including psoriatic arthritis)

### 244. VALIDITY OF COLOUR DOPPLER AND SPECTRAL DOPPLER ULTRASOUND OF SACROILICAC JOINTS AGAINTS PHYSICAL EXAMINATION AS GOLD STANDARD

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**Background:** Sacroiliac joints (SJ) involvement is a distinctive and charasteristic feature of Spondyloarthritis (SpA) and x-ray is the test routinely used to make a diagnosis. However, x-ray reveals late structural damage but cannot detect active inflammation. The objective of this study was to assess the validity of Doppler ultrasound in SJ.
**Methods:** Prospective blinded and controlled study of SJ, in which three populations were compared. We studied 106 consecutive cases, who were divided into three groups: a) 53 patients diagnosed with SpA who had inflammatory lumbar and gluteal pain assessed by a rheumatologist; b) 26 patients diagnosed with SpA who didn't have SJ tenderness and had normal physical examination; c) control group of 27 subjects (healthy subjetcs or with mechanical lumbar pain). All patients included that were diagnosed with SpA met almost the European Spondyloarthropathy Study Group (ESSG) classification criteria. Physical examination of the SJ included: sacral sulcus tenderness, iliac gapping, iliac compression, midline sacral thrust test, Gaenslen's test, and Patrick s test were used as gold standard. Both SJ were examined with Doppler ultrasound (General Electric Logiq 9, Wauwatosa WI, USA) fitted with a 9-14 Mhz lineal probe. The ultrasonographer was blinded to clinical data.

Doppler in SJ was assessed as positive when both Doppler colour and resistance index (RI) < 0.75 within the SJ area were present. Statistical analysis was performed estimating sensitivity and specificity against gold standard. The Kappa correlation coefficient was used for reliability study.
**Results:** 106 cases (53 female, 55 male; mean age 36 10 years) were studied. There were no statistical differences between groups related to age or sex. Physical examination of SJ was positive in 38 patients (59 sacroiliac joints). US detected Doppler signal within SJ in 37 patients (58 SJ): 33 of them were symptomatic SpA (52 SJ), one of them were asymptomatic SpA (1 SJ) and one was a healthy control (1 SJ). The accuracy of US when compared to clinical data as gold standard at subject level in the overall group was: sensitivity of 68.6% and specificity of 85.7%, positive predictive value of 70.5% and negative predictive value of 84.5%. A positive likelihood ratio of 4.8, a negative likelihood ratio of 0.36 and a kappa coefficient of 0.55 were achieved.
**Conclusions:** Doppler US of SJ seems to be a valid method to detect active SJ inflammation.
**Disclosure statement:** The authors have declared no conflicts of interest.