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Global chemokine expression in systemic sclerosis (SSc): CCL19 expression correlates with vascular inflammation in SSc skin

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Abstract

Objective—To characterise global chemokine expression in systemic sclerosis (SSc) skin in order to better understand the relationship between chemokine expression and vascular inflammation in this disease.

Methods—We investigated chemokine mRNA expression in the skin through quantitative PCR analysis comparing patients with diffuse cutaneous (dcSSc) or limited cutaneous (lcSSc) disease with healthy controls. We tested correlations between the most regulated chemokines and vascular inflammation and macrophage recruitment. CCL19 expression was examined in human primary immune cells treated with innate immune activators.

Results—The chemokines, CCL18, CCL19 and CXCL13, were upregulated in dcSSc skin, and CCL18 in lcSSc skin. Expression of CCL19 in dcSSc skin correlated with markers of vascular inflammation and macrophage recruitment. Immunofluorescence data showed CCL19 colocalisation with CD163 macrophages in dcSSc skin. In vitro studies on human primary cells demonstrated that CCL19 expression was induced after toll-like receptor activation of peripheral blood mononuclear cells and separated populations of CD14 monocytes.

Conclusions—CCL18, CCL19 and CXCL13—chemoattractants for macrophage and T cell recruitment—were three of six chemokines with the highest expression in dcSSc skin. Increased

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CCL19 expression in the skin suggests a role for CCL19 in the recruitment of immune cells to the peripheral tissue. Induction of CCL19 in macrophages but not structural cells indicates a role for skin-resident or recruited immune cells in perivascular inflammation. This study demonstrates that CCL19 is a sensitive marker for the perivascular inflammation and immune cell recruitment seen in dcSSc skin disease.

INTRODUCTION

The vascular injury in systemic sclerosis (SSc) occurs very early in the disease, even before the onset of fibrosis.^{1–3} It has been suggested that vessel permeability and injury coincide with the infiltration of immune cells,^{4–6} mainly consisting of T cells and macrophages localised at perivascular regions.^{36–8} This immune cell recruitment is facilitated by vascular endothelial cells which, upon injury, upregulate adhesion molecules such as VCAM-1, ICAM-1 and E-selectin,²⁵⁹¹⁰ ultimately leading to immune activation, cell recruitment and tissue damage.¹⁵ This process has been observed in SSc clinical–pathological studies, as there is a strong correlation between immune cell infiltration and the severity and progression of skin disease.¹¹ Several studies have suggested that vascular injury, resulting in vascular inflammation and ultimately fibrosis, is the primary mechanism driving pathogenesis in patients with SSc.^{112–14}

The recruitment of immune cells to peripheral tissues depends largely on chemokine gradients. Several chemokines show upregulated expression in SSc skin: CXCL9 (MIG) and CXCL10 (IP-10), which recruit T cells^{15–17}; CCL2 (MCP-1), which recruits macrophages^{18–21}; and other chemokines such as CCL5 (RANTES), CCL7 (MCP-3) and CCL20, which recruit many immune cell types, including T cells and macrophages.^{22–26} The redundancy of these and other chemokines demands a more comprehensive understanding of the role of chemokines in inflammation in SSc.

We assessed in this study expression of most known chemokines and chemokine receptors in diffuse cutaneous (dc)SSc skin. We observed increased expression of CCL18, CCL19 and CXCL13 in addition to the increased expression of CXCL9, CCL2 and CCL5 described in previous publications.^{1518–2024} Our results show a direct correlation between CCL19 expression and markers of vascular injury and macrophage recruitment, indicating that, of the limited subset of chemokines upregulated in SSc skin, only one, CCL19, correlates with vascular injury.

METHODS

Study participants

The Boston University Medical Center Institutional Review Board reviewed and approved the conduct of this study. Informed consent was obtained from all patients and healthy subjects. Skin biopsy samples were obtained from the dorsal mid forearm and immediately stored in RNeasy Lysis Buffer (Qiagen) at –80°C until RNA isolation. The modified Rodnan Skin Score (MRSS) was determined for each patient on the day of the biopsy. In addition, biopsy specimens were fixed in formalin and embedded in paraffin for histological analysis.

RNA isolation and quantitative real-time PCR (qPCR)

See online Supplementary methods.

Serum analyte analysis

Serum was isolated from blood collected in BD Vacutainer SST 8.5 mL tubes. CCL19 levels in sera were assessed using microsphere-based immune-multiplexing by Myriad-RBM as part of a Discovery array of analytes.

Microarray analysis

RNA isolated from skin biopsy samples from patients with dcSSc and healthy controls (HCs) were reverse transcribed into cDNA and analysed using U133A 2.0 Affymetrix arrays. Microarray data were clustered using Cluster 3.0 for Mac OSX, using unsupervised clustering by gene and by array.

Histology preparation and staining

See online Supplementary methods.

Peripheral blood mononuclear cell (PBMC) collection, separation and stimulation

Blood was collected from HCs in CPT tubes (Becton Dickinson), and PBMCs were isolated from buffy coats by Ficoll density-gradient centrifugation. PBMCs were plated in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin). Isolated PBMCs were separated by positive selection, first for CD14 cells and then CD3 cells using a MACS MicroBeads Column (Miltenyi Biotec). Cells were stimulated with 10 µg/mL poly(I:C) (PIC), 2 µg/mL Ultrapure *Escherichia coli* lipopolysaccharide (LPS) or 5 µg/mL CpGB class ODN 2006 (Invivogen). Cells were lysed in RLT lysis buffer (Qiagen) for RNA isolation and qPCR analysis.

Statistical analysis

qPCR data are normalised to mRNA expression of one HC, housekeeping gene (18S) expression, and average HC expression. All qPCR graph analyses include the mean expression with the SEM. p Values were calculated using Mann–Whitney non-parametric analyses. Correlations were calculated using Spearman non-parametric correlations, and data include graphing of the linear regression.

RESULTS

Patient characteristics

All patients selected for chemokine qPCR array analysis and gene expression assays met the criteria for dcSSc or limited cutaneous (lc)SSc according to diagnostic and subtype criteria.²⁷²⁸ As seen in table 1, the mean age was 48±11 years (mean±SEM) for patients with dcSSc and 58±12 for patients with lcSSc, with 70% and 83%, respectively, of those studied being female. Disease duration was longer in the lcSSc patient subset (79±76 vs 18±14 months). The mean MRSS was 23±10 for patients with dcSSc. The mean age of HCs (44±17 years) was similar to that of patients with dcSSc (48±11 years, p=0.15).

Upregulated chemokine expression in SSc skin

Skin samples from 10 patients with dcSSc and six with lcSSc were analysed for mRNA expression of 59 chemokines and chemokine receptors using RT² Profiler PCR Array: Human Chemokines & Receptors (SA Biosciences). Of all 59 genes, the average expression of only six genes was found to be increased over twofold in patients with dcSSc compared with HCs (figure 1A). All six were chemokines belonging to the C–C and C–X–C subfamilies: CCL2, CCL5, CCL18, CCL19, CXCL9 and CXCL13. The most highly upregulated genes were CCL19, CXCL13 and CXCL9, with an increase of 5.3 ± 4.5 -fold, 4.5 ± 6.8 -fold and 4.2 ± 4.9 -fold (mean $\pm$ SEM), respectively, compared with mean control expression. The mean expression of CCL18 (3.0 ± 2.8 -fold), CCL2 (2.8 ± 1.5 -fold) and CCL5 (2.0 ± 1.3 -fold) was modestly upregulated compared with mean control expression. These results confirm previous studies showing increased CCL2, CCL5 and CXCL9 expression in SSc skin.^{1518–2024} The expression of a few chemokine receptors was found to be increased >1.5 -fold in dcSSc skin, such as CXCR3 (1.7 ± 0.3) and CXCR4 (1.7 ± 0.4), which are expressed on T cells and monocytes, which is possibly biologically significant, but they were not studied further. Moreover, CCL18 was the only chemokine that showed >2 -fold increased expression in patients with lcSSc (2.8 ± 2.4). Complete data, including expression of all genes analysed by the chemokine array, can be found in online supplementary table S1.

To confirm the results seen in the chemokine array, gene mRNA expression levels were assessed on a larger cohort of dcSSc patients using Taqman gene expression assays (Applied Biosystems) for the six genes with >2 -fold increased expression on the array (figure 1B and online Supplementary figure S1). CCL19 showed a significant increase in expression in patients with SSc compared with HCs (6.2 ± 0.8 -fold change; $p<0.0001$). Expression of CXCL13 was increased very significantly in some patients with dcSSc, but with a large variability in expression across patients (71.3 ± 35 -fold change; $p<0.001$). CCL18 showed only a modest trend of increased expression in dcSSc skin compared with HCs (3.9 ± 1.0 -fold change; $p=0.06$). The other chemokines identified as having increased expression in the array, CCL2, CCL5 and CXCL9, were also confirmed to have statistically significantly increased gene expression in dcSSc compared with controls (see online Supplementary figure S1) in agreement with previously published studies (CCL2: 2.4 ± 0.2 -fold change, $p<0.001$; CCL5: 4.1 ± 1.4 -fold change, $p<0.0001$; CXCL9: 3.2 ± 0.8 -fold change, $p=0.01$).^{1518–2024}

CCL19 expression correlated with markers of vascular inflammation

Patients with dcSSc are known to have perivascular infiltrates at the main site of inflammation in the skin,¹⁵ and the degree of this inflammation corresponds to the trajectory of the skin disease.¹¹ To confirm this correlation in our patient cohort, changes in MRSS at 6 months were compared with histological evaluation of vascular inflammation (figure 2A). H&E-stained histological sections were examined in a blinded fashion and scored for perivascular inflammation (eg, see online Supplementary figure S2). The vascular inflammation scores showed a modest correlation with progressive skin disease, determined by a change in skin score, consistent with a previous report (figure 2A).¹¹

To investigate the association of upregulated chemokine expression and skin inflammation, we examined the relationship between chemokine expression and perivascular inflammation (figure 2B). These results showed a striking positive correlation between the inflammation seen on histology and CCL19, but not other chemokine mRNA expression ($r^2=0.42$, $p=0.01$). CCL19 expression did not correlate with skin score, disease duration or markers of fibrosis (data not shown). Furthermore, CCL19 expression did not show any significant differences when the dcSSc patient cohort was stratified by clinical features identified in table 1, and dichotomous stratification of CCL19 levels did not show any difference in clinical manifestations between groups showing high and low CCL19 expression.

Consistent with increased skin CCL19 mRNA expression, serum CCL19 levels were increased in patients with dcSSc compared with HCs (figure 2C; 1.9-fold increase, $p=0.0003$). Immunofluorescence staining of CCL19 showed increased inter-stitial staining in dcSSc, and double staining with macrophage marker, CD163, showed colocalisation with CCL19 only in dcSSc skin (figure 2D,E). In addition, positive staining was seen in the epidermal and glandular regions similar to HC skin.

The chemokine array results also identified CXCL13 as having upregulated expression in dcSSc skin, although its expression did not correlate with the skin score, markers of fibrosis or vascular inflammation (data not shown). We examined other CCL19-related chemokines and receptors by qPCR. Expression of CCR7, the receptor for CCL19, showed a trend toward a modest increase, as was seen in the chemokine array (figure 2F, 2.7 ± 0.6 -fold change, $p=0.08$). Expression of CCL21, a chemokine sharing the same receptor as CCL19, but not included in the chemokine array, was not found to be significantly increased in dcSSc skin (figure 2F).

To further explore the role of CCL19 in vascular inflammation, we examined CCL19 expression in relationship to expression of gene markers of vascular injury. Angiopoietin 2 is a vessel-destabilising cytokine that is increased in SSc and associated with disease activity.³²³³ Junctional adhesion protein 2 (JAM2) and von Willebrand factor (vWF) are additional markers of vascular activation and injury.³⁴³⁵ vWF expression has also been shown to be upregulated in dcSSc.³⁴ CCL19 expression correlated strongly with all of these markers of vascular activation and injury: angiopoietin 2 ($r^2=0.48$, $p<0.001$), JAM2 ($r^2=0.39$, $p<0.001$) and vWF ($r^2=0.32$, $p=0.003$) (figure 3A).

In addition, CCL19 expression correlated with the expression of markers of macrophages (figure 3B), which are found in areas of perivascular inflammation seen in dcSSc skin.³⁶ Macrophage surface antigens, Siglec-1 and CD163, and monocyte/macrophage chemoattractant, CCL2, have previously been shown to be upregulated in SSc,^{18–2036} and CCL19 expression correlated moderately with all these macrophage markers: Siglec-1 ($r^2=0.46$, $p<0.001$), CD163 ($r^2=0.25$, $p=0.009$) and CCL2 ($r^2=0.23$, $p=0.013$).

CCL19 has similar gene expression to markers of macrophages and vascular activation

To better understand the role for CCL19 in dcSSc, its expression was examined by microarray analysis comparing skin biopsy samples from HCs ($n=4$) and patients with early diffuse SSc ($n=20$) (figure 4). Expression was examined using U133A 2.0 Affymetrix

arrays, and results were analysed by complete linkage, unsupervised clustering of genes and arrays. These results showed a clear difference in the pattern of gene expression between the skin from HCs and patients with dcSSc. CCL19 clustered with several macrophage markers, such as CD14, CD163 and AIF1, in addition to genes known to be expressed during vascular inflammation, such as VCAM-1.²⁵

Innate immune activation induces CCL19 expression in PBMCs

We have previously shown that a subset of patients with dcSSc show upregulated interferon (IFN)-responsive genes, suggesting innate immune activation in these patients.³⁶ In addition, we have shown that the innate immune ligand, PIC, administered subcutaneously to mice, induces dermal fibrosis.¹⁵ Thus we tested if CCL19 expression could be induced after activation of innate immune receptors. PBMCs from HCs were treated for 0, 2, 6 or 16 h with toll-like receptor (TLR) ligands: PIC (TLR3), LPS (TLR4) or CpG (TLR9). All ligands induced CCL19 expression, with TLR4 (LPS) activation inducing the highest levels of expression, with a 647-fold increase at 16 h (figure 5A). Since we found that CCL19 expression strongly correlated with expression of macrophage markers in dcSSc skin, we determined if TLR activation induced CCL19 expression in monocytes. For this experiment, PBMCs were isolated from HCs and separated into CD14⁺, CD3⁺ and CD14⁺CD3⁺ populations. TLR ligands induced CCL19 expression in all the cell sub-populations (figure 5B), but monocytes treated with LPS showed the most robust induction of CCL19 expression (1264 ± 235-fold change, $p=0.002$).

The perivascular localisation of inflammatory cells suggested that CCL19 expression by endothelial cells or perhaps perivascular fibroblasts might be a stimulus for leucocyte migration in SSc. In contrast with PBMCs, these structural cells failed to show upregulated CCL19 expression in response to TLR ligands despite showing robust upregulated expression of IFN-responsive genes (data not shown).

DISCUSSION

This study provides the first comprehensive analysis of chemokine and chemokine receptor expression in SSc skin. This analysis newly identified upregulation of chemokines, CCL18, CCL19 and CXCL13, and confirmed upregulation of CCL2, CCL5 and CXCL9 as described previously.^{1518–2024} CCL19 expression correlated strongly with markers of vascular inflammation: angiopoietin 2, vWF and JAM2. In addition, its expression correlated with macrophage markers: Siglec-1, CD163 and CCL2. These results were further supported by microarray analysis that showed clustering of CCL19 with additional markers of vascular inflammation (VCAM-1) and macrophages (CD14 and AIF-1). Together, these data indicate that CCL19 is intimately involved in the process of vascular injury and inflammation in SSc.

Immunofluorescence data indicate that CD163 macrophages are the major source of CCL19 in dcSSc skin. Furthermore, *in vitro* data show that CD14 monocytes strongly upregulate CCL19 after innate immune activation. Expression of CCL19 is increased in other autoimmune diseases and found to have an important association in disease activity, such as rheumatoid arthritis, where production of CCL19 by macrophages is suggested to be important for the recruitment of leucocytes.^{37–39} Since macrophages are present in the skin

under non-inflammatory conditions, resident as well as recruited macrophages might be an important source of CCL19. Activation of skin-resident macrophages may lead to production of CCL19 early in the disease. The correlation of CCL19 expression with macrophage markers further supports this relationship between macrophage activation and CCL19.

CCL19 and CCL21 are the sole ligands for CCR7, which is expressed on many antigen-presenting cells and is important for their homing to lymph nodes from peripheral tissues during late-stage inflammation.⁴⁰⁴¹ In some diseases, increased expression of these chemokines marks the formation of tertiary lymph node structures. Such structures are not present in SSc skin, but instead lymphatic vessels are decreased compared with healthy skin.⁴²⁴³ Recent studies have identified a new role for CCL19 in the recruitment of cells to non-lymphoid tissues during early-stage inflammation.³⁷⁴⁴⁴⁵ This suggests that CCL19 plays a role in the recruitment of cells to the skin during SSc rather than emigration of antigen-presenting cells after activation. This is supported by the correlation of CCL19 with vascular inflammation, the major site of infiltrating immune cells in SSc skin.

CCR7 is mainly expressed on T cells and activated dendritic cells, and both of these populations are highly recruited to SSc skin.³⁷⁸ In addition to T cells and dendritic cells, previous studies have demonstrated that CCL19 is also chemotactic for CCR7 macrophages and can work in synergy with other chemokines to induce macrophage migration.⁴⁶⁴⁷ As our study and others have demonstrated a relationship between vascular inflammation and fibrosis, emphasising the importance of cell recruitment in the perpetuation of skin disease, increased CCL19 expression suggests an important role in immune cell infiltration in SSc skin and, ultimately, worsening of disease.¹¹

CCL19 can directly affect immune responses of T cells and dendritic cells by skewing them towards a proinflammatory (Th1) response.⁴⁸⁴⁹ However, CCL19 may have roles that depend on the species, as many of the CCL19 effects on Th1 skewing have been found in mice, some studies using CCL19/CCL21-deficient mice. We do not see coregulated increases in CCL21 in dcSSc skin. Whether the prototypic Th1 cytokine, IFN γ , is upregulated in SSc skin remains unclear, as the type of IFN leading to IFN-regulated gene expression in SSc skin has not been clearly defined.⁵⁰ While profibrotic (Th2) immune responses are dominant in this disease, these responses may still have an important role in pathogenesis.

We also found that CCL18 was elevated in dcSSc and lcSSc skin, and CXCL13 was also increased in dcSSc skin. CCL18, also known as PARC, is a chemoattractant for T cells and expressed at high levels in the lung.⁵¹ Serum levels of CCL18 have been shown to be an indicator of fibrotic activity in SSc pulmonary disease.⁵²⁵³ CXCL13, a lymphoid-organising chemokine, has an important role in germinal centre formation as a strong recruiter of B cells.⁴⁰⁵⁴ B cells and immunoglobulin genes are upregulated in SSc skin, although treatment of patients with a B cell-depleting agent had little effect on SSc skin disease.⁵⁵⁵⁶ The expression of CXCL13 was highly induced in a trimodal distribution. Further investigation into clinical data did not provide an explanation for this difference. In contrast with CCL19,

expression of CCL18 and CXCL13 did not correlate with markers of vascular inflammation or fibrosis, suggesting that they are not responsible for leucocyte perivascular migration.

Increased expression of CXCL9, CXCL13, CCL2, CCL5, CCL18 and CCL19 suggests a complex mechanism by which immune cells are recruited to the skin in dcSSc. Many of these chemokines have redundant roles, possibly to ensure recruitment of specific cell types. For example, CXCL9 recruits T cells and CCL2 recruits macrophages, while CCL19 and CCL5 are chemotactic for both, in addition to dendritic cells. Studies have shown a synergistic effect of combinations of these chemokines in cell recruitment, emphasising the importance of understanding global chemokine expression.⁴⁷⁵⁷ It is likely that this complex pattern of chemokine expression is responsible for the perivascular inflammation seen in SSc skin disease.

Our study shows a strong correlation of CCL19 with vascular inflammation and macrophage recruitment. In addition, the immunofluorescence and in vitro data identify macrophages as an important source of CCL19. Overall, this study suggests that CCL19 plays a role in the inflammation seen in dcSSc skin and is a sensitive marker for macrophage activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemokine expression in systemic sclerosis (SSc) skin. (A) Data from chemokine array analysis of healthy controls (HC) (n=4), patients with diffuse cutaneous SSc (dcSSc) (n=10) and patients with limited cutaneous SSc (lcSSc) (n=6). Only genes with a >2-fold change in expression comparing SSc with HC are shown. Data analysis of all genes on the chemokine array can be found in online supplementary table S1. (B) Quantitative PCR Taqman gene expression analysis of CCL19, CCL18 and CXCL13. HC (n=12); dcSSc (n=26). Horizontal lines represent the mean $\pm$ SEM.

Figure 2.

CCL19 expression correlates with vascular inflammation in systemic sclerosis (SSc) skin.

(A) Correlation between vascular inflammation score and change in skin score. Vascular inflammation was scored in a blinded fashion according to histological representation in online supplementary figure S2. Change in skin score was calculated as the difference in modified Rodnan Skin Score (MRSS) over 6 months ($n=12$). (B) Correlation of vascular inflammation with CCL19 expression ($n=14$). (C) Serum CCL19 levels in patients with diffuse cutaneous SSc (dcSSc) ($n=28$) compared with healthy controls (HC) ($n=12$). (D) Immunofluorescence staining of CCL19 (red) with CD163 (green) co-stain for both HC and dcSSc frozen skin sections. 10× magnification. (E) Double staining for CCL19 (red) and CD163 (green) for HC and dcSSc frozen skin sections. 20× magnification. (F) Gene expression analysis of CCL19-associated genes: CCL21 and CCR7. HC ($n=12$); dcSSc ($n=26$). Horizontal bars represent the mean $\pm$ SEM.

Figure 3.

CCL19 expression correlates with macrophage markers and vascular injury. (A) Correlation of CCL19 expression with expression of markers of vascular injury and activation: angiopoietin 2, junctional adhesion molecule 2 (JAM2) and von Willebrand factor. (B) Correlation of CCL19 expression with expression of macrophage markers: Siglec-1, CD163 and CCL2. For all gene analysis, $n=26$.

Figure 4.

Microarray analysis shows association between increased expression of CCL19 and macrophage markers. (A) Gene clustering in accordance with changes in expression in systemic sclerosis (SSc) skin compared with healthy controls. (B) Cluster of genes containing CCL19 including macrophage markers (*). Data were clustered by unsupervised, hierarchic clustering of genes and arrays. Colour variations green to red represent levels of gene expression from lower to higher, respectively (see inset). These data include four

arrays of skin samples from healthy controls and 20 arrays of skin samples from patients with SSc.

Figure 5.

CCL19 expression induced in peripheral blood mononuclear cells (PBMCs) and macrophages after toll-like receptor (TLR) activation. (A) PBMCs were isolated from blood of healthy donors and immediately treated with TLR ligands (poly(I:C) (PIC), TLR3; lipopolysaccharide (LPS), TLR4; CpG, TLR9). Cells were collected for RNA expression analysis at 0, 2, 6 and 16 h. For all samples, (n=2) (B) PBMCs were isolated from the blood of healthy donors and separated into CD14⁺, CD3⁺ and CD14⁻, CD3⁻ populations using positive selection. Cells were treated with TLR ligands (PIC, TLR3; LPS, TLR4; CpG, TLR9) overnight and then collected for gene expression analysis. For CD14⁺ and CD14⁻, CD3⁻ cells: media, PIC, LPS, n=4; CpG, n=2. For CD3⁺ cells: media, PIC, LPS, n=3; CpG, n=1. Data are expressed as the mean fold change after normalisation to mRNA expression of media-only treatment. Each bar represents the mean±SEM.

Table 1

Characteristics of healthy controls and patients with systemic sclerosis

Characteristic	dcSSc	lcSSc	Healthy controls
Age (years)	48±11	58±12	44±17
Gender	Female: 21 (70) Male: 9 (30)	Female: 5 (83) Male: 1 (17)	Female: 7 (50) Male: 7 (50)
MRSS	23±10	N A	N A
Disease duration (months) (range)	18±14 (2–66)	79±76 (6–204)	N A
Autoantibodies (n of number tested)	Anti-Scl70: 11 of 25 ANA: 26 of 28 Anti-centromere: 2 of 28	Anti-Scl70: 1 of 3 ANA: 4 of 5 Anti-centromere: 3 of 5	N A
ESR (mm)	19±12	N A	N A
Raynaud's phenomenon	28 (93)	6 (100)	N A
Raynaud's phenomenon duration (months) (range)	38±52 (2–216)	204±75 (96–264)	N A
Pulmonary arterial hypertension	6 (20)	0	N A
Digital ulcers	10 (33)	3 (50)	N A
Interstitial lung disease	15 (50)	2 (33)	N A
Renal crisis	2 (7)	0	N A
Arthritis	5 (17)	0	N A
Myositis	1 (3)	0	N A
Immunosuppressive treatments	Cellcept: 6 (20) Prednisone: 7 (23) Methotrexate: 4 (13) Rituximab: 2 (7) Other: 4 (13)	Cellcept: 1 (17)	N A

Values are mean±SD, number (%) or as indicated. All patients met the criteria for diffuse cutaneous systemic sclerosis (dcSSc) or limited cutaneous systemic sclerosis (lcSSc). Disease duration was defined by the time since the first non-Raynaud's symptom. For ESR data, dcSSc: n=6. For Raynaud's phenomenon duration data, dcSSc: n=21 and lcSSc: n=4. Autoantibody data are presented as number of patients positive out of the number of patients tested. The remainder of the data are presented as number and percentage of the total patient cohort. Pulmonary arterial hypertension was defined by a mean pulmonary artery pressure ≥25 mm Hg with capillary wedge pressure ≥15 mm Hg. Interstitial lung disease was defined by clinical history. Myositis was defined by elevated creatine kinase levels. Other treatments include: chemotherapy (n=1), Cytoxan (n=1), hydroxychloroquine (n=1) and Fresolimumab (n=1). Seven patients received more than one immunosuppressive treatment. ESR, erythrocyte sedimentation rate; MRSS, modified Rodnan Skin Score.