CCL2, CCL3 and CCL5 chemokines in systemic sclerosis: the correlation with SSc clinical features and the effect of prostaglandin E1 treatment

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ABSTRACT

Objective. *Chemokines favour leukocyte homing and participate actively in inflammation and accumulation of extracellular matrix. The aim of our work is to assess in patients with systemic sclerosis (SSc) the serum levels of CC chemokines: CCL2 monocyte chemotactic protein-1 (MCP-1/CCL2), CCL5 "regulated upon activation, normal T expressed and secreted" (RANTES/CCL5) and CCL3 "macrophage inflammatory protein 1 α" (MIP1α/CCL3), their associations with clinical characteristics and modulation by infusions of the prostaglandin E1 (PGE1) analogue, alprostadil alpha-cyclodextrin.*

Methods. *Serum levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 were studied by ELISA in 40 patients with SSc (34 lSSc, 6 dSSc) before and after 3 consecutive daily PGE1 infusions (60 μg) and compared to 30 healthy controls. We recorded clinical (age, duration of disease, ulcers, teleangectasias, calcinosis, skin score [mRSS], capillaroscopy pattern, heart and lung involvement) and immunological characteristics (ANA/ACA/Scl70) of patients.*

Results. *MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 levels were significantly higher in SSc patients than in controls and significantly decreased after PGE1 treatment. MCP-1 levels, higher in dSSc and Scl 70 positive patients, correlated with mRSS.*

Conclusions. *The high levels of circulating chemokines might support a role of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 in SSc pathogenesis and the correlation of MCP-1 with the extent of skin fibrosis might imply its involvement in the development of fibrosis in SSc. PGE1 down-regulates serum MCP1/CCL2 and RANTES/CCL5 levels, suggesting its possible additional effect on inflammation and cell trafficking in SSc.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disease, characterised by fibrosis and microvascular involvement of skin and internal organs. Some studies have hypothesised that cytokines and growth factors might have a role in the complex SSc pathogenesis, by modulating leukocytes and endothelial cells and by stimulating the synthesis of extra cellular matrix components (1, 2).

In fact, T lymphocytes and macrophages peri-vascular infiltrate is a hallmark of SSc early skin lesions, and correlates with the degree and progression of skin thickening (1).

According to this hypothesis, chemokines, defined as cytokines inducing chemotaxis in nearby responsive cells, released in early stages of SSc, might be critical in initiating and developing fibrosis, by attracting in the tissues leucocytes and mononuclear cells that, in turn, might release pro-fibrotic growth factors (3).

In particular, in animal models of SSc, the CC chemokines "monocyte chemoattractant protein 1" (MCP1/CCL2), "regulated upon activation, normal T expressed and secreted" (RANTES/CCL5) and "macrophage inflammatory protein 1 α" (MIP1/CCL3) – also called CCL2, CCL5 and CCL3, respectively – were shown to have an important role in disease pathogenesis, by recruiting monocytes (4) and T helper lymphocytes and up-regulating adhesion molecules expression, thus allowing diapedesis (2, 3, 5). Furthermore, they activate fibroblasts and up-regulate transforming growth factor (TGFβ), platelet growth factor (PDGF) (1), and connective tissue growth factor (CTGF) (6), ultimately stimulating collagen production (Fig. 1). TGFβ1, in turn, up-regulates MCP1/CCL2 and RANTES/CCL5 and down-regulates MIP1α/CCL3 in a complex model of feedback (7).

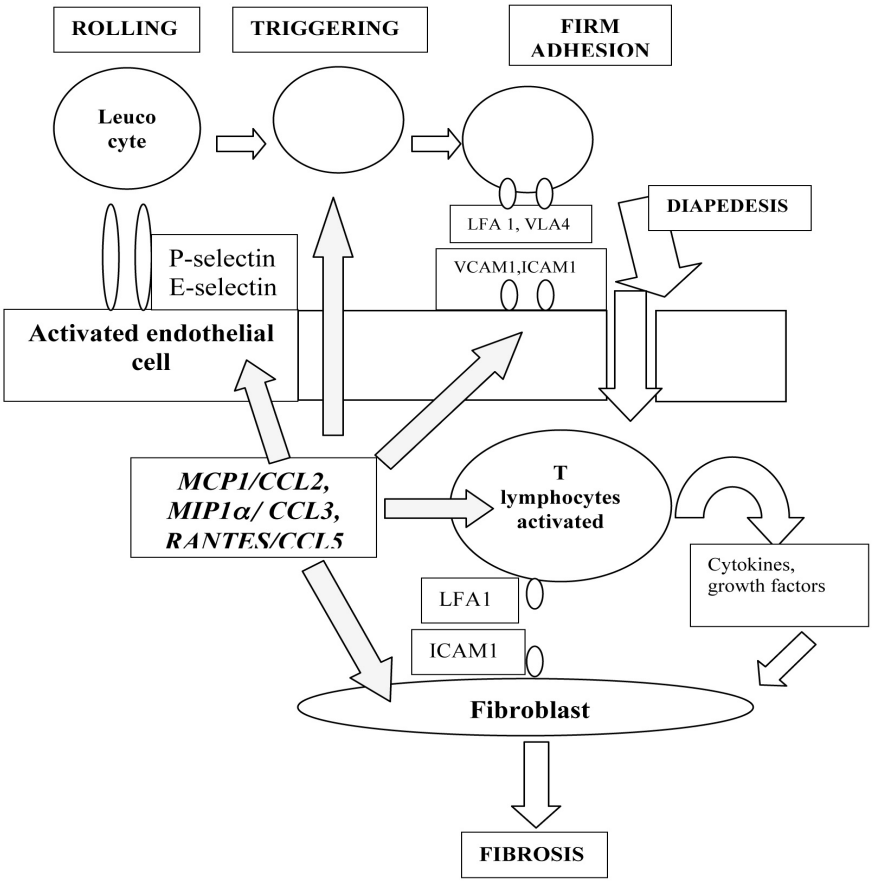

Fig. 1. The role of chemokines in inflammatory processes in SSc.

MCP1/CCL2 is more effective on monocytes than MIP1α/CCL3 (8), and RANTES/CCL5 has a more notable action on T lymphocytes. In SSc mice models, mRNA levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 are higher in very early phases of disease: RANTES/CCL5 increases earlier than MCP1/CCL2 and MIP1α/CCL3 but rapidly decreases, while MCP1/CCL2 and MIP1α/CCL3 levels remain unchanged (9). In SSc, MCP1/CCL2 and RANTES/CCL5 are over-expressed in skin biopsies (1, 10) and all C-C chemokines are increased in bronco-alveolar lavage (11-13).

From these evidences, MIP1α/CCL3, MCP1/CCL2 and RANTES/CCL5 seem to have a common role in fundamental pathogenic steps of the SSc such as recruitment of T cells and monocytes, leading to inflammation and fibroblast activation, and, ultimately, to fibrosis.

Prostanoids are currently used for their intensive vasoactive effect in the treatment of Raynaud's phenomenon and ischaemic skin ulcers in SSc (14). Alprostadil is an analogue of prostaglandin E1 (PGE1), that increases deformability of red cells (15), improving blood flow, inhibits activation and aggregation of platelets and modulates neutrophils activation (16). Furthermore, it regulates circulating endothelial adhesion molecules (17) and components of fibrinolytic system in SSc (18).

The first aim of our study is to evaluate the circulating levels of the CC chemokines MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 in SSc patients, their differences between patients with diffuse (dSSc) and limited (lSSc) SSc and their correlation with clinical data. The second aim is to evaluate the effect of the PGE1 analogue alprostadil alpha-cyclodextrin infusion on their levels.

Patients and methods

Patients

Forty consecutive Caucasian SSc (38 females and 2 males, 60.6±9.28 years old), diagnosed according to the American College of Rheumatology (ACR) classification criteria (19), were classified in lSSc and dSSc according to Le Roy (20), and 30 Caucasian healthy controls matched for age and sex (Table I), not suffering either from acute or from chronic diseases (including cardiovascular diseases and diabetes), were recruited at the Division of Rheumatology of the University of Florence, L'Aquila and Ancona after that a written informed consensus was signed. The study was approved by the local ethics committees.

Before sampling, in patients, there was a wash out period of 10 days from oral and topical vasodilators and for 40 days from alprostadil (the longest therapeutic interval between different cycles of treatment that we use in the clinical practise). Proton pump inhibitors and clebopride were allowed.

SSc patients treated with drugs potentially able to modify the evolution of the disease (corticosteroids, methotrexate, cyclophosphamide, D-penicillamine, iloprost) were excluded, as well as patients whose conditions didn't allow a complete pharmacology wash out (patients with severe ulcers, severe pulmonary hypertension, severe respiratory failure, congestive heart failure III–IV class of NYHA, creatinine values ≥1.5 mg/dl and mega-oesophagus and/or malabsorption).

Other exclusion criteria were: age <18 years, pregnancy, stroke and myocardial ischaemia in the 4 months preceding the study, chronic hepatitis, diabetes mellitus, malignancy and active infections.

Assessment

At baseline (the time of blood drawing), age and duration of disease [assessed from the first symptom after the onset of Raynaud phenomenon (RP)] were recorded. All patients underwent an extensive clinical work-out. Microvascular features were assessed by evaluating the presence of skin and fingertip ulcers, calcinosis and teleangectasias; nailfold capillaroscopy was performed in order to classify the patients into early, active, and late patterns (21). Skin involvement was scored by modified Rodnan skin score (mRSS) (22). Intersi-

Table I. Demographic and clinical features of SSc patients and healthy controls.

	SSc (n. 40)	LSSc (n. 34)	dSSc (n. 6)	Healthy controls (n. 30)
Age (years) mean ± SD (range)	60.6± 9.28 (37-77)	62 ± 8.5 (37-65)	51 ± 8 (39-77)	56.20 ± 11.4 (33-79)
Sex	F:38/M:2	F: 34/ M:0	F: 4/M:2	F:29/M:1
Disease Duration(years) mean ± SD (range)	8.± 7,6 (1-41)	9.3 ± 8 (7-41)	5.8 ± 3.6 (1-10)	n.a.
mRSS mean ±SD (range)	12.± 10.2 (3-47)	9.9 ± 5,9	28.5 ± 14.8	n.a.
Capillaroscopy patterns (early, active and late)	14/14/12	10/12/8	0/2/4	n.a.
ANA +	35/40 (87,5%)	29/34 (85.2%)	6/6 (100%)	n.a.
SCL 70+	10/40 (25%)	6/34 (17,6%)	4/6 (66.6%)	n.a.
ACA +	21/40 (52.5%)	28/34 (82.3%)	0/6 (0%)	n.a.
Skin ulcers	6/40 (15%)	6/34 (17.6%)	0/6 (0%)	n.a.
Fingertip ulcers	11/40 (27.5%)	10/34 (29.4%)	1/6 (16.6%)	n.a.
Teleangectasias	28/40 (70%)	24/34 (70,5%)	4/6 (66.6%)	n.a.
Calcinosis	6/40 (15%)	5/34 (14,7%)	1/6 (16.6%)	n.a.
FVC (%) mean ± SD (range)	99.0 ± 23.2 (55-120)	104.21 ± 20.53 (60-120)	70.3 ± 17.48 (55-90)	n.a.
DLCO (%) mean ± SD (range)	63.6 ± 23.0 (54-99)	66.72 ± 21.5 (75-99)	50.56 ± 26.75 (65-88)	n.a.
Intestitial disease at HRCT	19/40 (47,5%)	14/34 (41,1%)	5/6 (83,3%)	n.a.
Ecocardiography and EKG abnormalities	15/40 (37,5%)	13/34 (38,2%)	2/6 (33.3%)	n.a.

lSSc: limited SSc; dSSc: diffuse SSc; mRSS: modified Rodnan skin score; ANA: antinuclear antibodies; Scl70: anti-Scl70 antibodies positivity; ACA: anticentromere antibodies positivity; FVC: forced ventilatory capacity; DLCO: diffusing lung capacity for carbon monoxide; HRCT: high resolution computed tomography; EKG: Elettrocariography; n.a.: not assessed.

tial lung characteristics were evaluated by forced vital capacity (FVC), diffusing lung capacity for carbon monoxide (DLCO) and high resolution computed tomography (HRCT). Cardiovascular involvement was assessed by two dimensional ultrasound evaluation and standard EKG: a patient was referred as positive when abnormalities were found by one or both techniques.

Antinuclear antibodies (ANA) (by indirect immunofluorescence on rat liver), anticentromere antibodies (ACA) [by indirect immunofluorescence on Hep-2 cells and by enzyme-linked immunoabsorbent assay (ELISA) for CENP antigen] and anti-topoisomerase I antibodies (anti-Scl70) (by immunoblot analysis and by ELISA) were determined.

The assessment of clinical parameters, in particular mRSS, were performed just at baseline, because we supposed that the duration of the treatment could be too short in order to significantly modify these items.

Chemokine analysis

In SSc patients and controls subjects, blood was drawn in fasting state from the antecubital vein in the morning between 8:00 and 9:00 am and, in SSc, before the first infusion and after 3 consecutive daily infusions (60 μg in 250 cc of physiological solution) of Alprostadil (Alprostadil – Leiclodedrin®, Schwarz Pharm).

Samples were collected in vacutainers containing EDTA (1 mg/ml), maintained in ice for 30–60 minutes, centrifuged (5000 g for 15 minutes) at 4°C to obtain serum, conserved at -80° C until assay. MCP1/CCL2 (Chemicon international, range 15.6–1000 ng/ml), RANTES/CCL5 (Biosource International, range 3–2000 pg/ml), and MIP1α/CCL3 (Chemicon international, range 0.195–200 ng/ml) levels were determined by ELISA kits. The results were correlated to a standard curve, within the range of linearity. Each sample was evaluated in triplicate and with two different dilutions, in order to determine intra assay variability.

Statistics

Data were analysed by using SPSS for Windows. Normal distribution of each parameter was verified by Kolmogorov-Smirnoff test. Descriptive statistics were expressed as mean ± standard deviations (SD) (if normally distributed) and as median and range (if not normally distributed) for continuos variables and as number and percentage for categorical variables. A p-value <0.05 was considered statistically significant.

The statistical significance of the differences between the means of two groups was evaluated by the Student's t-test for paired or unpaired data and, when indicated, by the Wilcoxon's signed-rank test (paired data) or the U-test of Mann-Whitney (unpaired data).

The statistical significance of the differences between means of more than two groups was evaluated by ANOVA with Bonferroni correction test, and Kruskal Wallis test, when indicated. Fisher's exact test was used for comparison of categorical variables.

Non-parametric and parametric correlation analysis were performed with the Spearman's rank correlation test and Pearson test, respectively.

Results

All parameters were statistically normally distributed, as verified by Kolmogorov-Smirnoff test and are expressed in mean (standard deviation and range) and percentage.

The clinical and immunological characteristics of the 40 SSc patients (34 lSSc and 6 dSSs) are reported in Table I.

Baseline levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 in SSc and controls

Intra-assay coefficient of variation was 3.4–4.6% for MCP1/CCL2, 4.8–5.5% for RANTES/CCL5 and 4.5–5.2% for MIP1α/CCL3.

Baseline levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 in SSc, dSSc, lSSc *versus* controls are shown in Table II.

MCP1/CCL2 levels were higher in the whole group of SSc than in the

Table II. Basal Circulating levels of MCP1/CCL2, RANTES/CCL5, MIP1α/CCL SSc versus controls and after PGE1 infusions.

Mean and standard deviation of CCL2/CCL5/CCL3 chemokines and different levels of significance							
		MCP1/CCL2		RANTES/CCL5		MIP1α/CCL3	
		SSc *vs*. controls	Before *vs*. after PGE1	SSc *vs*. controls	Before *vs*. after PGE1	SSc *vs*. controls	Before *vs*. after PGE1
SSc	Before	585 ± 476 **	*	1377 ± 444 *	****	8 ± 2 *	*
	After	335 ± 332 *		992 ± 325 NS		8 ± 2 *	
Limited SSc	Before	449 ± 353 **	*	1431 ± 454 **	***	9 ± 2 *	*
	After	247 ± 282 *		1044 ± 324 ***		8 ± 2 *	
Diffuse SSc	Before	1356 ± 323 ****	****	1066 ± 214 NS	*	8 ± 2 NS	NS
	After	807 ± 80 ****		712 ± 139 *		8 ± 2 NS	
Healthy control		64 ± 15		921 ± 205		6 ± 2	

MCP1/CCL2: monocyte chemoattractant protein 1; RANTES/CCL5: regulated upon activation, normal T expressed and secreted; MIP1α/CCL3: macrophage inflammatory protein 1.
Statistical significance levels (from higher to lower) were: $p<0.0001$ (****), $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*) and not significant (NS).

controls ($p<0.01$), the difference is maintained *versus* controls both in dSSc ($p<0.0001$) and in lSSc ($p<0.01$); MCP1/CCL2 is higher in dSSc than in lSSc ($p<0.0001$).

RANTES/CCL5 was higher in SSc and in lSSc than in the controls ($p<0.05$ and <0.01, respectively), but not in dSSc (p=n.s.). No difference was shown between lSSc and dSSc.

With respect to the controls, MIP1α/CCL3 was higher in SSc ($p<0.05$), as well as in lSSc ($p<0.05$), but not in dSSc. No difference was found between lSSc and dSSc.

Correlation of baseline MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 levels with clinical characteristics

MCP1/CCL2 correlated moderately with mRSS (Pearson $r^2= 0.34$, $p<0.05$) (Fig. 2) and was higher in patients who were positive for Scl70 antibodies than in patients Scl70 negative ($p<0.01$) (Fig. 3). No significant correlation of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 levels with age and disease duration was found. No difference in chemo-kine levels in patients with/without ulcers, teleangectasias, calcinosis, lung and heart involvement, ANA/ACA and in patients stratified according to capillaroscopy patterns were shown. The levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 did not correlate either before or after therapy.

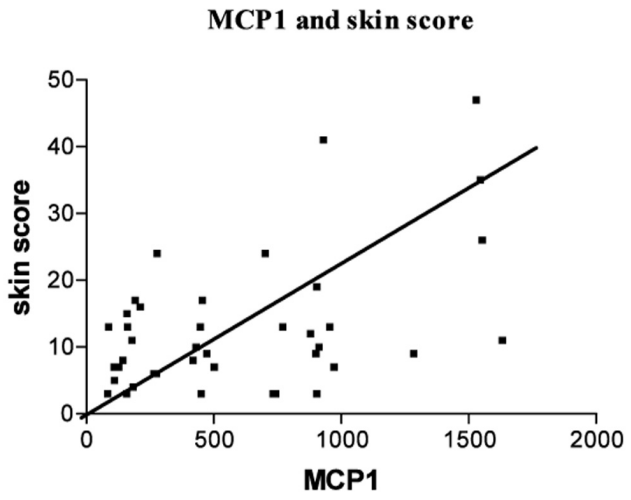

Fig. 2. Correlation between MCP1/CCL2 levels and mRSS.

Levels of MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 after alprostadil in SSc

Chemokines levels before and after alprostadil are shown in Table II.

In SSc patients, MCP1/CCL2 levels were reduced after alprostadil in respect to basal values ($p<0.01$), in dSSc ($p<0.01$) and lSSc ($p<0.05$).

Alprostadil reduced RANTES/CCL5 ($p<0.0001$), both in lSSc ($p<0.001$) and lSSc ($p<0.05$).

MIP1α/CCL3 levels were reduced by PGE1 ($p<0.05$), only in lSSc ($p<0.05$).

Discussion

Our study showed that serum levels of the C-C chemokines MCP1/CCL2, RANTES/CCL5 and MIP1α/CCL3 are increased in SSc patients in respect to healthy controls, according to previous *in vivo* and *in vitro* studies (1-2, 8-9, 10-12) that showed their involvement in early recruitment of immune cells (2, 8) and in successive development of fibrosis (1, 8) (Fig. 1).

Moreover, an association between SSc and genetic polymorphism varia-

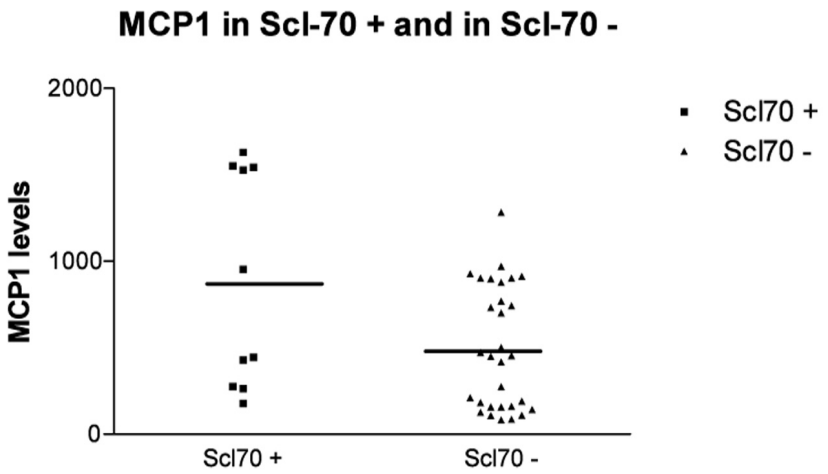

Fig. 3. MCP1/CCL2 levels are higher in anti-Scl70 positive than in negative SSc patients

tions either for MCP1/CCL2 (23) and for RANTES/CCL5 (24) was recently demonstrated.

Some studies have shown the increase of serum levels of MCP1/CCL2 (2, 25-28) and MIP1α/CCL3 (2) in SSc, but no data have been available till now on RANTES/CCL5 levels.

In early phase of SSc, in animal models, all these molecules are up-regulated (8), but while MCP1/CCL2 and MIP1α/CCL3 remained elevated during the time, RANTES/CCL5, after an initial marked peak, rapidly decreased, suggesting a more relevant role for MCP1/CCL2 and MIP1α/CCL3 (8).

For this reason, the up-regulation of these molecules demonstrated in our study is of great interest; however, other future studies should better elucidate their different role in SSc pathogenesis, the complex interaction between themselves, and the potential role of metalloproteinases, processing them during immune response.

In our study, only MCP-1/CCL2 correlates with the extent of skin fibrosis, as assessed by mRSS, even if this correlation has a moderate significant level, probably due to the preponderance, in our patients, of limited SSc. Otherwise, this datum is confirmed by the higher values of MCP1/CCL2 in the diffuse form of the disease (dSSc) and in patients positive for anti-Scl 70 antibodies, a serological marker for dSSc.

These finding are in agreement with experimental SSc models (6, 8, 29), histological (2, 3) and serological findings (26, 28), that demonstrated a role for MCP1/CCL2 in the deposition of extra cellular matrix.

In fact, MCP1/CCL2 has a crucial role in activating lymphocytes T helper 2 (27, 30-32) that drive the immune response towards fibrosis.

From these results, we might hypothesise its putative role as serum marker of fibrosis in SSc and, as already suggested, a possible target for specific drugs (26, 28, 33).

Finally, our study demonstrated a high down regulation of the three molecules evaluated after a brief course of PGE1 treatment, that was previously shown only in other chemokines in SSc (34).

Alprostadil is used in the management of vascular manifestations in SSc, but its action goes far beyond a simple vasodilatation effect, with interference between immune and microvascular system (3) and protective role on the endothelium, yielding a central role in chemotaxis (16, 17) . In SSc, it modulates the components of fibrinolytic system and restores microvascular function, as shown by the decrease of endothelial damage circulating markers (16-17) and down-regulated vascular leukocytes adhesion molecules -L selectins (35).

Furthermore, recently, other papers have shown that PGE1 has anti-inflammatory (inhibition on release of leukotriene B4 and anion super oxide by polimorphonuclear cells) (36, 37) and anti-fibrosis effects *in vitro* (38) .

The notable reduction of CC chemokines, especially of MCP1/CCL2, shown by our data support its role in interfering with processes of cell recruitment and fibrosis and confirm that PGE1 may be regarded as a potential disease modifier in SSc

Conclusions

The C-C chemokines MCP1/CCL2/CCL2, RANTES/CCL5/CCL5 and MIP1α/CCL3/XCL3 are increased in SSc and downregulated by PGE1 treatment. MCP1/CCL2 correlated with severity of skin involvement and was higher in diffuse SSc.

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