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전신성 경화증 환자에서 혈장 monocyte chemoattractant protein-1 수치와 폐고혈압과의 관련성

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전신성 경화증 환자에서 혈장 monocyte chemoattractant protein-1 수치와 폐고혈압과의 관련성

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근본적인 완치의 어려움에도 불구하고, 전신성 경화증의 효과적인 치료는 장기를 침범하는 합병증을 최소화하고 임상 증상을 조절하는 것을 목표로 하고 있다. 그래서 최근 연구들은 전신성 경화증의 발병기전에 관여한다고 알려진 신호 전달 물질이나 싸이토카인 등을 포함하여 중심적인 역할을 담당하는 매개물이나 연결통로를 차단하는 것에 목표를 두고 있다. 특히 폐나 신장을 침범하는 경우 예후가 불량해지며, 치료 또한 어려운 것으로 알려져 있다. 그러므로 치료의 측면에서 이러한 매개물과 임상 양상 사이의 연관성을 발견하는 것은 매우 중요하다할 수 있다.

본 연구는 건강한 대조군과 비교했을 때, 전신성 경화증 환자에서 질환의 발병기전과 관련 가능성이 높은 것으로 확인되었거나 추정되고 있는 몇 가지 매개물의 혈장 수치가 통계적으로 의미 있는 차이가 있는지에 대해 확인해보고자 하였고, 전신성 경화증 환자군의 임상양상과 이러한 매개물 수치 사이의 연관성을 알아보고자 하였다.

연구 대상은 60명의 전신성 경화증으로 진단된 환자와 30명의 건강 대조군, 23명의 질환 대조군으로 구성되었으며 전신성 경화증 환자는 유형에 따른 차이를 알아보고자 미만성과 제한성 전신성 경화증 환자를 각각 30명씩으로 구성하여 연구하였다. 혈장에서의 endothelin-1, monocyte chemoattractant protein-1,

interleukin-18, nitric oxide의 수치는 효소결합면역흡착검사 (ELISA) 를 이용하여 측정하였다. 임상 평가 항목으로는 류마티스 내과의사에 의해 확인된 수지 궤양, 관절통, 관절염이 평가되었고, 장기 침범을 평가하기위해 폐기능 검사, 흉부 고해상도 컴퓨터 단층촬영, 폐동맥압의 측정을 위한 심장 초음파, 식도압 검사, 뇨 검사와 신장 초음파, 혈액 생화학 검사가 시행되었다.

전신성 경화증 환자군에서 가장 흔한 증상은 레이노 현상이었고, 관절통, 폐 섬유화증, 위장관 증상 순으로 나타났다. 심장 초음파에서 확진된 30 mmHg를 초과하는 폐고혈압은 단지 3명의 환자에서만 관찰되었다. 전신성 경화증의 아형에 따른 소집단 분석에서는 피부 두께를 평가하기 위한 항목인 modified Rodnan score, 위장관 증상, 폐섬유화증, 수지 궤양이 제한 피부형에 비해 전신 피부형에서 통계적으로 의미 있게 높게 나타났다. Nitric oxide를 제외하고 혈장 endothelin-1, monocyte chemoattractant protein-1, interleukin-18의 수치는 건강 대조군에 비해 전신성 경화증 환자군에서 유의하게 높게 나타났으며, 특히 상승된 endothelin-1 수치는 질환의 활동성을 반영한다고 알려진 피부 경화, 수지 궤양, 폐고혈압과 연관성이 있었다. 또한 혈장 monocyte chemoattractant protein-1 수치는 폐고혈압이 합병된 전신성 경화증 환자에서 특히 상승된 소견을 보였다.

본 연구도 이전 연구들과 비슷하게 전신성 경화증 환자의 혈장에서 endothelin-1, monocyte chemoattractant protein-1, interleukin-18 수치의 상승된 결과를 보였으며, 특히 monocyte chemoattractant protein-1은 임상 양상 중 폐고혈압과의 통계적으로 의미 있는 관련성으로 보여 endothelin-1과 마찬가지로 monocyte chemoattractant protein-1도 폐고혈압을 일으키는데 기여한다고 사료된다.

핵심어 : Monocyte chemoattractant protein-1, Endothelin-1, 폐고혈압, 전신성 경화증

I. Introduction

Systemic sclerosis (SSc) is a disease characterized by excessive fibrosis and vascular change of skin and organ tissues.[1] Although the pathogenesis of SSc remains uncertain, many previous studies have suggested that some cytokines or chemokines regulate the induction of SSc by stimulating the synthesis of extracellular matrix components, injuring the endothelial cells, and modulating the function of leukocytes.[2] In addition, the imbalance of endothelial dependent vasoactive agents including endothelin-1 (ET-1) and nitric oxide (NO) ultimately lead to aggravating of chronic ischemia.[3]

Even though there is no curable therapy for SSc, the goal of treatment lies in the prevention of excessive fibrosis affecting major organs such as the lung, esophagus, or skin, and in minimizing microvascular injury to lessen the deterioration in the quality of life.[4,5] The identification of specific correlation between these mediators and organ involvement or clinical manifestation is very important for treatment of SSc. For this reason, recent studies are based on targeting key pathways or mediators including cytokines and signaling molecules involved in SSc pathogenesis. The known main mediators including cytokines and chemokines that release from fibroblasts, macrophages, lymphocytes and endothelial cells and induce fibrosis are transforming growth factor-β (TGF-β), interleukin-1 (IL-1), ET-1 and tumor necrosis factor-α (TNF-α).[4,6-8]

Recent studies have reported the correlation between the clinical manifestations of SSc and levels of monocyte chemoattractant protein-1 (MCP-1), interleukin-18 (IL-18), NO and vascular endothelial growth factor (VEGF) using different biological specimens.[7,9-11] We studied using serum that is more easily performed on outpatients and could be simultaneous assessment of multiple cytokine levels. But, not all studies on serum level of these mediators have shown consistent results like NO. MCP-1 is reported to

play an important role in the activation of monocytes and macrophages, leading to the induction of cytokine secretion and adhesion molecule expression incurring serious complications. Recent studies suggest that MCP-1 is a serological indicator of the activity of skin in patients with SSc and pulmonary fibrosis in patients with collagen vascular disease including SSc.[9,12,13] A study of plasma MCP-1 level in patients with idiopathic pulmonary arterial hypertension (PAH) suggested that MCP-1 is contributed to development of PAH.[14] In particular, it is the important to note that the primary cause of death in SSc is now the lung related complication including pulmonary fibrosis and PAH, and that's why we selected these mediators like ET-1 and MCP-1 on this study. In addition, IL-18 is known as having an influential role in inflammatory response. A study suggested that kidney involvement was related to IL-18 from peripheral blood mononuclear cells (PBMC) in SSc patients.[7]

This study aims to ascertain the plasma levels of several potential molecules involving SSc pathogenesis in SSc patients, compared to their levels in healthy control and to investigate the relationship between the plasma levels of these mediators and clinical manifestation or organ involvement in SSc patients.

II. Patients and Methods

A. Patients

Sixty patients (52 females and 8 males) with SSc who visited the outpatient clinic between May 2005 and April 2006 were included in this study. All patients fulfilled the American College of Rheumatology criteria for SSc.[15] For subgroup analysis, patients were grouped according to the degree of skin involvement, based on the classification system proposed by LeRoy et al.[16] They were sorted into two groups, diffuse and limited type of SSc and matched numbers of patient. Also, we selected 30 adults with no known medical history as the healthy controls and 23 patients with connective tissue disorders other than SSc as the disease controls. The disease control group comprised 14 patients with systemic lupus erythematosus (SLE), 4 with primary Sjögren syndrome (pSS), 2 with dermatomyositis (DM), 2 with mixed connective tissue disease (MCTD), and 1 with rheumatoid arthritis (RA).

B. Clinical assessments

All the patients and controls underwent physical examination and blood sampling from a peripheral vein. Blood was immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/mL), centrifuged at 4°C, and stored at -70℃ freezer (ULT-1386-5D-40) to measure the levels of ET-1, MCP-1, IL-18, and NO. The clinical manifestations in each patient group was evaluated using the following examinations: Manometry and gastrofiberscopy to determine gastric involvement. Abnormal finding including gastroesophageal reflux disease (GERD) in these studies is considered to have gastrointestinal manifestation. Pulmonary fibrosis is determined by pulmonary function test (PFT) and chest high resolution computed tomography scans (HRCT). PAH is defined over 30 mmHg in mean pulmonary arterial pressure measured by doppler echocardiography. Renal involvement is defined as existance of proteinuira in urinalysis and abnormal echogenicity in kidney sonogram. Symptomatically, we investigated the presence of arthralgia, arthritis, and digital ulceration. Skin sclerosis was assessed by an experienced rheumatologist, rating the severity by using the Modified Rodnan Score (MRS range: 0 - 51). The level of antinuclear antibody (ANA) was measured by ELISA (BIO-RAD, France) of immunofluorescence and the levels of anti-scl-70 antibody, anti-centromere antibody, and extractable nuclear antigen (ENA) were measured by immunoblotting.

C. Measurement of plasma endothelin-1, monocyte chemoattractant protein-1, interleukin-18, and nitric oxide

Plasma ET-1, MCP-1, IL-18, and NO were measured by ELISA.

1. We evenly mixed 500 μL plasma ET-1 in an EDTA tube with 750 μL extraction solvent (acetone:1 N HCl:water, 40:1:5), and the mixture was microcentrifuged. The supernatant was dried for 7 h using a speedVac concentrator, and an ET-1 ELISA kit (R&D, Minneapolis, MN) was used for measurement. The results were interpreted using a microplate reader set to 450 nm as the reference wavelength.

2. For the measurement of MCP-1, 100 μL of 2 μg/mL MCP-1 monoclonal antibodies (R&D, Minneapolis, MN) was added to a sandwich ELISA 96-well plate (NUNC, Roskilde, Denmark). This was incubated overnight at 4°C followed by addition of 200 μL of stop solution (1% BSA/Tween-20/PBS). The mixture was incubated at room temperature for 2 h. Recombinant human MCP-1 (R&D) was used and concentrations ranging from 2 ng/mL to 31.25 pg/mL were designated as the standard values. We then added 100 μL of plasma and incubated the mixture at room temperature for 2 h. Subsequently, it was irrigated 4 times with washing buffer (0.05% Tween-20/PBS), mixed with 100 μL of biotin-conjugated goat anti-human MCP-1 antibody (R&D) diluted to 50 ng/mL, and incubated at room temperature for 2 h. Following this, 100 μL of extraavidin-alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO) was diluted to 1:2000, and then added to the mixture. The mixture was incubated at room temperature for 2 h and irrigated. Phosphate disodium salt hexahydrate (Fluka, St. Louis, MO)/diethanolamine was

dissolved to a concentration of 1 mg/mL and 100 μL was added per well. The reaction was then stopped 20 minutes later with 0.2 N NaOH. The result was interpreted at a wavelength of 405 nm on a microplate reader.

3. In order to determine IL-18 levels, we used a human IL-18 ELISA Kit (MBL, Nagoya, Japan). Plasma and assay diluent were mixed at a ratio of 1:1 and added to a 96-well (U-shaped) plate in the kit, together with 100 μL of standard solution. The mixture was incubated at room temperature for 60 min and irrigated 4 times with washing buffer. Then, we added 100 μL each of the substrate and conjugate solutions, comprising anti-human IL-18 monoclonal antibody and peroxidase, respectively. The mixture was further incubated at room temperature for 30 min. Finally, 100 μL of stop solution was added. The result was interpreted at a wavelength of 450 nm on a microplate reader.

4. We mixed 100 μL of plasma NO with 80 μL of 75 mM $ZnSO_4$ for deproteination. The mixture was then centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant was mixed with 120 μL of 55 mM NaOH and recentrifuged at 14,000 rpm at 4°C for 5 min. Then, 210 μL of the resultant supernatant and 70 μL glycine buffer were applied to an NO measurement kit (Bioassay systems) and interpreted at a wavelength of 540 nm on a microplate reader.

D. Statistical analysis

The results were expressed in median values and interquartile ranges. SPSS ver. 12.0 for Windows (SPSS, Chicago, IL) was used for the statistical analysis. The Mann-Whitney U test was used for the comparison of continual variables of mediators including cytokines, and the chi-square and Fisher's exact tests were used for the comparison of clinical manifestations between the SSc group and the control groups. All the results were interpreted to be statistically significant when $p < 0.05$.

III. Results

A. Clinical characteristics

Thirty patients had the diffuse type of systemic sclerosis (dSSc) and 30, the limited type (lSSc). The mean age of the 60 SSc patients was 47 (12 - 66 years), of which 52 were women (84%), and the mean prevalence period was 4 years (0.1 - 18 years). Sexual ratio and age range were similar in both groups. The disease duration was measured from the onset of the first symptom other than Raynaud's phenomenon consistent with SSc. In SSc group, most frequent clinical manifestation was Raynaud's phenomenon, followed by arthralgia, pulmonary fibrosis, gastrointestinal manifestation. PAH measured by echocardiogram was shown only 3 patients (Table 1). The difference in clinical patterns due to SSc subtypes was significantly elevated in the outbreak frequency of MRS high scores, gastrointestinal dysfunction, pulmonary fibrosis, and digital ulcers in dSSc as compared to lSSc. Statistically, autoantibodies such as ANA, anti-scl-70 antibody, and ENA demonstrated a greater positive incidence in dSSc (Table 2).

B. Comparison of plasma endothelin-1, monocyte chemoattractant protein-1, interleukin-18 and nitric oxide levels in patients and control groups.

The median and ranges of the plasma ET-1, MCP-1, IL-18, and NO levels in SSc patients and control groups are presented in Table 3-1. The SSc and disease control group had higher plasma ET-1 levels than the normal control group. Also, the levels of MCP-1 and IL-18 in the SSc and diseased control group are statistically higher than those of the normal control group, and there was almost no difference in the level of plasma NO between the groups (Figure 1). No difference was observed in the plasma ET-1, MCP-1, IL-18, and NO levels among the subtypes in the SSc patient group (Table 3-2).

C. Correlation between the levels of plasma cytokines and clinical characteristics in patients with systemic sclerosis.

Comparison of the plasma ET-1, MCP-1, IL-18, and NO levels in the SSc patient group divided according to clinical characteristics revealed that plasma ET-1 is notably high in the group with digital ulcers and pulmonary hypertension ($p < 0.01$ and $p < 0.05$, respectively). It should be noted that plasma ET-1 is statistically proportional to MRS, PAH and digital ulcer. There was no notable difference in the NO and IL-18 levels in all the clinical characteristics; however, MCP-1 was statistically higher in the group with PAH ($p < 0.05$) (Table 4).

IV. Discussion

SSc is a generalized connective tissue disorder characterized by a wide spectrum of microvascular and immunological abnormalities, leading to progressive fibrosis of the skin and other internal organs such as the lungs, gastrointestinal tract, heart, and kidneys.[5,7,17] Several researches indicate that SSc presents deregulated production of cytokines that are important mediators in an aberrant recruitment of inflammatory cells into the perivascular dermal matrix of the skin and internal organs. [7,9,18] Although a number of cytokines and chemokines has been investigated as possible mediators implicated in the pathogenesis of SSc, only a few studies have been found specific correlation between cytokines and organ involvement in SSc. ET-1 is known as the representative cytokine that is responsible for the development of fatal diseases such as pulmonary fibrosis and pulmonary hypertension in SSc.[8,19] This is because ET-1 is also increased in other connective tissue disorders involving microvessel disruption due to vasoconstriction.[20-22] Further, in our study, the plasma ET-1 level in SSc was increased as compared to that in the healthy control group, and there was a statistic correlation with skin hardening, digital ulcers, and PAH, which well reflect the activity of the disease in general. Earlier studies have revealed that inflammatory processes may play an important role in the development of PAH and vascular remodeling.[23-25]

Scala *et al.* emphasized that investigation as possible mediators leading to fibrotic and vascular damage in SSc is adequate for comprehension of disease pathophysiology, and identification of correlation between these mediators and organ involvement is important to prevent disease progression.[7] On the other hand, it is difficult to constantly measure the changes in cytokines or chemokines, and their relationship to internal organ invasion is uncertain. MCP-1, a predominant monocyte chemoattractant and activator of

mononuclear cells, has been implicated in a variety of inflammatory and fibrotic diseases.[12,13] These chemoattractant properties stimulate collagen production by fibroblast.[12,26] MCP-1 has been implicated in the pathogenesis of SSc, with a role in recruiting monocytes from the skin circulation and development of pulmonary fibrosis.[9,14] Interestingly, we found a correlation between MCP-1 and PAH in SSc. PAH is not only correlated to ET-1, but it also has a correlation to the plasma MCP-1 levels; ET-1 is known to be the main cytokine in SSc. Therefore, plasma MCP-1 has optimal significance and it may be a factor that is responsible for the progression of PAH, fatal complication of SSc like as idiopathic PAH.

Several other studies reported the relationships between plasma NO and pulmonary hypertension, and between IL-18 and pulmonary fibrosis. In our study, significantly higher serum level of IL-18 was shown in SSc patients than that of healthy control. however, these relationships were not observed in our result. In addition, some studies of serum NO level in SSc patients showed conflicting results. In our study, serum NO level was not significantly high in SSc patients. Romero *et al.* explained that serum NO level might be dependent upon the disease stage, severity of tissue fibrosis and various circumstances of endothelial damage about previous different results.[27]

Similar to results of other studies, the plasma ET-1, MCP-1, and IL-18 levels in SSc in our study were increased as compared to the healthy control group, but there was no statistical difference as compared to the disease control group. This is because ET-1 is also increased in other connective tissue disorders involving microvessel disruption due to vasoconstriction. This is also why IL-18 and MCP-1 levels in SSc are not significantly different from those in the disease control group. This study revealed that plasma MCP-1 is elevated and significantly high in SSc patients with PAH. Therefore, our results suggest that similar to ET-1, MCP-1 may be closely related to the progression of PAH in SSc. By extension, like ET-1, we

attentively suggest the possibility of MCP-1 as therapeutic target for SSc patients with PAH. However, a longterm prospective study in a larger population will be needed to confirm its clinical utility as predictors of outcomes, and study on change of serum level depending on disease stage or clinical deterioration reflected on disease activity will be needed.

V. Conclusion

Higher levels of ET-1, MCP-1 and IL-18 in patients of SSc compared to healthy controls are in line with the previous studies in plasma. In the group of SSc patients with PAH, plasma MCP-1 was notably high and this finding suggests that MCP-1 is contributed to the development of PAH like ET-1.

Table 1. Clinical characteristics of controls and systemic sclerosis patients

	Healthy controls	Disease controls	Patients
	(N=30) (%)	(N=23) (%)	(N=60) (%)
Gender, female, n (%)	30 (100)	23 (100)	52 (83.9)
Age, years, median (IQR)	30 (24-53)	41 (18-57)	47 (12-66)
Disease duration (years) median (IQR)		5 (0.5-10)	4 (0.1-18)
Clinical manifestation			
Raynaud's phenomenon	0 (0)	10 (43.5)	57 (95)
Duration of Raynaud's phenomenon (months), Median (IQR)		6 (3-24)	12 (1-120)
Gastrointestinal manifestation	0 (0)	0 (0)	29 (48.3)*
Pulmonary fibrosis	0 (0)	2 (8.7)	33 (55)*
Pulmonary arterial hypertension	0 (0)	2 (8.7)	3 (5)
Renal disease	0 (0)	1 (4.4)	3 (5)
Arthralgia	0 (0)	11 (47.8)	45 (75)*
Arthritis	0 (0)	2 (8.7)	14 (23.3)*
Digital ulceration	0 (0)	5 (21.7)	27 (45)*

Abbreviations: N, number of patients; IQR, Interquartile range, median (range: minimum-maximum)

* $p < 0.05$ between patients group and the disease control group

Table 2. Clinical and serological findings according to type of systemic sclerosis

	Diffuse type of systemic sclerosis (N=30) (%)	Limited type of systemic sclerosis (N=30) (%)	p value
Clinical manifestation			
Raynaud's phenomenon	28 (93.3)	29 (96.7)	NS
Modified Rodnan score, IQR	10.5 (4-33)	4 (2-18)	$p < 0.05$*
Gastrointestinal manifestation	19 (63.3)	10 (33.3)	$p < 0.05$*
Pulmonary fibrosis	19 (63.3)	14 (46.7)	$p < 0.05$*
Pulmonary arterial hypertension	1 (3.3)	2 (6.7)	NS
Renal disease	1 (3.3)	2 (6.7)	NS
Arthralgia	23 (76.7)	22 (73.3)	NS
Arthritis	7 (23.3)	7 (23.3)	NS
Digital ulceration	17 (56.7)	10 (33.3)	$p < 0.05$*
Serological positivity			
Antinuclear antibody (>1:160)	30 (100)	24 (80)	$p < 0.05$*
Anti scl-70 antibody	18 (60)	12 (40)	$p < 0.05$*
Anticentromere antibody	3 (10)	4 (13.3)	NS
Extractable nuclear antigen	15 (50)	6 (20)	$p < 0.05$*

Abbreviations: N, number of patients; IQR, Interquartile range, median (range: minimum-maximum); NS, not significant

$p < 0.05$* is the significant value

Table 3-1. Levels of cytokines in the controls and patients with systemic sclerosis

	Healthy controls	Disease controls	Patients
	(N =30)	(N =23)	(N =30)
Cytokine (pg/ml), median (IQR)			
Endothelin-1	1.7 (0.5-2.5)	2.1 (1.2-4.1)**	2.4 (0.9-6.3)***
MCP-1	51.0 (17.8-102.3)	67.3 (14.0-741.8)**	61.2 (21.1-474.1)***
IL-18	82.7 (38.2-220.7)	109.1 (48.5-1373)**	104.0 (62-251.9)***
Nitric oxide	59.5 (41.7-71.9)	70.2 (35.3-116.3)	64.1 (42.9-89)

Table 3-2. Levels of cytokines in patients with diffuse and limited type of systemic sclerosis

	dSSc	lSSc	
	(N =30)	(N =30)	p value
Cytokine (pg/ml), median(IQR)			
Endothelin-1	2.6 (0.9-4.5)	2.2 (1.1-6.3)	NS
MCP-1	57.3 (27.7-474.1)	75 (21.1-206.9)	NS
IL-18	94.4 (62-177)	123.7 (62.4-251.9)	NS
Nitric oxide	61.4 (42.9-89)	64.5 (51.4-86)	NS

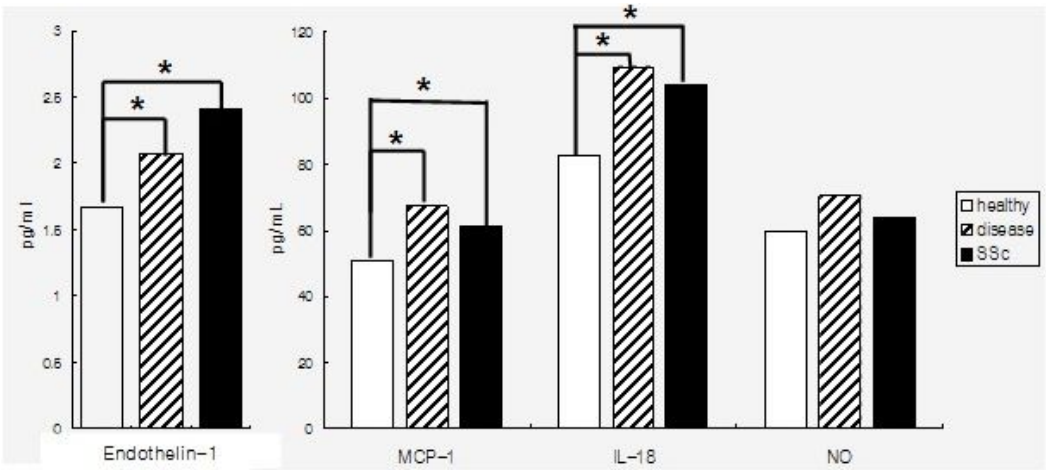
Abbreviations: SSc, systemic sclerosis; dSSc, diffuse type of SSc; lSSc, limited type of SSc; N, number of patients; IQR, Interquartile range, median (range: minimum-maximum); MCP-1, monocyte chemoattractant protein-1; IL-18, interleukin-18; NS, not significant

*$p < 0.05$ is the significant value

**$p < 0.05$ between disease and the healthy control group

***$p < 0.05$ between SSc and the healthy control group

Figure 1. Plasma levels of endothelin-1, monocyte chemoattractant protein-1, interleukin-18 and nitric oxide in healthy control, disease control, and systemic sclerosis patient groups



Abbreviations: Healthy, control group comprising healthy subjects; Disease, control group comprising patients with disease; SSc, patient group comprising patients with systemic sclerosis

*$p < 0.05$ by Mann-Whitney U test

Table 4. Correlation between the levels of plasma cytokines and clinical characteristics in patients with systemic sclerosis

	Cytokines			
	Endothelin-1	MCP-1	IL-18	NO
Skin hardening (MRS)	$p < 0.05$*	NS	NS	NS
Gastrointestinal manifestation	NS	NS	NS	NS
Pulmonary fibrosis	NS	NS	NS	NS
Pulmonary arterial hypertension	$p < 0.05$*	$p < 0.05$*	NS	NS
Digital ulcer	$p < 0.01$*	NS	NS	NS
Renal disease	NS	NS	NS	NS
Arthralgia	NS	NS	NS	NS
Arthritis	NS	NS	NS	NS

Abbreviations: MRS, modified Rodnan score; NS, not significant
$p < 0.05$* is the significant value

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