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Surfactant protein D and KL-6 serum levels in systemic sclerosis: correlation with lung and systemic involvement

F. Bonella[1,4], A. Volpe[2], P. Caramaschi[2], C. Nava[1], P. Ferrari[1], K. Schenk[1], S. Ohshimo[3], U. Costabel[4], M. Ferrari[1]

[1] Department of Biomedical and Surgical Sciences, Respiratory Physiopathology, University of Verona, Italy; [2] Department of Clinical and Experimental Medicine, Rheumatology, University of Verona, Italy; [3] Department of Molecular and Internal Medicine, Division of Clinical Medical Science, Programs for Applied Biomedicine, Hiroshima University, Hiroshima, Japan; [4] Department of Pneumology and Allergy, Ruhrlandklinik, University of Duisburg-Essen, Essen, Germany

Abstract. *Objective:* The aim of this study was to investigate the relationship between SP-D and KL-6 serum concentrations and the extent of interstitial lung involvement, as measured by a quantitative HRCT score and the functional impairment, in patients with systemic sclerosis (SSc). Moreover we analysed the association between these lung-specific biomarkers and skin involvement, anti-Scl-70 antibody titres and an index of disease activity. *Methods:* Serum SP-D, KL-6 and anti-Scl-70 concentrations were determined by ELISA in 25 SSc patients. Disease activity and lung function parameters were assessed, and the extent of ILD was measured by a HRCT score. *Results:* SP-D and KL-6 concentrations were higher in patients with SSc and lung fibrosis than in healthy controls. KL-6 correlated positively with the HRCT-fibrosis score ($r=0.68$, $p<0.001$), SP-D showed a weaker correlation ($r=0.44$, $p=0.025$). Increased KL-6 concentrations were associated with decreased DLCO and decreased FVC in SSc patients, SP-D showed no association. Furthermore KL-6, but not SP-D, showed a strong association with skin involvement as expressed by the modified Rodnan skin score ($r=0.71$, $p<0.0001$) and a disease activity index ($r=0.73$, $p<0.0001$). *Conclusion:* KL-6 is more strongly associated than SP-D with the HRCT-fibrosis score, and, different from SP-D, it correlates with skin involvement and disease activity. We suggest that KL-6 may be a useful biomarker in the assessment of scleroderma patients. *(Sarcoidosis Vasc Diffuse Lung Dis 2011; 28: 27-33)*

Key words: systemic sclerosis, SP-D, KL-6, anti-Scl-70**,** lung fibrosis

Abbreviations:

ACA	Anticentromere antibodies
anti-Scl-70	Anti topoisomerase I antibodies
BALF	Bronchoalveolar lavage fluid
DLCO	Diffusing capacity of the lung for carbon monoxide
ELISA	Enzyme-linked immunosorbent assay
FEV1	Forced expiratory volume in one second
GG	Ground glass
HRCT	High resolution computed tomography
IPF	Idiopathic pulmonary fibrosis
KL-6	Krebs von den Lungen 6
mRss	Modified Rodnan skin score
PF	Pulmonary fibrosis
ROC	Receiver operating characteristic
SP-D	Surfactant protein D
Ssc	Systemic sclerosis
dSsc	Diffused systemic sclerosis
lSsc	Limited systemic sclerosis
VC	Vital capacity

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Correspondence: Ulrich Costabel, MD
Department of Pneumology/Allergy,Ruhrlandklinik,
Tüschener Weg 40, 45239, Essen, Germany
Tel. 0049 (0)2014334021
Fax 0049 (0)2014334029
E-mail ulrich.costabel@ruhrlandklinik.uk-essen.de

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Introduction

Systemic sclerosis (SSc) is a generalized disorder with abnormalities of the microvasculature and the connective tissues, characterized by scarring (fibrosis) and vascular obliteration in the skin, gastrointestinal tract, lungs, heart and kidney. Its pathogenesis is complex and still largely unknown (1). Interstitial lung disease occurs in 50-90% of patients with SSc and constitutes the major cause of death (2, 3).

In order to identify the presence and to quantify the extent of pulmonary fibrosis in SSc patients, previous studies have revealed several potential markers, such as areas with ground-glass or reticular appearance on high resolution computed tomography (HRCT), and a neutrophilic alveolitis determined by bronchoalveolar lavage (BAL) analysis (3). However, simpler and easier, non-invasive serological markers would be helpful to more closely monitor the activity of pulmonary fibrosis in SSc. In the past, the positivity of anti-Scl-70 or of anti histone antibodies has been demonstrated to be serologic indicators of the presence or the extent pulmonary fibrosis (4). The serum concentrations of SP-D (a collectin of the CC-type lectin superfamily) (5) and KL-6 (a glycoprotein antigen expressed mainly on type II pneumocytes in alveoli and respiratory bronchiolar epithelial cells) (6, 7) have been shown to be increased in SSc patients with pulmonary fibrosis, and to be associated with functional lung derangement as measured by vital capacity and diffusion capacity for carbon monoxide (8). More recently, a study of the Scleroderma Lung Study Research Group showed that SP-D and KL-6 are markers of "alveolitis" in a cohort of 66 patients (9).

Concerning the association between these serum markers and the extent of lung involvement, only in one study HRCT was used to quantify the presence and the degree of either "fibrosis" or "alveolitis" (9). Furthermore, the relationship between serum concentrations of pneumoproteins such as SP-D and KL-6 and the type, systemic extension and activity of scleroderma disease was not systematically explored.

The aim of this study was to investigate the relationship between SP-D and KL-6 serum concentrations and the extent of lung involvement as measured by a quantitative HRCT-fibrosis score as well as the degree of functional impairment. Moreover we studied the association between these lung-specific biomarkers and skin involvement, anti-Scl-70 antibody titres and an index of disease activity.

Materials and methods

Patients

Twenty-five patients with SSc consecutively admitted to our institution with or without clinical signs of respiratory involvement were included in the study. SSc was defined according to the American College of Rheumatology criteria (10). No patient presented comorbidity such as lung, pancreas and gastric neoplasm, severe liver, kidney and heart diseases (excluding the occurrence of internal organ involvement of SSc), or was pregnant. The patients were grouped according the classification system proposed by LeRoy and Medsger (11): 10 patients had limited cutaneous SSc and 15 had diffuse cutaneous SSc. Treatment consisted of vasodilators, cyclophosphamide, low dose prednisone (<10 mg/daily). Demographics, clinical characteristics and lung function data of the patients are presented in table 1. Twenty five healthy age and sex matched Caucasian subjects were used as controls (20 women, 5 men, age 54.9±12.2 years). The study protocol was approved by the local Ethic Committee and all patients signed the informed consent.

Duration of disease was calculated from the time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. To evaluate the skin involvement the modified Rodnan total skin score (mRSS) (12) was used. Briefly, the mRss uses physical examination to measure dermal thickening. Seventeen anatomic sites were evaluated by the same operator using a score from 0 to 3 (0 indicates normal), thus resulting in a total score from 0 to 51 (13).

To assess the disease activity we used the Valentini disease activity index (14**)**. It consists of 10 items, each of which has its own weight (mRss (1), scleredema (0.5), digital necrosis (0.5), arthritis (0.5), DLCO<80% (0.5), ESR>30 mm (1.5), hypocomplementemia (1) and any deterioration as evaluated by the patient, with respect to the previous

Table 1. Clinical and laboratory characteristics of the 25 enrolled patients affected by systemic sclerosis [data expressed as absolute number (%) unless otherwise indicated]

Characteristic	N (%)
Sex (M/F)	5/20 (20/80)
Age *	55,52±15,5
Duration of disease, years*	6.7±4.7
SSC Type	
lSSc	10 (40)
dSSc	15 (60)
Smokers	2 (8)
Therapy at time of blood collection	
Prostacyclin	24 (96)
Corticosteroids	11 (44)
Autoantibodies pattern	
ACA	4 (16)
Anti-Scl-70	18 (72)
No Antibodies	3 (12)
Skin involvement	
mRss mean score *	10,6±5.9
Interstitial involvement at HRCT	
Absent (no evidence)	4 (16)
Reticular pattern	15 (60)
Ground glass pattern	12 (48)
Mixed pattern	9 (36)
Lung function	
FVC (% predicted)*	86±19
TLC (% predicted)*	87±24
DLCO (% predicted)*	62±20
Respiratory impairment	
Restriction (TLC <80% predicted)	11 (44)
Diffusion defect (DLCO/VA < 80% predicted)	15 (60)
Both present	6 (24)
Dyspnea grade	
MRC scale <3	18 (72)
MRC scale ≥3	7 (28)
Pulmonary hypertension	
Absent	20 (80)
Present	5 (20)
Laboratory	
ESR > 38 mm/h	19 (76)
CRP > 6 mg/L	9 (36)

* Values expressed as mean ± SD
lSSc, limited systemic sclerosis; dSSc, diffuse systemic sclerosis; ACA, anticentromere antibodies; Anti-Scl70, anti-Scl70 antibodies; ESR, erythrocyte sedimentation rate; mRss, modified Rodnan skin score; FVC, forced vital capacity; DLCO/VA, diffusing capacity for carbon monoxide/alveolar volume

month, in the conditions of skin (2), vessels (0.5) heart, or lung (2). The disease is considered active when the value of the index, (i.e. the sum of the values of the items detected in the patient) is 3 or higher.

Anticentromere antibodies (ACA) and Anti-Scl-70 antibodies

ACA were determined by indirect immunofluorescence using human epithelial cells (HEp-2) as the substrate.

Anti-Scl-70 Antibodies were determined by a commercially available ELISA (BMD; Marne la Valleé, France) according to the manufacturer's instructions. The upper limit of the normal range (30 U) was defined on the basis of previous studies (15).

SP-D and KL-6 serum levels

Patients' serum was stored at -80° before analysis. ELISA Kits were used to determine SP-D (Yamasa, Chiba, Japan) and KL-6 (Eitest KL-6, Eisai, Tokyo, Japan) according to the manufacturer's instruction (16).

High Resolution CT Scanning

All patients underwent CT with a collimation of 1.5–3.0 mm and an interspace of 10 mm. Images were reconstructed with a high-spatial-frequency algorithm and photographed at window settings appropriate for viewing the lung parenchyma (17). In order to quantify the lung involvement by ILD, the HRCT scoring system proposed by Kazerooni and co-workers was used (17). CT scans were reviewed separately by two observers (F.B. and M.F) who were blinded to clinical data. Briefly, each lung lobe was scored for the extent (expressed as percent of the surface of the lobe) of ground-glass (GG) opacity (HRCT-GG score), and of reticular opacities or honeycombing (HRCT-fibrosis score) on a scale of 0-5. The values of the score were as follows: 0 (absent), 1 (<5%), 2 (5-25%), 3 (25-50%), 4 (50-75%), and 5 (>75%). For the purpose of analysis, each lobe score measured by the two readers was averaged, and the sum of the means of all lobes was incorporated into a fibrosis, a ground glass, and a total score for each patient.

Pulmonary Function Tests

In all patients, pulmonary function tests were performed within 1 month of CT. Lung volumes and the single breath carbon monoxide diffusing capaci-

ty were measured using Vmax 229 Bodybox PFT Unit (Sensor Medics, Jorba-Linda, USA). The results of pulmonary function tests were expressed as percentage of predicted (18).

Statistical Analysis

Data were expressed as mean values ± standard deviation (SD). Group comparisons were made by using the Wilcoxon rank sum test. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS (version 13.0) statistical package (SPSS, Chicago, IL).

Results

The results of pulmonary function tests and the distribution of the patterns of interstitial lung involvement as derived by HRCT are reported in table 1.

Serum levels of SP-D and KL-6 in patients affected by SSc and in healthy controls are shown in figure 1. KL-6 serum concentrations were significantly higher in SSc patients with interstitial reticular pattern and/or honeycombing (1358.2±952.5 U/ml) than in those without (274.1±85.3 U/ml) ($p<0.01$) and in healthy controls (202.5±63.0 U/ml, $p<0.0001$). SP-D serum concentrations were also significantly elevated in the same subgroups (SSc with lung fibrosis 362.0±285.2 ng/ml, SSc without lung fibrosis 267.5±223.3 ng/ml, $p<0.01$), compared with healthy controls (22.9±28.7 ng/ml, $p<0.0001$).

To evaluate the association between serum levels of the two biomarkers and the extent of ILD, we determined how SP-D and KL-6 serum levels were correlated with the HRCT score. KL-6 correlated positively with the HRCT-fibrosis score ($r=0.68$, $p<0.001$) and the HRCT-total score ($r=0.67$, $p<0.001$) but not with the HRCT-GG score ($r=0.33$, $p=0.08$) (Figure 2). SP-D showed a weak correlation with the fibrosis score ($r=0.44$, $p=0.025$), but not with the total score or the GG score.

Concerning the lung function, KL-6 correlated inversely with FVC ($r=-0.47$ $p<0.05$) and DLCO ($r=-0.58$ $p=0.003$) (Figure 3). SP-D showed no significant relationship with FVC and DLCO.

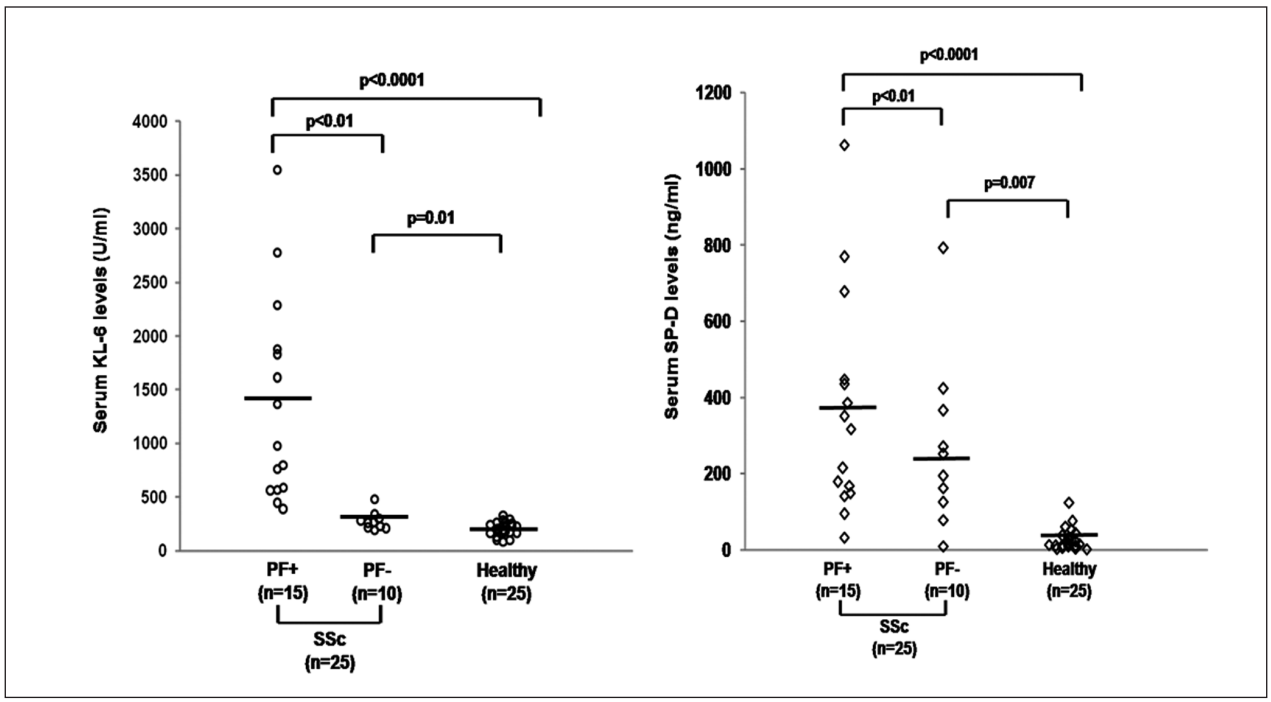

Fig. 1. Scatter plot graph showing the distribution of serum KL-6 and serum SP-D in patients affected by SSc and in healthy controls. Bars indicate average values; PF=pulmonary fibrosis

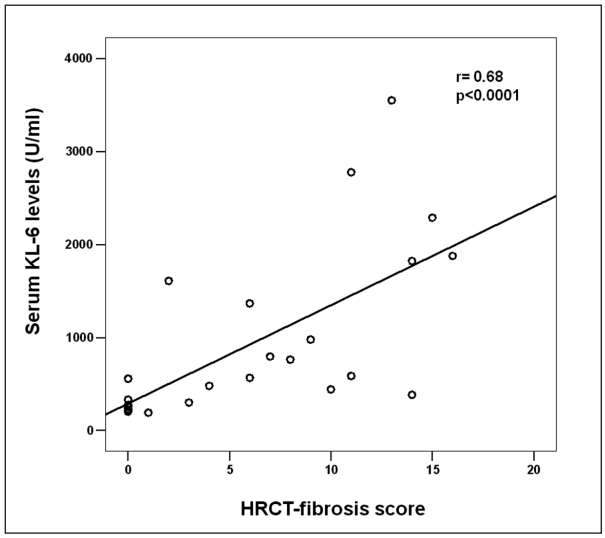

Fig. 2. Correlation between serum levels of KL-6 and HRCT-fibrosis score (score 0 to 25)

With respect to the extrapulmonary involvement, there was no relationship with gastrointestinal, cardiac, or vascular involvement. Interestingly KL-6, but not SP-D, showed a strong association with skin involvement as expressed by the mRss (r= 0.71, p<0.0001).

Serum levels of both biomarkers were higher in anti-Scl-70 positive than in ACA positive patients (KL-6: 1159±992 vs 305±153, p=0.003; SP-D: 365±297 vs 186±136 p=0.08). We found a strong relationship between serum levels of anti-Scl-70 and the HRCT-fibrosis score (r=0.63, p=0.001), and between anti-Scl-70 and KL-6 (r=0.62, p=0.009) but not with SP-D serum levels (r=0.25, p=0.39).

KL-6 showed a strong correlation with the Valentini disease activity index (r=0.73; p<0.0001), whereas anti-Scl-70 showed only a moderate correlation (r=0.42; p=0.03), and SP-D no correlation (r= 0.16; p=0.44).

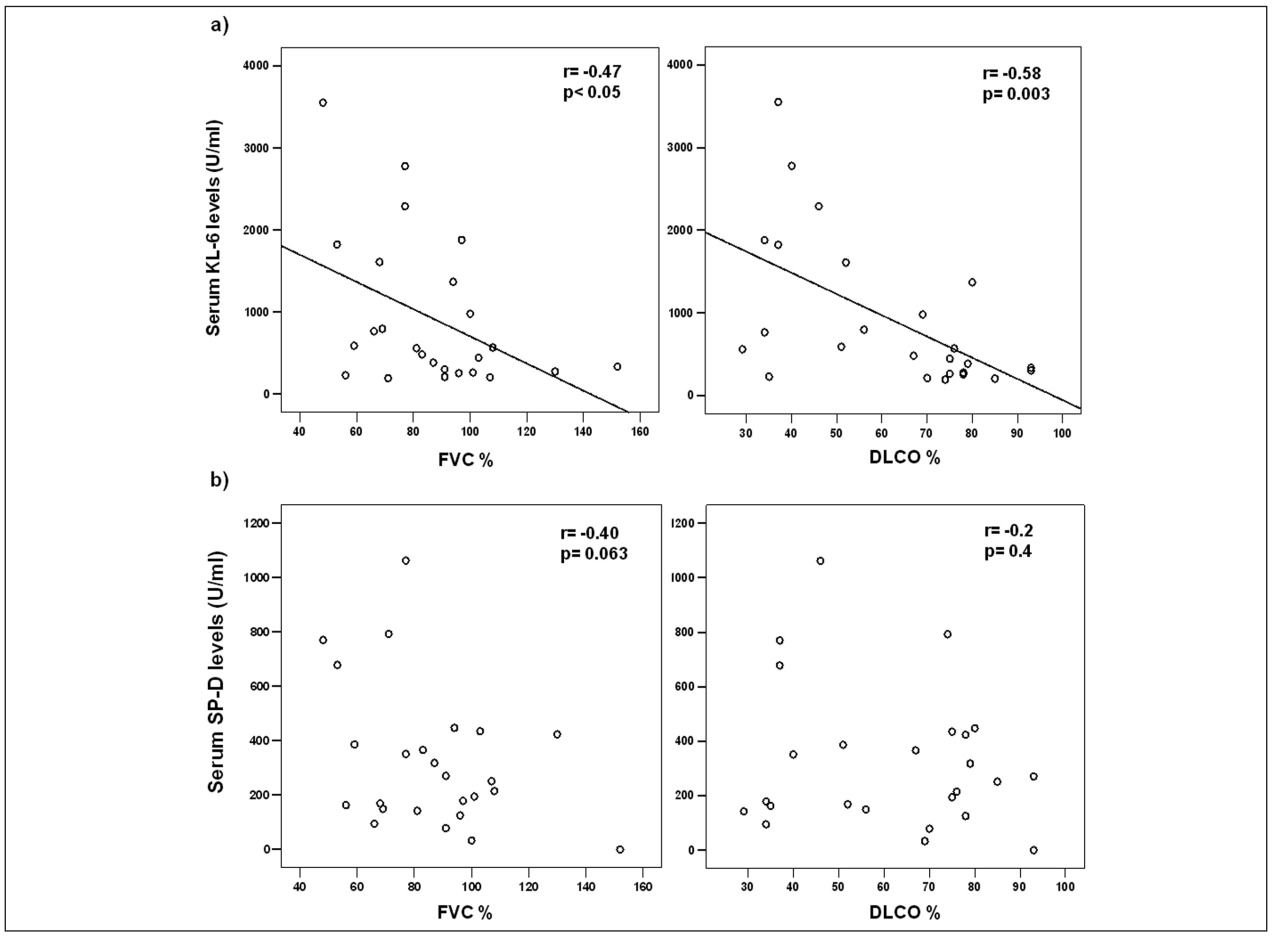

Fig. 3. Correlation of Serum KL-6 (**a**) and SP-D (**b**) with FVC and DLCO (percent of predicted).

Discussion

In the present study, serum concentrations of KL-6 and SP-D were higher in SSc patients with pulmonary fibrosis than in those without or in healthy controls. This is in agreement with previous reports (7-9, 16, 19). KL-6 was more strongly correlated with the HRCT fibrosis score, as defined in the Methods section, than SP-D ($r=0.68$, $p<0.0001$ vs $r=0.44$, $p<0.001$). This seems to indicate that KL-6 is more directly linked than SP-D to the late phase of epithelial damage, characterized by the fibrosing process. This hypothesis is also supported by the strong correlation of KL-6, but not of SP-D, with functional lung impairment as expressed by DLCO reduction. In fact, in previous reports, HRCT images were mostly used to define the presence or the absence of pulmonary fibrosis rather than to construct a continuous variable of pulmonary involvement (8, 16). In idiopathic pulmonary fibrosis (a disease with a different pulmonary involvement with respect to scleroderma lung), Takahashi et al (20) showed a strong correlation between SP-D and the GG area extension, but not with honeycombing. Similar to the results of Hant et al (9), we did not find any correlation between these pneumoproteins and the GG score on HRCT. The GG pattern on HRCT rather reflects the presence of alveolitis and is not related to fibrosis, although this point still represents an area of uncertainty.

In regard to the potential relationship of the pneumoproteins with extrapulmonary manifestations, KL-6, different from SP-D, was also associated with mRss ($r=0.713$, $p<0.0001$), a clinical index of the cutaneous extent of the disease (21, 22) related to visceral involvement (9, 21, 22) and to collagen deposition, as recently suggested by Verecchia et al (23). Yanaba et al. (8) also compared serum KL-6 levels with the presence of organ involvement other than pulmonary fibrosis in SSc patients and found no significant association with the mRss and with anti-Scl-70 antibody levels. These data are not shown in detail in their study (8), making it difficult to explain why our results are different. The recent study of Hant et al (9) did not find any correlation between serum levels of SP-D and KL-6 with clinical and patient-reported symptoms, and no data are shown related to the activity of systemic disease.

Anti-Scl-70 and ACA are found in patients with manifestations of the scleroderma spectrum of disease (24). Correlations have been reported between specific antibodies and specific features of SSc, some of which influence survival (25). Thus, ACA occur chiefly in limited cutaneous scleroderma and are more frequently associated with the absence of visceral involvement. In contrast, anti-Scl-70 are seen in patients with diffuse cutaneous extent of the disease, and in patients with severe visceral involvement, mostly interstitial lung involvement (8, 25-29). In the present study we confirm the association between anti-Scl-70 and lung involvement, and demonstrate for the first time that anti-Scl-70 is directly correlated not only with the presence but also with the extent of lung involvement as determined by HRCT. We also demonstrate a positive relationship between anti-Scl-70 titres and KL-6 ($r=0,62$, $p=0,009$), but not with SP-D. The mechanisms of these associations are not clear. We speculate that KL-6 and anti-Scl-70 could be markers of the fibrotic remodelling process occurring in the lung and in the skin.

Since the receptors for KL-6 have not been identified, it remains unclear whether or not the increased levels of KL-6 are co-causative of also extrapulmonary fibrosis or simply an epiphenomenon. Ohshimo et al. (30) demonstrated that the pro-proliferative and anti-apoptotic activities of KL-6 in an experimental system with human lung fibroblasts were comparable to those of bFGF, PDGF-BB, TFG-b1, and -b2. Leask (31), analyzing the transcriptional profiling of the scleroderma fibroblast, pointed out the existence of a TGFb-independent CTGF expression, characteristic of lung fibrotic lesions and perpetuating the fibrotic response. Recently Liu et al (32) confirmed the similarity between the experimental model of bleomycin induced skin scleroderma and of bleomycin induced lung fibrosis. The skin myofibroblasts show the same phenotype as the lung after migration in response to fibrotic stimuli. Even if speculative, it cannot be excluded that high circulating levels of KL-6 in scleroderma patients, similarly to other pleonastic molecules, may also directly contribute to chronic, persistent systemic fibrosis in a TGFb-additive way.

There are several limitations of our study. The number of scleroderma patients is small, affecting the power of the statistic analysis. We were unable to

perform follow-up HRCTs in each patient, but only if a clinical deterioration occurred. Due to the lack of longitudinal data on the correlation between the HRCT score and both biomarkers, their prognostic role could not be assessed.

In conclusion, our study confirms that KL-6 is more strongly associated than SP-D with the HRCT-fibrosis score, and, different from SP-D, it correlates with the cutaneous extent of sclerosis and with a disease activity index. We suggest that KL-6, but not SP-D, could be a useful biomarker in the assessment of scleroderma patients.

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