Comprehensive investigation of novel serum markers of pulmonary fibrosis associated with systemic sclerosis and dermato/polymyositis

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Abstract

Objective

To investigate the association between serum levels and clinical signs of lung fibrosis in patients with systemic sclerosis and inflammatory myopathies.

Methods

ELISA tests for a mucin-like glycoprotein KL-6, von Willebrandt factor (vWF), soluble E-selectin (sES) and surfactant protein D (SP-D) were performed in sera of 104 patients with systemic sclerosis, 31 patients with poly/dermatomyositis) and 24 patients with Raynaud's phenomenon as controls. The clinical and laboratory data were evaluated by a simple standard protocol including chest x-ray, lung function tests, echocardiography and, in selected cases, high resolution computer tomography (HRCT). Clinically significant pulmonary fibrosis (PF) defined as a simultaneous presence of radiological sign of pulmonary fibrosis and restrictive impairment. Severe PF was established if HRCT scans showed diffuse interstitial lung disease with low diffusing capacity. End stage PF was determined as severe PF with very low diffusing capacity.

Results

Patients with pulmonary fibrosis on chest x-ray showed significantly elevated serum levels of KL-6, SP-D and vWF. Inverse correlation was found between serum levels of KL-6/SP-D and lung function parameters, such as DLCO% and FVC. With regard to HRCT findings, patients with elevated serum level of KL-6 showed significantly more frequently ground glass opacity, diffuse and honeycombing fibrosis than patients with normal level of KL-6. The sensitivity of KL-6 for PF in SSc is increased with the severity of PF (PF on chest x-ray < severe PF < end stage of PF). Lung fibrosis occurred more frequently in patients with simultaneously elevated KL-6 and sES compared to cases with a single positivity of either KL-6 or sES.

Conclusion

KL-6, SP-D, vWF and ES are good surrogate factors of pulmonary fibrosis but can not replace conventional diagnostic procedures. However, these markers are suitable for the assessment of progression and severity of pulmonary fibrosis in systemic autoimmune disorders once the diagnosis is established.

Key words

KL-6, SP-D, vWF, E-selectin, alveolitis, pulmonary fibrosis, systemic sclerosis, dermato/polymyositis.

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Introduction

Systemic sclerosis (SSc), and dermato/polymyositis (DM/PM) are connective tissue diseases of unknown origin. Lung involvement plays an important role in the prognosis and outcome of these disorders, therefore the early detection and appropriate follow up of lung abnormalities are of high priority (1). Pulmonary fibrosis (PF) is one of the most frequent and serious complication causing unfavorable outcome both in SSc and DM/PM (1-5).

Knowing the type of the alveolitis is required to determine the therapeutic and prognostic aspects of patients with connective tissue disease. For this purpose, bronchoalveolar lavage is the gold standard, as the non-invasive diagnostic procedures can not adequately determine the type of alveolitis (1). We wanted to find new non-invasive methods for the detection of the lung fibrosis to predict the outcome of these disorders.

KL-6 (mucin-like glycoprotein) is reported to be excreted from the lung alveolar and bronchial epithelial cells, and it can be measured from serum (6). Its serum level may indicate the damage to alveolar type II cells. It has already been described that KL-6 may be an appropriate marker for monitoring the severity of interstitial lung diseases (7, 8). Surfactant protein D (SP-D) is also produced by type II cells (9). This glycoprotein is a useful marker for the diagnosis of pulmonary fibrosis (10, 11). High titer of von Willebrand factor (vWf) – a vascular endothelial cell product – can be measured in sera of patients with systemic autoimmune disorders, and is indicative of an acute phase reaction (12, 13). Adhesion molecules, such as E-selectin, are thought to contribute to the pathogenesis of interstitial inflammation. Soluble forms of selectins may play a regulatory role in inflammatory responses that are key to the pathophysiology of rheumatic diseases. Serum levels of soluble E-selectin (sES) may provide a useful additional marker for disease activity and severity in connective tissue disorders (14). Elevated vWf and sES levels may reflect vascular damage as well (12, 14, 15).

Our aim was to measure these serum markers in various systemic autoimmune diseases and to determine their applicability for monitoring disease activity and severity. The goal of our investigation was the detection and characterization of pulmonary fibrosis in various systemic autoimmune diseases with noninvasive methods.

Methods

Clinical protocol

We evaluated the clinical laboratory findings of 135 patients with systemic sclerosis and inflammatory myopathy, and 24 patients with Raynaud's phenomenon as controls. Informed consent was obtained from all patients.

ESR, CRP, rheumatoid factor, creatine kinase and antinuclear antibody test were tested in all cases. Anti-centromere, anti-topoisomerase I, anti-Jo-1, anti-Rnp, anti-SS-A, anti-SSB, anti-cardiolipin IgG and IgM were investigated by conventional ELISA tests. Urine analysis (proteinuria, hematuria, casts), and the presence of hematological changes were investigated in all cases as well. Increased sedimentation rate was recorded, if the ESR value was above 40 mm/hr for a period of at least 6 weeks without any other known cause. Signs of scleroderma capillary pattern and modified Rodnan skin score were also investigated.

Disease definitions and patient groups

From the 104 patients with SSc seventy patients belonged to the limited cutaneous systemic sclerosis (lcSSc), and 34 patients to the diffuse cutaneous systemic sclerosis (dcSSc) subset (16). The mean age of the patients was 54±12years. The mean disease duration was 12±9 years. All but 12 patients were females (Table I).

All 31 patients in the inflammatory myopathy group (patients with DM, PM, juvenile myositis (JM), cancer associated myositis /CAM/) fulfilled the diagnostic criteria for DM-PM (17). The diagnosis was also supported by electromyography in 23 cases, and by muscle biopsy in 17 patients. The mean age of the patients was 50±14 years. The mean disease duration was 7±5 years, and the mean follow up was 6±4

Table I. Clinical characteristics of investigated patient's groups.

	SSc	DM/PM	Raynaud's phenomenon
Number of patients	104	31	24
Age[1]	54 ± 12	50 ± 14	48 ± 11
Sex (female/male)	92 / 12	26 / 5	22 / 2
Subgroups[2]	DcSSC: 34 LcSSc: 70	PM: 14 DM: 14 JM: 2 CAM: 1	Primary: 10 Secondary: 14
Antibodies[3]	Scl70: 42 ACA: 7	Jo-1: 5	ANA: 5, ACA: 0, Scl70: 3, SSA: 0, SSB: 0
Pulmonary fibrosis on radiographs[4]	67	15	0
Clinically significant pulmonary fibrosis[5]	23	6	0
GGO on HRCT[6]	48	6	Not tested
Diffuse fibrosis on HRCT	27	5	Not tested
Honeycombing fibrosis	17	2	Not tested
Severe pulmonary fibrosis[7]	16	4	Not tested
End stage pulmonary fibrosis[8]	9	1	Not tested

[1]mean age in years ± standard deviation; [2]number of patients in main subgroups of disorders (see *Methods*); DcSSc: diffuse cutaneous systemic sclerosis; LcSSc: limited cutaneous systemic sclerosis, PM: polymyositis; DM: dermatomyositis; JM: juvenile myositis; CAM: cancer associated myositis; [3]number of patients with characteristic antibodies in a patient's group; [4]number of patients with signs of pulmonary fibrosis on chest x-ray (see *Methods*); [5]number of patient with fibrosis on radiographs and diffusing impairment (see *Methods*); [6]number of patients with ground glass opacity on high resolution computer tomography; [7]number of patient with diffuse fibrosis on HRCT and severe diffusing impairment (see *Methods*); [8]number of patient with diffuse fibrosis on HRCT and very severe diffusing impairment (see *Methods*).

years. Fourteen patients had DM, 14 cases exhibited PM, 2 patients showed JM and in one case, the inflammatory myopathy was associated with adenocarcinoma of rectum. None of the patients described above showed overlap symptoms to other systemic autoimmune disorders (Table I).

Twenty-four cases with Raynaud's phenomenon were also investigated as a control group. With regard to the patients with primary Raynaud's phenomenon (pRp), the criteria of Medger and LeRoy were used (18). Patients with the following characteristics were sorted into this category: episodic attacks of acral pallor or cyanosis, and strong symmetric peripheral pulses. Beside the 10 patients with primary Raynaud's phenomenon who did not show any clinical or laboratory signs of the presence of a systemic autoimmune disease, 14 patients with secondary Raynaud's phenomenon were also included (18, 19). Clinically, all of these cases exhibited Raynaud's phenomenon as the sole predominant clinical symptom. These cases also had either high titer of antinuclear antibody, scleroderma capillary pattern or ulcerations/gangrene, but they did not exhibit any internal organ manifestation (like pulmonary interstitial changes, esophageal dysmotility, colonic abnormalities, renal kidney symptoms, etc).

Investigation of the lung involvement

Lung involvement was investigated as previously described (3). Briefly, cases with signs of lung fibrosis on chest x-ray, and/or restrictive ventilatory failure, and/or decreased diffusing capacity were investigated. Pulmonary function tests were analyzed, which included forced vital capacity (FVC), total lung capacity (TLC), diffusing capacity for carbon monoxide (DLCO), and carbon monoxide diffusing capacity adjusted for alveolar volume (DLCO/VA). Patients were considered to have a restrictive ventilatory impairment if FVC was less than 80% of the predicted normal. Diffusing capacity impairment was diagnosed if the DLCO was less than 80% of the normal value. Cases with either signs of lung fibrosis on chest x-ray and/or restrictive ventilatory/diffusing impairment underwent a high-resolution computed tomography (HRCT). HRCT scans were performed on Siemens Somatom AR HP (non-spiral, third generation) computer tomograph in supine AP patient position, under normal breathing, with HiRes Lung scan type, basically in native mode without contrast material. Clinical significant pulmonary fibrosis (CSPF) defined as a pulmonary fibrosis on chest x-ray with decreased DLCO% (<80%). Severe PF was established if HRCT scans showed diffuse interstitial lung disease with low DLCO% (<67%). End stage PF determined as diffuse pulmonary fibrosis with very low DLCO% (<51%).

Measurements

Blood samples were collected during routine clinic consultations or during hospitalization, and chest radiographs and respiratory functions tests were performed within 3 months of the day blood was collected. Serum levels of sES, SP-D and vWF were analyzed by ELISA, using commercial immunoassays according to the manufacturer's instructions (E-selectin: Bender MedSystems GmbH, Austria; SP-D: Antibodyshop Grusbakken, Denmark; vWF: BioGene, USA). Evaluation of KL-6 was performed using an ELISA kit (Sanko-Junyako, Tokyo, Japan), as described (20). All other routinely investigated marker (anti-nuclear antibody (ANA), anti-centromere antibody (ACA), anti-DNA topoisomerase I antibody (anti-Scl70), anti-Jo-1, anti-SSA, anti-SSB, anti-dsDNA, anti-CCP) were measured by commercial immunoassays too. Modified Rodnan skin score (mRSS) were blindly evaluated by two independent investigators, the interobserver variation was less than 5%.

Data analysis

The values in patients were regarded as abnormally increased if they exceeded the median value observed in control subjects by 2 or more standard deviations. Non-parametric analysis was used for group comparisons of the quantitative data. The Mann-Whitney U-test was used for comparisons between two groups. The Fisher's exact correlation-test was also used as indicated.

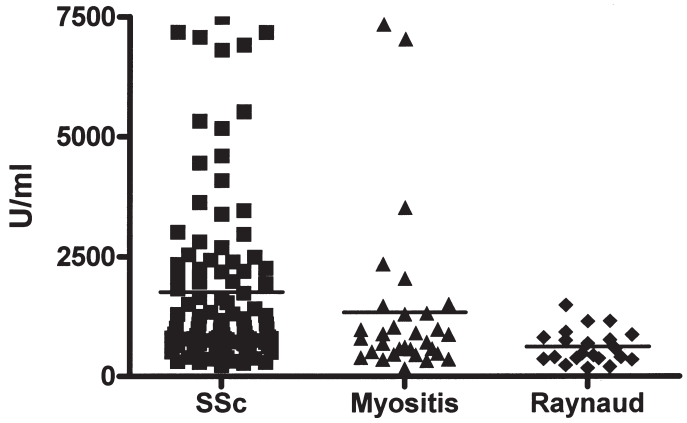

Fig. 1. Serum levels of KL-6 glycoprotein in SSc and inflammatory myo-pathy. Significantly higher level of KL-6 was measured in sera of patients with SSc (■ $p<0.0005$) and myositis (▲ $p<0.05$) compared to levels of control group (◆ Raynaud's phenomenon).

Results

There was no significant difference in the serum levels of investigated serological markers between patients with primary and secondary Raynaud's phenomenon (data not shown). Significantly higher levels of KL-6 antigen were measured in sera of patients with SSc and myositis than in patients with Raynaud's phenomenon (SSc: $p<0.0005$, myositis: $p<0.05$) (Fig. 1). Serum levels of vWF, SP-D and sES were significantly elevated only in the SSc group ($p<0.0001$, $p<0.05$ and $p<0.05$, respectively) (data not shown). The 26 patients with elevated serum levels of SP-D significantly more frequently exhibited diffuse cutaneous form of SSc compared to the 78 patients with normal level of SP-D (Odds ratio /OR/: 3.38, $p<0.05$). Serum levels of other investigated markers were independent of disease subgroups: there was no statistically significant difference between patients with lcSSc and dcSSc, nor between PM and DM. Significantly more patients with Scl70 antibody positivity showed elevated serum level of KL-6 compared to cases without the presence of Scl70 (OR: 2.96, $p<0.01$). Serum levels of other investigated markers were not statistically different between Scl70, or Jo-1 positive and negative patients.

We did not find any correlation between modified Rodnan skin score and serum levels of investigated markers in SSc group, or between levels of serological markers and creatine kinase in the myositis group (not shown). Nor did we find any association between serum markers and ESR (data not shown). Patients with PF on chest x-ray showed significantly elevated serum levels of KL-6, SP-D and vWF compared to patients with no signs of PF (KL-6: $p<0.0005$, SP-D: $p<0.0001$, vWF: $p<0.001$, data not shown). Similar changes without statistical significance were observed in relation to sES ($p=0.1352$). On the other hand, we observed notable number of patients with pulmonary fibrosis on chest x-ray without elevated level of these markers: 32 in SSc and 10 patients in myositis group had normal level of KL-6 with PF on chest x-ray (from 67 and 15 patients respectively, not shown). Normal level of SP-D was detected in 45 (from 67 patients with SSc) and 15 (from 15 patients with myositis) patients with PF on chest x-ray. This ratio for vWF was 35/67 in SSc, 10/15 in myositis group. The ration for sES was 53/67 in SSc, and 13/15 in myositis (Table II).

Significantly higher level of KL-6 was established in patients with clinically significant degree of PF (CSPF) than in patients with only radiologic signs of fibrosis ($p<0.005$) (signs of fibrosis on radiographs with normal lung function tests). Significant difference was found between serum levels of KL-6 in patients with fibrosis on radiographs and patients without fibrosis too ($p<0.05$) (Fig. 2). Similar difference was found

Table II. Sensitivity and specificity of investigated markers for pulmonary fibrosis.

		KL-6		SP-D		vWF		sES	
		SSc	Myositis	SSc	Myositis	SSc	Myositis	SSc	Myositis
Pulmonary fibrosis on chest x-ray	Sensitivity	52%	33%	33%	0%	48%	33%	21%	13%
	Specificity	78%	69%	92%	100%	73%	75%	89%	75%
Ground glass opacity	Sensitivity	54%	33%	33%	0%	52%	33%	17%	33%
	Specificity	82%	75%	86%	100%	68%	88%	89%	88%
Diffuse fibrosis on HRCT	Sensitivity	67%	40%	41%	0%	59%	40%	30%	40%
	Specificity	76%	78%	84%	100%	61%	89%	92%	89%
Honey combing	Sensitivity	82%	50%	47%	0%	59%	100%	29%	50%
	Specificity	73%	75%	81%	100%	59%	92%	90%	83%
PF with restrictive impairment	Sensitivity	78%	27%	48%	0%	65%	18%	30%	27%
	Specificity	67%	70%	81%	100%	65%	65%	85%	85%
Severe PF	Sensitivity	94%	25%	50%	0%	69%	50%	38%	50%
	Specificity	66%	70%	80%	100%	64%	74%	85%	85%
End stage PF	Sensitivity	100%	100%	56%	0%	67%	100%	22%	100%
	Specificity	62%	73%	78%	100%	61%	73%	82%	83%

Pulmonary fibrosis on chest x-ray and lung function test were performed in all patients (SSc of 104, myositis of 31). HRCT scans were carried out in cases of 90 patients with systemic autoimmune diseases: 76 patients with SSc and 14 patients with myositis.

between CSPF and PF, and between PF and no fibrosis in relation to only one other serological marker, to SP-D too (CSPF versus PF on chest x-ray: $p<0,05$; PF on chest x-ray versus no fibrosis: $p<0.05$). However, the differences between serum levels of sES and vWF in patient were not statistically significant. On the other hand, not all of patients with CSPF show marker positivity: We detected normal serum level of KL-6 in 21.7% (5/23) of sera of patient with SSc compounded with CSPF, this ratio for SP-D was: 52.2% (12/23). Patients with CSPF in myositis group showed normal level of KL-6 in 72.7% (8/11), normal level of SP-D in 100% (11/11) (Table II). Within the group of patients with CSPF, who have more severe interstitial pulmonary disease (severe or end stage pulmonary fibrosis), also show higher serum levels of investigated markers but these differences do not obtain the level of significance (not shown).

Negative correlation was found between serum levels of KL-6 and DLCO% (r=-0.55, $p<0.0001$) (Fig. 3). Analysis of data about SPD and DLCO% showed similar result (r=-0.52, $p<0.0001$) (not shown). Weak, but statistically significant inverse correlation was found between vital capacity and KL-6, SP-D (r=-0.3189, $p<0.0005$; r=-0.4176, $p<0.0001$ respectively), and between vWF and DLCO% (r=-0.3618, $p<0.0001$). Other associations between investigated serum markers and lung function tests could not be found. Similar results were found in groups of patients with SSc but not with myositis. Serum levels of KL-6 and DLCO% correlated very well (r= -0.52, $p<0.0001$) in SSc, whereas in myositis group this correlation was not significant (not shown).

With regard to HRCT findings, patients with elevated serum level of KL-6 had significantly more frequently GGO than cases with normal level of KL-6 (OR: 2.80, $p<0.05$) (not shown). Patients with elevated serum level of KL-6 had significantly higher risk for diffuse PF on HRCT scans than patient with normal titer of KL-6 (OR: 3.70, $p<0.01$). The significance was further increased if data of patients with honeycombing fibrosis was analyzed (OR: 7.83, $p<0.0005$) (Fig. 4). Similar observations were not seen with

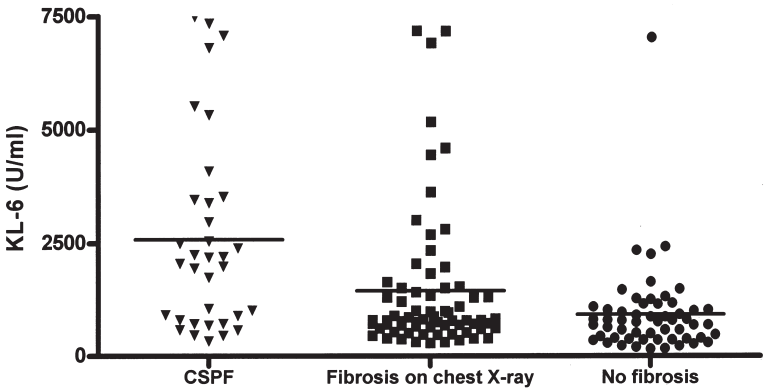

Fig. 2. Average serum level of KL-6 increases with the severity of pulmonary fibrosis in patients with systemic autoimmune disorders.
Clinically significant pulmonary fibrosis (CSPF, ▼): fibrosis on chest x-ray and restrictive impairment together. Number of patients: 34 – 23 cases with SSc, 11 cases with myositis. Fibrosis on chest x-ray ■ : fibrosis on radiographs with normal lung function tests. Number of patients: 69 – 61 cases with SSc, 8 cases with myositis. No fibrosis ● : no sign of fibrosis on radiographs and normal lung function tests. Number of patients: 56 – 20 cases with SSc, 12 cases with myositis, 24 cases with Raynaud's syndrome. Levels of significance between diseases groups were $p<0.005$ (significant versus fibrosis on chest x-ray), $p<0.05$ (fibrosis versus no fibrosis on chest x-ray), respectively.

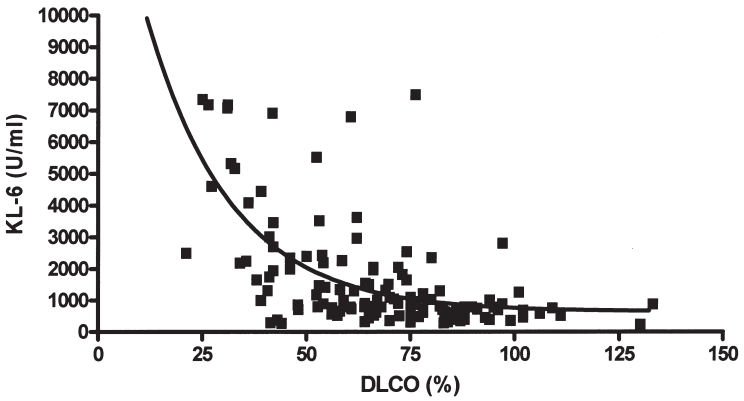

Fig. 3. Correlation between serum levels of KL-6 and DLCO% in 141 patients with systemic autoimmune disorders.
A negative correlation was established between serum levels of KL-6 and diffusion capacity in percent of predicted normals (r = -0.55, $p<0.0001$).

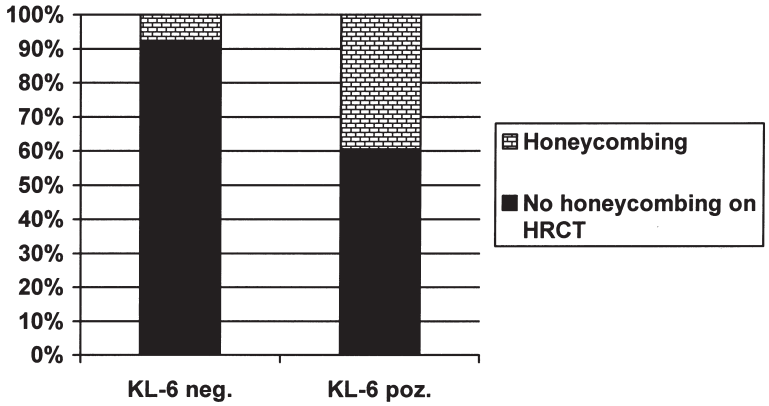

Fig. 4. Occurrence of honeycombing on HRCT scans in patients with elevated and normal level of KL-6. HRCT scans were performed in cases of 90 patients with systemic autoimmune diseases. Patients with elevated serum levels of KL-6 (number of patient: 38) exhibited honeycombing on HRCT scans significantly more frequent than patient with normal level of KL-6 (number of patients: 52) (OR=7.83, $p<0.0005$).

the other three markers except a weak association between sES and diffuse PF (OR: 3.31, $p<0.05$) (data not shown). Sensitivity of KL-6 was increased from ground glass opacity through diffuse PF honey combing, severe PF to the end stage of PF (54%, 67%, 82%, 94%, 100% respectively, Table II). The highest sensitivity and specificity we detected for honey combing in case of vWF (100% and 92% respectively), these parameters in other cases were much lower (Table II). Patients with diffuse PF and simultaneous GGO (26 patients, 22 with SSc and 4 with myositis) have significantly higher serum level of investigated markers compared with patients who had either diffuse PF or GGO or neither of them (KL-6: $p<0.0005$, SP-D: $p<0.001$, sES: $p<0.01$, vWF: $p<0.05$, respectively) (data not shown). However, the sensitivity and specificity of these markers were not better for patients with diffuse PF and simultaneous GGO (KL-6: 65%/67%, SP-D: 35%/88%, sES: 35%/86%, vWF: 62%/64%, respectively) then for patients with one of them (Table II).

We investigated the utility of various combinations of serum markers too. The double positivity for KL-6 and SP-D increased the risk for clinical significant pulmonary fibrosis: Odds ratio and level of significance was higher for double positive patients group than for patients with single positivity (OR for double positive cases: 6.73, $p<0.001$, whereas for only KL-6 OR: 4.71, $p<0.001$; for SP-D OR: 3.20, $p<0.05$). If an increased level of KL-6 and sES is detected, than the risk for diffuse PF on HRCT is higher compared to patients with only one marker positivity (OR: 13.13, <0.005 (data not shown); whereas for KL-6 OR: 3.70, $p<0.01$; for sES OR: 3.31, $p<0.05$).

Discussion

PF plays an important role in the prognosis and outcome of SSc and inflammatory myopathies (2-4). Our aim was to investigate any possible association between various serum markers and clinical signs of pulmonary manifestation of these disorders. We demonstrated significantly higher serum levels for all investigated markers (KL-6, SP-D, vWF, sES) in patients with SSc compared to patients with Raynaud's syndrome (Fig. 1). Only KL-6 was found to be elevated in both SSc and myositis. SP-D, KL-6 and vWF exhibited significantly higher serum levels in patients with PF irrespectively of disease groups when compared with patient without fibrosis on chest x-ray. In this context, SP-D was the most significant marker. These findings are in accordance with previous reports (20-23). We think that the best marker for x-ray signs of PF is KL-6, because of its highest sensitivity (Table II) and marked difference between serum levels of patients with PF and without PF. On the other hand, the assessment of pulmonary fibrosis on chest x-ray is not reliable. The clinical judgment depends on results of lung function tests and HRCT findings. If we define PF as a combination of fibrosis signs on radiographs and in lung function tests, than serum levels of KL-6 and SP-D were significantly higher in patients with fibrosis than in patients with only radiographic changes (Fig. 2). Similar to previous findings (10, 11, 23, 24), serum levels of KL-6 and SP-D are inversely correlated with vital and diffusion capacity (Fig. 3). Levels of significance were high, but correlation coefficients – in contrast of DLCO (in our study r=-0.55, in the publication from Yanaba *et al*. r=-0.52) – were not impressive in considerations of vital capacity (r=-0.32, r=-0.43 respectively) (24). It is well known that the decrease of DLCO is more sensitive and earlier parameter of restrictive lung disease than FVC. We believe that the closer correlation between KL-6 or SP-D and DLCO than FVC supports this hypothesis. In relation to the HRCT findings, patients with elevated level of KL-6 exhibit GGO more frequently – as a possible sign of disease activity – than patients with normal level of KL-6. This observation is also true for diffuse and honeycombing fibrosis (Figure 4); however, the levels of significance were higher in these relationships than in case of GGO and odds ratio for honeycombing was much higher than for GGO. This could be if KL-6 is more of a serum marker for tissue damage (fibrosis) than for disease activity (alveolitis). This hypothesis is supported by our other results as well: the sensitivity of KL-6 for PF in SSc is increased with the severity of PF (PF on chest x-ray ≈ GGO on HRCT < clinically significant pulmonary fibrosis < severe PF < end stage of PF) (Table II). Within the group of patients with CSPF serum levels of KL-6 increases with the severity of pulmonary fibrosis further, but the difference between severe and end stage pulmonary fibrosis was not statistically significant – probably by reason of low number of patients. These findings are also in accordance with previous reports (24, 25). However, patients with diffuse PF and simultaneous GGO on HRCT scans have higher serum level (but not the sensitivity) of investigated markers than other patients. This result supported the theory that these serum molecules are markers of active pulmonary inflammation. It was established in a longitudinal analysis of serum KL-6 levels that the serum level of KL-6 is increasing rapidly and parallel to the progression of PF (24). However, a significant number of patients with PF in our study had normal levels of these markers (Table II); therefore, we conclude that the use of these markers can not replace the conventional diagnostic techniques. In this study, we did not find any correlation between conventional signs of disease activity – such as erythrocyte sedimentation rate, mRSS in SSc – and novel serum markers. All of these findings support our opinion that these markers are useful determining the severity of PF and they have lower importance in assessment of disease activity. However, to establish the usefulness of these markers in disease progression we need further prospective investigations based on multicentre cooperation.

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