

# Surfactant Protein D and KL-6 as Serum Biomarkers of Interstitial Lung Disease in Patients with Scleroderma

FAYE N. HANT, ANNA LUDWICKA-BRADLEY, HE-JING WANG, NING LI, ROBERT ELASHOFF, DONALD P. TASHKIN, and RICHARD M. SILVER, for the Scleroderma Lung Study Research Group

**ABSTRACT. Objective.** To assess whether serum concentrations of surfactant protein D (SP-D) and Krebs von den Lungen-6 (KL-6), glycoproteins expressed by type II pneumocytes, correlate with the presence of “alveolitis” and measures of lung function in patients enrolled in the Scleroderma Lung Study (SLS).

**Methods.** Serum obtained at baseline screening of patients with systemic sclerosis (SSc, scleroderma) in the SLS was assayed. “Alveolitis” was defined by either bronchoalveolar lavage or thoracic high-resolution computed tomography (HRCT) by SLS criteria. SP-D and KL-6 levels were measured by ELISA in 66 SSc patients (44 with “alveolitis,” 22 without “alveolitis”) and in 10 healthy controls. These were compared to clinical measures of lung disease and “alveolitis” in the SLS patients.

**Results.** SP-D levels were  $300 \pm 214$  ng/ml (mean  $\pm$  SD) in the SSc patients compared to  $40 \pm 51$  ng/ml in controls ( $p < 0.0001$ ). KL-6 levels were  $1225 \pm 984$  U/ml in the SSc patients and  $333 \pm 294$  U/ml in controls ( $p < 0.0001$ ). SSc patients with “alveolitis” had higher levels of both SP-D and KL-6 than those without “alveolitis.” The level of SP-D was  $353 \pm 219$  ng/ml in patients with “alveolitis” and  $161 \pm 143$  ng/ml without “alveolitis” ( $p = 0.0002$ ). The level of KL-6 was  $1458 \pm 1070$  U/ml in patients with “alveolitis” and  $640 \pm 487$  U/ml without “alveolitis” ( $p = 0.0001$ ). Receiver operator characteristic curve analysis demonstrated high sensitivity and specificity of both SP-D and KL-6 for the determination of “alveolitis.” KL-6 and SP-D were positively correlated with maximum fibrosis scores, but not with maximum ground-glass opacities, on HRCT.

**Conclusion.** Serum levels of SP-D and KL-6 appear to be indicative of “alveolitis” in SSc patients as defined by the SLS, and are significantly higher than in SSc patients without “alveolitis.” Serum SP-D and KL-6 may serve as noninvasive serological means of assessing interstitial lung disease in patients with SSc. (J Rheumatol First Release March 15 2009; doi:10.3899/jrheum.080633)

## Key Indexing Terms:

INTERSTITIAL LUNG DISEASE    SYSTEMIC SCLEROSIS    SURFACTANT PROTEIN D  
KL-6    BIOLOGICAL MARKERS    SCLERODERMA

Scleroderma (systemic sclerosis, SSc) is an autoimmune connective tissue disease of unknown etiology, characterized by 3 major processes: appearance of disease-specific autoantibodies, organ fibrosis, and small-vessel vasculopathy<sup>1</sup>. Organ fibrosis can involve several body systems,

including the pulmonary, integument, cardiac, gastrointestinal, and renal systems<sup>1</sup>. As therapies to improve the various organ-specific manifestations related to SSc have developed, involvement of the pulmonary system in SSc has emerged as the leading cause of mortality in this disease. Pulmonary interstitial lung disease (ILD) and associated fibrosis, and pulmonary arterial hypertension are the leading causes of death in SSc, and contribute much to the overall morbidity associated with SSc<sup>1</sup>.

Pulmonary involvement can take many forms in SSc, where the spectrum of pneumonitis can range from potentially reversible alveolar inflammation (“alveolitis”) to irreversible homogenous pulmonary fibrosis. The exact etiology of the pathogenesis that induces and maintains progression of ILD and fibrosis in SSc is not well known. Silver, *et al* first reported that, among patients with SSc and restrictive lung disease, a subset of patients with lung inflammation had bronchoalveolar lavage (BAL) fluid containing elevated levels of eosinophils and/or neutrophils (PMN)<sup>2</sup>. Studies have demonstrated increased concentrations of thrombin, platelet-derived growth factor, and transforming growth fac-

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From the Department of Medicine, Medical University of South Carolina, Charleston, South Carolina; and Department of Biomathematics and Department of Medicine, David Geffen School of Medicine, University of California at Los Angeles (UCLA), Los Angeles, California, USA.

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F.N. Hant, DO, MSCR; A. Ludwicka-Bradley, PhD, Department of Medicine, Medical University of South Carolina; H-J. Wang, PhD; N. Li, PhD (currently Department of Epidemiology and Biostatistics, College of Public Health and Health Professions, University of Florida); R. Elashoff, PhD, Department of Biomathematics, David Geffen School of Medicine, UCLA; D.P. Tashkin, MD, Department of Medicine, David Geffen School of Medicine, UCLA; R.M. Silver, MD, Department of Medicine, Medical University of South Carolina.

Address reprint requests to F.N. Hant, Division of Rheumatology and Immunology, Medical University of South Carolina, 96 Jonathan Lucas Street, Suite 912, Charleston, SC 29425-6370. E-mail: hant@musc.edu  
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tor- $\beta$  in the BAL fluid of patients with SSc<sup>3,4</sup>. Why some patients progress to severe lung involvement with or without treatment, while some respond to treatment or never progress further without treatment, remains an important unanswered question. Currently the diagnosis of "alveolitis" is made by BAL or high resolution computed tomography (HRCT). These tests are costly, and in the case of BAL, invasive. Thus, there is a critical need for less invasive, clinically applicable biomarkers to improve the prospective evaluation of patients having SSc-related ILD.

Two promising biomarker candidates, surfactant protein D (SP-D) and Krebs von den Lungen-6 (KL-6) are glycoproteins secreted by type II pneumocytes, and have emerged as potential surrogate markers of "alveolitis." We measured and compared these 2 glycoproteins in a unique, large, well characterized population of patients with SSc enrolled in the Scleroderma Lung Study (SLS). The SLS was a National Institutes of Health sponsored multicenter, randomized, double-blind, placebo-controlled trial of oral cyclophosphamide (CYC) versus placebo for the treatment of SSc lung disease<sup>5</sup>.

SP-D and KL-6 concentrations were compared to BAL and HRCT in the diagnosis of "alveolitis," and to pulmonary function test (PFT) measures. We show through receiver-operator characteristic (ROC) curve analysis the impressive sensitivity and specificity of SP-D and KL-6 in the ability to diagnose active "alveolitis." Our study adds to the existing literature by evaluating both glycoproteins concomitantly in a cohort of North American patients with and without "alveolitis" from the SLS study, a unique group in one of the largest randomized trials involving patients with SSc.

It is known that serum levels of SP-D rise when there is damage to the alveolar epithelium, such as in the "alveolitis" seen with SSc-related ILD<sup>6-8</sup>. SP-D levels appear to correlate with the presence of pulmonary fibrosis in patients with SSc<sup>9</sup>. KL-6 levels directly reflect alveolar damage and inflammation, and may enter the circulation in a soluble form during lung disease secondary to increased vascular and epithelial permeability, suggesting its use as a serologic marker to mirror the extent of alveolar damage<sup>10</sup>. Yanaba, *et al* found that SP-D and KL-6 levels were associated with the activity of pulmonary fibrosis in patients with SSc, and that SP-D levels changed more quickly than KL-6 levels and in parallel with the activity of pulmonary fibrosis<sup>11</sup>. In that study, 4 patients were treated with intravenous (IV) CYC and steroids. In 3 of these 4 cases, after CYC was started, SP-D and KL-6 levels decreased or stabilized and there was stabilization of PFT findings [DLCO and forced vital capacity (FVC)], and in 2 of these, no progression of ground-glass opacities by HRCT<sup>11</sup>. In the remaining case, prevention of disease progression did not occur and the glycoprotein levels increased, along with worsening ground-glass opacities by HRCT and decline of PFT<sup>11</sup>. Certainly, the utility of

using biomarkers to attempt to correlate and assess clinical response to treatment is a promising area, and more information is needed to prove their true effectiveness in aiding the diagnosis of SSc-related ILD.

To evaluate the role of SP-D and KL-6 as serum biomarkers in SSc-related ILD, and to assess their correlation with "alveolitis," lung function, and HRCT findings, we performed a cross-sectional assessment using data from patients with SSc enrolled in the SLS.

## MATERIALS AND METHODS

**Patients.** We obtained and evaluated 66 de-identified randomly selected serum samples that were archived as part of the SLS. This number was selected due to limited resources to perform the assays. The study population consisted of patients with SSc who were screened and/or enrolled in the SLS between September 2000 and January 2004<sup>5</sup>. Serum obtained at baseline screening from patients with or without "alveolitis," as defined by entry criteria for the SLS, was assayed<sup>5</sup>. Serum samples of 10 random healthy controls (without SSc) not associated with the SLS were also evaluated for comparison. The control samples were obtained from volunteer subjects under a protocol approved by the Institutional Review Board of the Medical University of South Carolina.

A total of 76 serum samples were assayed, comprising 66 patients with SSc: 44 with "alveolitis" and 22 without "alveolitis," as defined by SLS criteria, and 10 healthy controls. In the SLS, evidence of active "alveolitis" was defined by either BAL analysis or thoracic HRCT. Definitions included a BAL fluid sample with  $\geq 3\%$  neutrophils,  $\geq 2\%$  eosinophils, or both on a cell differential count, or any ground-glass opacity on HRCT<sup>5</sup>.

Both BAL and HRCT were performed in 62 of the 66 (94%) patients with SSc, and 4 of the 66 (6%) patients had only one of these tests performed, with 1 patient having only HRCT and 3 patients having only BAL. HRCT scan data were available for 42 (95%) of the 44 "alveolitis" patients. Both lungs were assessed by 6 regional zones (upper, mid, lower) for maximum honeycombing, maximum ground-glass, and maximum fibrosis, and the scores were correlated with levels of SP-D and KL-6.

Patients with SSc were an average age of  $48 \pm 12$  (mean  $\pm$  SD) years. Overall, there were 52 women and 9 men, with 5 patients having no data available for age or sex. There were 34 Caucasians, 14 African Americans, 7 Hispanics, 2 Asians, and 4 classified as "other," with 5 patients missing information on race. Of the 44 patients with SSc who had "alveolitis," the average age was  $48 \pm 11$  years, with 36 women and 8 men. There were 29 Caucasians, 4 African Americans, 7 Hispanics, 1 Asian and 3 classified as "other" in the SSc "alveolitis" group. Of the 22 patients with SSc who did not have "alveolitis," the average age was  $49 \pm 14$  years, with 16 women and 1 man. There were 5 Caucasians, 10 African Americans, no Hispanics, 1 Asian, and 1 "other" in the SSc non-"alveolitis" group. The 5 patients for whom there were missing age, race, and sex data were all in the non-"alveolitis" group.

There was no missing information on the 10 controls. Data for controls yielded an average age of  $27 \pm 4$  years, with 5 women and 5 men; there were 7 African Americans and 3 Caucasians.

PFT data were available for all the patients in the "alveolitis" group ( $n = 44$ ). There were 5 patients missing data for FVC% predicted ( $n = 17$ ), and 6 patients missing DLCO% predicted data in the non-"alveolitis" group ( $n = 16$ ). The overall average FVC% predicted ( $n = 61$ ) and DLCO% predicted ( $n = 60$ ) were  $69\% \pm 13\%$  predicted, and  $49\% \pm 15\%$  predicted, respectively.

**Assays.** Specific ELISA kits determined the levels of SP-D and KL-6 from SSc and control serum samples (KL-6 ELISA kits were kindly provided by Sanko Junyaku Co., Ltd., Tokyo, Japan). Assays were performed on all 76 serum samples in duplicate according to the manufacturer's protocol.

Serum concentrations of SP-D were measured with specific SP-D ELISA kits (Biovendor LLC, Candler, NC, USA)<sup>12</sup>. Calibrators, quality

controls, and samples were incubated in microtitration 96-well dishes coated with a monoclonal anti-human SP-D antibody. After overnight incubation and washing, horseradish peroxidase conjugate was added and incubated 2 h with captured SP-D. After washing, the remaining conjugate was allowed to react with H<sub>2</sub>O<sub>2</sub>-tetramethylbenzidine substrate solution. The reaction was stopped by addition of sulfuric acid solution. Absorbance of the color change was measured spectrophotometrically at 450 nm. The absorbance is proportional to the SP-D concentration. A standard curve was constructed by plotting absorbance values against concentrations of SP-D standards, and the concentrations in unknown samples were determined using this standard curve.

Serum concentrations of KL-6 were measured with a specific KL-6 ELISA kit (kindly provided by Sanko Junyaku Co., Ltd.). 96-well plates were coated with mouse anti-KL-6 monoclonal antibodies. Serum samples diluted 1:200 were added to duplicate wells for 2 h. After washing, bound antibodies were detected with peroxidase-conjugated mouse anti-KL-6 monoclonal antibodies. The reaction was stopped by addition of sodium azide solution. Absorbance of the color change was measured spectrophotometrically at 405 nm. The absorbance is proportional to the KL-6 concentration. A standard curve was constructed by plotting absorbance values against concentrations of KL-6 standards, and the concentrations in unknown samples were determined using this standard curve.

The following data were obtained as part of the SLS<sup>5</sup>: baseline measurements of PFT (FVC% predicted, DLCO% predicted), measures of quality of life (QOL), and clinical symptoms such as Health Assessment Questionnaire (HAQ) data, cough index (severity, frequency, phlegm), Short Form-36 (SF-36) indicators (health transition, physical function, social function, body pain, general health, vitality, role emotional, mental health, standardized physical component score, standardized mental component score), and dyspnea score (Mahler Dyspnea Index).

**Statistical analysis.** All data analysis was performed by the SLS biostatistical group (RE, H-JW, and NL) using SAS software (version 9.1). SP-D and KL-6 levels were compared to measures of lung disease and “alveolitis” through PFT and HRCT in the SLS patients. Descriptive statistics, including mean, standard deviation, median, minimum and maximum, were used to summarize the measurements of SP-D and KL-6. Since the distributions of SP-D and KL-6 did not follow normal distribution, Wilcoxon rank-sum test was used to compare SP-D and KL-6 levels between patients with and without active “alveolitis,” and also to compare patients with SSc with controls. All p values were 2-tailed and p values < 0.05 were considered significant. Box plots were produced to show the differences of SP-D and KL-6 levels between patients with and without active “alveolitis” and patients with SSc versus controls.

The relationships of SP-D and KL-6 with PFT (DLCO% predicted and FVC% predicted) and measures of QOL and clinical symptoms (HAQ, cough index, SF-36, and dyspnea score) were examined using Kendall's Tau-b correlation coefficients. Correlations between the glycoproteins and PFT were analyzed for the entire group of patients with SSc, as well as individual analyses for the “alveolitis” and non-“alveolitis” groups. Scatterplots of SP-D and KL-6 against PFT were also generated with a Loess regression curve. Loess regression is a robust regression method that does not need parametric assumptions, and therefore can better delineate the true relationship between the 2 variables. The relationship of SP-D and KL-6 with HRCT scores (maximum honeycombing, ground-glass, and fibrosis) was examined using Kendall's Tau-b correlation coefficients. ROC curve analysis was carried out to evaluate the accuracy of SP-D and KL-6 for the diagnosis of active “alveolitis.” The area under the curve (AUC) was calculated, and sensitivity and specificity at different cutoff points were estimated.

Our study was approved by the Medical University of South Carolina Institutional Review Board.

## RESULTS

Overall, patients with SSc had higher SP-D and KL-6 con-

centrations than controls. SP-D levels were  $300 \pm 214$  ng/ml (mean  $\pm$  SD) in SSc patients compared to  $40 \pm 51$  ng/ml in controls ( $p < 0.001$ ; Figure 1A). KL-6 levels were  $1225 \pm 984$  U/ml in SSc patients compared to  $333 \pm 294$  U/ml in controls ( $p < 0.001$ ; Figure 1B). Overall, SSc patients with “alveolitis” (BAL-positive and/or HRCT-positive) had higher levels of both SP-D and KL-6 than patients without “alveolitis” (both BAL and HRCT-negative). SP-D levels were  $353 \pm 219$  ng/ml in SSc patients with “alveolitis” and  $161 \pm 143$  ng/ml in patients without “alveolitis” ( $p < 0.001$ ; Figure 1C). KL-6 levels were  $1458 \pm 1070$  U/ml in SSc patients with “alveolitis” and  $640 \pm 487$  U/ml in patients without “alveolitis” ( $p < 0.001$ ; Figure 1D).

Overall, patients with an HRCT that showed evidence for any ground-glass opacity had significantly higher levels of SP-D and KL-6 compared to patients with no evidence for ground-glass. SP-D levels were  $368 \pm 218$  ng/ml in patients with HRCT evidence for any ground-glass ( $n = 42$ ) and  $167 \pm 145$  ng/ml in patients with no HRCT evidence for ground-glass ( $n = 21$ ;  $p < 0.001$ ). KL-6 levels were  $1469 \pm 1077$  U/ml in patients with HRCT evidence for any ground-glass ( $n = 42$ ) and  $764 \pm 633$  U/ml in patients with no HRCT evidence for ground-glass ( $n = 21$ ;  $p = 0.002$ ). Assessing the SSc patients with alveolitis for whom HRCT data were available ( $n = 42$ ), there were significant positive correlations between maximum fibrosis score and SP-D ( $r = 0.27$ ,  $p = 0.024$ ) and KL-6 ( $r = 0.30$ ,  $p = 0.012$ ; Table 1). KL-6, but not SP-D, was also found to correlate positively with maximum honeycombing ( $r = 0.31$ ,  $p = 0.014$ ). Although levels of each glycoprotein were higher in patients with any ground-glass opacity, neither SP-D nor KL-6 was found to correlate with maximum ground-glass on HRCT ( $r = 0.12$  and  $r = 0.11$ , respectively; both nonsignificant; Table 1).

Patients with BAL evidence of “alveolitis” had significantly higher KL-6 levels than patients with a negative BAL. KL-6 levels were  $1489 \pm 1090$  U/ml in patients with BAL evidence of “alveolitis” ( $n = 36$ ), and  $868 \pm 718$  U/ml in patients without BAL evidence for “alveolitis” ( $n = 29$ ;  $p = 0.008$ ). SP-D levels were also higher in patients with BAL evidence of “alveolitis” ( $n = 36$ ) versus negative BAL ( $n = 29$ ),  $332 \pm 216$  ng/ml versus  $252 \pm 207$  ng/ml, respectively; these did not reach statistical significance ( $p = 0.134$ ).

Assessing the total SSc patient group (non-“alveolitis” and “alveolitis” groups in combination,  $n = 66$ ), there was a significant correlation between DLCO% predicted ( $n = 60$ ) and SP-D ( $r = -0.21$ ,  $p = 0.02$ ), and DLCO% predicted ( $n = 60$ ) and KL-6 ( $r = -0.29$ ,  $p = 0.001$ ). There was a trend toward an inverse correlation between FVC% predicted ( $n = 61$ ) and SP-D ( $r = -0.15$ ,  $p = 0.078$ ) and FVC% predicted ( $n = 61$ ) and KL-6 ( $r = -0.17$ ,  $p = 0.060$ ; Table 2). Correlations in the “alveolitis”-only group ( $n = 44$ ) revealed a significant correlation between DLCO% predicted ( $n = 44$ ) and KL-6 ( $r = -0.25$ ,  $p = 0.019$ ), but not for DLCO% predicted ( $n = 44$ ) and SP-D ( $r = -0.13$ ,  $p = 0.232$ ). Neither

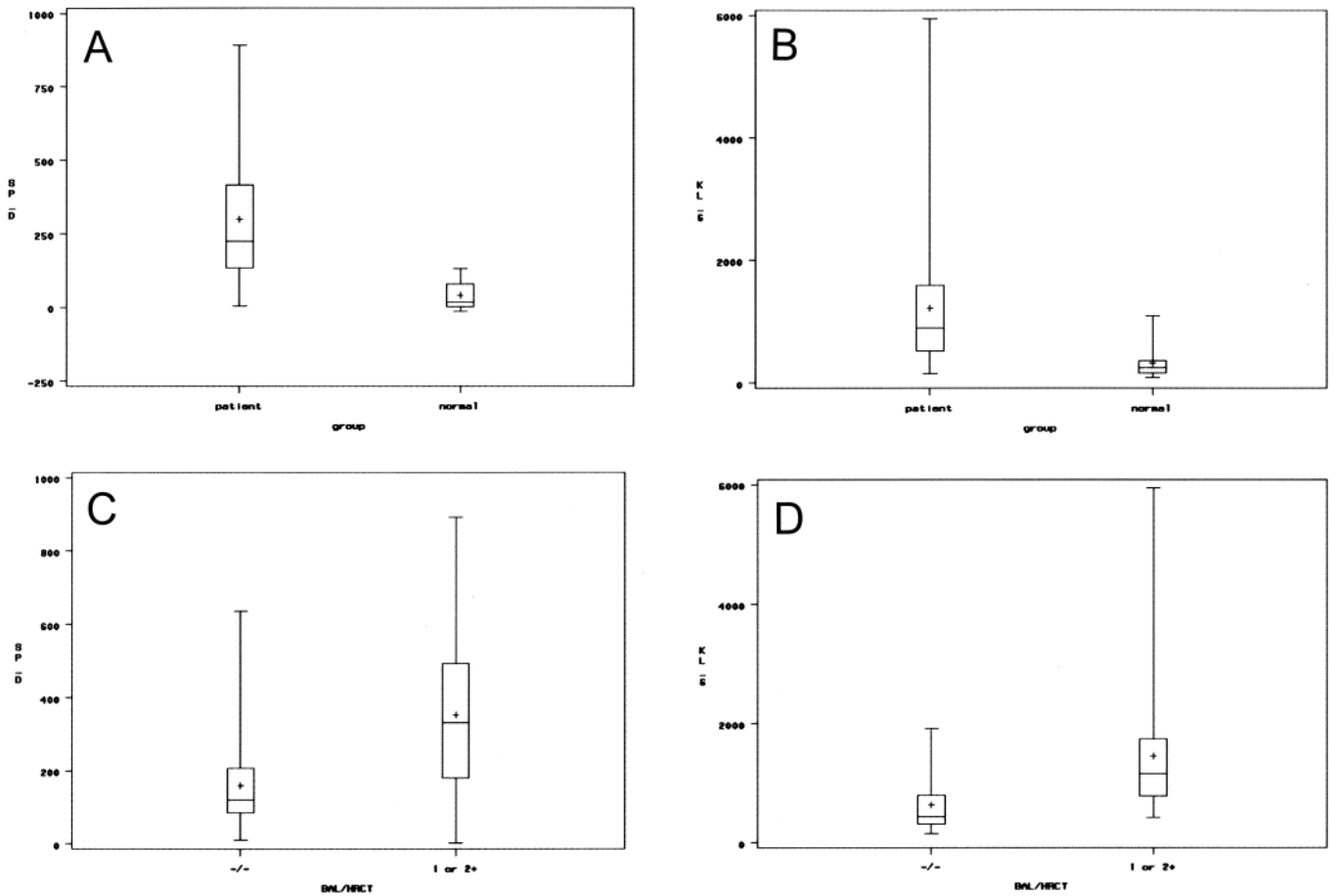


Figure 1. A. SP-D levels in SSc patients (with and without “alveolitis”) and healthy controls. B. KL-6 levels in SSc patients (with and without “alveolitis”) and healthy controls. C. SP-D levels in SSc patients with and without “alveolitis.” D. KL-6 levels in SSc patients with and without “alveolitis.” Boxes show 75th and 25th percentiles; plus sign = mean; horizontal bar = median; outliers show maximum and minimum.

Table 1. Correlation of high resolution computed tomography results with SP-D and KL-6 in patients with SSc with alveolitis.

	Kendall Tau b Correlation Coefficients	
	KL-6	SP-D
Maximum honey combing, n = 42	0.31 p = 0.0138	0.002 p = 0.9900
Maximum ground-glass opacity, n = 42	0.11 p = 0.3677	0.12 p = 0.3088
Maximum fibrosis score, n = 42	0.30 p = 0.0121	0.27 p = 0.0241

SP-D ( $r = -0.03$ ,  $p = 0.793$ ) nor KL-6 ( $r = -0.03$ ,  $p = 0.746$ ) correlated with FVC% predicted ( $n = 44$ ) in the “alveolitis”-only group (Table 2). Among the non-“alveolitis”-only patients ( $n = 22$ ), neither SP-D ( $r = -0.31$ ,  $p = 0.084$ ) nor KL-6 ( $r = -0.22$ ,  $p = 0.217$ ) correlated with FVC% predicted ( $n = 17$ ). Neither SP-D ( $r = -0.34$ ,  $p = 0.071$ ) nor KL-6 ( $r = -0.27$ ,  $p = 0.149$ ) correlated with DLCO% predicted ( $n = 16$ ) in the same group (Table 2).

None of the other variables tested, such as HAQ, cough index (severity, frequency, and phlegm), SF-36, or dyspnea index, correlated with SP-D or KL-6 levels.

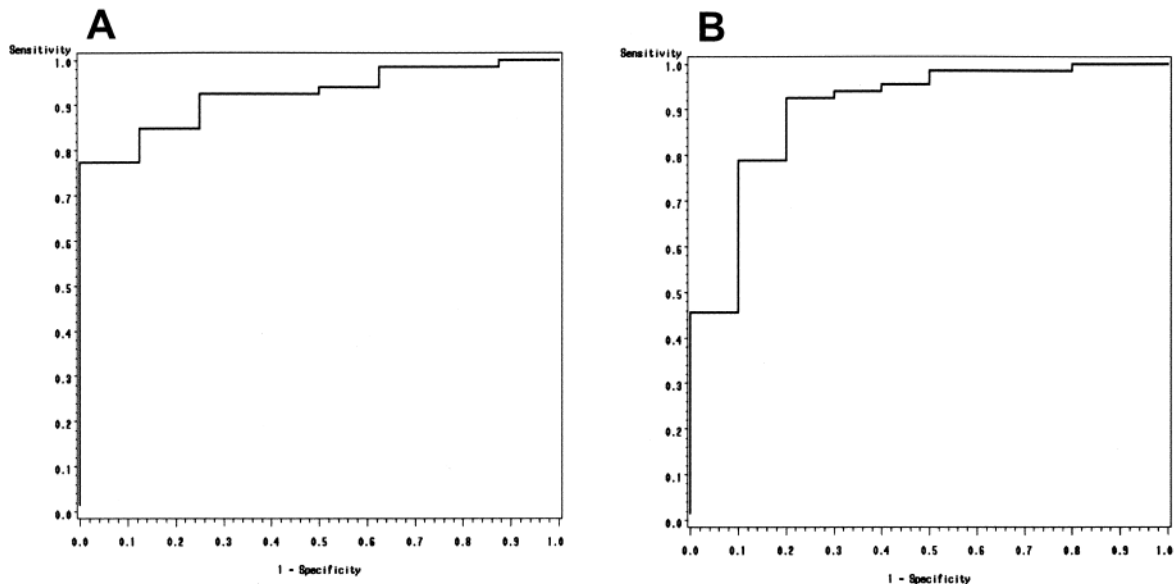
To evaluate the accuracy of KL-6 and SP-D for diagnosis of “alveolitis,” ROC curve analysis was performed. ROC curve is a plot of sensitivity versus  $1 - \text{specificity}$  of a screening test, where different points on the curve correspond to different cutoff points used to designate a test positive<sup>13</sup>. The area under the curve is a reasonable summary of the overall diagnostic accuracy of the test. In general, of 2 screening tests for the same disease, the test with the higher area under ROC curve is considered the better test, unless some particular level of sensitivity or specificity is especially important in comparing the 2 tests.

ROC curves for determination of cutoff levels of SP-D and KL-6 are shown in Figures 2A, 2B, respectively. When tentative cutoff levels for SP-D were measured in the 66 patients with SSc and 10 healthy controls at intervals of 10 ng/ml from 0 to 100 ng/ml, 90 ng/ml was found to be the best value in the relationship between sensitivity (89.4%)

Table 2. Correlation of pulmonary function tests with SP-D and KL-6.

		Kendall Tau b Correlation Coefficients	
		KL-6	SP-D
All SSc “alveolitis” + non-alveolitis	FVC, n = 61	-0.17	-0.15
		p = 0.0602	p = 0.0782
SSc with “alveolitis”	DLCO, n = 60	-0.29	-0.21
		p = 0.0012	p = 0.0198
SSc without “alveolitis”	FVC, n = 44	-0.03	-0.03
		p = 0.7462	p = 0.7926
	DLCO, n = 44	-0.25	-0.13
		p = 0.0188	p = 0.2321
	FVC, n = 17	-0.22	-0.31
		p = 0.2165	p = 0.0836
	DLCO, n = 16	-0.27	-0.34
		p = 0.1488	p = 0.0711

SP-D: surfactant protein D; SSc: systemic sclerosis; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide.



Cut-Off Point*	Sensitivity	Specificity
60	93.9% (62/66)	60% (6/10)
70	92.4% (61/66)	60% (6/10)
80	92.4% (61/66)	70% (7/10)
90	89.4% (59/66)	80% (8/10)
100	84.9% (56/66)	80% (8/10)

Cut-Off Point*	Sensitivity	Specificity
300	95.5% (63/66)	60% (6/10)
350	92.4% (61/66)	70% (7/10)
375	92.4% (61/66)	80% (8/10)
400	90.9% (60/66)	80% (8/10)
500	78.8% (52/66)	90% (9/10)

Figure 2. A. SP-D, sensitivity and specificity; area under ROC curve (AUC) = 0.983. Subject will be diagnosed with active “alveolitis” if SP-D value is greater than or equal to cutoff value. B. KL-6, sensitivity and specificity; AUC = 0.901. Subject will be diagnosed with active “alveolitis” if KL-6 value is greater than or equal to cutoff value.

and specificity (80%). When tentative cutoff levels for KL-6 measured in the same subjects were evaluated at intervals of 25 U/ml from 0 to 500 U/ml, 500 U/ml was found to be the best value in the relationship between sensitivity (78.8%) and specificity (90%). The AUC for SP-D and KL-6 are 0.98 and 0.90, respectively.

## DISCUSSION

The pathogenesis of ILD, including that associated with scleroderma, remains uncertain. Earlier concepts, including those prevailing when the SLS was designed and initiated, invoked an inflammatory process or “alveolitis” characterized by an increase in neutrophils and/or eosinophils in the

lower airways detectable by BAL, or a ground-glass opacification detectable by HRCT. Thus, our subjects were defined as having or not having “alveolitis” on the basis of BAL and HRCT findings set forth in the SLS.

Recently, the significance of neutrophils/eosinophils on BAL and ground-glass opacification on HRCT has been questioned. Indeed, an increase in neutrophils on BAL may be an indication of the severity of fibrotic lung disease and might represent a secondary inflammatory response rather than the primary event preceding fibrosis. Similar uncertainty regarding the specificity of ground-glass on HRCT scans also exists. Until these concerns are resolved, the definition and implications of “alveolitis” will remain controversial.

Due to the high morbidity and mortality associated with SSc pulmonary involvement, noninvasive means to assess its presence are needed. The ability to find and utilize a serologic noninvasive means of diagnosing SSc-related ILD, specifically “alveolitis,” is necessary and eagerly anticipated. For this reason, the use of biomarkers in SSc-related ILD appears promising to aid in diagnosis, monitoring, and progression. The ability to assess these biomarkers longitudinally and study whether changes of glycoprotein levels parallel clinical progression, response to treatment, and patient outcomes appears to be worthwhile.

The ability to correlate serum SP-D and KL-6 levels with BAL and HRCT findings and lung function in a large, well characterized group of patients with SSc from the SLS is the distinctive strength of our study. Previous studies examining the role of SP-D and KL-6 glycoproteins in SSc-related ILD were limited to smaller numbers of patients with SSc<sup>9,11,14-16</sup>.

We chose to examine SP-D and KL-6 because of studies demonstrating that these glycoproteins reflect underlying alveolar damage in various forms of ILD, including SSc-related ILD<sup>6-11,14-19</sup>. SP-D is a hydrophilic glycoprotein that belongs to the family of innate immune molecules, collectins, members of the mannose-type subfamily of C-type lectins<sup>20</sup>. SP-D is produced and secreted by alveolar type II pneumocytes and Clara cells and has a low molecular weight of 43 kDa<sup>21</sup>. It is important for innate immune response and inflammation regulation within the lung<sup>20</sup>. KL-6 is a mucin-like glycoprotein discovered by Kohno, *et al* in 1985<sup>22</sup>. It is also expressed mainly on type II pneumocytes in alveoli and bronchiolar epithelial cells<sup>17</sup>, and is found more strongly on proliferating and regenerating type II pneumocytes in interstitial disease than in normal type II pneumocytes. KL-6 levels are increased in BAL fluid and correlate with serum KL-6 levels<sup>17,18</sup>. In our study, baseline sera of patients enrolled in the Scleroderma Lung Study were assayed for levels of SP-D and KL-6.

Although the initiating event(s) in SSc-associated ILD remains unknown, there is accumulating evidence for both epithelial cell and endothelial cell injury. Endothelial cell injury is widespread in SSc, including the pulmonary capil-

lary endothelium. Functional studies indicate the presence of pulmonary capillary endothelial dysfunction prior to the occurrence of pulmonary hypertension or pulmonary fibrosis<sup>23</sup>. 99m-Tc-DTPA clearance scans demonstrate increased radionuclide clearance consistent with an abnormality at the alveolar epithelial cell barrier<sup>24</sup>. Ultrastructural analysis of SSc lung tissue confirms the presence of epithelial injury as well as endothelial cell injury, both occurring together with interstitial edema and excess collagen deposition<sup>25</sup>. Additionally, histochemical studies of scleroderma lung tissues demonstrate alveolar epithelial cell injury characterized by increased expression of tissue factor and, of particular relevance to our studies, increased expression of surfactant protein<sup>26</sup>. Elevated plasma concentrations of SP-D and KL-6 in patients with SSc-ILD, as observed in our study, are consistent with reported functional and histopathologic data, and provide additional evidence of epithelial cell injury as an important event in the pathogenesis of SSc-ILD.

We found significantly higher levels of SP-D and KL-6 in patients with SSc compared to healthy controls and in patients with “alveolitis” as compared to disease control subjects. We found a statistically significant positive correlation between each lung glycoprotein and the maximum fibrosis score on HRCT. Additionally, levels of KL-6 correlated with the maximum honeycombing score on HRCT. Neither glycoprotein correlated with maximum ground-glass scores, although both were higher in patients with any ground-glass finding on HRCT. Since baseline HRCT fibrosis was shown in the SLS to be an independent predictor of response to CYC, our data suggest that these serum markers might also prove to be useful in selecting patients for treatment.

We also found that SP-D and KL-6 levels were inversely correlated with DLCO% predicted, but not with FVC% predicted. We found no correlation between serum levels of SP-D and KL-6 and clinical and patient-reported symptoms (e.g., HAQ, cough index, SF-36, and dyspnea index). Unfortunately, only baseline data were available in the SLS, so we were unable to assess whether serum levels of KL-6 and SP-D might predict which patients would have progression of lung disease, or which would have significant clinical improvement after 1 year of treatment with CYC, or whether levels correlated with treatment response.

In order to evaluate the utility of these glycoproteins as diagnostic tests for the presence of “alveolitis,” we performed ROC curve analysis, and found impressive AUC sensitivity and specificity for both glycoproteins in the assessment of “alveolitis.” Using a cutoff value of 90 ng/ml, serum SP-D had a sensitivity of 89.4% and specificity of 80% for the diagnosis of “alveolitis.” Using a cutoff value of 500 U/ml, serum KL-6 had a sensitivity of 78.8% and specificity of 90% for diagnosis of “alveolitis.” Yanaba, *et al*, in a comparative, retrospective, longitudinal study, assessed the clinical significance of SP-D and KL-6 levels in the diagno-

sis and monitoring of pulmonary fibrosis in SSc patients, and evaluated differences between them<sup>11</sup>. They defined pulmonary fibrosis as bibasilar interstitial fibrosis on chest radiographs, and ground-glass opacities, reticular opacities, or honeycombing on HRCT. Forty-two SSc patients, 20 SLE disease controls, and 30 healthy matched controls were studied, and 83 serum samples from 6 SSc patients were analyzed during a followup period of 0.6–6.3 years<sup>11</sup>. They found a sensitivity of 91% and specificity of 88% for SP-D for the diagnosis of pulmonary fibrosis. In contrast, the sensitivity of KL-6 for pulmonary fibrosis was 39% and specificity was 100%<sup>11</sup>. Yanaba and colleagues concluded that SP-D was a more sensitive marker and KL-6 a more specific marker for pulmonary fibrosis. They believed their combined use would be more helpful to diagnose and monitor pulmonary fibrosis in patients with SSc<sup>11</sup>. Our study also suggests that SP-D is a more sensitive, and KL-6 a more specific marker for “alveolitis” in patients with SSc.

There are specific strengths to our study. The SLS afforded a unique, well characterized patient population. Strict definitions for the diagnosis of “alveolitis” had to be met for SSc patients with “alveolitis,” as well as for non-“alveolitis” disease controls<sup>5</sup>. Data regarding PFT results, specifically FVC% predicted and DLCO% predicted, and QOL measures were also obtained and evaluated. As the largest randomized, double-blind clinical trial assessing the treatment of SSc-related ILD with CYC versus placebo, the SLS is a landmark investigation in the field of SSc. Although previous studies showed these glycoproteins were increased in interstitial lung diseases, most studies of SP-D and KL-6 involved Asians, and thus our study provides evidence of these biomarkers in a group of North American, mostly Caucasian, patients. Our study is also the first to include African American and Hispanic groups. Additionally, we were able to determine levels of both SP-D and KL-6 in serum, as few previous studies assessed both glycoproteins concomitantly.

Several limitations exist in our study. We were unable to assess longitudinal serum samples over the course of the SLS study. As the study was a 1-year treatment trial of oral CYC versus placebo followed by 1 year of followup, it would have been ideal to determine whether serum levels of SP-D and KL-6 could predict significant improvements in PFT, HRCT findings, and symptoms after 1 year of CYC treatment. In this way, changing or fluctuating levels of the 2 glycoproteins could be analyzed to monitor the activity of “alveolitis,” assess efficacy of treatment, and determine which patients with SSc-related ILD are likely to progress. Tanaka, *et al* recently studied 16 patients with SSc to evaluate concentrations of SP-D, KL-6, and surfactant protein-A (SP-A) as markers for evaluating ILD during CYC therapy<sup>27</sup>. SSc-related ILD was assessed using BAL and HRCT, and all patients received 6 monthly cycles of IV CYC and oral prednisolone. Among 10 patients with improved HRCT

results after 6 cycles of IV CYC, KL-6 and SP-A levels tended to decrease, and SP-D levels decreased more quickly than either, after 3 cycles. In 4 patients with stable HRCT findings after 6 cycles of IV CYC, none of the 3 biomarker concentrations appeared to change. In the remaining 2 patients with worsening HRCT findings, KL-6 and SP-D increased quickly, and there was no effect on SP-A levels. Tanaka, *et al* proposed that measurements of these serum markers might be useful to monitor the efficacy of pulse therapy with CYC in SSc patients with ILD, and that SP-D appeared to be the most sensitive marker<sup>27</sup>. These findings support the need for further research in the area of serum biomarkers in SSc-related ILD, to assess their utility to correlate with clinical findings and response to treatment.

A recent report by Strange, *et al* evaluated whether BAL cellularity in patients enrolled in the SLS might predict response to treatment with CYC, and/or related to specific subsets of disease in patients with SSc<sup>5,28</sup>. They found abnormal BAL results in patients with advanced SSc-related ILD, but results did not predict response to CYC at 1 year, or predict disease progression<sup>5,28</sup>. Although the role of BAL in SSc-related ILD requires further study, these results make newer, less invasive diagnostic tests, such as serum biomarkers, highly desirable.

We detected high levels of SP-D and KL-6 in SSc patients compared to healthy controls, with even higher levels in patients who fulfilled the SLS working definition of “alveolitis.” It is conceivable that in patients with SSc-ILD the presence of neutrophils and eosinophils in BAL represents a secondary inflammatory response to lung tissue injury, rather than a primary pathogenic process, and higher lung glycoprotein levels are indicative of greater epithelial cell injury associated with fibrosis. Similarly, it is conceivable that the presence of ground-glass findings on HRCT may not represent reversible inflammation, but might indicate another process, e.g., “fine fibrosis,” and the higher lung glycoprotein levels reflect tissue injury leading to lung fibrosis.

Despite the controversy about the terminology of “alveolitis” and whether inflammation might be a primary or secondary event, our findings support the notion that epithelial cell injury is an important aspect of SSc-ILD, and measurement of serum lung glycoproteins may provide a useful, noninvasive biomarker of such alveolar epithelial cell injury. Further study of these glycoproteins will define their role in the diagnosis of SSc-related ILD and in monitoring of disease activity and therapeutic efficacy.

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#### REFERENCES

1. Mayes MD, Reveille JD. Epidemiology, demographics and

- genetics. In: Clements PJ, Furst DE, editors. Systemic sclerosis. 2nd ed. Philadelphia: Lippincott Williams and Wilkins; 2004:1-15.
2. Silver RM, Miller KS, Kinsella MB, Smith EA, Schabel SI. Evaluation and management of scleroderma lung disease using bronchoalveolar lavage. *Am J Med* 1990;88:470-6.
  3. Ohba T, McDonald JK, Silver RM, Strange C, LeRoy EC, Ludwicka A. Scleroderma bronchoalveolar lavage fluid contains thrombin, a mediator of human lung fibroblast proliferation via induction of platelet-derived growth factor alpha-receptor. *Am J Respir Cell Mol Biol* 1994;10:405-12.
  4. Ludwicka A, Ohba T, Trojanowska M, et al. Elevated levels of platelet derived growth factor and transforming growth factor-beta 1 in bronchoalveolar lavage fluid from patients with scleroderma. *J Rheumatol* 1995;22:1876-83.
  5. Tashkin DP, Elashoff R, Clements PJ, et al. Cyclophosphamide versus placebo in scleroderma lung disease. *N Engl J Med* 2006;354:2655-66.
  6. Honda Y, Kuroki Y, Matsuura E, et al. Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. *Am J Respir Crit Care Med* 1995;152:1860-6.
  7. Kuroki Y, Takahashi H, Chiba H, Akino T. Surfactant proteins A and D: disease markers. *Biochim Biophys Acta* 1998;1408:334-45.
  8. Greene KE, Wright JR, Steinberg KP, et al. Serial changes in surfactant-associated proteins in lung and serum before and after onset of ARDS. *Am J Respir Crit Care Med* 1999;160:1843-50.
  9. Asano Y, Ihn H, Yamane K, et al. Clinical significance of surfactant protein D as a serum marker for evaluating pulmonary fibrosis in patients with systemic sclerosis. *Arthritis Rheum* 2001;44:1363-9.
  10. Kohno N, Awaya Y, Oyama T, et al. KL-6, a mucin-like glycoprotein, in bronchoalveolar lavage fluid from patients with interstitial lung disease. *Am Rev Respir Dis* 1993;148:637-42.
  11. Yanaba K, Hasegawa M, Takehara K, Sato S. Comparative study of serum surfactant protein-D and KL-6 concentrations in patients with systemic sclerosis as markers for monitoring the activity of pulmonary fibrosis. *J Rheumatol* 2004;31:1112-20.
  12. Nagae H, Takahashi H, Kuroki Y, et al. Enzyme-linked immunosorbent assay using F(ab')<sub>2</sub> fragment for the detection of human pulmonary surfactant protein D in sera. *Clin Chim Acta* 1997;266:157-61.
  13. Rosner B. Fundamentals of biostatistics. 6th ed. Pacific Grove: Duxbury Press; 2006.
  14. Sato S, Nagaoka T, Hasegawa M, Nishijima C, Takehara K. Elevated serum KL-6 levels in patients with systemic sclerosis: association with the severity of pulmonary fibrosis. *Dermatology* 2000;200:196-201.
  15. Yamane K, Ihn H, Kubo M, et al. Serum levels of KL-6 as a useful marker for evaluating pulmonary fibrosis in patients with systemic sclerosis. *J Rheumatol* 2000;27:930-4.
  16. Yanaba K, Hasegawa M, Hamaguchi Y, Fujimoto M, Takehara K, Sato S. Longitudinal analysis of serum KL-6 levels in patients with systemic sclerosis: association with the activity of pulmonary fibrosis. *Clin Exp Rheumatol* 2003;21:429-36.
  17. Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity. Sialylated carbohydrate antigen KL-6. *Chest* 1989;96:68-73.
  18. Hamada H, Kohno N, Akiyama M, Hiwada K. Monitoring of serum KL-6 antigen in a patient with radiation pneumonia. *Chest* 1992;101:858-60.
  19. Kobayashi J, Itoh Y, Kitamura S, Kawai T. Establishment of reference intervals and cut-off value by an enzyme immunoassay for KL-6 antigen, a new marker for interstitial pneumonia. *Jpn J Clin Pathol* 1996;44:653-8.
  20. Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annu Rev Physiol* 2001;63:521-54.
  21. Persson A, Rust K, Chang D, Moxley M, Longmore W, Crouch E. CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins. *Biochemistry* 1988;27:8576-84.
  22. Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. *Jpn J Clin Oncol* 1988;18:203-16.
  23. Orfanos SE, Psevdi E, Stratigis N, et al. Pulmonary capillary endothelial dysfunction in early systemic sclerosis. *Arthritis Rheum* 2001;44:902-11.
  24. Nakano A, Yamaguchi E, Naitoh S, et al. [Assessment of alveolar epithelial permeability in progressive systemic sclerosis (PSS) using 99mTc-DTPA (diethylene triamine penta acetate) aerosol inhalation]. *Nihon Kyobu Shikkan Gakkai Zasshi* 1992;30:45-50.
  25. Harrison NK, Myers AR, Corrin B, et al. Structural features of interstitial lung disease in systemic sclerosis. *Am Rev Respir Dis* 1991;144:706-13.
  26. Imokawa S, Sato A, Hayakawa H, Kotani M, Urano T, Takada A. Tissue factor expression and fibrin deposition in the lungs of patients with idiopathic pulmonary fibrosis and systemic sclerosis. *Am J Respir Crit Care Med* 1997;156:631-6.
  27. Tanaka C, Hasegawa M, Fujimoto M, Yasui M, Takehara K. Comparative study of serum KL-6, surfactant protein-A and -D concentrations in patients with systemic sclerosis as markers for evaluating the activity of interstitial pneumonia during cyclophosphamide pulse therapies [abstract]. In: Prof. E. Carwile Leroy Memorial International Workshop on Scleroderma; May 18-20, 2007, Tokyo, Japan. Tokyo: Scleroderma Study Conference; 2007;67, p-037.
  28. Strange C, Bolster MB, Roth MD, et al. Bronchoalveolar lavage and response to cyclophosphamide in scleroderma interstitial lung disease. *Am J Respir Crit Care Med* 2008;177:91-8.