Biomarkers to identify ILD and predict lung function decline in scleroderma lung disease or idiopathic pulmonary fibrosis

Barry Kennedy[1], Peter Branagan[2], Fiachra Moloney[3], Muhammad Haroon[4], Oisin J O'Connell[2], Terence M O'Connor[1], Kevin O'Regan[3], Sinead Harney[4], Michael T Henry[2]

[1] Department of Respiratory Medicine, Mercy University Hospital, Cork, Ireland; [2] Department of Respiratory Medicine, Cork University Hospital, Cork, Ireland; [3] Department of Radiology, Cork University Hospital, Cork, Ireland; [4] Department of Rheumatology, Cork University Hospital, Cork, Ireland

Abstract. *Background:* SSc-ILD and IPF demonstrate significant morbidity and mortality. Predicting disease progression is challenging in both diseases. *Objectives:* We sought a serum biomarker that could identify patients with SSc-ILD or IPF and prospectively predict short-term decline in lung function in these patients. *Methods:* 10 healthy controls, 5 SSc w/o ILD, 6 SSc-ILD and 13 IPF patients underwent venesection. An array of cytokines including KL-6, SP-D and MMP7 were measured. PFTs were obtained at baseline and six months. Cytokine measurements were correlated with PFTs. *Results:* KL-6 in IPF patients (633ng/ml, IQR 492-1675) was significantly elevated compared to controls (198ng/ml, IQR 52-360, $p<0.01$) and SSc w/o ILD patients (192ng/ml, IQR 0-524, $p<0.05$); KL-6 in SSc-ILD patients (836ng/ml, IQR 431-1303) was significantly higher than in controls ($p<0.05$). SP-D was significantly higher in IPF patients (542ng/ml, IQR 305-577) compared to controls (137ng/ml, IQR 97-284, $p<0.01$) or to SSc w/o ILD patients (169ng/ml, IQR 137-219, $p<0.05$). In comparison with controls (0.0ng/ml, IQR 0.0-0.6), MMP7 was significantly higher in both IPF patients (2.85ng/ml, IQR 1.5-3.6, $p<0.05$) and SSc-ILD patients (5.41ng/ml, IQR 2.6-7.2, $p<0.001$). Using a cut-off level of 459ng/ml for KL-6 and of 1.28ng/ml for MMP7, 18 out of 19 patients with ILD had a serum value of either KL-6 or MMP7 above these thresholds. For all ILD patients, baseline serum SP-D correlated with ΔFVC %pred over six months ($r=-0.63$, $p=0.005$, 95% CI -0.85 to -0.24). *Conclusions:* Combining KL-6 with MMP7 may be a useful screening tool for patients at risk of ILD. SP-D may predict short-term decline in lung function. *(Sarcoidosis Vasc Diffuse Lung Dis 2015; 32: 228-236)*

Key words: biomarkers, scleroderma, pulmonary fibrosis, pulmonary function

Glossary of abbreviations

CPI	Composite physiological index
HRCT	High Resolution Computerised Tomography
IPF	Idiopathic Pulmonary Fibrosis
ROC	Receiver Operating Characteristics
SSc	Scleroderma
SSc-ILD	Scleroderma-associated interstitial lu disease
SSc w/o ILD	Scleroderma without interstitial lung disease

Introduction

Scleroderma (SSc) is a connective tissue disease characterised by organ fibrosis, destruction of vasculature and autoantibodies targeting cellular antigens (1). Scleroderma-associated interstitial lung disease (SSc-ILD) occurs in up to 80% of these patients (2) and is a leading cause of morbidity and mortality. Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial pneumonia of unknown aetiology resulting in progressive fibrosis of lung parenchyma and median survival of 2 to 3 years (3).

There is considerable overlap in the clinical, radiological and histological features of SSc-ILD and IPF. In addition, many of the pathogenetic processes that drive interstitial changes in IPF have also been described in SSc-ILD (i.e. epithelial cell injury, alternative activation of monocytes/macrophages and TGFβ1-mediated fibroblast responses) (4) indicating that the pathogenesis of IPF may be similar to that of SSc-ILD.

Both SSc-ILD and IPF exhibit heterogeneity in their clinical course (5, 6); predicting disease progression in either condition remains challenging. To date, no biomarkers have been validated for the detection of ILD or for the identification of ILD patients likely to develop progressive disease. Although several serum and physiological markers have been investigated, the data are retrospective and undermined by a lack of reproducibility or a lack of specificity for lung function decline (7).

We aimed to identify a serum biomarker which could identify patients with ILD and predict short-term decline in lung function in patients with SSc-ILD or IPF. A panel of serum cytokines was measured at baseline in healthy controls, patients with SSc but without ILD (SSc w/o ILD), patients with SSc-ILD and IPF patients. We then correlated these cytokine measurements with changes in pulmonary function tests (PFTs) over six months and radiological data.

Methods

Patients

Venous blood was obtained from 10 healthy controls, 5 SSc w/o ILD patients, 6 SSc-ILD pa-

tients and 13 IPF patients. The diagnosis of IPF accorded with international guidelines (8). The diagnosis of SSc was based on the diagnostic criteria of the American College of Rheumatology (9). SSc-ILD was defined as evidence of ILD on high resolution CT imaging (HRCT) of the thorax in patients who met the criteria for SSc.

This study was conducted in accordance with the amended Declaration of Helsinki. The protocol was approved by the Clinical Research Ethics Committee of University College Cork (reference number ECM 4 (ww) 02/03/10). Written informed consent was obtained from all patients.

Biomarkers

Pathogenetic mechanisms that contribute to aberrant pulmonary fibrosis in IPF and/or SSc-ILD were identified; cytokines whose serum levels likely reflected the activity or severity of these processes were then selected for measurement. These processes and their respective cytokines were epithelial cell injury/repair (KL-6, Surfactant Protein-D (SP-D), MMP7) (7, 10-12), fibroblast activation/survival (TGF-β1, CCL18, PDGF-AA, TNF-α) (13-18), vascular remodelling (VEGF) (19-21), abnormal coagulation (Thrombomodulin, PAI-1) (22, 23) and leukocyte recruitment (VCAM-1, ICAM-1, P-Selectin, L-Selectin, CCL2) (24-27).

CCL18, TGF-β1, KL-6, SP-D, Thrombomodulin, MMP-7, PAI-1, and PDGF-AA were measured by the quantitative sandwich enzyme immunoassay technique (Biovendor, Czech Republic) as were serum VCAM-1, ICAM-1, P-Selectin, L-Selectin, VEG-F, TNF-α, and CCL2 (Randox, UK).

Measurements of disease activity

All patients with IPF or SSc-ILD had spirometry and carbon monoxide gas transfer measured at baseline and six months. These measurements were then used to calculate a Composite Physiological Index (CPI); this score is a strong correlate of disease extent on CT with higher scores indicating more severe fibrosis (28). The score is calculated as:

extent of disease on CT = $91 - (0.65 \times DL_{CO}\ \%pred) - (0.53 \times FVC\ \%pred) + (0.34 \times FEV1\ \%pred)$

Baseline HRCT scans of the thorax were assessed by 2 independent pulmonary radiologists with a specialist interest in interstitial lung disease. Disease extent was determined using the scoring system described by Lopes *et al* (29); higher scores indicate more extensive disease.

Statistics

Descriptive data are presented as Mean ± SD. Continuous variables are presented as median, IQR. The Kruskal-Wallis test was used to assess for differences across the four patient groups; Dunn's multiple comparison post-test was then applied between groups. All p values have been adjusted for post-testing except where otherwise stated. Comparisons between two groups were made using the Mann-Whitney U test. Cut-off levels of KL-6, SP-D and MMP7 were based on Receiver Operating Characteristics (ROC) curve for all ILD patients. Correlations between two continuous variables were calculated using Spearman's non-parametric correlation. p values were two-tailed and values <0.05 were considered significant.

RESULTS

Baseline characteristics of the study patients are shown in Table 1.

Serum levels of biomarkers

Median values for serum KL-6 were 198ng/ml (IQR 52-360) for controls, 192ng/ml (IQR 0-524) for SSc w/o ILD patients, 836ng/ml (IQR 431-1303) for SSc-ILD patients and 633ng/ml (IQR 492-1675) for IPF patients. Serum KL-6 was significantly higher in IPF patients compared to controls (p<0.01) and to SSc w/o ILD patients (p<0.05). SSc-ILD patients demonstrated significantly higher serum KL-6 levels than controls (p<0.05) (Figure 1A). Of note, SSc-ILD patients had significantly higher serum KL-6 compared to SSc w/o ILD patients (p=0.03) but this difference was not maintained after adjustment for multiple post-tests.

Median values for serum SP-D were 137ng/ml (IQR 97-284) in controls, 169ng/ml (IQR 137-219) in SSc w/o ILD patients, 398ng/ml (IQR 190-727) in SSc-ILD patients and 542ng/ml (IQR 305-577)

Table 1. Baseline clinical, physiological and serological characteristics

	SSc w/o ILD (n=5)	SSc-ILD (n=6)	IPF (n=13)
Age (years)	52.4 ± 7.9	69.7 ± 8.4	72.6 ± 10.7
Sex (M/F)	2 (40%) / 3 (60%)	0 (0%) / 6 (100%)	10 (77%) / 3 (23%)
Non-smoker, n (%)	3 (60%)	5 (83%)	2 (15%)
X-smoker, n (%)	2 (40%)	0	11 (85%)
Current smoker, n (%)	0	1 (17%)	0
FEV1 %pred	N/A	86.9 ± 20.7	83.4 ± 27.23
FVC %pred	N/A	89.9 ± 18.5	83.3 ± 26.9
DL_{CO} %pred	N/A	37.1 ± 18.2	39.1 ± 16.1
Organ involvement, n (%)			
Skin	4 (80%)	6 (100%)	N/A
Raynaud's	5 (100%)	5 (83%)	N/A
Digital Ulcers	4 (80%)	1 (17%)	N/A
Oesophagus	4 (80%)	4 (66%)	N/A
Heart	1 (20%)	0 (0%)	N/A
Kidneys	0 (0%)	0 (0)%	N/A
Joint	2 (40)%	0 (0)%	N/A
Muscle	0 (0)%	0 (0)%	N/A
Anti-nuclear Ab, n (%)	5 (100%)	6 (100%)	N/A
Anti-centromere Ab, n (%)	3 (60%)	1 (17%)	N/A
Anti-topoisomerase Ab, n (%)	1 (20%)	3 (50%)	N/A
Immunomodulators, n (%)	5 (100%)	4 (66%)	10 (84%)

Data presented as mean ± SD unless otherwise stated. SSc w/o ILD = scleroderma without lung disease, SSc-ILD = scleroderma lung disease, IPF = idiopathic pulmonary fibrosis, FEV1 = forced expiratory volume in 1 second, FVC = forced vital capacity, DL_{CO}= diffusion capacity of the lung for carbon monoxide
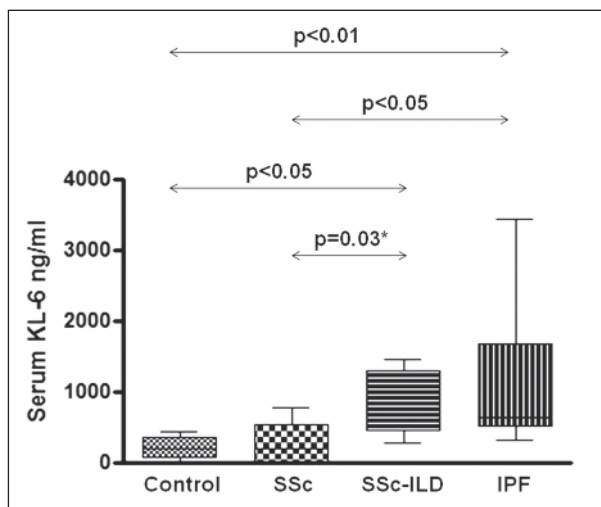
Fig. 1A. Comparison of serum KL-6 in controls, SSc w/o ILD, SSc-ILD and IPF patients (* indicates unadjusted analysis only)
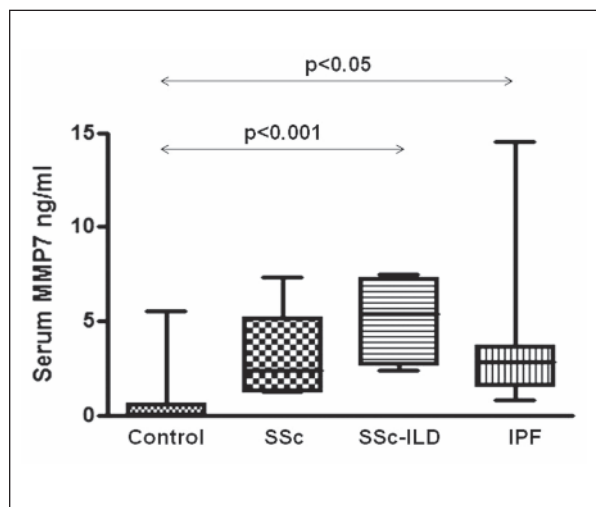

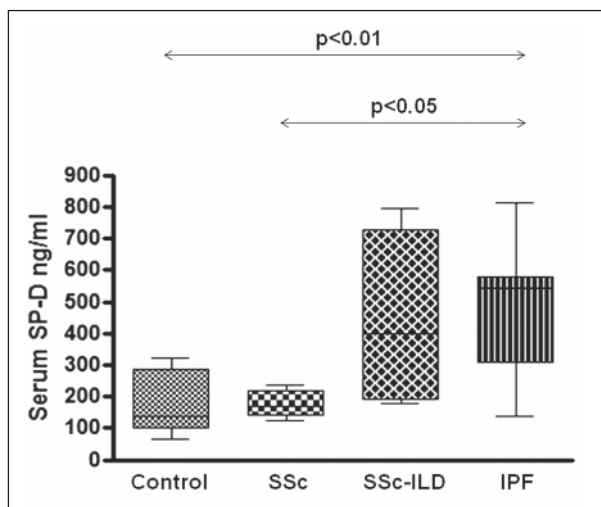**Fig. 1C.** Comparison of serum MMP7 in controls, SSc w/o ILD, SSc-ILD and IPF patients

Fig. 1B. Comparison of serum SP-D in controls, SSc w/o ILD, SSc-ILD and IPF patients

in IPF patients. Serum SP-D was significantly higher in IPF patients compared to controls (p<0.01) or to SSc w/o ILD patients (p<0.05) (Figure 1B).

Median values for serum MMP7 were 0.0ng/ml (IQR 0.0-0.6) for controls, 2.36ng/ml (IQR 1.23-5.11) in SSc w/o ILD patients, 5.41ng/ml (IQR 2.6-7.2) for SSc-ILD patients and 2.85ng/ml (IQR 1.5-3.6) for IPF patients. In comparison with controls serum MMP7 was significantly higher in both IPF

patients (p<0.05) and SSc-ILD patients (p<0.001) (Figure 1C).

There were no significant differences in the expression of the other potential biomarkers (Table 2).

When all patients with ILD were compared to all patients without ILD, a KL-6 cut-off level of 459ng/ml identified the presence of ILD with a sensitivity of 84.2% (95% CI 60.4% to 96.62%) and a specificity of 93.33% (95% CI 68% to 99.8%). A serum SP-D threshold of 321.8ng/ml identified the presence of ILD with a sensitivity of 73.68% (95% CI 48.8% to 90.8%) and a specificity of 100% (95% CI 78.2 to 100%). Using a serum MMP7 threshold of 1.28ng/ml, sensitivity and specificity for ILD were 89.5% (95% CI 66.9% to 98.7%) and 73.3% (95% CI 44.9% to 92.21%) respectively.

For fifteen of the nineteen patients with ILD, serum measurements of both KL-6 and MMP7 were above the threshold. In one patient serum KL-6 but not MMP7 was above the threshold; 2 patients had serum MMP7 but not KL-6 above the threshold. Only one patient with ILD had serum measurements of both KL-6 and MMP7 that were below the threshold. Thus 18 out of 19 patients with ILD had a serum value of either KL-6 or MMP7 above the threshold. Combining both measurements resulted in a sensitivity of 94.7% which was superior to KL-6 (84.2%) or MMP7 (89%) alone.

Table 2. Comparison of putative serum biomarkers as measured by Kruskal-Wallis Test. Data are presented as median (IQR)

	Controls	SSc w/o ILD	SSc-ILD	IPF	p-value
KL-6 (ng/ml)	198 (52-360)	192 (0-525)	836 (431-1303)	633 (492-1675)	0.0003
SP-D (ng/ml)	137 (97–284)	169 (137-219)	398 (190-727)	542 (305-577)	0.0012
MMP7 (ng/ml)	0 (0-0.6)	2.36 (1.2-5.1)	5.4 (2.6-7.25)	2.85 (1.5-3.6)	0.0009
TGF-β (pg/ml)	7251 (5654-10034)	2986 (2483-4029)	3743 (1855-5500)	2388 (1501-7367)	0.07
CCL18 (ng/ml)	46.85 (34.6-153.1)	49.1 (43.65-65.05)	62.05 (52.3-137.4)	48.4 (36.8-90.5)	0.58
PDGF-AA (pg/ml)	1011 (605-2989)	437 (314.5-649)	554 (328-935)	405 (167.5-1222)	0.057
TNF-α (pg/ml)	2.73 (2.18-3.39)	2.53 (2.43-3.21)	3.41 (2.24-10.06)	2.78 (1.9-5.3)	0.84
VEGF (pg/ml)	60.32 (23.3-209.6)	22.9 (11.88-29.28)	24.96 (20.5-33.46)	24.14 (11.45-37.28)	0.053
Thrombomodulin (ng/ml)	3.07 (1.84-4.45)	1.36 (1.1-2.57)	1.63 (1.05-3.07)	2.57 (1.72-6.2)	0.054
PAI-1 (ng/ml)	37.2 (26.7-61.35)	21.3 (9.15-49.15)	40.55 (21.55-56.5)	32.7 (15.75-56.2)	0.35
VCAM-1 (ng/ml)	467.5 (397.1-686.6)	700.1 (567-969.5)	706.1 (583.2-801.3)	753.7 (444.5-916.3)	0.12
ICAM-1 (ng/ml)	297.7 (206.5-742.7)	259.5 (210.4-361.8)	431.4 (325.3-504.80)	416 (289.7-561.9)	0.18
P-Selectin (ng/ml)	168.5 (91.35-224.6)	131.3 (110-137.3)	133.9 (115.4-167.1)	119.1 (100.9-170.3)	0.51
L-Selectin (ng/ml)	1397 (914.3-1878)	1385 (1032-1679)	1329 (818.1-1746)	1203 (891.4-1784)	0.9
CCL2 (pg/ml)	84.9 (78.3-121.1)	86.7 (43.85-121.7)	145.2 (118.8-189.5)	159.4 (103.7-180.3)	0.06

Lung function

Table 3 demonstrates baseline lung function and the changes that occurred over the course of the study. There were no significant differences in any measurement of lung function between IPF and SSc-ILD patients.

In the IPF group, serum SP-D demonstrated significant negative correlations with FVC %pred at six months (r=-0.6364, p=0.0261, 95% CI -0.8906 to -0.07907) and ΔFVC %pred (r=-0.7483, p=0.0051, 95% CI -0.9277 to -0.2878). There were no significant correlations noted between serum SP-D and any parameter of lung function in the SSc-ILD group.

When we pooled the IPF and SSc-ILD patients into a single group, there were significant negative correlations between serum SP-D and FVC %pred at six months (r=-0.59, p=0.01, 95% CI -0.83 to -0.156) (Figure 2A), ΔFVC %pred (r=-0.63, p=0.005, 95% CI -0.85 to -0.24) (Figure 2B) and DL_{CO} %pred at six months (r=-0.48, p=0.04, 95% CI -0.79 to -0.006) (Figure 2C).

CPI Score

Baseline and six month changes in CPI score are illustrated in Table 4.

Serum SP-D correlated with CPI score at base-

Table 3. Baseline and changes in lung function for SSc-ILD, IPF and all ILD patients

	SSc-ILD (n=6)	IPF (n=13)	All ILD (n=19)
Baseline FVC % pred	89.9 ± 18.5	83.3 ± 26.9	85.4 ± 24.2
Δ FVC % pred after six months	-6.2 ± 7.2	+0.7 ± 6.8	-1.6 ± 7.5
Baseline DL_{CO} % pred	37.1 ± 18.2	39.1 ± 16.1	38.5 ± 16.3
Δ DL_{CO} % pred after six months	-3.4 ± 6.6	-4.3 ± 7.8	-4 ± 7.2

Data presented as mean ± SD. See Table 1 legend for expansion of abbreviations

Table 4. Baseline and change in CPI score in SSc-ILD, IPF and all ILD patients

	SSc-ILD (n=6)	IPF (n=13)	All ILD (n=19)
Baseline CPI score	49.6 ± 14.1	49.68 ± 15	49.66 ± 14.28
ΔCPI Score after six months	2.35 ± 3.9	1.18 ± 6.0	1.6 ± 5.2

Data presented as mean ± SD. CPI = composite physiological index. See Table 1 legend for expansion of abbreviations
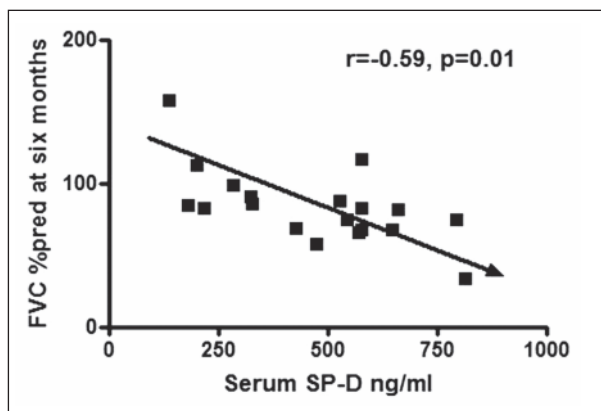
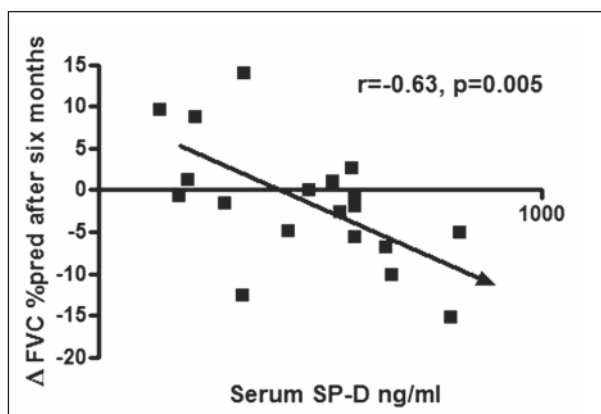**Fig. 2A.** Correlation between baseline SP-D and six month FVC %pred in pooled analysis

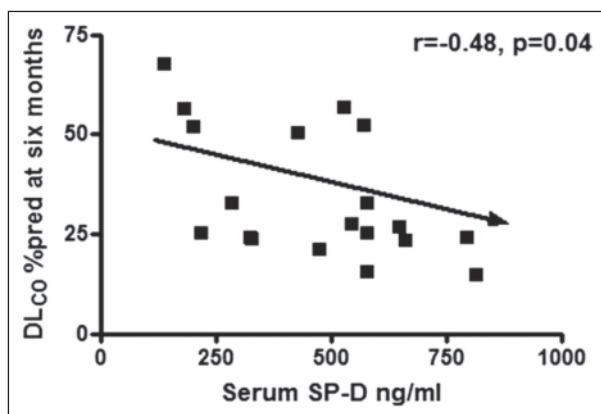**Fig. 2B.** Correlation between baseline SP-D and FVC %pred in pooled analysis

Fig. 2C. Correlation between baseline SP-D and six month DL-CO %pred in pooled analysis

line (r=0.5874, p=0.0446, 95% CI 0.0009 to 0.87) in the IPF group but not with any CPI score in the SSc-ILD group. In the pooled analysis, there were significant positive correlations between serum SP-D and baseline CPI score (r=0.54, p=0.01, 95% CI 0.10 to 0.8) (Figure 3A) as well as CPI score at six months (r=0.6, p=0.01, 95% CI 0.18 to 0.83) (Figure 3B). There was a trend for a positive correlation with ΔCPI score (r=0.43, p=0.064, 95% CI -0.04 to 0.75).

No other potential biomarker correlated with any CPI score.

HRCT Scores

Baseline disease extent as measured by HRCT was 29 ± 12.4 in the IPF patients, 19 ± 12.2 in the SSc-ILD patients and 25.5 ± 12.1 in the combined
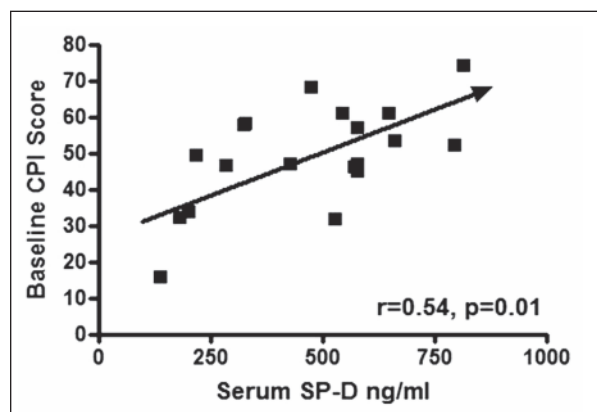

Fig. 3A. Correlation between baseline serum SP-D and baseline CPI score in pooled analysis
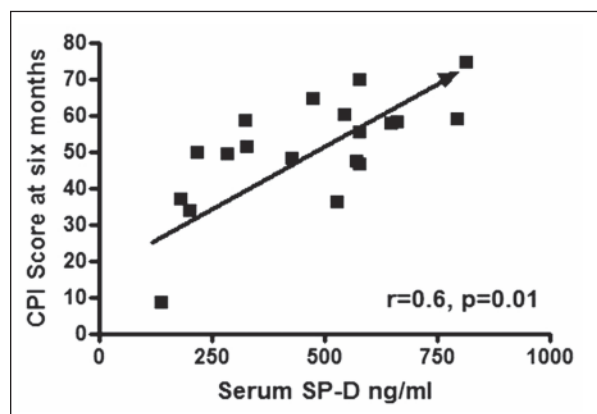

Fig. 3B. Correlation between serum SP-D and six month CPI score in pooled analysis
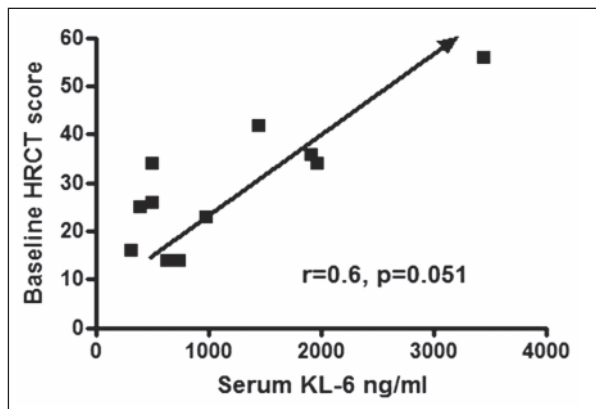
Fig. 4. Correlation between serum KL-6 and baseline disease extent as measured by HRCT in IPF patients (n=11)

cohort. IPF and SSc-ILD patients did not differ significantly in their baseline HRCT scores.

In IPF patients, there was a trend for correlation between serum KL-6 and baseline HRCT score (r=0.6073, p= 0.051, 95% CI -0.008962 to 0.8892) (Figure 4). Baseline HRCT score did not correlate significantly with serum SP-D, KL-6 or MMP7 in IPF, SSc-ILD patients or in the pooled analysis of all ILD patients.

Discussion

We conducted an analysis of serum cytokines in healthy controls, SSc patients without ILD, SSc patients with ILD and IPF patients to identify a biomarker that could identify patients with ILD and prospectively predict lung function decline in these patients. Our results demonstrate that patients with IPF or SSc-ILD have significantly altered peripheral blood expression of KL-6 and MMP7 while IPF patients exhibit altered SP-D expression. Furthermore baseline measurement of serum SP-D correlates with prospective decline of lung function as measured by pulmonary function testing and composite physiological index. We also show a trend for a correlation between serum KL-6 and severity of lung fibrosis on HRCT in patients with IPF.

We detected significant differences in the serum measurement of KL-6 and MMP7 in IPF and SSc-ILD patients compared to controls while serum SP-D was significantly different in IPF pa-

tients compared to controls or SSc alone patients. These findings are consistent with data from previous studies showing elevations of these peptides in SSc-ILD (30-32) and IPF patients (33, 34). Furthermore 95% of patients with ILD had a serum KL-6 or MMP7 above a cut-off threshold determined by ROC curve; this indicates that combining these two markers may be a sensitive screening tool for identifying patients at risk of ILD.

We assessed the clinical relevance of these potential biomarkers by correlating their serum measurements with lung function. Our study was unique in that we correlated serum markers with prospective changes in lung function over six months. For all ILD patients, baseline serum SP-D had a significant negative correlation with prospective change in FVC %pred. The association of serum SP-D with change in lung function has been reported previously in IPF, but only in a retrospective analysis (35).

In addition to lung function tests, serum cytokine measurements were correlated with a composite physiological index. This score uses PFT measurements to derive a score which correlates with extent of fibrosis on CT; this scoring system has been shown to predict IPF mortality more accurately than single PFT parameters such as baseline FVC or DLco (28). It may also offer logistical advantages over current methods for predicting disease progression (10% drop in FVC %pred or 15% drop in DLco over 6 months) (3) as it is measured at a single time-point.

Our analysis showed that baseline serum SP-D had a significant positive correlation with CPI score at baseline and at six months; there was a trend for correlation with prospective change in CPI score. A recent retrospective study describes a correlation between baseline serum SP-D and baseline CPI score in patients with a combination of IPF and emphysema (36). However our study differs in that our correlation is based on prospective data and also suggests a relationship between baseline SP-D and change in CPI score over time.

In the IPF cohort, a trend was noted for a correlation between baseline extent of disease on HRCT and serum KL-6. Previous studies have demonstrated an association between serum KL-6 and HRCT features of ILD in patients with NSIP (37), SSc-ILD (31) and rheumatoid associated-ILD (38). However we are unaware of any previous stud-

ies suggesting a possible correlation between serum KL-6 and HRCT scores in IPF. It is unclear why serum KL-6 correlated with extent of disease activity in IPF patients but not SSc-ILD patients.

There are limitations to our study. Firstly, the number of patients in our study is relatively small. Nevertheless, many of our results are consistent with that reported in other studies (increased serum KL-6, SP-D and MMP7 in ILD patients compared to controls (7, 39)); this indicates that our findings may be reproducible in a larger population. Secondly although we correlated baseline serum SP-D with change in FVC %predicted over six months, the actual change in FVC %pred was small (a 6.2% decrease in the SSc-ILD group and a 0.7% increase in the IPF group). However these modest short-term changes are comparable to what has been previously reported for both SSc-ILD and IPF; the placebo arm of Scleroderma Lung Study lost 4.2% of FVC %pred over 12 months (6) while the placebo arm of the interferon ^{3}-1b trial in IPF lost 0.8% over 24 weeks (5). Thus the changes observed in our study are what can be anticipated in any trial evaluating short-term changes in lung function in either SSc-ILD or IPF. Thirdly although we found a correlation between baseline SP-D and CPI scores, it must be acknowledged that the CPI score has not been validated in a SSc-ILD population. Finally while we noted a trend for a correlation between baseline serum SP-D and change in CPI score over six months, the minimal meaningful change in CPI score has yet to be determined.

In summary we demonstrated that patients with IPF or SSc-ILD have higher serum KL-6 and MMP7 than healthy controls while KL-6 is higher in IPF and SSc-ILD patients compared to SSc patients without ILD; combining measurements of KL-6 and MMP7 identified ILD patients with 95% sensitivity. We have also shown that baseline serum SP-D predicts change in FVC %pred over six months. Baseline SP-D also correlated with CPI score, a predictor of mortality in IPF. This study is unique in that these findings were demonstrated with prospective data. Larger studies are now required to investigate the efficacy of KL-6, SP-D or MMP7 as a screening tool for detecting ILD in at risk populations and whether serum SP-D can be used to identify ILD patients at risk of an acute decline in lung function. Ultimately this may improve outcomes by facilitating more prompt initiation of immunosuppressives in SSc-ILD or timelier referral for transplant in IPF.

Key messages

A combination of serum KL-6 and MMP7 may identify patients at risk of ILD with high sensitivity

Baseline serum SP-D correlates with prospective lung function decline in a pooled cohort of IPF and SSc-ILD patients

Authorship

Dr. Kennedy contributed to data acquisition, statistical analysis, writing the manuscript and reading and approving the final manuscript.

Dr. Branagan contributed to study design, data acquisition and reading and approving the final manuscript.

Dr. Moloney contributed to data acquisition, analysis and interpretation of data and reading and approving the final manuscript.

Dr. Haroon contributed to study design, data acquisition and reading and approving the final manuscript.

Dr. O'Connell contributed to study design, data acquisition and reading and approving the final manuscript.

Dr. O'Connor contributed to study design and revision of manuscript for important intellectual content, and read and approved final manuscript.

Dr. O'Regan contributed to data acquisition, analysis and interpretation of data and reading and approving the final manuscript.

Dr. Harney contributed to study design, data acquisition, statistical analysis, writing the manuscript and revision of manuscript for important intellectual content and reading and approving the final manuscript.

Dr. Henry contributed to study design, data acquisition, statistical analysis, writing the manuscript and revision of manuscript for important intellectual content and reading and approving the final manuscript.

Dr. Kennedy, Dr. Harney and Dr. Henry had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

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