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Surfactant protein-D predicts survival in patients with idiopathic pulmonary fibrosis

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Abstract. *Background:* Idiopathic pulmonary fibrosis is a progressive interstitial lung disease with a high mortality rate. As lung transplantation is the only therapeutic option, it is important to predict survival. *Objective:* This study evaluates the clinical value of surfactant protein-D as a marker of prognosis in patients with idiopathic pulmonary fibrosis. *Design:* Surfactant protein-D was measured in serum of 72 patients and 305 healthy controls. The optimal cut-off level to define unfavourable prognosis was determined using a ROC analysis. A Cox's proportional Hazards model was used to evaluate variables that were significant predictors of survival. *Results:* Serum levels of surfactant protein-D were significantly higher in patients than in controls. ROC analysis showed 460 ng/ml to be the optimal cut-off level to discriminate survivor from non-survivors after 1 year. Patients with high levels (> 460 ng/ml) had a median survival time of 13 months, compared to 67 months in the group with low levels (< 460 ng/ml). Surfactant protein-D showed to be a significant predictor of prognosis, even when corrected for age, sex, smoking, and lung function. *Conclusion:* The measurement of surfactant protein-D in serum of patients with idiopathic pulmonary fibrosis might be a clinically relevant tool to predict survival. *(Sarcoidosis Vasc Diffuse Lung Dis 2009; 26: 155-161)*

Key words: idiopathic pulmonary fibrosis, interstitial lung disease, surfactant protein, biomarker, prognosis

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lung parenchyma. Clinically, it is characterized by dyspnea and worsening of lung function. The clinical course of IPF is unpredictable and median survival time varies between 2.4 and 4.2 years (1-4). To date, no therapy has been proven to prolong survival (5). Lung transplantation seems to be the only option for those who meet the appropriate criteria. Unfortunately, IPF patients have the highest mortality rate on the transplant waiting list (6). Optimal timing of referral for lung transplantation is therefore crucial and dependent on predicting survival. In this respect it is of great importance to study new biomarkers that can predict survival.

Lung-specific secretory proteins, also referred to as pneumoproteins, are potential biomarkers to assess disease severity and progression in interstitial lung disease (7). These proteins are secreted by the respiratory tract epithelium and their occurrence in serum is probably due to leakage through the lung

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parenchyma. Surfactant protein (SP)-D is mainly synthesized in type II pneumocytes, but it is also detected in Clara cells and in other extrapulmonary epithelial cells (8). SP-D contributes to the function of surfactant in the alveoli (9).

SP-D has been studied as a marker in patients with bird fanciers' lung and sarcoidosis and has been shown to be associated with lung function impairment and disease severity (10, 11). In IPF patients, serum SP-D is elevated compared to sarcoidosis, beryllium disease and healthy controls (12). In a series of Japanese IPF patients, concentrations of SP-D were significantly increased and related to disease extent and progression (13). Although previous studies have shown that serum SP-D is a potentially useful marker, there is still insufficient information to use serum SP-D in clinical practise as a biomarker to predict prognosis.

Different single nucleotide polymorphisms (SNPs) of the SP-D gene (SFTPD) have been described previously. The rs721917 SNP results in an alteration of the codon corresponding to amino acid 11, where a methionine (Met) is exchanged for a threonine (Thr). The Met11Thr polymorphism results in significantly different serum SP-D levels in healthy controls. The Met variant (T allele) is associated with higher serum SP-D levels. Constitutional SP-D serum levels are approximately 80% under control of genetic factors and the Met11Thr polymorphism determines half of this genetic component (14). Because the value of some markers can improve when serum levels were corrected for genotype (15, 16), it might be important to assess the relationship between serum SP-D levels in IPF patients and the Met11Thr polymorphism.

As SP-D in serum is a potential biomarker in interstitial lung diseases and high levels might indicate worse prognosis, it was our aim to evaluate the clinical value of SP-D measurements in IPF at the time of diagnosis in order to find a cut-off level for defining unfavourable prognosis.

Methods

Patients and healthy controls

Patients with IPF who presented to the Department of Pulmonary Medicine of the St. Antonius Hospital in Nieuwegein between 1998 and 2007 were retrospectively included in this study. Medical records were retrieved and patients were included according to current ATS/ ERS guidelines: a histologic or radiologic pattern typical of usual interstitial pneumonia (UIP) (17). Diagnoses made before 2002 were reviewed by a clinician and only included when current ATS/ ERS criteria were met. Other causes of UIP (drugs, collagen vascular diseases) were ruled out. Serum and BALf were collected from all ILD patients, and were systematically enrolled in our database used for scientific research. Serum samples of 72 IPF patients were available at the time of diagnosis. Bronchoalveolar lavage fluid (BALf) was available from 54 IPF patients and was obtained using fibreoptic bronchoscopy according to a previously described method (18). Serum and BALf samples were stored at -80°C until analysis. Serum from healthy controls was obtained from 305 self-reported healthy employees of the St. Antonius Hospital. Bronchoalveolar lavage was performed on 30 healthy controls. The study protocol was approved by the Ethical Committee of the St. Antonius Hospital, and all subject gave their written informed consent.

Pulmonary function tests

Pulmonary function tests were performed according to ERS recommendations (19). Vital capacity (VC), forced expiratory volume in 1 second (FEV_1) and diffusing capacity for carbon monoxide (D_{Lco}) were measured with a Jaeger System. All values were expressed as percentage of predicted value. The interval between pulmonary function testing and collection of the serum and BAL samples was less than three months.

Analysis of SP-D and genotyping of the polymorphism

The concentrations of SP-D in serum and bronchoalveolar lavage fluid (BALf) were detected by monoclonal anti-human SP-D antibody using a commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Biovendor; Heidelberg, Germany). Serum was diluted to a concentration of 1:11 and bronchoalveolar lavage 1:40; the detection limit was 1.2 ng/ml. The Met11Thr polymorphism in the surfactant protein-D gene, corresponding to rs721917,

was analyzed with a custom Illumina goldengate bead SNP assay using sequence specific primers. The assay was performed in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, CA, USA)

Statistical analysis

Data were expressed as median and interquartile ranges (IQR). Differences in serum or BALf concentrations between independent groups were analyzed using a Mann-Whitney U test. Differences between more than two groups were analyzed using one-way analysis of variance (ANOVA). The relationship between markers in serum and BALf and clinical data was assessed using Spearman's correlation coefficients. To find the optimal cut-off level to discriminate survivors from non-survivors after one year, receiver operating curves (ROC) were used. The Kaplan-Meier method was used to describe survival time and the log-rank test to evaluate statistical significance between groups. Transplants and non-IPF deaths were censored. In order to determine the patients' status and cause of death we retrieved medical records and when inconclusive, we contacted the patients' general practitioner. A considerable part of our cohort would not meet the criteria to undergo lung transplantation, due to age restrictions. Therefore, a subanalysis of the group of patients with age < 65 years was performed. A Cox's proportional Hazards model was used to determine covariates that influence survival. Statistical analysis was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc; San Diego, CA, USA). Statistical significance was considered at a value of $p < 0.05$.

Results

Clinical characteristics

Seventy-two IPF patients (56 male and 16 female, mean age 62.9 years [SD 12.9]) were included in the study. Fifteen IPF patients were treated with low-dose oral corticosteroids at the time of serum and/or BAL sampling. In 50 IPF patients (69%) the histological diagnosis of UIP was confirmed by open lung biopsy. Table 1 shows the clinical characteristics of patients and controls.

Table 1. Characteristics of patients and healthy controls

	IPF Patients	Healthy controls
Number of subjects	72	305
Sex M/F	56/16*	115/190
Age, yr (mean, SD)	62.9 (12.9)*	40.4 (11.7)
Smoking status		
Smoker	3	61
Non-smoker	19	192
Ex-smoker	50	52
Lung function, (median, IQR)		
% pred FEV_1	77 (64 - 95)	-
% pred VC	75 (60 - 87)	-
% pred $D_{L_{co}}$	43 (33 - 56)	-

*p < 0.05 compared to healthy controls

SP-D levels

Median serum and BALf levels of SP-D in patients and healthy controls are shown in table 2. The median serum SP-D level in healthy controls was 57.5 ng/ml (IQR 41.2 - 77.5). There was no difference in serum SP-D levels between men and women. Serum SP-D levels were weakly correlated with age ($r = 0.18$ $p < 0.05$). In healthy controls, no influence of smoking was seen in relation to serum SP-D levels (data not shown). Serum SP-D levels in IPF patients were significantly higher (365 ng/ml, IQR 226 - 527) than in healthy controls (57.5 ng/ml, IQR 41.2 – 77.5) ($p < 0.0001$), but SP-D in BALf of IPF patients (385 ng/ml, IQR 290 - 530) was significantly lower than in controls (504 ng/ml, IQR 357 - 734). Serum and BALf levels in IPF patients were not significantly different between smokers (serum: 353 ng/ml, 227 - 506; BALf: 380 ng/ml, IQR 246 - 519) and non-smokers (serum: 357

Table 2. BALf and serum SP-D levels in IPF patients and healthy controls

SP-D levels (median, IQR)	IPF patients	Healthy controls
BALf	n = 54 385 (290 - 530)*	n = 30 504 (357 - 734)
Serum	n = 72 365 (226 - 527)†	n = 305 57.5 (41.2 - 77.5)

*p < 0.05 compared to healthy controls
†p < 0.0001 compared to healthy controls

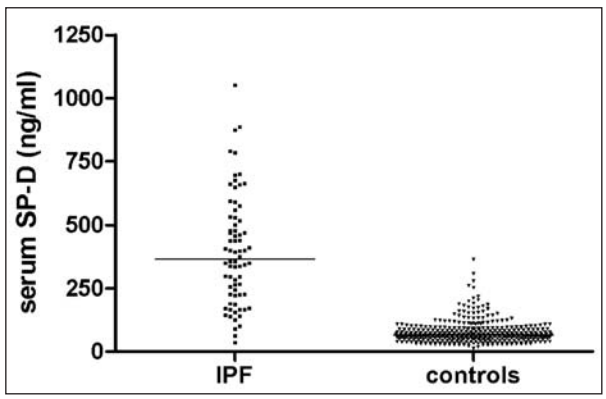

Fig. 1. Serum SP-D levels in 72 IPF patients and 305 healthy controls (HC).

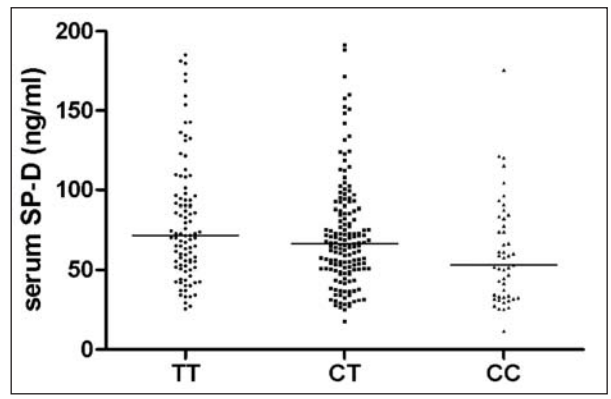

Fig. 2. Scatterplot illustrating the association between the Met11Thr polymorphism in the SFTPD gene and serum SP-D levels in healthy controls (n = 305). Horizontal bars represent median values. ANOVA: p = 0.002.

ng/ml, IQR 164 - 530; BALf: 322 ng/ml, IQR 195 - 504). Figure 1 shows serum SP-D levels at the time of diagnosis in IPF patients and healthy controls. In patients and healthy controls, serum levels of SP-D did not correlate with BALf levels. Serum SP-D levels in IPF patients were correlated with $D_{L_{co}}$ (r = -0.315, p = 0.04). There were no correlations between serum SP-D and any other lung function parameters, BALf parameters or age. Furthermore, SP-D in BALf did not show a correlation with lung function, BALf parameters, survival or age either.

Met11Thr polymorphism and serum SP-D levels

The distribution of the SFTPD genotype in patients and healthy controls was in Hardy-Weinberg equilibrium. There was no significantly different allele frequency in IPF patients (T 56%; C 44%) compared to healthy controls (T 58%; C 42%). Similarly, no significant difference was seen in genotype counts: IPF patients TT 17 (30%), CT 30 (53%), CC 10 (17%); healthy controls TT 99 (32%), CT 157 (52%) and CC 49 (16%) respectively. Figures 2 and 3 show the influence of the Met11Thr polymorphism and corresponding serum SP-D levels in healthy controls and IPF patients. Significant differences in serum SP-D levels were found within the population of healthy controls when groups were formed according to their genotype: TT 71.7 ng/ml (IQR 52.5 - 97.7), CT 66.6 ng/ml (IQR 50.8 - 88.3), and CC 53.0 ng/ml (IQR 33.1 - 83.0), ANOVA: p = 0.002. When serum SP-D levels of IPF patients were grouped according to genotype, no significant difference between these groups was observed, ANOVA: p = 0.9.

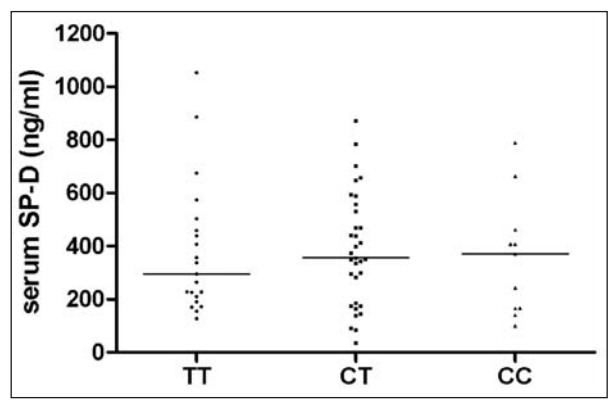

Fig. 3. Scatterplot illustrating the absence of association between the Met11Thr polymorphism in the SFTPD gene and serum SP-D levels in IPF patients (n = 57). Horizontal bars represent median values. ANOVA: p = 0.9.

Survival

The median follow-up period for IPF patients was 39 months (range: 1 - 114). Within the study period, 48 from the total of 71 IPF patients died, while one patient was lost to follow-up. The cause of death was respiratory failure due to progressive IPF (n = 37), lung carcinoma (n = 4), pneumonia (n = 5) or pulmonary embolism (n = 1). One patient died from an extrapulmonary cause and two patients un-

derwent lung transplantation; those cases were censored in the survival analysis. Figure 4 illustrates that patients with a survival less than 6 months have significantly higher serum SP-D levels than patients who lived longer (survival < 6 months: 661 ng/ml [IQR 573 - 886]; survival 6 –12 months: 465 ng/ml [IQR 265 - 567]; survival > 12 months: 250 ng/ml [IQR 163 - 370]), ANOVA: p < 0.0001.

To find an optimal cut-off level for serum SP-D to discriminate survivors from non-survivors after one year, ROC curves were used. According to the ROC curves, the optimal cut-off level for SP-D was 460 ng/ml (sensitivity 0.625, specificity 0.783, AUC 0.690). Patients were divided into two groups according to the cut-off level of 460 ng/ml. Median survival in the low SP-D group (n = 47) was 67 months (SE 7.5) compared to a median of 13 (SE 12) months in the high SP-D group (n = 25, Log-rank test p = 0.001, figure 5A). Patients within the group of serum SP-D levels > 460 ng/ml did not show significant differences in age, duration of symptoms until diagnosis, smoking behaviour, lung function parameters or therapy compared to the group with serum levels < 460 ng/ml (data not shown). A subgroup analysis was performed for patients with age under 65 years and a similar result could be shown. Median survival in the low SP-D group (n = 22) was 50 months (SE 9.6) compared to a median of 11 (SE 1.8) months in the high SP-D group (n = 17, Log-rank test p = 0.02, figure 5B).

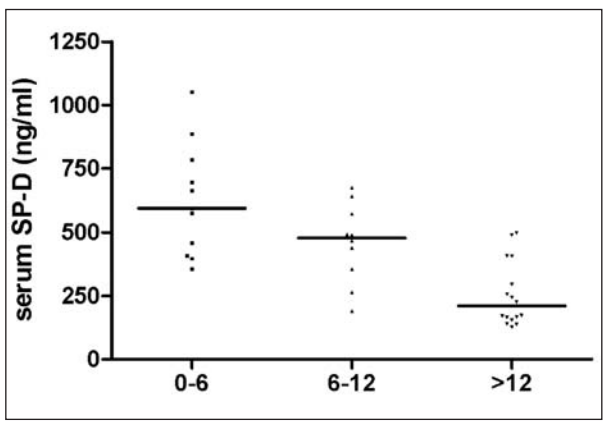

Fig. 4. Scatterplot illustrating serum SP-D levels in patients who died from progressive IPF (n = 37). Patients were categorized according to survival time. ANOVA: p < 0.0001

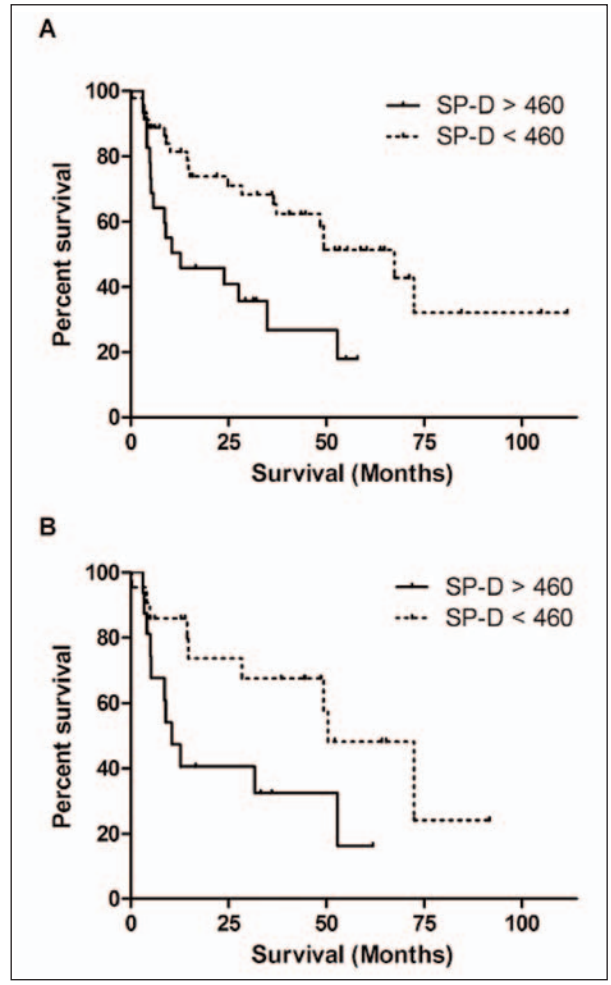

Fig. 5. A) Kaplan-Meier curve showing a median survival of 13 months in IPF patients with high serum SP-D levels (> 460 ng/ml, n = 25) and a median survival of 67 months in patients with low (< 460 ng/ml, n = 47) serum SP-D levels. The difference between the two curves is statistically significant, p = 0.001.
B) Kaplan-Meier curve for the subgroup of patients with age < 65 years. Patients with SP-D levels > 460 ng/ml (n = 17) have a median survival of 11 months, compared to 50 months in the group of patients with low SP-D levels (n = 22), p = 0.02

Cox proportional hazards models were used to examine the influence of serum SP-D levels on survival while adjusting for known predictors of prognosis such as age (20), smoking status (20, 12), VC (21, 22), $D_{L_{co}}$ (23, 22) and BAL fluid neutrophilia (24). In the univariate analysis (table 3), both $D_{L_{co}}$ and SP-D levels were significantly related to survival. In the multivariate analysis only SP-D levels were associated with increased mortality, Hazard ratio 3.22 (95% CI 1.33 - 7.81), p = 0.01 (table 4).

Table 3. Univariate Cox's proportional Hazards model, describing hazard ratios of covariates in relation to survival

Covariate	Hazard ratio	CI	p-value
Age	1.01	0.98 - 1.04	0.32
Smoking	0.89	0.57 - 3.00	0.53
VC	1.08	0.70 - 2.23	0.40
% neutrophils in BALf	1.03	0.99 - 1.06	0.14
$D_{L_{co}}$	0.05	0.01 - 0.717	0.05
SP-D (< 460 vs ≥ 460 ng/ml)	3.01	1.55 - 5.87	<0.01

Table 4. Multivariate Cox's proportional Hazards model, describing hazard ratios of covariates in relation to survival

Covariate	Hazard ratio	CI	p-value
Age	1.04	0.99 - 1.08	0.38
Smoking	0.78	0.21 - 2.82	0.77
VC	0.98	0.95 - 1.02	0.41
% neutrophils in BALf	1.03	0.97 - 1.03	0.33
$D_{L_{co}}$	0.14	0.01 - 2.02	0.15
SP-D (< 460 vs ≥ 460 ng/ml)	3.22	1.33 - 7.81	0.01

CI: confidence interval; VC: Vital capacity; $D_{L_{co}}$: Diffusion capacity for carbon monoxide; SP-D: surfactant protein-D

Discussion

The present study showed that SP-D in serum can predict mortality in IPF patients, and that the value of SP-D remains stable after adjustment for known predictors of mortality. A serum SP-D level higher than 460 ng/ml indicates a significantly worse prognosis compared to levels lower than 460 ng/ml. This cut-off value can be useful in clinical practice. It might help in estimating survival time, which is important for optimal timing of referral for lung transplantation in selected candidates. Furthermore, the study showed that the Met11Thr polymorphism influences serum SP-D levels in healthy controls, but not in IPF patients.

Part of our results are in agreement with data from Takahashi et al (13). Our study, however, adds to these findings by providing a cut-off levels for prognosis and shows a clear relationship of high serum SP-D levels and short survival. This can facilitate interpretation of serum SP-D levels in clinical practice, and helps the identification of patients with the worst prognosis. The Kaplan-Meier curve (fig. 5) shows us that the difference between the two lines is mainly caused by the rapid decline in the first 12 months in the group with SP-D levels higher than 460 ng/ml. After 12 months, the two lines run parallel. This means that high serum SP-D levels predict a rapid deterioration and that high serum SP-D levels mainly predict short-term survival (i.e. < 12 months). In figure 4 this is supported by the fact that patients with a shorter survival time show higher SP-D levels. Furthermore, we performed a Cox proportional Hazards model to evaluate serum SP-D while adjusting for patient characteristics and lung function parameters. Even after adjustment, serum SP-D levels at the cut-off value of 460 ng/ ml remain a significant predictor of mortality. This strengthens the recommendation of using SP-D in clinical practice as a new marker for prognosis in IPF.

The source of increased serum concentrations of SP-D has to be further elucidated but it seems likely that it is at least in part the result of increased alveolar-capillary permeability (7). It is also assumed that it correlates with the total amount of damaged epithelium in the alveolar compartment. In contrast, lower SP-D levels in BALf were found compared to controls, and this might be related to the replacement of alveolar epithelial cells by scar tissue. As alveolar type II epithelial cells are the major producers of SP-D, a reduced number of these cells could lead to decreased amounts of SP-D in BALf. However, this does not explain the lack of correlation between serum and BALf SP-D levels. Fujii et al. (25) suggested that the leakage from the alveolar to the vascular compartment is superior to the secretion of SP-D into the alveolar lining fluid. Whether an increased local clearance of SP-D by alveolar macrophages or other alveolar cells might also play a role is unclear.

Although increased serum SP-D levels are most likely the result of increased secretion and/or leakage of these molecules across the alveolar-capillary membrane, it can not be ruled out that there are other cells in the circulation that secrete SP-D (26). For example, it has recently been reported that SP-D is expressed in vascular smooth muscle cells, and plays a role in the local regulation of inflammatory processes and innate host defense (27). Therefore, increased serum SP-D levels in IPF might also be partly due to SP-D released by vascular endothelial cells, and reflect a local inflammatory response involving the vascular endothelium.

In healthy controls, Sorensen et al. illustrated that the Met11Thr polymorphism accounted for

marked differences in serum SP-D levels (14). We confirmed their findings, and in addition showed that in IPF patients serum SP-D levels were independent of the Met11Thr polymorphism. A possible explanation could be that functional effects of SNPs on protein levels in healthy controls may become less prominent in pathological conditions.

One of the limitations of this study is that it is a retrospective study. A prospectively conducted study is needed to determine whether serum SP-D levels rise as lung function declines. Currently, a prospective study with serial measurements of serum SP-D and lung function is being conducted in our centre. As such, we can validate our results and test the cut-off level in a prospective manner.

In summary, SP-D is a marker that can be easily determined in serum and has been proven to be a prognostic marker in IPF patients. This study adds clinically useful cut-off levels that could identify patients with a significantly worse prognosis. This prognostic value of SP-D persists after adjustment for known predictors of mortality. Taken all previously published studies into account, we encourage the implication of routine measurement of SP-D at the time of diagnosis in IPF patients.

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