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Serum Krebs von den Lungen-6: Promising biomarker to differentiate CPFE from IPF

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Abstract. *Background:* Combined pulmonary fibrosis and emphysema (CPFE) has been recognised as a phenotype of pulmonary fibrosis. We aimed to compare serum surfactant protein-A (SP-A), surfactant protein-D (SP-D) and Krebs von den Lungen-6 (KL-6) levels, functional parameters, in CPFE and IPF (idiopathic pulmonary fibrosis) patients. *Methods:* Patients diagnosed with 'CPFE' and 'IPF' were consecutively included in 6 months as two groups. The patients with connective tissue diseases are excluded. *Results:* In this study, 47 patients (41 males, 6 females) with CPFE (n = 21) and IPF (n = 26) with a mean age of 70.12 ± 8.75 were evaluated. CPFE patients were older, had more intense smoking history, had lower DLCO/VA, lower FVC, and worse six-minute walking distance than the IPF group (p=0.005, p=0.027, p=0.02, p<0.001, p=0.001, respectively). Serum KL-6 levels were higher in CPFE group compared to IPF group [264.70 U/ml (228.90-786) vs 233.60 (101.8-425.4), p<0.001]. Serum KL-6 levels of 245.4 U/ml and higher have 81% sensitivity and 73% specificity for the discrimination of CPFE from IPF. *Conclusions:* Our study has shown that serum KL-6 level is a promising biomarker to differentiate CPFE from IPF. In CPFE cases respiratory and functional parameters are worse than those of pure fibrosis cases.

Key words: Combined pulmonary fibrosis emphysema, Idiopathic pulmonary fibrosis, Krebs von den Lungen-6, Surfactant protein-A, Surfactant protein-D, Pulmonary function tests, Echocardiography

Introduction

Combined pulmonary fibrosis and emphysema (CPFE) has been recognised as a phenotype of pulmonary fibrosis that is characterised by upper lobe emphysema and lower lobe fibrosis (1). The prevalence of emphysema in idiopathic pulmonary fibrosis (IPF) cohorts ranged from 28% to 50% (2-4). While emphysema is characterised pathologically by loss of the extracellular matrix and enlargement of pulmonary alveoli, IPF involves accumulation of mesenchymal cells and extracellular matrix components in the lungs (5). Pulmonary function test results of CPFE cases differ from those of pure emphysema or pure fibrosis cases (6). In CPFE cases impaired gas exchange is found alongside relatively normal spirometry and lung volume (7, 8). Frequently, CPFE develops when a known history of emphysema becomes superimposed with fibrosis that may alter its course. In contrast, occurrence of pulmonary emphysema altering the outcomes in IPF patients has also been observed (9). Both emphysema and pulmonary fibrosis may result from different responses to the

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same lung injury (10). Research has also revealed increased lung collagen content in emphysema cases, indicating fibrotic changes occurred as part of connective tissue remodelling in their lungs (11).

Surfactant protein-A (SP-A), surfactant protein-D (SP-D) and Krebs von den Lungen-6 (KL-6) are reported to be highly specific, sensitive markers for interstitial lung diseases (ILDs) (12-18). KL-6 is a circulating high molecular weight glycoprotein and, expressed on the surface membranes of alveolar and bronchial epithelial cells (19). Studies have indicated KL-6 to be a sensitive and useful biomarker that can be utilised for differential diagnosis of ILD, assessment of disease activity and prediction of disease outcome (5, 14, 20-24). We aimed to investigate, whether serum SP-A, SP-D and KL-6 levels are useful biomarkers in differentiating CPFE and IPF.

The primary outcome was to compare serum SP-A, SP-D, KL-6 levels in CPFE patients and in patients with IPF without emphysema. Secondary outcomes were to compare the functional parameters, transthoracic echocardiographic and radiological findings, and quality of life in CPFE and IPF patients.

Methods

Patients diagnosed with 'CPFE' and 'IPF without emphysema' that were over the age of 18 and had given written informed consent were prospectively recruited consecutively from the respiratory out-patient clinic, over a period of 6 months (October 2015 to March 2016). Patients were diagnosed as CPFE if they fulfilled the following criteria identified by Cottin et al (1): CT shows (a) emphysema, defined as localized low attenuation areas with very thin (<1 mm) or no surrounding walls and/or multiple bullae, with upper lobe predominance and (b) significant pulmonary fibrosis, characterized by the presence of peripheral and basal predominance reticular opacities with or without traction bronchiectasis. Patients with connective tissue disease and occupational or drug related ILD were excluded. The study population was divided into two groups as "CPFE" and "IPF (IPF without emphysema)". The diagnostic criteria for IPF were applied according to the 2011 ATS/ERS/JRS/ALAT guidelines for diagnosis and management of IPF (25). Demographic features, functional parameters, echocardiography (ECHO) findings, quality of life and serum SP-A, SP-D and KL-6 measurements of the two groups were compared. Spirometry and single-breath carbon monoxide diffusion tests were performed by using Vmax Encore System (Sensormedics, Viasys, Yorba Linda, CA, USA) in accordance with ATS/ERS recommendations (26, 27). Predicted normal values were derived from standard equations recommended by the European Community for Steel and Coal and European Respiratory Society (28, 29). The Six-minute walking test (6MWT) was performed according to the ERS/ATS technical standards, by using a 30-m corridor (30).

Quality of life was assessed using the Short-Form 36 (SF-36) questionnaire, validated for Turkish populations (31). Consisting of 36 statements, this questionnaire measures the quality of life for eight domains under two main factors (physical and mental factors). Each main factor and domain of the measure is scored between 0 and 100. In the SF-36, which uses a positive scoring system, higher scores for each health-related domain indicate better quality of life in terms of health.

Echocardiographic assessment

Echocardiographic evaluation was made by the same cardiologist for all patients. Left ventricular diameters were measured in parasternal long axis and ejection fraction were calculated by using the Simpson method. Mitral e and a waves were measured by pulse wave mode. Right atrium and ventricular diameters were measured in apical four chamber view. Maximum pulmonary velocity was calculated in parasternal short axis. Pulmonary artery pressure was calculated by measuring tricuspid regurgitant velocity. Tricuspid annular place systolic excursion (TAPSE) was measured using M-mode echocardiography. Tissue Doppler measurements were done from basal free RV wall during cardiac cycle. S wave was measured during the systolic phase, while e' and a' waves were measured during the diastolic.

Serum KL-6, SP-A, SP-D analysis

Serum samples were stored at −80°C until biomarker assays were performed. Human KL-6, Pulmonary SP-A and Pulmonary SP-D levels were measured using commercially available enzyme-linked immunosorbent assay kits (*KL-6;*

Bioassay Technology Laboratory, China, SP-A and SP-D; Sunred Biological Technology, China), according to the manufacturer's instructions. Tests were performed in duplicate for each sample and the protein concentrations were calculated using standard curves. The sensitivity of the protein detection system of the assays were 1.12 U/ml for KL-6, 0.217 ng/ml for SP-A and 4.153 ng/ml for SP-D.

This study was approved by the "Ethics Committee of Bursa Uludag University (decision number=2015/24)".

Statistical analysis

The data was examined by the Shapiro Wilk test whether or not it presents normal distribution. The results were presented as mean±standard deviation or median (minimum-maximum) for continuous variables. Categorical variables were described as frequency. Continuous variables were compared using Student t-test and Mann-Whitney U test. Categorical variables were compared using Pearson's chi-squared test and Fisher's exact test.

Statistically significance level was accepted as α=0.05. Statistical analyses were performed with IBM SPSS ver.23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). The receiver operating curve (ROC) at the optimal cut-off threshold score for serum KL-6, SP-A and SP-D levels as derived using the MedCalc Statistical Software version 19.1.5 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2020). The sensitivity and specificity at the optimal cut-off threshold score were also derived from the ROC.

Results

Forty-seven patients, diagnosed with CPFE (n=21) and IPF (n=26) by their CT scan images, were prospectively enrolled in this study. The study population comprised 47 patients (41 male, 6 female) with a mean±SD age of 70.12±8.75 years. Of the patients, none of the CPFE and, 11 of the IPF cases were diagnosed through surgical lung biopsy. CPFE patients were older, had more intense smoking history, had lower diffusion capacity divided by the alveolar volume ratio (DLCO/VA), lower forced vital capacity (FVC), and worse six-minute walking distance (6MWD) than the IPF group (p=0.005, p=0.027, p=0.02, p<0.001, p=0.001, respectively) (Table 1). Diffusing capacity of the lung for carbon monoxide (DLCO) was low in both groups, no significant difference was found between the two. Comparison of demographic, clinical and radiological features are summarized in Table 1. At the time of diagnosis, CPFE patients were older in comparison to IPF patients, [70 (42-79) vs 59 (47-76), p<0.001, respectively]. According to SF-36 scores, CPFE group had more physical role limitation, which is a domain of the physical component and, social functioning, a domain of mental scoring (Table 1).

Cardiovascular evaluation revealed that patients with CPFE had significantly more comorbidities, such as hypertension (Table 2). Brain natriuretic peptide (BNP), a biomarker secreted in response to ventricular dysfunction and wall stretch indicating heart failure, is significantly higher in CPFE patients (Table 2). Transthoracic ECHO findings in two groups are summarized in Table 2. Left ventricular systolic (ejection fraction) and diastolic functions (mitral e/a ratio) showed no difference between CPFE and IPF groups [62.7±8.5 vs 66±6.03 and 0.73 (0.66-0.80) vs 0.83 (0.66-0.88) mm; p=0.129, p=0.49, respectively]. Right ventricular diameter was not statistically different in CPFE and IPF patients [36.1±7.1 vs 33.6±6.3 mm, p=0.098]. Tissue Doppler ECHO revealed similar muscular functioning in systolic and diastolic phase in both groups (Table 2). Systolic pulmonary artery pressure was in normal limits and similar between CPFE and IPF patients [39 (22-90) vs 37 (20-128) mmHg, p=0.648].

When we compared CPFE and IPF groups, serum KL6 levels were significantly higher in the CPFE group [264.70 U/ml (228.90-786) vs 233.60 U/ml (101.8-425.4), p<0.001, respectively]. Serum SP-A [28.09 ng/ml (19.17-63.4) vs 25.08 ng/ml (19.09-63.11), p=0.514] and SP-D levels [452.3 ng/ml (367.5-1332.80) vs 415.15 ng/ml (350.4-1175.60), p=0.066] were similar in CPFE and IPF groups, respectively (Table 3). According to multivariate logistic regression analysis, the significant difference of serum KL-6 levels in two groups, is independent of age and FVC values.

In order to assess the distinguishing value of serum KL-6 for CPFE from IPF, the receiver operating characteristic curve (ROC) analysis was performed and yielded a cut-off level of 245.4 IU/L. At this cut-off value, the serum KL-6 level had a sensitivity of 81% and specificity of 73% for the discrimination of

Table 1. Comparison of demographics features, functional-radiological parameters and serum KL-6, SP-A and SP-D levels between two groups.

	Study population	IPF patients n=26	CPFE patients n=21	p
Gender (M/F)	41/6	22/4	19/2	0.678
Age (years) †	70.12±8.75	66.96±7.51	74.04±8.74	0.005
BMI (kg/m^2) †	29.38±4.88	30.29±5.03	28.37±4.61	0.195
Smoking history (n) (non-smokers /ex-smokers/smokers)	8/34/5	7/17/2	1/17/3	0.140
Smoking (pack-years) †	35 (0-110)	25 (0-90)	40 (10-110)	0.027
FVC (% predicted) †	77.34±22.84	88.4±20.15	62.68±17.49	<0.001
FEV_1 (% predicted) †	80.95±24.02	89.8±22.48	69.31±21.28	0.004
FEV_1/FVC (%)†	81.99±9.22	79.12±6.48	85.7±10.9	0.016
DLCO Adj (% predicted) †	54 (33-108)	55.5 (33-108)	51 (34-74)	0.743
DLCO/VA Adj (%predicted) †	83 (47-146)	90.5 (47-146)	78 (50-103)	0.020
6MWD (m) †	327 (60-510)	360 (100-510)	180 (60-430)	0.001
Short form-36 (n) †				
Physical functioning (36)	50 (5-100)	55 (20-100)	35 (5-100)	0.066
Role-physical (37)	100 (0-100)	100 (0-100)	0 (0-100)	0.001
Body pain (35)	90 (33-100)	85 (58-100)	90 (33-100)	0.933
General health (35)	45 (5-80)	41.5 (5-80)	45 (15-80)	0.775
Vitality (35)	60 (15-95)	62.5 (35-95)	55 (15-95)	0.533
Social functioning (35)	88 (13-100)	100 (75-100)	75 (13-100)	0.007
Role-emotional (35)	100 (0-100)	100 (100-100)	100 (0-100)	0.139
Mental health (35)	72 (24-100)	72 (32-100)	68 (24-96)	0.699
Thoracic CT findings n (%)				
Honeycombing	39 (88.6)	22(95.7)	17 (81)	0.176
Reticulation	36 (81.8)	16(69.6)	20 (95.2)	0.048
Traction bronchiectasis	6 (13.6)	3 (13)	3 (14.3)	1
Ground glass opacities	7 (15.9)	4 (17.4)	3 (14.3)	1

† Data are presented as mean±SD or median(min-max).
Abbreviations: BMI:body mass index, FVC: forced vital capacity, FEV_1: forced expiratory volume in one second, DLCO: diffusing capacity of the lung for carbon monoxide, DLCO/VA: diffusion capacity divided by the alveolar volume ratio, 6MWD: six minute walking distance, SF-36 score: Short Form-36 score.

CPFE from IPF patients (area under the curve [AUC] = 0.805, p<0.001) (Fig 1). Serum KL-6 levels and cut of value in are presented in IPF and CPFE patients as a dot diagram (Fig 2). The ROC curves for serum SPA and SPD ([AUC]=0.556, sensitivity= 52.38%, specificity= 73.03%, p= 0.523; [AUC]=0.658, sensitivity= 71.43%, specificity=61.54%, p= 0.053, respectively) are also shown in Fig. 1. Between October 2015 and March 2016, the mortality rate was 19% (n=4) in CPFE group, while no deaths were observed in IPF group (p<0.001).

Discussion

In our study, serum KL-6 level greater than ≥245.4 U/ml had 81% sensitivity and 73% specificity in discriminating CPFE from IPF. In this study, we observed that serum KL-6 levels are higher in CPFE compared to pure IPF group. In functional assessment, FVC (% predicted), DLCO/VA measurements and 6MWD were lower in CPFE group compared to IPF patients.

As is known, vital capacity is a robust predictor of mortality in IPF patients (32). In CPFE patients, though, lung volumes are generally preserved. Contrary to the findings of our study, previous studies have found higher FVC values in CPFE as opposed to pure fibrosis cases, while observing milder restrictive impairment (1, 4, 33-34). Hence, it was reported that absolute FVC or FVC% values are not robust predictors of disease in CPFE, as they are in IPF cases. However, there are studies that have,

Table 2. Comparison of cardiac comorbidities and ECHO findings in groups.

	Study population	IPF patients N=26	CPFE patients N=21	p
Comorbidities, n(%)	35 (74.5)	16 (61.5)	19 (90.5)	0.024
Hypertension n(%)	24 (51.1)	9 (34.6)	15 (71.4)	0.012
Coronary artery disease n(%)	11 (23.4)	4 (15.4)	7 (33.3)	0.181
BNP (pg/ml)†	22.5 (10-678.9)	13.7 (10-138)	48 (10-678.9)	0.006
Ejection fraction†	64.3 ± 7.3	66 ± 6.03	62.7 ± 8.5	0.129
Right ventricular diameter (mm)†	33.7 ± 6.1	32.2 ± 4.6	35.5 ± 7.3	0.098
Right atrium diameter (mm)†	34.7 ±6.7	33.6 ± 6.3	36.1 ± 7.1	0,254
Max. pulmonary velocity (mm)†	0.89 ±0.1	0.87 ± 0.07	0.92 ±0.12	0.117
IVC diameter (mm)†	14.5 ± 3.4	14 ± 2.8	15.1 ± 4.06	0.309
TAPSE (mm)†	20.6 ± 3.3	21.1 ± 3	19.8 ± 3.7	0.224
Mitral e/a ratio†	0.75 (0.66-0.86)	0.83 (0.66-0.88)	0.73 (0.66-0.80)	0.49
PAB (mmHg) †	38 (20-128)	37 (20-128)	39 (22-90)	0.648
TDI- s'	0.17(0.07-0.31)	0.18(0.12-0.25)	0.16 (0.07-0.31)	0.373
TDI- e'	0.10(0.04-0.21)	0.10(0.05-0.18)	0.11 (0.04-0.21)	0.74
TDI- a'	0.15(0.07-0.23)	0.15(0.08-0.21)	0.14 (0.07-0.23)	0.576

† Data are presented as mean±SD or median(min-max).
Abbreviations: BNP: brain natriuretic peptide, IVC: inferior vena cava, TAPSE: Tricuspid annular place systolic excursion, PAB: pulmonary artery pressure, TDI: Tissue Doppler Imaging

Table 3. Comparison of serum KL-6, SP-A and SP-D levels in both groups.

	Study population	IPF patients n=26	CPFE patients n=21	p
SP-A (ng/ml)	25.5 (19.09-63.4)	25.5 (19.09-63.11)	28.09 (19.17-63.4)	0.514
SP-D (ng/ml)	427.5 (350.4-1332.8)	415.15 (350.4-1175.6)	452.3 (367.5-1332.8)	0.066
KL-6 (IU/L)	247.3 (101.8-786)	233.6 (101.8-425.4)	264.7 (228.9-786)	<0.001

Data are presented as Med (min-max)
Abbreviations: SP-A: surfactant protein-A, SP-D: surfactant protein-D, KL-6: Krebs von den Lungen-6.

similarly to ours, identified lower vital capacity, and severe restrictive impairment than those found in other CPFE series (2, 8). It is considered that the divergence of our findings might be due to potentially high fibrosis scores of our cases. Existence of fewer emphysematous areas or patients being at different phases of CPFE may also explain the discrepancy. Another mechanism posited as a probable explanation is the greater volume loss of the lower lung field due to severe fibrosis (35). In line with the studies by Mura et al and Jacob J. et al, our study found reduced DLCO in both groups, without a statistically significant difference between the two (34, 36).

SF-36 appears to be a valid instrument to measure health-related quality of life in IPF (37). Studies have shown that in addition to worsened physical health, IPF patients' general health, energy level, respiratory symptoms and level of independence also deteriorate (38, 39). Compared to pure emphysema patients, marked reductions were observed in exercise tolerance and the quality of life in CPFE cases (40). In contrast to previous studies, in our study both groups scored high in SF-36, despite fibrosis (37, 38). Nonetheless, in the CPFE group, role limitation due to physical problems and social functioning scores were significantly lower than those of the IPF group. These results indicate that CPFE patients have, in certain respects, poorer quality of life.

A common complication that develops during the clinical course of CPFE is pulmonary

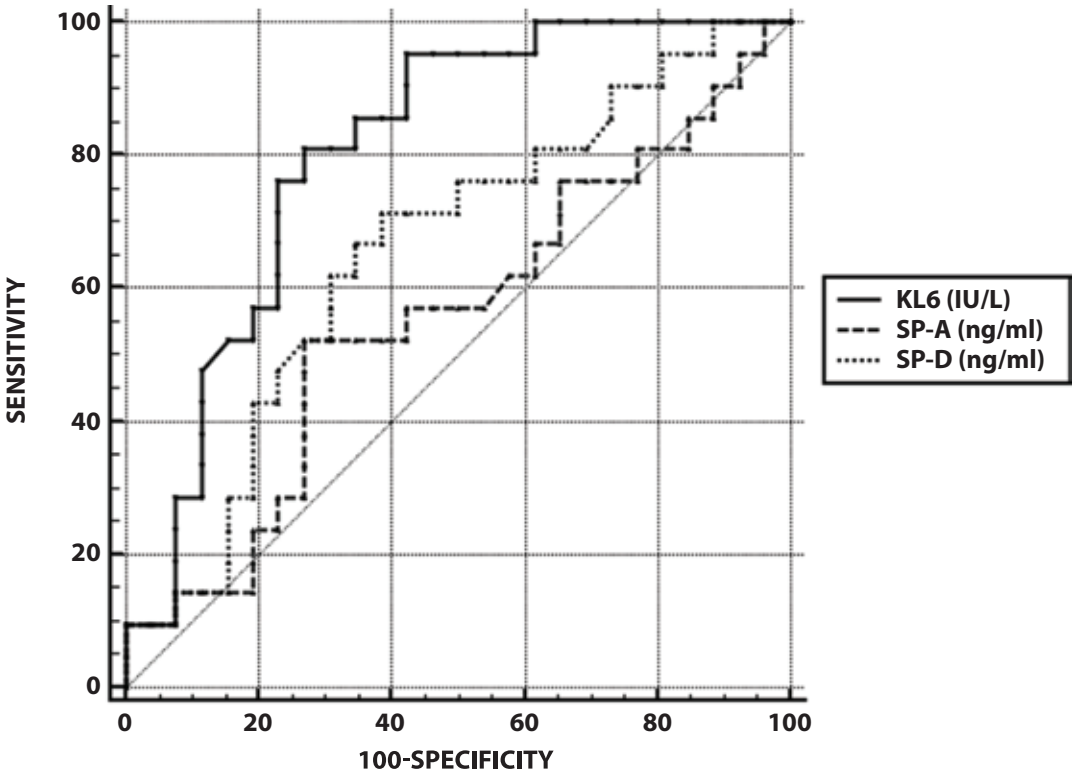

Figure 1. ROC curves for serum KL6, SPA, SPA (KL-6 cut off value= 245.4 IU/L, AUC=0.805, sensitivity=0.80,95, specificity=0.73,08, p<0.001; SP-A AUC=0.556, sensitivity=0.52,38, specificity=0.73,03, p=0.523; SP-D AUC=0.658, sensitivity=0.71,43, specificity=0.61,54, p=0.053).
Abbreviations: SP-A: surfactant protein-A, SP-D: surfactant protein-D, KL-6: Krebs von den Lungen-6.

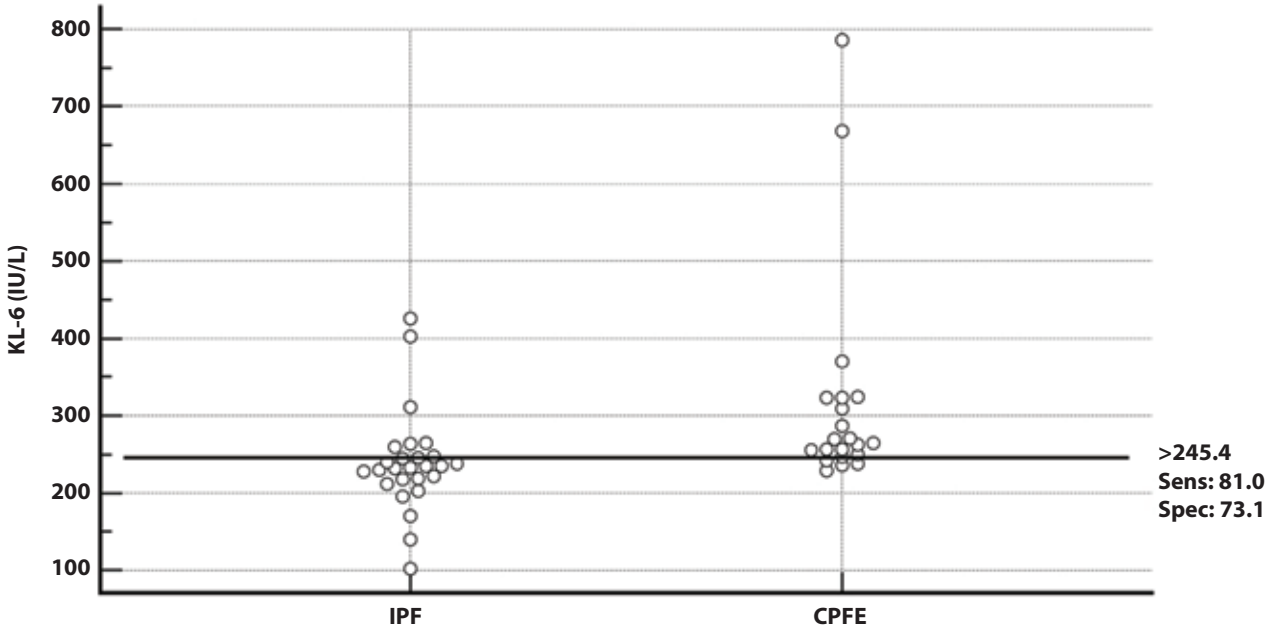

Figure 2. Serum KL-6 levels dot diagram in IPF and CPFE patients.
Abbreviations: IPF: idiopathic pulmonary fibrosis, CPFE: combined pulmonary fibrosis emphysema

hypertension (PH). PH prevalence increases in CPFE cases (1, 41). Studies have reported PH prevalence ranging from 47% to 90% in CPFE patients, which is considerably high when compared to its prevalence in chronic obstructive lung disease or IPF only (2, 41, 42). Mejía M. et al's study has also found strong correlation between the extent of emphysema and estimated systolic pulmonary artery pressure (2). Similar to Jankowich et al., our study found no statistically significant difference between the systolic pulmonary artery pressure and right ventricular diameters of the two groups (4). Tissue Doppler Imaging (TDI) which is a useful echocardiographic tool for quantitative assessment of left ventricular systolic and diastolic function revealed the similar muscular function in CPFE and IPF groups.

KL-6, SP-A and SP-D are useful biomarkers in the diagnosis of various types of ILD and, associated with the extent of pulmonary fibrosis and also were found in elevated levels in IPF patients (12, 19, 43). Ohnishi et al have demonstrated that KL-6 was superior to SP-A, SP-D and MCP-1, as a pathological marker in ILD (12). It is thought that increases in serum KL-6, SP-A, SP-D and MCP-1 are in part related to enhanced permeability or disintegration of the air-blood barrier in the lungs. The said study's findings also supported the previous notion that SP-D is superior to SP-A in the diagnosis of ILD (17, 18). Molecular weight of KL-6 is much higher than SP-A and SP-D. SP-D is much more soluble than SP-A, from which it differs by being a lipid-free form (18). Leakage of these markers may be dependent on these factors. Furthermore, serum KL-6 levels, which can be used to predict acute exacerbation and survival in IPF, were also associated with acute exacerbation and mortality in CPFE cases (8, 22, 44). None of the patients were in acute exacerbation in our study. The underlying mechanism of increased serum KL-6 levels is believed to be the over expression of KL-6 due to regenerating or injured alveolar type II cells and/or enhanced permeability of the air-blood barrier following its disintegration in affected lungs (45). Contrary to Xu L, et al., our study has found significantly higher levels of serum KL-6 in CPFE cases than IPF patients (5). In the study by Kokuho et al, serum KL-6 levels were found to be significantly higher only in the emphysema group, in comparison to the smoker controls (46). This observation is in support of another study that found higher concentrations of serum KL-6 in emphysema patients compared with healthy controls (14). In a study by Ishii H. et al, serial serum KL-6 measurements in untreated usual interstitial pneumonia (UIP) cases revealed a progressive decline and basal KL-6 levels of decreased patients were found to be significantly lower when compared with the survivors (45). In the said study, it was stressed that the decline in KL-6 levels would not necessarily rule out the possibility of disease activity, as KL-6 levels followed a heterogeneous time course and could naturally decline as the disease progressed. In complete contradiction to these findings, another study has shown that in IPF patients KL-6 levels increased, particularly after pulse steroid treatment (15). The mechanisms underlying the decline in KL-6 levels in advanced stage UIP are unclear. One hypothesis suggests that the reduction of the normal lung area, as chronic IPF progresses, leads to a gradual depletion of KL-6 producing cells, resulting in lower levels of serum KL-6 (45). Studies that revealed higher KL-6 levels in emphysema cases as opposed to healthy controls are in support of our view that this difference might be attributed to the existence of emphysema. Moreover, it should also be kept in mind that the difference observed might be completely unrelated to emphysema and due to a natural decline associated with the course of IPF, despite a lack of sufficient evidence in the literature. In order to distinguish patients with ILDs from healthy subjects and patients with lung diseases other than ILDs, the clinical cut-off value was set at 500 U/mL (positive rate 70-100%) (23). In their study, Satoh et al have stated that in ILD cases with 1000 U/mL and higher initial serum KL-6 levels disease progression would be more rapid (47). No cut-off value determined by comparing CPFE and IPF cases exists in the literature, before our study.

There were several limitations to our study. We did not measure the exact areas of emphysema and fibrosis. Therefore, our cases may have been at a different stage in comparison to previous CPFE patients. Since CPFE patients cannot tolerate invasive procedures, such as video-assisted thoracic surgery, it was not possible to compare identical numbers of biopsy proven CPFE cases to IPF cases. Nearly half of IPF cases and none of the CPFE cases were diagnosed with biopsy.

CPFE is an increasingly recognized condition. Higher KL-6 levels are important to differentiate from IPF and, also characterize the role of KL-6 in CPFE pathogenesis. Our study has shown that in

CPFE cases respiratory and functional parameters are worse than those of pure fibrosis cases. Larger series investigating the impact of emphysema and fibrosis, on progression of the disease, quality of life, respiratory functional parameters and, predictions of biomarkers are required for this heterogeneous group. CPFE is a heterogeneous disease, and may have distinct phenotypes.

Conflicts of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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