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ORIGINAL ARTICLE

Calgranulin B and KL-6 in Bronchoalveolar Lavage of Patients with IPF and NSIP

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Abstract— Idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP) are the most frequent idiopathic interstitial pneumonias. The aim of this study was to evaluate concentrations of calgranulin B and Krebs von den Lungen 6 (KL-6) in bronchoalveolar lavage (BAL) fluid of patients with IPF and idiopathic NSIP (i-NSIP) with fibrotic pattern. Thirty patients with IPF (68.73 ± 8.63 years), 30 with i-NSIP (68.33 ± 7.45 years), and healthy controls were included in the study. Calgranulin B and KL-6 both proved to be significantly higher in BAL of IPF and i-NSIP patients than in healthy controls ($p < 0.05$). Calgranulin B showed several significant correlations with functional parameters (oxygen demand at rest, 6-min walking test (6MWT), and PFTs); KL-6 was correlated with oxygen demand at rest and during 6MWT. Patients with higher concentrations of both biomarkers (> 75th percentile) had more advanced disease with lower values of FEV1%, FVC%, RV%, TLC%, DLCO% of predicted, distance walked in 6MWT, and BAL neutrophil percentage. Calgranulin B and KL-6 in BAL proved to be reliable biomarkers of IPF and i-NSIP and to have prognostic meaning, discriminating severe and advanced patients. The combination of the two biomarkers can facilitate the stratification of severity.

KEY WORDS: biomarkers; bronchoalveolar lavage; calgranulin B; KL-6; idiopathic pulmonary fibrosis; non-specific interstitial pneumonia.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP) are the most frequent entities among the idiopathic interstitial pneumonias [1]. Idiopathic pulmonary fibrosis is defined as a specific form of chronic progressive fibrosing interstitial pneumonia of unknown cause, associated with the histopathologic and/or radiological pattern of usual interstitial pneumonia [2]. Non-specific interstitial pneumonia can present with two main variants: the fibrotic form or the cellular form dominated by inflammation, CT evidence of ground glass pattern, and histological evidence of abundant cell infiltration. It can be idiopathic (i-NSIP), an expression of lung involvement in systemic diseases (particularly connective tissue diseases) or due to exposure to exogenous factors [1, 3]. Prognosis is usually better than for IPF; however, the fibrotic form of NSIP can also be very aggressive [1].

Several biomarkers in peripheral blood or bronchoalveolar lavage (BAL) fluid have been proposed in

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patients with idiopathic interstitial pneumonias [4]; however, at the moment, there are no biomarkers universally recognized in clinical practice. Krebs von den Lungen 6 (KL-6) (mucin 1), a glycoprotein encoded by the MUC1 gene and expressed on the outer surface of alveolar epithelial type II cells and airway epithelial cells, is currently considered a promising biomarker of IPF [5]. Its precise function is unknown, but it is believed to promote fibroblast migration, proliferation, and survival in the lungs [5]. High levels of serum KL-6 have been found in patients with IPF, NSIP, and other interstitial lung diseases [5–8], correlated with aggressive clinical phenotypes and predicting episodes of acute exacerbation [9, 10]. High levels of KL-6 have also been found in BAL fluid of patients with different interstitial lung diseases, including IPF [11].

Calgranulin B (S100A9/MRP14) is a small calcium-binding protein with various immunological functions, mainly involved in chronic inflammation [12]. It can contribute to neutrophil recruitment, oxidant-antioxidant balance, neutrophil adhesion to fibronectin, and apoptosis regulation [12]. Higher concentrations of calgranulin B have been reported in BAL from IPF patients than from patients with other interstitial lung diseases (*e.g.*, sarcoidosis, NSIP, pulmonary fibrosis associated with connective tissue diseases) and its possible use as a biomarker with clinical significance is intriguing [12–15].

The aim of the present study was to evaluate the utility of Calgranulin B, in combination to KL-6, in BAL fluid as clinical biomarkers of IPF and fibrotic i-NSIP, with particular regard to disease severity stratification and clinical behavior.

MATERIALS AND METHODS

Population

The present exploratory study included 60 patients, 30 with IPF (24 males, age 68.73 ± 8.63 years) and 30 with idiopathic NSIP (fibrotic pattern) (17 males, age 68.33 ± 7.45 years), and 14 healthy controls (11 males, age 61.36 ± 15.20 years). The study was conducted at the Regional Referral Centre for Sarcoidosis and other Interstitial Lung Diseases, Respiratory Diseases and Lung Transplant Unit, Department of Medical, Surgical and Neurological Sciences, University Hospital of Siena, Italy. The local ethics committee approved the study (180712) and patients and controls gave written informed consent to participation.

Diagnosis of IPF and i-NSIP was according to ATS guidelines [1–3]. A multidisciplinary team including at least two pulmonologists, a radiologist, and a pathologist, all experts in interstitial lung diseases, reviewed the data. All clinical, radiological, and functional data and bronchoalveolar lavage (BAL) samples were gathered from the time of diagnosis; the patients enrolled had never been treated with steroids or other immunosuppressants or antifibrotic drugs. Demographic data, smoking history, respiratory functional data [pulmonary function tests (PFTs), diffusing capacity of the lung for carbon monoxide (DLCO), arterial blood gas analysis (BGA), 6-min walking test (6MWT)], and comorbidities at time of diagnosis were collected. PFT data was also collected and analyzed at 6-, 12-, and 18-month follow-up. PFTs and 6MWT were performed according to the corresponding guidelines [16, 17].

Bronchoalveolar Lavage

BAL was performed for diagnostic reasons according to specific ATS guidelines [18]. Differential cell count and lymphocyte phenotype were performed in all patients. BAL was obtained and analyzed as previously described [12].

Biomarker Determination

Concentrations of calgranulin B (also known as S100A9 or MRP-14) in BAL supernatant were determined for patients and controls with a commercial ELISA kit (Promokine Human MRP14 ELISA) and expressed in nanogram per milliliter of recovered BAL fluid. A modified sandwich enzyme immunoassay (EIA) kit was used to assay KL-6 concentrations (MICRO Cup-TYPE ENZYME Immunoassay Test Kit). Samples were diluted 1:10 to bring the signal (OD) into the range of the method; results are expressed in international units per milliliter of recovered BAL fluid.

Statistical Analysis

Parametric statistics was applied in the present study. *T* test was utilized to compare two groups, for three or more groups, the ANOVA analysis test (Holm-Sidak's test was applied for multiple comparisons) was used. Correlations were studied through the Pearson's (r) test. Significance was set at $p < 0.05$. All data was expressed as mean ± standard deviation. The statistical analysis softwares used were StatSoft (2001) and Graph Pad Prism 4.0.

RESULTS

Demographic characteristics, BAL data, and biomarker concentrations in BAL of patients and healthy controls are reported in Table 1. Patients with IPF were predominantly male (80%) and most had a history of cigarette smoking (60%); 63.3% of i-NSIP patients were smokers or former smokers; 56.6% of patients were male and 43.7% female. No statistically significant differences in demographic characteristics or smoking history were found between controls and IPF or i-NSIP patients (Table 1).

Pulmonary function tests at baseline generally showed moderate restrictive ventilatory defects and a moderate-to-severe reduction in DLCO with no significant differences between the IPF and i-NSIP groups. In the i-NSIP group, significant sparing of residual volume was noted with respect to IPF patients with the same degree of restriction ($p = 0.047$) (Table 1). There were no significant differences in 6MWT and BGA parameters between the two groups (Supplementary Material, Table 1 SM).

Patients with IPF and i-NSIP showed a significantly lower percentage of macrophages in BAL than healthy controls ($p = 0.004$); no other significant differences were found except for an increasing trend of neutrophil and eosinophil percentages in both groups of patients compared with controls (the latter reached significance between i-NSIP and controls, $p = 0.047$) (Table 1).

BAL Calgranulin B and KL-6 Analysis

Significantly higher concentrations of calgranulin B and KL-6 were found in BAL of IPF and i-NSIP patients with respect to controls ($p = 0.0005$ and $p = 0.002$, respectively) (Table 1, Figs. 1 and 2). The concentrations of the two biomarkers were not significantly different in the two groups of patients, nor were any differences in levels of

Table 1. Demographic Data, Smoking History, BAL Parameters, Baseline Pulmonary Functional Test (PFT) Data, and Calgranulin B and KL-6 Concentrations in BAL of Patients with IPF and i-NSIP *Versus* Healthy Controls

	IPF	i-NSIP	CONTROLS	*p*
N	30	30	14	
Age (years)	68.73 ± 8.63	68.33 ± 7.45	61.36 ± 15.20	$p = 0.052$; *$p = 0.87$; #p = 0.066; ^$p = 0.066$
Gender, males (%)	24 (80%)	17 (56%)	11 (78.5%)	$p = 0.10$
Smoke history (*n*):				
Yes	N 18	N 19	N 8	$p = 0.91$
No	N 12	N 11	N 6	
Pack/year:				
Current (P/Y)	N 2 (48.50 ± 16.26)	3 (33.33 ± 5.77)	4 (30.15 ± 5.34)	p = 0.10; *$p = 0.17$; #$p = 0.12$; ^$p = 0.63$
Former (P/Y)	N 16 (33.0 ± 23.65)	16 (24.06 ± 21.62)	4 (26.24 ± 12.34)	$p = 0.51$;*$p = 0.58$; #$p = 0.82$; ^$p = 0.85$
Biomarker concentration in BAL				
• Calgranulin B (ng/ml)	49.69 ± 15.46	54.14 ± 21.45	28.12 ± 23.64	$p = 0.0005$; *$p = 0.67$; #$p = 0.001$; ^$p = 0.0006$
• KL-6 (U/ml)	721.1 ± 481.7	775.36 ± 410.22	309.24 ± 220.02	$p = 0.002$; *$p = 0.61$; #$p = 0.006$; ^$p = 0.003$
BAL				
• Cells/ml	135,740.90 ± 63, 956.42	122,736.67 ± 84, 887.64	115,016.66 ± 56, 465.26	$p = 0.63$; *p = 0.75; #$p = 0.75$; ^$p = 0.75$
• Macrophages %	69.4 ± 17.72	63.23 ± 24.61	83.16 ± 8.61	$p = 0.004$; *$p = 0.19$; #$p = 0.042$; ^$p = 0.003$
• Lymphocytes %	10.71 ± 7.59	9.37 ± 8.84	10.50 ± 7.91	$p = 0.80$; *$p = 0.89$; #$p = 0.93$; ^$p = 0.89$
• Neutrophils %	10.33 ± 8.39	9.82 ± 10.26	4.83 ± 4.44	$p = 0.13$; *$p = 0.82$; #p = 0.15; ^$p = 0.15$
• Eosinophils %	5.06 ± 5.61	6.07 ± 6.88	1.50 ± 1.76	$p = 0.005$; *$p = 0.49$; #$p = 0.11$; ^$p = 0.047$
• CD4/CD8 lymphocytes	2.55 ± 2.12	2.30 ± 2.85	2.35 ± 0.13	$p = 0.9$; *$p = 0.9$; #$p = 0.9$; ^$p = 0.9$
Baseline PFT (% pred.)				
• FEV1 (%)	82.07 ± 21.46	77.67 ± 19.9	–	$p = 0.41$
• FVC (%)	79.63 ± 22.11	76.69 ± 20.17	–	$p = 0.59$
• FEV1/VC (%)	80.61 ± 6.54	80.92 ± 7.18	–	$p = 0.86$
• RV (%)	81.62 ± 23.35	99.77 ± 43.13	–	$p = 0.03$
• TLC (%)	77 ± 18.77	84.08 ± 23.93	–	$p = 0.20$
• DLCO (%)	44.42 ± 15.53	50.44 ± 19.95	–	$p = 0.20$

*p value for IPF *vs.* i-NSIP; #p value for IPF *vs.* controls; ^p value for i-NSIP *vs.* controls

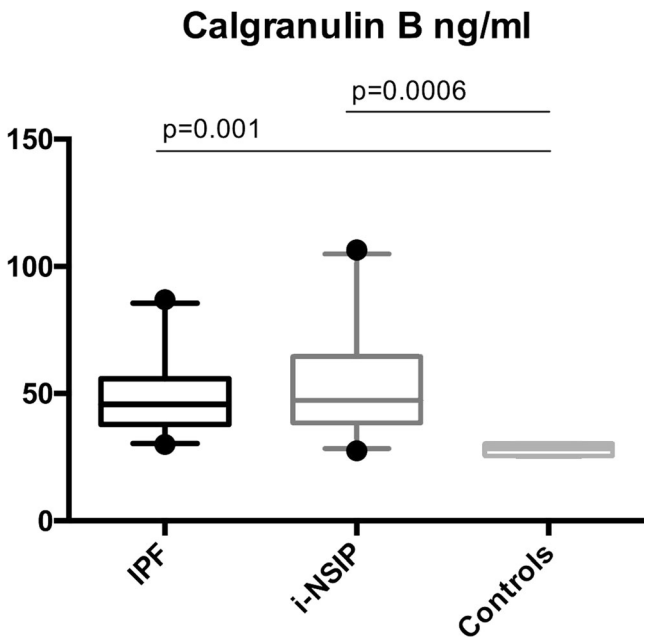

Fig. 1. Concentrations of calgranulin B (ng/ml, boxplot, median, and 5–95° percentile) in BAL of IPF and i-NSIP patients and healthy controls.

calgranulin B and KL-6 found in relation to smoking habits, age, or gender.

We found significantly higher concentrations of calgranulin B and KL-6 in the pool of IPF and i-NSIP patients with advanced disease exhibiting chronic respiratory failure ($n = 15$) than in patients without respiratory failure (calgranulin B 66.18 ± 23.52 *vs.* 48.16 ± 15.92 ng/ml, $p = 0.003$; difference between means -18.02 ± 5.91 ng/ml

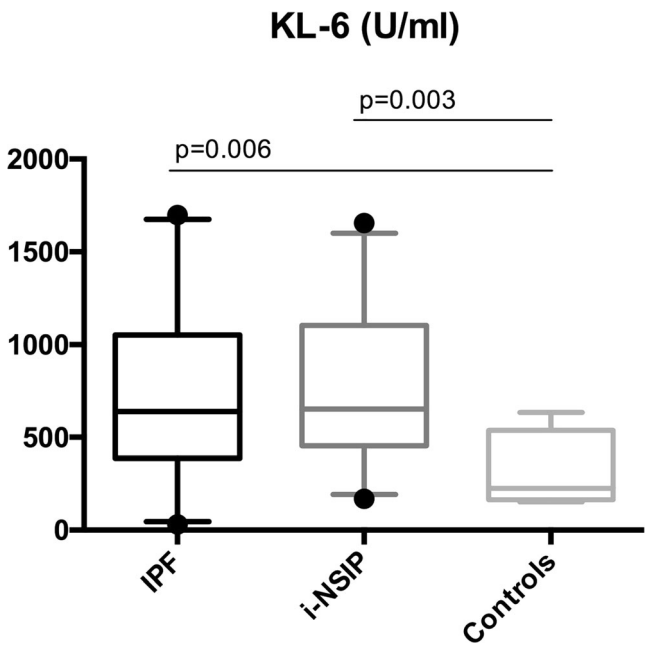

Fig. 2. Concentrations of KL-6 (IU/ml, boxplot, median, and 5–95° percentile) in BAL fluid of IPF and i-NSIP patients and healthy controls.

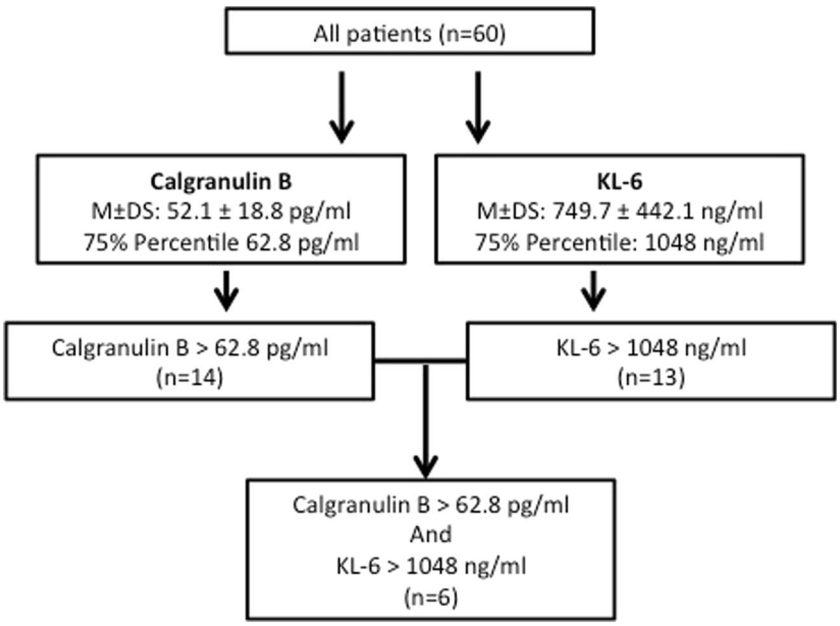

Fig. 3. Population of patients (IPF + i-NSIP) with calgranulin B and KL-6 concentrations in BAL fluid above 75th percentile.

(95% CI − 29.87; − 6.17); KL-61022.0 ± 672.80 *vs.* 522.2 ± 397.6 U/ml, $p = 0.017$; difference between means − 346.5 ± 142.4 U/ml (95% CI − 635.0; − 63.83), respectively).

Moreover, when we divided the entire population (IPF + i-NSIP) according to specific cutoffs for each biomarker, *i.e.*, 75th percentile (1048 IU/ml for KL-6 and 62.8 ng/ml for calgranulin B) (Fig. 3), we observed significantly lower values of FEV1%, FVC%, RV%, TLC%, and DLCO% of predicted, a higher percentage of neutrophils in BAL and a higher prevalence of chronic respiratory failure in patients showing simultaneous over-expression (> 75th percentile) of both calgranulin B and KL-6 ($n = 6$) (Table 2).

Correlations with Clinical, BAL, and Respiratory Function Data

Calgranulin B and KL-6 showed a direct statistically significant correlation in both groups of patients. In IPF patients, calgranulin B showed significant inverse correlations with deterioration of FVC, TLC, RV, and DLCO at 12-month follow-up (% of decrement from baseline); among i-NSIP patients, it was inversely correlated with baseline FEV1%, FVC%, TLC%, RV%, DLCO%, and KCO% of predicted, O_2 demand at rest and PaO_2 at rest, and with final SpO_2, distance walked at 6MWT and neutrophil and eosinophil

Table 2. Variables Found Significantly Different in the Pool of Patients (IPF + i-NSIP) Divided According to Calgranulin B and KL-6 Concentrations in BAL Above or Below 75th Percentile

	Calgranulin B and KL-6 < 75th percentile	Calgranulin B and KL-6 > 75th percentile	*p*
FEV1 (% pred.)	83.5 ± 19.6	59.7 ± 16.3	0.007
FVC (% pred.)	81.8 ± 19.6	56.3 ± 14.8	0.002
RV (% pred.)	93.7 ± 35.5	62.2 ± 19.5	0.005
TLC (% pred.)	83.8 ± 19.5	56.7 ± 13.1	0.03
DLCO (% pred.)	48.8 ± 17.0	29.3 ± 9.5	0.05
Walked distance 6MWT (meters)	288.9 ± 131.3	200.0 ± 75.3	0.05
CRF prevalence	22.7%	66.6%	0.02
BAL neutrophils %	10.6 ± 12.9	26.8 ± 24.5	0.05

FEV1 forced expiratory volume, *FVC* forced vital capacity, *RV* residual volume, *TLC* total lung capacity, *DLCO* diffusing lung capacity for carbon monoxide, *CRF* chronic respiratory failure, *6MWT* 6-min walking distance

Table 3. Significant Correlations Between Concentrations of Calgranulin B and KL-6 in BAL Fluid of IPF and i-NSIP Patients Together with the Corresponding Statistics

IPF ($n = 30$)		
• KL-6 (ng/ml)	Calgranulin B	$r = 0.41$; $p = 0.03$
• FVC worsening at 12-month follow-up (% of decrement from baseline)	Calgranulin B	$r = -0.44$; $p < 0.05$
• TLC worsening at 12-month follow-up (% of decrement from baseline)	Calgranulin B	$r = -0.66$; $p < 0.01$
• RV worsening at 12-month follow-up (% of decrement from baseline)	Calgranulin B	$r = -0.57$; $p = 0.01$
• DLCO worsening at 12-month follow-up (% of decrement from baseline)	Calgranulin B	$r = -0.54$; $p < 0.05$
• FiO2 at rest (%)	KL-6	$r = 0.55$; $p < 0.01$
• O2 at 6MWT (flow l/min)	KL-6	$r = 0.62$; $p < 0.001$
• Distance walked at 6MWT (meters)	KL-6	$r = -0.41$; $p < 0.05$
i-NSIP ($n = 30$)		
• KL-6 (ng/ml)	Calgranulin B	$r = 0.52$; $p = 0.003$
• Baseline FEV1% pred.	Calgranulin B	$r = -0.44$; $p = 0.001$
• Baseline TLC% pred.	Calgranulin B	$r = -0.54$; $p < 0.05$
• Baseline RV% pred.	Calgranulin B	$r = -0.54$; $p < 0.01$
• Baseline DLCO% pred.	Calgranulin B	$r = -0.51$; $p < 0.001$
• Baseline KCO% pred.	Calgranulin B	$r = -0.46$; $p < 0.05$
• Oxygen-need at rest (flow l/min)	Calgranulin B	$r = -0.424$, $p = 0.02$
• PaO2 at rest (mmHg)	Calgranulin B	$r = -0.55$; $p = 0.01$
• Final heart rate at the 6MWT (bpm)	Calgranulin B	$r = 0.377$, $p = 0.04$
• Final SpO2% at the 6MWT	Calgranulin B	$r = -0.424$, $p = 0.02$
• Distance walked at the 6MWT (meters)	Calgranulin B	$r = -0.37$, $p = 0.04$
• Neutrophils % BAL	Calgranulin B	$r = 0.56$; $p < 0.01$
• Lymphocytes % BAL	Calgranulin B	$r = -0.42$; $p < 0.05$
• Eosinophils % BAL	Calgranulin B	$r = 0.45$; $p < 0.01$
• Baseline FEV1% pred.	KL-6	$r = -0.43$; $p < 0.05$
• Baseline FVC% pred.	KL-6	$r = -0.43$; $p = 0.01$
• Baseline TLC% pred.	KL-6	$r = -0.46$, $p = 0.015$
• Distance walked at the 6MWT (meters)	KL-6	$r = -0.469$, $p = 0.01$
• Final SpO2% at the 6MWT	KL-6	$r = -0.51$; $p < 0.05$

percentages in BAL, and directly with final heart rate in 6MWT and macrophage and lymphocyte percentages in BAL.

In IPF patients, KL-6 showed significant direct correlations with FiO_2 at rest and oxygen demand during 6MWT, and an inverse correlation with distance walked (meters) in 6MWT. In i-NSIP patients, KL-6 was inversely correlated with baseline FEV1%, FVC%, and TLC% of predicted, with final SpO_2 and distance walked at 6MWT. Table 3 shows all the correlations found.

DISCUSSION

Calgranulin B is a small calcium-binding protein with various immunological functions. It is mainly involved in chronic inflammation, particularly in neutrophil activity, and has also been implicated in the regulation of apoptosis [12]. Its involvement as a mediator of tumor cell aggressiveness in the development of several types of cancer was recently described [19]. Our research group first suggested a role of calgranulin B in IPF [12]. The molecule was found upregulated in BAL in proteomic studies and later confirmed by immunohistochemistry in lung tissue of patients with IPF [12, 13].

Calgranulin B in BAL of patients with IPF may be released by activated neutrophils and macrophages; by interacting with endothelial surface, it may participate to phagocytes adhesion and tissue migration thus contributing to fibrotic process [12].

In the present study, we analyzed concentrations of calgranulin B and KL-6 in BAL fluid of patients with IPF and i-NSIP for insights into their role as clinical biomarkers in these diseases and their potential use in combination. We found that both molecules occurred in higher concentration in patients with IPF and i-NSIP than in healthy controls and were directly correlated with each other.

Significant correlations between calgranulin B and several functional parameters (FEV1%, TLC%, DLCO% of predicted, PaO_2, and FiO_2 at rest) are described here for the first time, confirming the hypothesis that its levels are correlated with disease

severity. Calgranulin B was also significantly higher in advanced IPF and i-NSIP patients with chronic respiratory failure requiring long-term oxygen-therapy; an indirect correlation with deterioration of DLCO at 18-month follow-up was also found in i-NSIP patients.

Various authors have described higher levels of KL-6 in serum and BAL of patients with IPF [5, 9–11]. In the present study, we confirmed that KL-6 concentrations are higher in BAL of patients with pulmonary fibrosis (IPF and i-NSIP) than in healthy controls, and were correlated with several parameters of disease severity, including BAL neutrophils, DLCO, and the need for oxygen-therapy. Data on the use of KL-6 in the differential diagnosis of IPF and other interstitial lung diseases (ILDs) is controversial [4]. Almost all the data in the literature on KL-6 is based on serum levels, increases in which have been described not only in IPF, but also in fibrotic NSIP and other ILDs [4, 6–8]. Zhu et al. [11] also recently reported an increase in KL-6 in BAL of patients with various ILDs, including IPF. In our study, KL-6 and calgranulin B were significantly higher in IPF and i-NSIP patients than in healthy controls, but did not differ between the two diseases. The patients enrolled in the study were carefully selected: in the case of i-NSIP, only patients with a fibrotic pattern were included and we were careful to exclude patients with cellular NSIP. This may explain why the two groups of patients had overlapping clinical, functional, and BAL characteristics. A French multicenter study showed idiopathic NSIP survival rates comparable to those commonly reported in IPF [20]. These aspects may be why similar concentrations of the two biomarkers (KL-6 and calgranulin B) were found in BAL of IPF and i-NSIP patients. However, the specificity of these two biomarkers in different ILDs needs further investigation.

Ten percent of our patients showed simultaneous overexpression of both calgranulin B and KL-6 (> 75th percentile) and significant correlations with advanced disease stages. The combined use of panels of biomarkers can be useful in a clinical setting, facilitating stratification of severity and identifying patients with poor prognosis requiring urgent therapy or assessment for lung transplant [21].

The relatively small number of patients enrolled in the study was its main limit. Further studies are needed to validate our results more generally and for a complete understanding of their utility in routine clinical practice.

In conclusion, calgranulin B concentrations in BAL are confirmed to be a reliable biomarker in patients with IPF and i-NSIP, being correlated with major prognostic variables and discriminating patients with severe or advanced disease. Their combination with concentrations of KL-6 in BAL facilitates the stratification of severity. The next objective of our research will be to evaluate the role that these two biomarkers can play in identifying patients who respond to new antifibrotic therapies.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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