

Multiplex protein profiling of bronchoalveolar lavage in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis

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Abstract:

CONTEXT: Idiopathic pulmonary fibrosis (IPF) and chronic hypersensitivity pneumonitis (HP) are diffuse parenchymal lung diseases characterized by a mixture of inflammation and fibrosis, leading to lung destruction and finally death.

AIMS: The aim of this study was to compare different pathophysiological mechanisms, such as angiogenesis, coagulation, fibrosis, tissue repair, inflammation, epithelial damage, oxidative stress, and matrix remodeling, in both disorders using bronchoalveolar lavage (BAL).

METHODS: At diagnosis, patients underwent bronchoscopy with BAL and were divided into three groups: Control ($n = 10$), HP ($n = 11$), and IPF ($n = 11$), based on multidisciplinary approach (clinical examination, radiology, and histology): Multiplex searchlight technology was used to analyze 25 proteins representative for different pathophysiological processes: Eotaxin, basic fibroblast growth factor (FGFb), fibronectin, hepatocyte growth factor (HGF), interleukine (IL)-8, IL-12p40, IL-17, IL-23, monocyte chemotactic protein (MCP-1), macrophage-derived chemokine (MDC), myeloperoxidase (MPO), matrix metalloproteinase (MMP)-8, MMP-9, active plasminogen activating inhibitor 1 (PAI-1), pulmonary activation regulated chemokine (PARC), placental growth factor (PIGF), protein-C, receptor for advanced glycation end products (RAGE), regulated on activation normal T cells expressed and secreted (RANTES), surfactant protein-C (SP-C), transforming growth factor- β 1 (TGF- β 1), tissue inhibitor of metalloproteinase-1 (TIMP-1), tissue factor, thymic stromal lymphopoietin (TSLP), and vascular endothelial growth factor (VEGF).

RESULTS: All patients suffered from decreased pulmonary function and abnormal BAL cell differential compared with control. Protein levels were increased in both IPF and HP for MMP-8 ($P = 0.022$), MMP-9 ($P = 0.0020$), MCP-1 ($P = 0.0006$), MDC ($P = 0.0048$), IL-8 ($P = 0.013$), MPO ($P = 0.019$), and protein-C ($P = 0.0087$), whereas VEGF was decreased ($P = 0.0003$) compared with control. HGF was upregulated in HP ($P = 0.0089$) and active PAI-1 was upregulated ($P = 0.019$) in IPF compared with control. Differences in expression between IPF and HP were observed for IL-12p40 ($P = 0.0093$) and TGF- β 1 ($P = 0.0045$).

CONCLUSIONS: Using BAL, we demonstrated not only expected similarities but also important differences in both disorders, many related to the innate immunity. These findings provide new clues for further research in both disorders.

Key words:

Bronchoalveolar lavage, enzyme-linked immunosorbent assay, hypersensitivity pneumonitis, interstitial lung disease, idiopathic pulmonary fibrosis

Diffuse parenchymal lung diseases (DPLDs) are a group of more than 200 diverse chronic pulmonary disorders characterized by progressive fibrosis leading to destruction of lung architecture resulting in respiratory failure and finally death. Being mostly non-specific, distinguishing between different DPLDs is difficult. The classification is often based on differences in etiology with IPF being the most common of DPLDs of unknown origin and chronic HP is an important representative for DPLDs of known cause.^[1]

Both IPF and chronic HP lead to end-stage pulmonary fibrosis with excessive collagen deposition, extracellular matrix deposition, and destruction of lung architecture, leading to respiratory failure.^[2] Although they share similarities in clinical presentation, prognosis and treatment are different, indicating the importance to differentiate between these disorders for which BAL may be used.^[3,4]

The pathophysiological mechanism of HP is accepted to be a hypersensitivity reaction,

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induced by a T-cell-mediated reaction after sensitization with antigens such as organic dusts, bacteria, and molds,^[5] but the exact mechanisms are unresolved. Despite intensive research, the pathophysiological mechanisms of IPF remain enigmatic. Complicating factors are (1) the majority of current knowledge is based on the mouse model of bleomycin-induced fibrosis, which is an useful model but not specific for IPF, (2) the late diagnosis of IPF precludes the ability to study early mechanisms and etiology, (3) the discrepancy between clinical, histological, and radiological findings for diagnosis, and (4) the scarcity of the disease.

In the pathogenesis of pulmonary fibrosis, many contributing mechanisms have been described, including angiogenesis, coagulation, fibrogenesis, tissue repair, inflammation, epithelial damage, matrix remodeling, and oxidative stress. As both disorders progress toward end-stage parenchymal lung fibrosis, an important question is whether similar mechanisms drive this fibrosis. BAL represents the closest sampling tool of the lung besides biopsies and the earliest time point possible to research these disorders. Therefore, our aim was to study the differences and similarities in mechanistic pathways involved in IPF and chronic HP using BAL fluid. For this study, we focused on 25 proteins related to different mechanisms using multiplex SearchLight[®] Assay System. Its use helps to provide new information to unravel mechanisms triggering pulmonary fibrosis.^[3]

Methods

Study design

This study was approved by the Ethics Committee (S51293) and Biosafety (MS20101568) of the University Hospital UZ Leuven, Belgium. At the time of diagnosis, patients underwent spirometry tests and bronchoscopy with BAL as part of the diagnostic work-up. The total lung capacity (TLC), forced vital capacity (FVC), forced expiratory volume in 1 s (FEV₁), and diffusing capacity (DL_{CO}) were measured according to international guidelines.^[6] During bronchoscopic evaluation, BAL was performed following standard guidelines.^[7,8] BAL consisted of four aliquots of sterile saline (50 ml) instilled in the right middle lobe or lingula; returned fraction 1 was used for microbiological evaluation, fractions 2, 3 and 4 were pooled and used to assess cellular differentiation, BAL return and protein analysis.^[9]

After multidisciplinary consultation, patients with IPF ($n = 11$) and HP ($n = 11$) were selected and compared with a control group ($n = 10$) without evidence for a pulmonary disease. BAL samples were analyzed by means of multiplex searchlight technology to unravel different pathological mechanisms.

Patient selection

Patients were diagnosed within a multidisciplinary consensus as suggested by international guidelines. IPF patient diagnosis was based on clinical, radiological, and/or pathological data following international guidelines.^[10] Patients were diagnosed with chronic HP based on their clinical course and insidious onset over a period of months, history of antigen exposure, radiological, and/or histopathological data.^[11,12] Both disorders showed the presence of fibrosis in radiological data. For the control group, history was recorded and BAL, biopsy, and

radiological imaging were performed to exclude pulmonary illness/fibrosis (e.g., esophageal cancer, connective tissue disease without pulmonary affection). Medical history provided information regarding age, gender, and smoking history (pack years and amount of current-/ex-/non-smokers). Plasma C-reactive protein (CRP) levels were measured to assess systemic inflammation.^[13] The selection was refined by excluding patients with lack of clinical data, no full pulmonary function data, lack of sufficient BAL sample, and highly increased CRP levels (>20 mg/l). Finally, 10 controls, 11 IPF, and 11 HP patients were included.

Bronchoalveolar lavage protein measurement

Protein expression was analyzed in undiluted or 1:10 diluted BAL supernatant using the custom multiplex SearchLight[®] Assay System (Aushon, Billerica, MA, USA). Twenty-five proteins based on the different pathophysiological mechanisms were selected: Eotaxin, basic fibroblast growth factor (FGFb), fibronectin, HGF, IL-8, IL-12p40, IL-17, IL-23, MCP-1, MDC, MPO, MMP-8, MMP-9, active PAI-1, PARC, PIGF, Protein-C, RAGE, RANTES, SP-C, TGF- β 1, TIMP-1, tissue factor, TSLP, and VEGF (for abbreviations, see Table 1). If concentration was below detection limit, a value of 50% of the detection limit was attributed (detection limits: FGFb = 2.0 pg/ml; IL-12p40 = 0.6 pg/ml; IL-17 = 0.8 pg/ml; IL-23 = 19.5 pg/ml; RANTES = 0.4 pg/ml; SP-C = 65.4 pg/ml; TGF- β 1 = 19.5 pg/ml; and TSLP = 2.4 pg/ml).

Analysis

Results are expressed as median (interquartile range [IQR]). Significances among the groups were tested by Kruskal–Wallis one-way analysis of variance (ANOVA) in combination with Mann–Whitney U test. Correlation analysis using the Spearman rank test was performed on both HP and control and on both the IPF and control. Statistical analysis was performed using Prism 4.1 software (GraphPad, San Diego, CA, USA). Results were considered significant if $P < 0.05$.

Table 1: Abbreviations for factors analyzed in bronchoalveolar lavage fluid

Basic fibroblast growth factor	FGFb
Hepatocyte growth factor	HGF
Interleukine-12p40	IL-12p40
Interleukine-17	IL-17
Interleukine-23	IL-23
Monocyte chemotactic protein-1	MCP-1
Macrophage-derived chemokine	MDC
Matrix Metalloproteinase-8	MMP-8
Matrix Metalloproteinase-9	MMP-9
Myeloperoxidase	MPO
Plasminogen activating inhibitor-1 Active	active PAI-1
Pulmonary activation regulated chemokine	PARC
Placental growth factor	PIGF
Receptor for advanced glycation end products	RAGE
Regulated on activation normal T cells expressed and secreted	RANTES
Surfactant protein-C	SP-C
Transforming growth factor- β 1	TGF- β 1
Tissue inhibitor of metalloproteinase-1	TIMP-1
Thymic stromal lymphopoietin	TSLP
Vascular endothelial growth factor	VEGF

Results

Patient characteristics

Patient characteristics are summarized in Table 2. No significant differences were observed for age, sex, smoking history, plasma CRP level, or BAL return fraction between the control, IPF, and HP groups.

Pulmonary function and bronchoalveolar lavage cell differentiation

Pulmonary function measurements are described in Table 2 and BAL cell differentiation in Table 3. Both IPF and HP patients showed significant decrease in pulmonary function parameters (TLC, FVC, FEV₁, and DL_{CO}) compared with the

control group. No differences were found between IPF and HP patients.

In IPF, an increase in percentage and total number of neutrophils ($P = 0.0025$; $P = 0.0017$) and eosinophils ($P = 0.0002$; $P = 0.0002$) was observed compared with the control group.

In HP, total white blood cell number was significantly increased compared with the control group ($P = 0.0067$). The number of macrophages did not change but a decrease in their percentage ($P = 0.0167$) was observed. An increase in percentage and total number of lymphocytes ($P = 0.0412$; $P = 0.0067$), neutrophils ($P = 0.0017$; $P = 0.0008$), and eosinophils ($P = 0.0002$; $P = 0.0002$) was observed compared with control.

When comparing IPF with HP, an increase in percentage of lymphocytes ($P = 0.00216$) was observed in HP.

Table 2: Patient characteristics

	Control	IPF	HP	ANOVA
Characteristics				
N	10	11	11	
Age (Years)	45 (41-75)	65 (61-72)	57 (54-72)	0.16
Gender (Females)	5	2	5	0.27
Number of current-/ex-/non-smokers	0/2/8	0/4/7	0/5/6	0.48
Smoking pack years	0 (0-0)	5 (0-20)	0 (0-25)	0.25
C-reactive protein (mg/l)	3 (1-15) <i>n</i> =7	3 (1-5) <i>n</i> =10	5 (2-8) <i>n</i> =9	0.49
BAL return (ml)	132 (96-145)	109 (97-136)	103 (90-118)	0.19
Pulmonary function (% of predicted value)				
TLC	91 (81-103)	61 (60-72)***	68 (48-80)**	0.0012
FEV ₁	95 (82-114)	81 (65-84)*	69 (47-97)*	0.023
FVC	97 (89-114)	75 (66-85)**	73 (54-98)**	0.0049
DL _{CO}	70 (68-77)	46 (39-50)**	46 (37-51)*	0.0044

The differences in patient characteristics and pulmonary function, shown as median (IQR), between the control, IPF and HP groups were calculated with Kruskal–Wallis analysis of variance with Mann–Whitney U-test. Significances: Compared with control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IPF = Idiopathic pulmonary fibrosis, HP = Hypersensitivity pneumonitis, BAL = Bronchoalveolar lavage, TLC = Total lung capacity, FEV₁ = Forced expiratory volume in 1 s, DL_{CO} = Diffusing capacity of carbon monoxide

Bronchoalveolar lavage protein measurement

Protein measurements are summarized in Table 4. ANOVA statistics were not significant for RAGE, SP-C, TIMP-1, fibronectin, eotaxin, IL-17A, IL-23, PARC, RANTES, TSLP, PlGF, FGFb, and tissue factor and will therefore not be further discussed.

When compared with the control group, an increase in MMP-8 ($P = 0.038$; $P = 0.0083$), MMP-9 ($P = 0.010$; $P = 0.0006$), MCP-1 ($P = 0.0011$; $P = 0.0014$), MDC ($P = 0.0044$; $P = 0.018$), IL-8 ($P = 0.018$; $P = 0.0083$), MPO ($P = 0.015$; $P = 0.022$), and Protein-C ($P = 0.045$; $P = 0.0054$) was observed, whereas VEGF was decreased ($P = 0.0014$; $P = 0.0005$) in both disorders.

In IPF, active PAI-1 was increased ($P = 0.0022$), a trend toward increase for HGF ($P = 0.053$) and a decrease for IL-12p40 ($P = 0.072$) was found compared with control.

In HP, compared with control, HGF was significantly upregulated ($P = 0.0022$) and a trend toward increase for active PAI-1 ($P = 0.091$) and decrease for TGF-β1 ($P = 0.057$) was found.

In HP, compared with IPF, TGF-β1 ($P = 0.0045$) was decreased and IL-12p40 ($P = 0.072$) was increased.

Table 3: Cellular differentiation of bronchoalveolar lavage fluid

	Control	IPF	HP	ANOVA
Total cell number (×10 ³ /ml)	91 (58-156)	173 (62-392)	169 (140-232)**	0.038
Macrophages				
%	90 (82-94)	84 (77-88)	56 (35-89)*	0.016
Number (×10 ³ /ml)	79 (49-135)	120 (60-301)	111 (68-135)	0.28
Lymphocytes				
%	7.7 (3.9-13.2)	10.4 (2.0-14.6)	28.0 (4.4-44.0)* ^o	0.036
Number (×10 ³ /ml)	5.0 (3.9-14.1)	9.0 (5.7-30.4)	56.8 (12.3-81.1)** ^o	0.0083
Neutrophils				
%	0.8 (0.2-1.7)	3.6 (1.6-6.8)**	6.6 (2.6-15.0)**	0.0011
Number (×10 ³ /ml)	0.9 (0.1-1.2)	7.3 (8.0-18.4)**	15.3 (3.6-25.4)***	0.0006
Eosinophils				
%	0.0 (0.0-0.2)	1.8 (1.2-2.6)***	1.2 (0.8-2.4)**	$P < 0.0001$
Number (×10 ³ /ml)	0.0 (0.0-0.1)	2.9 (0.9-11.0)***	2.3 (1.1-4.4)***	$P < 0.0001$

BAL cellular differentiation, shown as median (IQR), between the control, IPF and HP groups were calculated with Kruskal–Wallis analysis of variance with Mann–Whitney U-test as post-hoc test. Significances: Compared with control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with IPF: ^o $P < 0.05$, ^{oo} $P < 0.01$. IPF = Idiopathic pulmonary fibrosis, HP = Hypersensitivity pneumonitis

Table 4: Protein profile in bronchoalveolar lavage fluid

	Control	IPF	HP	ANOVA
Epithelial damage				
RAGE (×10 ³)	1.0 (0.4-1.9)	0.6 (0.6-0.9)	1.0 (0.7-2.1)	0.31
Matrix remodeling				
MMP-8 (×10 ³)	4.5 (1.3-8.9)	11.0 (6.3-22.6)*	10.9 (4.8-13.8)*	0.022
MMP-9 (×10 ³)	4.9 (1.2-9.2)	17.3 (6.5-47.0)*	19.2 (10.8-56.2)**	0.002
SP-C	64.8 (32.7-128.9)	85.1 (51.5-187.8)	32.7 (31.5-32.7)	0.096
TIMP-1 (×10 ³)	6.2 (3.8-8.4)	9.6 (5.8-18.9)	10.6 (5.9-17.8)	0.096
Tissue repair				
HGF	219.3 (131.1-376.6)	318.6 (275.9-1172.0)	513.8 (392.3-661.9)**	0.0089
Fibrogenesis				
TGF-β1	58.2 (9.8-107.4)	54.3 (18.0-104.2)	9.8 (9.8-15.2) ^{oo}	0.012
Fibronectin (×10 ³)	112.7 (41.9-254.6)	250.2 (70.3-406.9)	231.2 (83.8-478.5)	0.25
Inflammation				
MCP-1	51.8 (33.0-107.5)	294.1 (109.6-1307.0)**	187.7 (133.0-578.2)**	0.0006
MDC	2.9 (1.1-6.5)	15.6 (9.0-42.5)**	7.7 (4.9-15.8)*	0.0048
Eotaxin	0.7 (0.5-2.0)	1.4 (0.8-2.2)	2.0 (0.3-3.5)	0.55
IL-8	29.2 (13.3-58.5)	67.2 (48.7-156.8)*	70.0 (61.1-196.3)**	0.013
IL-12p40	0.3 (0.3-0.6)	0.3 (0.2-0.3)	0.3 (0.3-4.3) ^o	0.0067
IL-17A	0.4 (0.4-0.8)	0.6 (0.4-2.1)	1.2 (0.4-2.5)	0.1
IL-23	7.9 (0.7-9.8)	9.8 (1.7-9.8)	9.8 (1.9-9.8)	0.55
PARC (×10 ³)	2.8 (1.6-6.7)	6.6 (2.5-11.2)	7.4 (3.2-11.7)	0.11
RANTES	8.4 (0.6-17.2)	7.2 (2.6-68.9)	21.5 (10.5-99.1)	0.15
TSLP	1.2 (1.2-1.2)	1.2 (1.2-1.2)	1.2 (1.2-2.1)	0.12
Oxidative stress				
MPO (×10 ³)	1.4 (0.3-2.2)	6.2 (1.8-21.6)*	4.9 (1.9-12.5)*	0.019
Angiogenesis				
PlGF	5.2 (3.6-7.3)	5.6 (5.1-7.6)	4.7 (2.7-8.7)	0.55
FGFb	5.4 (5.0-7.4)	6.4 (5.5-9.9)	6.4 (3.5-12.1)	0.56
VEGF	686.6 (358.2-967.0)	226.2 (202.5-288.3)**	188.8 (123.8-237.7)***	0.0003
Coagulation				
Protein-C	52.4 (24.4-131.1)	92.1 (74.4-267.5)*	192.6 (102.0-658.6)**	0.0087
Active PAI-1	2.9 (2.2-4.6)	5.7 (4.0-17.0)**	7.7 (2.1-16.7)	0.019
Tissue factor	99.7 (60.5-156.0)	108.5 (75.8-152.4)	100.6 (53.1-149.5)	0.86

The variation in protein expression levels, shown as median (IQR) (for Abbreviations, see Table 1) between the control, IPF and HP groups were calculated with Kruskal-Wallis ANOVA and Mann-Whitney U-test used as post-hoc test. Significances: Compared with control: **P*<0.05, ***P*<0.01, ****P*<0.001, compared with IPF: ^o*P*<0.05, ^{oo}*P*<0.01. IPF = Idiopathic pulmonary fibrosis, HP = Hypersensitivity pneumonitis, RAGE = Receptor for advanced glycation end products, IPF = Idiopathic pulmonary fibrosis, HP = Hypersensitivity pneumonitis, MMP = Matrix Metalloproteinase, SP = Surfactant protein, TIMP = Tissue inhibitor of metalloproteinase, TGF = Transforming growth factor, MCP = Monocyte chemoattractant protein, MDC = Macrophage-derived chemokine, IL = Interleukine, PARC = Pulmonary activation regulated chemokine, RANTES = Regulated on activation normal T cells expressed and secreted, TSLP = Thymic stromal lymphopoietin, MPO = Myeloperoxidase, VEGF = Vascular endothelial growth factor, PAI = Plasminogen activating inhibitor, PlGF = Placental growth factor, FGFb = Fibroblast growth factor

Correlation analysis

Correlation results are summarized in Table 5. Regarding IPF, there was a significant correlation between DL_{CO} and BAL protein expression of HGF (*R* = -0.49; *P* = 0.023), MCP-1 (*R* = -0.47; *P* = 0.030), MDC (*R* = -0.58; *P* = 0.0064), IL-8 (*R* = -0.45; *P* = 0.040), IL-12p40 (*R* = 0.50; *P* = 0.020), VEGF (*R* = 0.60; *P* = 0.0039), Protein-C (*R* = -0.50; *P* = 0.022), and active PAI-1 (*R* = -0.49; *P* = 0.024). In addition, a correlation was found between FVC and MDC (*R* = -0.48; *P* = 0.029), IL-8 (*R* = -0.47; *P* = 0.031), VEGF (*R* = 0.56; *P* = 0.0087), and Protein-C (*R* = -0.67; *P* = 0.0008).

In patients with HP, there was a significant correlation between DL_{CO} and TGF-β1 (*R* = 0.55; *P* = 0.010), MCP-1 (*R* = -0.58; *P* = 0.0062), IL-8 (*R* = -0.47; *P* = 0.030), and VEGF (*R* = 0.72; *P* = 0.0002). Additionally, there was a significant correlation between FVC and MMP-9 (*R* = -0.44; *P* = 0.049), IL-8 (*R* = -0.60; *P* = 0.0042), and VEGF (*R* = 0.51; *P* = 0.018).

Discussion

In this study, we examined the involvement of different pathophysiological mechanisms in BAL in IPF and HP, using multiplex protein analysis of 25 different proteins. In both IPF and HP, we observed differences in key factors of angiogenesis (VEGF), coagulation (protein-C and active PAI-1), fibrogenesis (TGF-β1), tissue repair (HGF), inflammation (MCP-1, MDC, and IL-8), matrix remodeling (MMP-8 and MMP-9), and oxidative stress (MPO). Patients with IPF or HP demonstrated decreased pulmonary function combined with altered differential cell counts of BAL. Although IPF and HP differ in onset and clinical presentation, only a few significant differences between them were found.

The first observation in the well-characterized groups was that BAL cellular pattern in both disorders was different, as patients with HP suffer from lymphocytic inflammation compared with

Table 5: Correlation analysis of bronchoalveolar lavage protein expression with pulmonary function

Correlations	IPF		HP	
	DL _{co}	FVC	DL _{co}	FVC
MMP-8				
<i>R</i> value	-0.35	-0.41	-0.36	-0.37
<i>P</i> value	0.13	0.065	0.11	0.1
MMP-9				
<i>R</i> value	-0.25	-0.33	-0.36	-0.44
<i>P</i> value	0.28	0.14	0.1	0.049
HGF				
<i>R</i> value	-0.49	-0.40	-0.23	-0.29
<i>P</i> value	0.023	0.07	0.31	0.2
TGF-β1				
<i>R</i> value	-0.0034	-0.14	0.55	0.4
<i>P</i> value	0.99	0.56	0.01	0.069
MCP-1				
<i>R</i> value	-0.47	-0.38	-0.58	-0.42
<i>P</i> value	0.03	0.091	0.0062	0.056
MDC				
<i>R</i> value	-0.58	-0.48	-0.37	-0.30
<i>P</i> value	0.0064	0.029	0.1	0.18
IL-8				
<i>R</i> value	-0.45	-0.47	-0.47	-0.60
<i>P</i> value	0.04	0.031	0.03	0.0042
IL-12p40				
<i>R</i> value	0.5	0.35	-0.14	0.0065
<i>P</i> value	0.02	0.12	0.55	0.98
MPO				
<i>R</i> value	-0.23	-0.29	-0.36	-0.41
<i>P</i> value	0.33	0.2	0.11	0.062
VEGF				
<i>R</i> value	0.6	0.56	0.72	0.51
<i>P</i> value	0.0039	0.0087	0.0002	0.018
Protein-C				
<i>R</i> value	-0.50	-0.67	-0.12	-0.17
<i>P</i> value	0.022	0.0008	0.6	0.46
Active PAI-1				
<i>R</i> value	-0.49	-0.39	-0.13	-0.11
<i>P</i> value	0.024	0.084	0.58	0.62

All significant correlations for BAL protein expression (for Abbreviations, see Table 1) with pulmonary function are displayed with their respective *P* value and Spearman coefficient. IPF = Idiopathic pulmonary fibrosis, HP = Hypersensitivity pneumonitis, DL_{co} = diffusing capacity, FVC = Forced vital capacity, MMP = Matrix Metalloproteinase, HGF = Hepatocyte growth factor, TGF = Transforming growth factor, MCP = Monocyte chemotactic protein, MDC = Macrophage-derived chemokine, IL = Interleukine, MPO = Myeloperoxidase, VEGF = Vascular endothelial growth factor, PAI = Plasminogen activating inhibitor

IPF and control. This might suggest a discriminative role for BAL in diagnosis of IPF and HP.

The role of innate immunity in IPF and HP has been shown by the upregulation of MCP-1 and MDC. MCP-1, involved in macrophage recruitment, was negatively correlated with DL_{co} for IPF and HP.^[14,15] Suga *et al.* investigated MCP-1 in HP and found no increase in its expression. This difference might be due to the high lymphocytosis (average > 70%) found in their patients.^[13] Alternatively, this can be explained by a higher number of patients with acute HP in comparison

with our study. In seasonal summer-type HP, an increase in MCP-1 was interpreted as a possible causative mechanism following repeated stimulations.^[16] MDC was negatively correlated with DL_{co} and FVC in IPF and negatively correlated with DL_{co} in HP. MDC contributes to pulmonary fibrosis by recruiting macrophages and triggering T-helper cell 2 inflammation, further inducing fibrogenic proteins by stimulating macrophages.^[17]

IL-8 functions as a major chemoattractant for neutrophils and has a role in angiogenesis.^[18] In both IPF and HP, IL-8 was significantly upregulated and correlated negatively with DL_{co} and FVC. Neutrophil numbers and percentages were increased in both disorders, indicating an important role for IL-8 as neutrophil attractant. IL-8 has been proposed as an early phase marker for IPF and an indicator of continuous exposure to provoking agents for HP.^[19]

MPO expression, a marker for oxidative stress produced by neutrophils, was increased in IPF and HP, corroborating with literature.^[20] A shift in the redox balance of IPF has been demonstrated to favor oxidants involving an IL-8-dependent mechanism.^[21] In addition, increased levels of MMP-8 and MMP-9, expressed by neutrophils, were found without a compensatory increase in TIMP-1 in both disorders, corroborating the findings of Henry *et al.*^[22,23] This imbalance enhances the activity of MMPs influencing remodeling and development of fibrosis.

Despite current issues on BAL and neutrophils, we observed an important role for immunological mechanisms including neutrophilia and associated oxidative stress and matrix remodeling, in perpetuating both disorders. The activation of the innate arm of the immune system can be linked with reflux,^[24] *Pseudomonas* colonization,^[25] and air pollution.^[26] Although there is no direct proof for the involvement of innate activation and its different activators, our results might point to a certain role in the process. We recently demonstrated in lung transplant patients that traffic-related air pollution can cause substantial airway immunological reactions with an increase in mortality. Yet very importantly, it was observed that macrolide therapy was protective for this immunological reaction and related development of chronic rejection and mortality. With the studied disorders, HP and IPF, showing similar mechanisms regarding innate immunity in chronic rejection after lung transplantation, one should consider decreasing the innate immune response. In this regard, as in chronic rejection, a potential agent might be the macrolide azithromycin, which was recently studied in the bleomycin mouse model where it modulated both innate and adaptive immunity and consequently reduced parenchymal fibrosis.^[27]

Previous studies have shown an increased ratio of angiogenic and angiostatic chemokines in BAL of IPF and HP patients, indicating upregulation of angiogenesis.^[28,29] In this study, we observed a downregulation of VEGF in IPF and HP. It has been suggested that VEGF is increased in non-fibrotic, more inflammatory regions and decreased in severe fibrosis.^[30,31] This decrease of VEGF at the site of fibrosis may be mediated by proteolytic degradation or may result from epithelial cell apoptosis or injury.^[32] Thickening of the barrier between the epithelial surface and the intravascular space due to fibrosis

may alter the secretion of VEGF. This also suggests the reason for positive correlation of VEGF with DL_{CO} and FVC in both disorders.^[33]

Protein-C was upregulated in both disorders and when activated, it has shown a potential anti-fibrotic effect by diminishing inflammation and coagulation.^[34,35] This increased expression was remarkably higher in patients with HP compared with IPF. This is in agreement with literature where protein-C has shown to be increased in IPF, but its activated form was decreased.^[36] Enhanced plasminogen activity provides an environment supporting tissue repair in the lung by increasing levels of active HGF. We are the first to report an upregulation of HGF in HP. HGF stimulates the proliferation of alveolar type II epithelial cells and inhibits epithelial to myofibroblast transition.^[37,38] It has been shown that production and activation of HGF are reduced in fibroblasts in IPF secondary to a defect in prostaglandin E2 secretion.^[39] The expression of HGF may also be restrained by the anti-fibrinolytic effect of PAI-1, which was upregulated and correlated with DL_{CO} in patients with IPF, as shown in literature.^[40] These data show that the role of the coagulation pathway may be different between IPF and chronic HP. The enhanced plasminogen activity may provide an environment explaining a slower progression of pulmonary fibrosis in chronic HP when compared to IPF.

Apart from similarities, significant differences between IPF and HP were also found. IL-12p40 was upregulated in HP compared with IPF. IL-12p40 directs the immune response toward the appropriate location and correct pathogen.^[41] This points toward the role of the hypersensitivity reaction present in patients with HP.^[42] TGF-β1, known as the fibrotic molecule in IPF and experimental fibrosis, was not significantly different in both disorders when compared with control,^[43] due to large variance in samples. A significant decrease in patients with HP

compared with IPF was observed. This difference between IPF and HP has been documented previously by Hagimoto *et al.*^[44] The increase in TGF-β1 is thought to be produced by alveolar macrophages, in an early inflammatory phase and by epithelial cells during the later fibrotic phase.^[45] This could explain the lower concentration of TGF-β1 in HP as they are in a chronic phase of the disorder, with less fibrosis than in IPF.

VEGF (angiogenesis), IL-8, MCP-1, MDC, IL-12p40 (inflammation), HGF (tissue repair), TGF-β1 (fibrogenesis), MMP-9 (matrix remodeling), protein-C, and active PAI-1 (coagulation) were correlated with DL_{CO} and/or FVC for IPF and HP, reflecting the degree of severity. This indeed suggests that pulmonary fibrosis is the result of a combination of different pathological mechanisms.

The major limitation of this study is the low number of samples that were used. Both IPF and HP are orphan diseases and to establish homogenous groups well-documented cases are needed. We are convinced that with higher number of patients, the results would be more pronounced and might have less variability. BAL sampling was performed at the time of diagnosis, potentially reflecting different stages of pulmonary fibrosis, which might explain the broad range of protein expression in each group [Figure 1]. However, time of diagnosis presents the earliest possible phase for BAL sampling in the clinic. This study confirms the usefulness of BAL for research purposes uncovering similarities and differences between these disorders.

This research provides a better understanding of the involvement of underlying mechanisms and differences between these diseases are compared with the use of animal models. This study demonstrates that though common mechanisms appear, some mechanistic discrepancies were

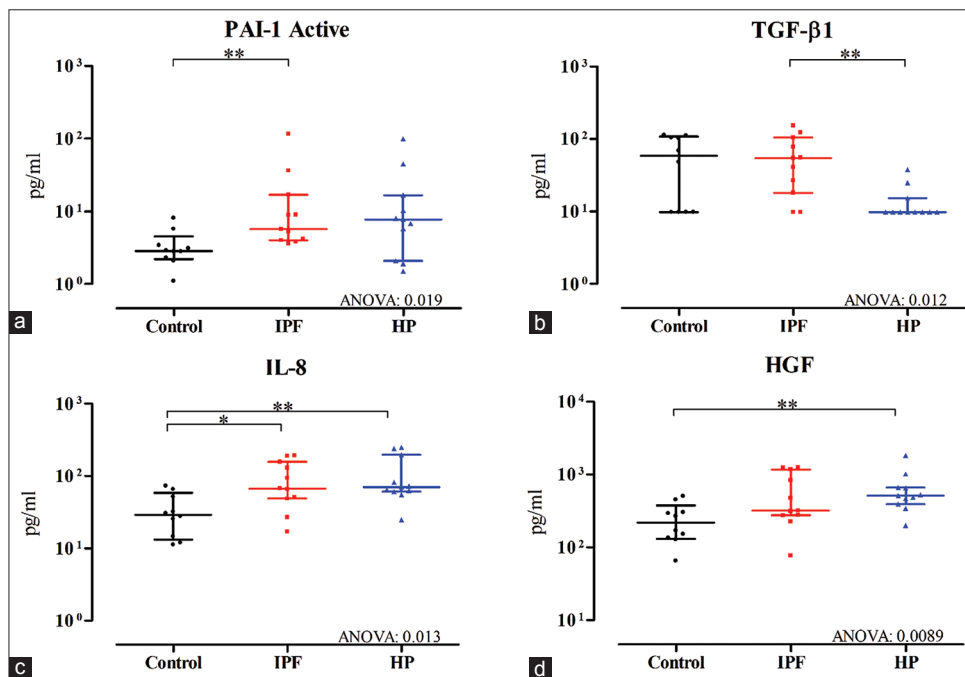


Figure 1: The expression of (a) active PAI-1, (b) TGF-β1, (c) IL-8, and (d) HGF shown as median with IQR (for Abbreviations, see Table 1) in BAL fluid of 10 controls, 11 IPF, and 11 HP patients. Significances: **P*<0.05, ***P*<0.01 remove: , and ****P*<0.001

discovered, providing new clues for further research in both disorders.

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