

CLUSTERIZATION OF PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS WITH CHEMOKINE RECEPTORS: A POSSIBLE ROLE IN THE DIAGNOSTIC WORK-UP OF IDIOPATHIC PULMONARY FIBROSIS?

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ABSTRACT. *Background and objective:* Idiopathic pulmonary fibrosis (IPF) is a chronic and irreversible interstitial lung disease whose diagnosis often requires surgical lung biopsies (SLB) in cases without consistent radiological findings. We previously published that the expression of the chemokine receptors CXCR3 and CCR4 on T cells is significantly different in bronchoalveolar lavage (BAL) of IPF patients from other interstitial lung diseases. The aim of the study was to evaluate cut-off values of CXCR3 and CCR4 receptors expressed on bronchoalveolar lavage (BAL) and peripheral blood (PB) T cells useful for a differential diagnosis. *Methods:* Ninety-three patients were enrolled: 35 IPF, 36 interstitial lung diseases (nIPF) and 22 sarcoidosis. CXCR3 and CCR4 were evaluated on BAL and PB T lymphocytes with flow cytometry. *Results:* Among PB and BAL variables considered, the values of the ratio of BAL and PB CXCR3 on CD4 cells were clustered in the most informative way to obtain a classification rule for the diagnosis of patients without steroid therapy (n = 66/93). Patients with a CXCR3 ratio BAL/PB on CD4 T cells lower or equal than 1.43 were assigned to the IPF group with sensitivity = 0.87 and specificity = 0.90. All the other variables considered showed lower sensitivity and specificity in discriminating IPF patients. *Conclusions:* The evaluation of chemokine receptors on BAL and PB T lymphocytes could aid to discriminate IPF in subjects without steroid therapy, particularly in those patients with a high-resolution computed tomography (HRCT) non typical for Usual Interstitial Pneumonia (UIP). (*Sarcoidosis Vasc Diffuse Lung Dis* 2018; 35: 35-43)

KEY WORDS: pulmonary fibrosis, bronchoalveolar lavage, chemokines

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible fibrosing lung disease char-

acterized by remodeling of the lung parenchyma and collagen deposition (1).

The gold standard for the diagnosis of idiopathic interstitial pneumonias (IIPs) and IPF is the multidisciplinary approach, matching the clinical, radiological and histopathological data (2). The international guidelines on IPF assigned a secondary role to the fibrobronchoscopy and bronchoalveolar lavage (BAL) in the diagnostic work up, considering BAL and differential cell count not useful for IPF diagnosis due to the scarce accuracy of these data and limit-

Received: 2 February 2017

Accepted after revision: 25 May 2017

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ing their use to the exclusion of infective, neoplastic or occupational diseases (3).

CXCR3 and CCR4 are two chemokine receptors expressed on Th1 and Th2 switched lymphocytes, respectively (4). We previously published that the expression of these chemokine receptors on BAL and peripheral blood (PB) CD4T cells was significantly different in IPF compared to other interstitial lung diseases (ILDs) which are frequently considered for differential diagnosis (5).

The aim of this study was to define cut-off values of CXCR3 and CCR4 on PB and BAL T cells sensitive and specific in distinguishing IPF subjects from patients with other ILDs and to compare the obtained accuracy with that of other BAL cellular markers usually considered in literature for IPF (BAL lymphocytes, neutrophils, CD4 and CD8 T cells).

MATERIAL AND METHODS

Patients

Ninety-three patients were enrolled: 35 IPF, 36 interstitial lung disease other than IPF (nIPF), 22 sarcoidosis (SAR). Thirty IPF subjects received the diagnosis according to 2002 ATS/ERS guidelines (2) revised on the light of the 2011 ATS/ERS/JRS/ALAT statement on IPF(3) and 5 patients according to 2011 Statement(3). nIPF group included: 9 subjects with asbestosis, 4 with drug-induced ILDs, 4 with Idiopathic Organizing Pneumonia (OP), 2 with idiopathic non specific interstitial pneumonia (NSIP), 2 with Pulmonary Langerhans cell Histiocytosis (PLCHC), 1 with Desquamative Interstitial Pneumonia (DIP), 1 patient with Hypersensitivity Pneumonitis (HP), 1 patient showing radiologic and histologic features of unclassifiable IIP and 12 subjects with connective tissue disease-associated ILDs (5 Progressive Systemic Sclerosis, 3 with Undifferentiated Connective Tissue Disease, 1 with Mixed Connective Tissue Disease, 2 with Sjogren Syndrome, and 1 with Rheumatoid Arthritis).

HRCT

In IPF group, the HRCT evaluation showed an usual interstitial pneumonia (UIP) pattern in 24 cases and a probable UIP pattern in 11 cases. Four out

of 35 patients showed HRCT features of IPF combined pulmonary emphysema. In the nIPF group, the HRCT showed a reticular pattern with honeycombing in 4 cases, a reticular/ground glass pattern in 8 cases, consolidative pattern in 8 cases, diffuse ground glass in 1 case. Various radiological signs, like non-specific interstitial peripheral thickening, nodules, cysts, pleural plaques and pulmonary consolidations have been observed in the other nIPF patients. Among sarcoidosis patients, 9 showed subpleural peribronchovascular nodules, 8 parenchymal consolidations, 2 upper lobe central fibrosis. All subjects showed mediastinal-hilar lymph node enlargement.

Lung biopsies

Transbronchial or surgical lung biopsies have been performed in 12 IPF patients, resulting in a definitive UIP pattern, in 13 patients with sarcoidosis, confirming the diagnosis in all cases and in 8 nIPF patients showing OP (3 cases), NSIP (2 cases), DIP (1 case), PLCHC (1 case), undefined IIP (1 case).

Subjects without steroid or other immunosuppressive therapy were 22/35 in IPF group, 26/36 in nIPF and 18/22 in sarcoidosis group (SAR).

Patients gave their informed consent to extra blood drawing during routine venipuncture. Written informed consensus was obtained for BAL procedure. The study conformed to the Declaration of Helsinki and was approved by the internal review board of our Institute (Fondazione Salvatore Maugeri).

Bronchoalveolar lavage

BAL was performed and analysed following international guidelines (6,7). Total, differential cell count and lymphocyte subset (CD4/CD8), CXCR3 and CCR4 expression was determined on PB and BAL CD4 and CD8 cells by flow-cytometry as previously reported (5). In BAL and PB T cells the following variables were measured: CD4CXCR3%, CD4CCR4%, CD8CXCR3%, CD8CCR4% and the ratios: CD4CXCR3/CCR4, CD8CXCR3/CCR4, CD4CXCR3% BAL/PB, CD8CXCR3% BAL/PB, CD4CCR4% BAL/PB, CD8CCR4% BAL/PB.

Statistical analyses

Since most of the quantitative variables' distribution deviated significantly from the normality as-

assumptions (Shapiro $p < 0.05$), these were described by median (25th - 75th percentiles). Categorical variables distributions were described by count (%). The presence of statistically significant differences between binary subgroups was tested by the Wilcoxon rank-sum test or by the Kruskal-Wallis in the case of categorical variables with >2 subgroups. The presence of statistically significant differences between categorical variables distributions was assessed by the Fisher's exact test. Unsupervised Principal Components Analysis (PCA) was performed after data scaling and centering.

The most informative threshold to discriminate IPF from nIPF/SAR was identified for each variable on a subset of patients representing ~70% of the whole cohort (training set). The generalization performances were then tested on the remaining ~30% of the data that were not used to identify the optimal thresholds (test set) to avoid overfitting.

The following steps summarize the described approach:

1. Split the whole sample randomly into *training set* (~70% of the whole data) and *test set* (~30% of the whole data) with outcome stratification (i.e., both training and test sets must be characterized by the same proportion of IPF and nIPF/SAR patients).
2. On the *training set*:
 - a. Identify the most informative threshold for each variable as the cut-off value guaranteeing the greatest Matthews correlation coefficient (MCC) in discriminating IPF from nIPF/SAR patients.
 - b. Compare the classification performances of all variables discretized as reported at point 2a and identify the most informative one.
3. On the *test set*:

Test the predictive performances of the most informative discretized variable identified at point 2b.

As terms of comparison, the following classification algorithms were also applied to the training set to identify informative multivariate models: i) Classification Trees (CT), ii) Conditional Inference Trees (CIT), iii) Random Forests (RF), iv) Naïve Bayes (NB), v) Logistic Regression (LR) with and without stepwise selection algorithm and vi) Least Absolute Selection and Shrinkage Operator (LASSO). The

classification performances of the identified multivariate models were then tested on the test set.

The significance threshold was set to $p < 0.05$. All statistical procedures were performed by the R statistical software (www.r-project.org). CT, RF, CIT, NB and LASSO functions are implemented in the R packages called "*rpart*", "*randomForest*", "*party*", "*e1071*" and "*glmnet*" respectively. Shapiro test for normality, LR, Fisher's Exact Test and Wilcoxon rank-sum tests are implemented in the package "*stats*".

RESULTS

Clinical and functional characteristics of the subjects

The clinical-functional data and the BAL cell composition of the studied patients are shown in Table 1 and Table 2 respectively. Considering the effect of the steroid and/or immunosuppressive drugs on the expression of CXCR3 and CCR4 receptors previously shown (5), a comparison of the distribution of each variable among IPF, nIPF and SAR groups, has been performed for therapy and off therapy groups, (Table 2).

Principal component analysis

The unsupervised Principal Components Analysis (PCA) based on BAL variables was performed independently for the subgroups of subjects on - and off -therapy: the density plots reported in Figure 1 describe the distribution of the first 5 Principal Components (PCs) that, taken together, account for ~80% of the data variability in both cohorts. Results show that PC1 and PC4 derived from the subgroup of off-therapy patients had the greatest power in discriminating subjects according to their disease ($p < 0.001$ and $p < 0.05$ respectively). The scatterplot representing the distribution of subjects according to PC1 vs. PC4 values (Figure 2) reveals that the majority of IPF subjects are clustered and localized on the low-left quarter of the scatterplot, while SAR subjects are localized on upper-right quarter of the plot, defining two independent and well separated groups of subjects. On the opposite, nIPF subjects are spread and partially superimposed to both groups. Further, it was possible to observe that PC1 variability was

Table 1. Characteristics of the patients with idiopathic pulmonary fibrosis (IPF), heterogeneous interstitial lung diseases (nIPF), and sarcoidosis (SAR). Categorical variables distributions are described by count (%); quantitative variables distributions are described by median (25th, 75th percentiles). p = p-value from the Kruskal-Wallis test (for quantitative variables) or by the Fisher's Exact Test (categorical variables)

	IPF (n=35)	nIPF (n=36)	SAR (n=22)
Age (years) ^s	65.0 (60.0-73.0)	68.5 (59.5-73.2)	38.5 (35.2-51.5)
Sex (M/F)	28/7	17/19	11/11
Smoking habit (yes/ex/no)	5/19/11	3/19/14	6/3/13
VC % pred. ^s	65.8 (53.9-78.0)	80.7 (63.3-94.9)	90.9 (82.2-96.7)
DLCO % ^s	48.1 (36.2-61.4)	79 (49-99)	98.8 (78.2-116.8)
A-aPO ₂ (mmHg) ^s	35.1 (28.3-45.1)	31.8 (24.7-39.1)	21.5 (18.1-31.6)
6MWT distance (meters) ^s	400 (313-444)	417 (270-504)	549 (450-625)
Disease duration (months)	12.5 (8.2-34.0)	12.0 (6.0-24.0)	6.0 (3.0-21.0)
Steroid therapy (yes/no)	13/22	10/26	4/18

^s Statistically significant difference among the three subgroups (p<0.05)

* 13 IPF patients received supplemental oxygen during 6MWT

° 8 nIPF patients received supplemental oxygen during 6MWT

heavily influenced by the following set of variables: CD4CCR4, CD4CCR4 BAL/PB, neutrophils BAL, CD4CXCR3BAL, CD4CXCR3/CCR4% BAL and CD4CXCR3BAL/PB. To be noted, these variables were the ones that showed the strongest association with the disease condition by univariate tests (Table 2). None of the PCs derived from on-therapy subjects were informative with respect to the disease condition subjects (p >0.05) (Figure 1).

Identification of informative thresholds with respect to the disease

The most informative threshold to be applied to discretize each variable was identified for subjects on therapy and off therapy independently as described in the Statistical Methods section.

Considering subjects without immunosuppressive therapy, the ratio CD4CXCR3 BAL/PB discretized according to the identified threshold 1.43 reached the greatest power in discriminating IPF from nIPF and SAR individuals on the *training set* (45 patients off therapy: 15 IPF, 18 nIPF and 12 SAR) (Table 3). In detail, individuals characterized by the ratio CD4CXCR3 BAL/PB ≤ 1.43 have a ~49 folds increase in the probability of being IPF compared to the rest of the cohort (OR = 49, 95% CI = 7 - 669.9, p = 6.7 x 10⁻⁷), reaching sensitivity = 0.87 and specificity = 0.90 (Table 3). Thus, the discriminative performances of the variables discretized according to the thresholds defined on the *training set* were tested on the independent *test set* (20 patients off therapy: 7 IPF, 8 nIPF and 5 SAR). Most signifi-

cative results are reported in Table 3 (supplementary Table 1S for all the variables considered) and confirm that patients with the ratio CD4CXCR3BAL/PB ≤ 1.43 have a statistically significant increase in the probability of being IPF with respect to the rest of the cohort (OR = 45.92, 95% CI = 2.76 - 3437.43, p = 0.004) with discriminative performances that are equal to or higher than those observed in the screening cohort for the same variable (sensitivity = 0.86, specificity = 0.92) Figure 3.

Since from the diagnostic point of view it is certainly more useful to have mediators able to distinguish IPF and nIPF than IPF from sarcoidosis patients, the process of threshold identification was repeated on a subset of the original cohort that excluded SAR subjects (48 subjects off therapy: 22 IPF and 26 nIPF) using the procedure described in the methods section. Table 4 shows the results of the most significant variables that confirmed that the ratio CD4CXCR3BAL/PB discretized using a cut-off corresponding to 1.43 is the most predictive variable among the tested predictors to discriminate IPF from nIPF subjects even excluding SAR subjects. The results corresponding to the complete set of analyzed variables are reported in supplementary material Table 2S results.

Comparison against multivariate models

A further comparison between the discriminative performances of the ratio CD4CXCR3 BAL/PB discretized according to the identified threshold (1.43) vs. more complex multivariate classification

Table 2. Results from the univariate tests evaluating the association between the set of BAL - related variables considered in the three groups of subjects that were on therapy (n = 27) and off therapy (n = 66). * = statistically significant difference in terms of variable's distribution between treated and untreated subjects (p < 0.05)

Variable	Group	On-Therapy			Off-Therapy		
		n	Median (25th, 75th percentiles)	p	n	Median (25th, 75th percentiles)	p
Macrophages (%) BAL	IPF	13	72.3 (59.1-81.7)	0.45	22	75.75 (58.55-85.38)	0.35
	nIPF	10	65.05 (55.78-84.32)		26	76.7 (60.82-90.38)	
	SAR	4	72.2 (57.95-83.85)		18	75.75 (60.12-88.77)	
Lymphocytes (%) BAL	IPF*	13	5.2 (3.9-8.6)	0.01	22	3.3 (1.55-5.72)	0.0003
	nIPF	10	11.4 (5.12-29.65)		26	13.95 (3.42-26.67)	
	SAR	4	7.3 (4.05-16.15)		18	5.3 (2.72-15.95)	
Neutrophils (%) BAL	IPF	13	17.4 (4.7-21.2)	0.39	22	12.5 (7.35-18.95)	9 x 10 ⁻⁷
	nIPF	10	5.95 (4.75-14.45)		26	4.05 (1.7-5.88)	
	SAR	4	10.9 (4.55-18.5)		18	5.85 (2.55-13.32)	
Eosinophils (%) BAL	IPF	13	2.1 (1.6-3.9)	0.03	22	4.55 (2.15-7.95)	0.003
	nIPF	10	2.75 (0.55-6.5)		26	1.4 (0.4-4.73)	
	SAR	4	2.1 (1-6.4)		18	3.05 (0.78-7.65)	
CD4/CD8BAL	IPF*	13	0.7 (0.3-1)	0.27	22	1.38 (0.94-1.95)	0.009
	nIPF	10	0.7 (0.62-1.5)		26	1.5 (0.65-2.18)	
	SAR*	4	0.7 (0.32-1.1)		18	1.48 (0.8-2.12)	
CD4CXCR3 (%) BAL	IPF*	13	67.2 (54.8-73.6)	0.49	22	51.05 (40.95-60.4)	2 x 10 ⁻⁶
	nIPF	10	71.25 (53.23-87.17)		26	76.95 (65.05-84.7)	
	SAR	4	67.2 (53.45-80.75)		18	65.1 (50.73-78.95)	
CD4CCR4 (%) BAL	IPF	13	41.2 (34.2-53)	0.05	22	45.45 (31.27-52.7)	0.0009
	nIPF	10	26.2 (18.08-36.33)		26	28.2 (17.38-41.92)	
	SAR	4	36.2 (24.35-47.1)		18	36.1 (19.85-49.58)	
CD4CXCR3/CCR4BAL	IPF	13	1.48 (1.06-2.15)	0.13	22	1.02 (0.84-1.86)	5 x 10 ⁻⁶
	nIPF	10	2.51 (1.44-4.79)		26	2.71 (1.63-4.26)	
	SAR	4	1.99 (1.14-2.98)		18	1.82 (1.16-3.32)	
CD8CXCR3 (%) BAL	IPF	13	81.8 (61.1-93.5)	0.56	22	71.4 (52.38-81.4)	0.14
	nIPF	10	73.45 (58.95-82.82)		26	78.75 (66.55-89.3)	
	SAR	4	77.5 (59.3-90.35)		18	75.5 (61.53-84.5)	
CD8CCR4 (%) BAL	IPF	13	14.5 (6.1-22.4)	0.08	22	7.7 (5.03-12.85)	0.50
	nIPF	10	9.65 (4.67-13.5)		26	7.65 (3.52-10.78)	
	SAR	4	13.2 (5.35-18.3)		18	7.65 (3.82-12.12)	
CD8CXCR3/CCR4BAL	IPF	13	4.25 (3.5-15.33)	0.13	22	9.73 (4.3-11.97)	0.48
	nIPF	10	7.05 (4.53-10.62)		26	9.45 (6.93-21.57)	
	SAR	4	5.62 (3.6-14.15)		18	9.55 (6.18-14.86)	
CD4CXCR3BAL/PB	IPF*	13	1.89 (1.34-2.9)	0.82	22	1.08 (0.76-1.3)	1 x 10 ⁻⁷
	nIPF	10	1.88 (1.38-3.25)		26	1.91 (1.55-2.42)	
	SAR*	4	1.89 (1.35-3.07)		18	1.44 (1.09-2.01)	
CD4CCR4BAL/PB	IPF	13	1.5 (1.22-1.76)	0.03	22	1.52 (1.19-1.83)	0.0002
	nIPF	10	0.9 (0.62-1.5)		26	1.02 (0.72-1.51)	
	SAR	4	1.4 (0.76-1.6)		18	1.22 (0.88-1.68)	
CD8CXCR3BAL/PB	IPF	13	1.36 (1.12-1.72)	0.45	22	1.24 (0.75-1.42)	0.009
	nIPF	10	1.68 (1.25-1.98)		26	1.46 (1.21-1.9)	
	SAR	4	1.46 (1.15-1.84)		18	1.37 (1.14-1.78)	
CD8CCR4BAL/PB	IPF	13	1.39 (1.02-1.91)	0.13	22	0.69 (0.51-1.41)	0.07
	nIPF	10	1 (0.73-1.23)		26	0.53 (0.32-0.97)	
	SAR*	4	1.21 (0.73-1.88)		18	0.64 (0.4-1.14)	

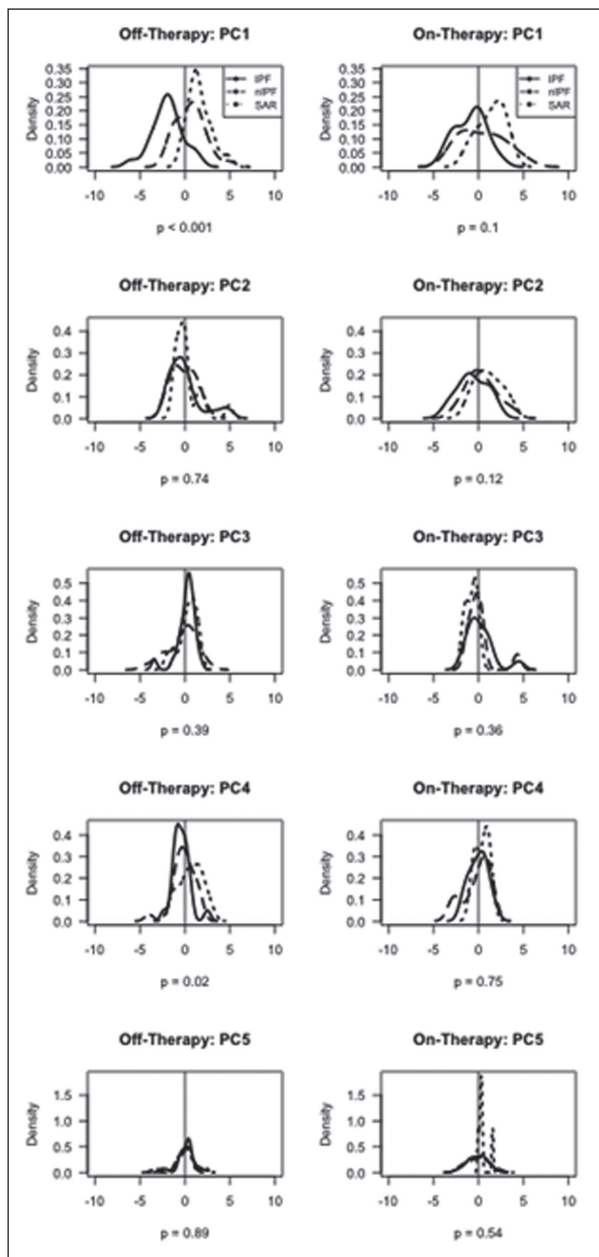


Fig. 1. Density plots of the first 5 Principal Components estimated using BAL variables on off-therapy (leftmost) and on-therapy subjects (rightmost). p = p -value from the Kruskal – Wallis test comparing PC distribution in the three groups of patients

models including clinical and non-clinical/epidemiological predictors (age, gender, smoking status and disease duration) was performed on off-therapy subjects (supplementary Table 3S). Results show that the ratio $CD4CXCR3_{BAL}/PB \leq 1.43$ was the most informative decisional rule to identify IPF patients.

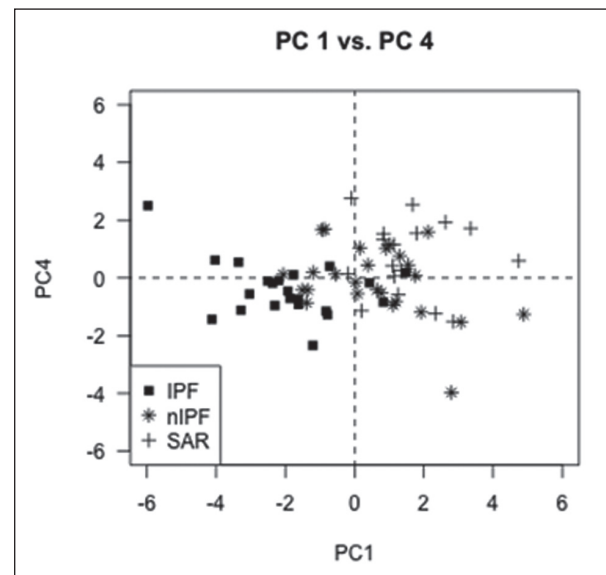


Fig. 2. Scatterplots of PC1 (x-axis) vs. PC4 (y-axis) deriving from the PCA on off-therapy subjects. Each dot represents a patient

The only multivariate models able to outperform the ratio $CD4CXCR3_{BAL}/PB \leq 1.43$ on the training set were those defined by Random Forests and Logistic Regression. However, when tested on the independent test set, the discriminative performances of these models were remarkably lower than those reached by $CD4CXCR3_{BAL}/PB \leq 1.43$. This is due to the fact that complex models based on a large number of variables tend to have a high capability to describe the analyzed set of patients but poor generalization performances when applied to independent cohorts (overfitting issue).

DISCUSSION

BAL, as a source of cellular and soluble mediators from the alveolar part of the lung, has always represented a valuable tool in research applied to the study of ILD.

However, BAL does not play the same relevant role in clinical practice for various reasons (7, 8): invasiveness of the test with associated additional risks and costs, mainly in IPF patients; scarce possibility to repeat the test, dilution factor which makes it difficult to accurately evaluate the soluble components of BAL particularly; cellular data specific only for a few pathologic conditions, and as a consequence,

Table 3. Most significant analysed variables and corresponding threshold in - off-therapy subjects. Threshold = identified threshold; Set = training or test sets; AUC = Area Under the Receiver Operating Characteristics curve; MCC = Matthew's Correlation Coefficient; Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value; F = F-measure; CA = Classification Accuracy.

Variable	Threshold	Set	AUC	MCC	Sens	Spec	PPV	NPV	F
CD4CXCR3BAL/PB	≤ 1.43	Training Set	0.91	+0.76	0.87	0.90	0.81	0.93	0.84
		Test Set	0.96	+0.78	0.86	0.92	0.86	0.92	0.86
CD4CXCR3/CCR4BAL	≤ 1.39	Training Set	0.98	+0.69	0.67	0.97	0.91	0.85	0.77
		Test Set	0.96	+0.30	0.43	0.85	0.60	0.73	0.50
Neutrophils (%) BAL	> 6.2	Training Set	0.85	+0.64	0.73	0.90	0.79	0.87	0.76
		Test Set	0.88	+0.60	0.86	0.77	0.67	0.91	0.75
Lymphocytes (%) BAL	≤ 6.9	Training Set	0.82	+0.57	0.93	0.67	0.58	0.95	0.72
		Test Set	0.79	+0.60	1.00	0.62	0.58	1.00	0.74
CD4/CD8BAL	≤ 1.6	Training Set	0.68	+0.38	0.73	0.67	0.52	0.83	0.61
		Test Set	0.46	+0.03	0.57	0.46	0.36	0.67	0.44

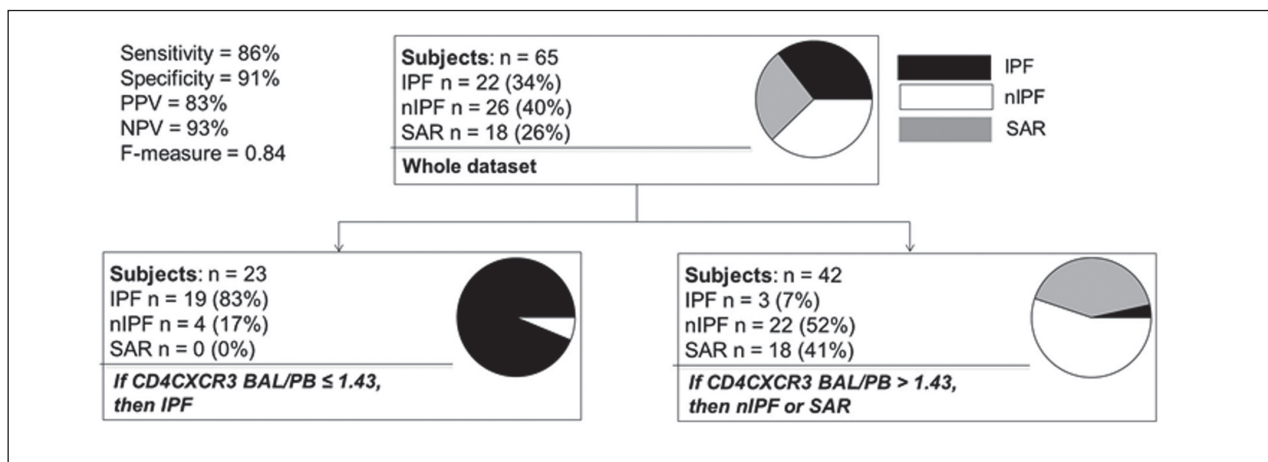


Fig. 3. Discriminative performances of the decisional rule based on the CD4CXCR3 BAL/BP value. The tree describes the distribution of IPF, nIPF and SAR off-therapy subjects in the analysed population (n = 65) and in subjects with CD4CXCR3 BAL/BP ≤ 1.43 (n = 23) and > 1.43 (n = 42) respectively.

the lack of specific indicators useful for diagnostic procedures. All these considerations determined the criticism regarding the use of BAL in the IPF-diagnostic procedures as reported in the recently published guidelines “BAL cellular analysis should not be performed in the diagnostic evaluation of IPF in the majority of patients, but may be appropriate in a minority” (3).

Nowadays, the use of BAL in the diagnostic work up of IPF is limited to exclude infective or neoplastic diseases, and to diagnose HP in patients with an alveolar lymphocytosis (lymphocytes >30–40%) (3, 9).

We previously reported a decrease in the expression of the chemokine receptor CXCR3, and an in-

crease in the expression of CCR4 on BAL CD4 T cells of IPF subjects (5) compared to patients with other ILDs (Sarcoidosis, Organizing Pneumonia, Nonspecific Interstitial Pneumonia, Mixed Connective Tissue Diseases associated ILDs and Asbestosis).

The expression of CXCR3 by CD4 T cells was associated with a Th1 switch of these cells as the expression of CCR4 with a Th2 switch. The low BAL levels of CXCL10 (IP10), the chemokine attracting CXCR3 positive cells, supported the data of a decrease in the recruitment of CXCR3 positive cells from the blood to the lung in IPF subjects (5). Recent studies on the possible mechanisms causing IPF, switched from the inflammatory theory to the theory

Table 4. Variables and corresponding thresholds analysed in off-therapy subjects (excluding SAR subjects). Variable = analysed variable and corresponding threshold; Set = training or test sets; AUC = Area Under the Receiver Operating Characteristics curve; MCC = Matthew's Correlation Coefficient; Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value; F = F-measure; CA = Classification Accuracy.

Variable	Threshold	Set	AUC	MCC	Sens	Spec	PPV	NPV	F
CD4CXCR3BAL/PB	≤ 1.43	Training Set	0.91	0.70	0.87	0.83	0.81	0.88	0.84
		Test Set	0.96	0.73	0.86	0.88	0.86	0.88	0.86
CD4CXCR3/CCR4BAL	≤ 1.39	Training Set	0.89	0.65	0.67	0.94	0.91	0.77	0.77
		Test Set	0.76	0.19	0.43	0.75	0.60	0.60	0.50
CD4CXCR3(%)BAL	≤ 60.5	Training Set	0.88	0.63	0.80	0.83	0.80	0.83	0.80
		Test Set	0.88	0.46	0.71	0.75	0.71	0.75	0.71
Neutrophils (%) BAL	> 6.2	Training Set	0.85	0.57	0.73	0.83	0.79	0.79	0.76
		Test Set	0.88	0.61	0.86	0.75	0.75	0.86	0.80
Lymphocytes (%) BAL	≤ 6.9	Training Set	0.82	0.56	0.93	0.61	0.67	0.92	0.78
		Test Set	0.79	0.66	1.00	0.63	0.70	1.00	0.82
CD4/CD8BAL	≤ 1.6	Training Set	0.68	0.29	0.73	0.56	0.58	0.71	0.65
		Test Set	0.46	-0.19	0.57	0.25	0.40	0.40	0.47

of fibroblastic degeneration (10), limiting the role of inflammation to that of bystander activation. Which role the defect in the lung-recruitment of CXCR3 positive T cells could play in the development or follow up of IPF is still to be elucidated. CXCR3 is a key receptor in the regulation of fibroblast stasis and apoptosis, and signaling through CXCR3 blocks the growth factor induced motility of fibroblasts and endothelial cells (11). Therefore, the decreased amount of CXCR3 positive T cells in the lung of IPF subjects could mirror the decrease in CXCR3 expression on fibroblasts and their deregulated response, which occurs in the lung of these subjects.

Our previous data obtained in cells from BAL have been confirmed by Yoshinouchia et al. who reported a reduced expression of CXCR3 in T cells from lung biopsies of IPF patients compared with NSIP subjects (12).

These data prompted us to compare the expression of CXCR3 and CCR4 with other BAL cellular markers in the diagnostic procedures of IPF in order to obtain sensitive and specific cut-off values for IPF diagnosis. We found that the most discriminative variable among those considered was the ratio between the expression of CXCR3 on BAL and peripheral blood CD4 T cells, and a cut-off of <1.43 allowed us to discriminate, with high specificity, IPF subjects from patients with other ILDs. The discriminative effect of such cut-off was also confirmed in the analysis without sarcoidosis patients who are characterized by the highest amount of Th1 cells.

Noteworthy, the ratio between the expression of CXCR3 on BAL and PB CD4 T cells had higher

sensitivity/specificity in discriminate IPF patients than the ratio between CXCR3 and CCR4 on BAL CD4 T cells we previously considered in our previous study (5) and than the percentage of each cellular component of BAL.

In the majority of cases, the BAL cellular profile of IPF patients is characterized by an increase in neutrophils, a modest increase in eosinophils, and a low percentage of lymphocytes. A mild increase in BAL lymphocytes is reported in 10-20% of IPF patients (13) but when BAL lymphocytosis exceeds 30% an alternative diagnosis, namely HP, should be suspected (9). However, the use of differential cell count of BAL for the differential diagnosis could be rather confusing due to the discordant findings (14-16).

Our data confirm the biological relevance of the presence of CXCR3 positive T lymphocytes in the alveolar compartment for the modulation of lung fibrosis, since the down-modulation of CXCR3 expression might favor the fibrotic process as demonstrated by Jiang et al. These authors showed a marked increase in the development of lung fibrosis in CXCR3-deficient mice compared to wild type mice in bleomycin-induced fibrosis, the experimental model of lung fibrosis (17).

From the clinical point of view, our data suggest the possible use of CXCR3 and CCR4 evaluation in BAL and PB CD4 T cells in order to strengthen or to exclude an IPF diagnosis, especially in those subjects for whom, due to a HRCT not consistent with a UIP pattern, a surgical lung biopsy is needed. In part of these subjects, the biopsy could be not advis-

able since the risks may be too high compared to the benefits of a certain diagnosis of IPF (18). However, the new promising technique of bronchoscopic lung cryobiopsy could offer a high diagnostic yield with less side effects (19).

In daily practice, cases of HRCT not diagnostic of UIP according with the ATS/ERS/JRS/ALAT 2011 IPF guidelines can occur, thus, the evaluation of BAL markers might be useful, particularly taking into consideration that the radiological diagnosis of UIP is highly specific, although low sensitive (20, 21), as suggested by a recent retrospective study from large cohort trials showing that a high percentage of patients with probable or inconsistent features of UIP had histologically definite or probable UIP (22).

In our study, 3 steroid untreated patients with possible UIP pattern and 1 patient with inconsistent UIP pattern on HRCT, received the histological diagnosis of IPF after surgical lung biopsy. All had the ratio CD4/CXCR3 BAL/PB < 1,43, consistent with the rule generated in the current study.

The main concern regarding the evaluation of CXCR3 and CCR4 on BAL and PB T cells is the effect of steroid intake in the expression of these receptors. As we previously reported the steroid therapy reduces the imbalance of these two receptors causing an increase in CXCR3 and a decrease in CCR4 expression, therefore, the evaluation of these chemokine receptors should be limited to "steroid-naïve" subjects.

In conclusion, our study suggests that the evaluation of chemokine receptors on BAL and PB T lymphocytes could help to support IPF diagnosis in subjects without steroid therapy. Further validation studies on larger cohorts of patients are needed to confirm the usefulness of these evaluations, particularly in those patients with a HRCT non-typical for UIP.

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