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Circulating fibrocytes as prognostic biomarkers of autoimmune interstitial lung disease

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Abstract

Background: Autoimmunity is a common cause of pulmonary fibrosis and can present either as a manifestation of an established connective tissue disease or as the recently described entity of interstitial pneumonia with autoimmune features. The rate of progression and responsiveness to immunosuppression in these illnesses are difficult to predict. Circulating fibrocytes are bone marrow-derived progenitor cells that home to injured tissues and contribute to lung fibrogenesis. We sought to test the hypothesis that the blood fibrocyte concentration predicts outcome and treatment responsiveness in autoimmune interstitial lung diseases.

Methods: We compared the concentration of circulating fibrocytes in 50 subjects with autoimmune interstitial lung disease and 26 matched healthy controls and assessed the relationship between serial peripheral blood fibrocyte concentrations and clinical outcomes over a median of 6.25 years.

Results: As compared to controls, subjects with autoimmune interstitial lung disease had higher circulating concentrations of total fibrocytes, the subset of activated fibrocytes, and fibrocytes with activation of PI3K/AKT/mTOR, TGF-beta receptor, and IL4/IL13 receptor signaling pathways. Over the follow-up period, there were episodes of marked elevation in the concentration of circulating fibrocytes in subjects with autoimmune interstitial lung disease but not controls. Initiation of immunosuppressive therapy was associated with a decline in the concentration of circulating fibrocytes. For each 100,000 cell/ml increase in peak concentration of circulating fibrocytes, we found a 5% increase in odds of death or lung function decline.

Conclusion: In patients with autoimmune interstitial lung disease, circulating fibrocytes may represent a biomarker of outcome and treatment response.

Introduction

Autoimmunity is among the most common causes of interstitial lung diseases (ILD). Approximately 40% of patients with a diagnosis of rheumatoid arthritis, systemic sclerosis, autoimmune myopathies, mixed connective tissue disease, and primary Sjogren syndrome have ILD [1], and conversely, many of the patients who present with an undiagnosed ILD have evidence of a previously undiagnosed connective tissue disease [2]. In addition, the recently defined entity of interstitial pneumonia with autoimmune features describes patients with evidence of autoimmunity who do not meet the diagnostic criteria for a connective tissue disease [3], and represents another important subset of autoimmune ILD. Together, these illnesses comprise a large proportion of patients seen by ILD specialists and present unique challenges relating to diagnosis, therapy, and prognosis.

The rate of progression among autoimmune ILD is notoriously heterogeneous and difficult to predict, with many patients displaying mild lung disease or a slowly progressive course, but a subset presenting with rapid progression to respiratory failure and death [4]. The rate of response to immunosuppressive therapies can also be unpredictable [5]. Clinical measures of ILD -- including pulmonary function tests (PFT), CT imaging pattern, and histology -- provide aggregate evidence of past episodes of inflammatory and fibrotic damage to the lungs, but have limited value in predicting future events [6]. As such, identification of biomarkers to predict prognosis and response to therapy in these illnesses would constitute an important clinical advance and is an area of research priority [7].

Fibrocytes are bone marrow-derived circulating progenitor cells that can home to sites of injury and contribute to scar formation [8]. In mouse models of fibrotic lung disease, fibrocytes rapidly exit the bone marrow to enter the bloodstream after tissue injury and home to the lungs; blocking this migration attenuates lung fibrosis [9-11]. In human ILD, elevated circulating fibrocyte concentration is associated with subsequent poor outcome in idiopathic pulmonary fibrosis and in a monogenic form of ILD [12, 13]. Elevation of circulating fibrocytes has previously been observed in fibrotic lung diseases associated with rheumatoid arthritis and scleroderma [14-17], but, to our knowledge, the value of longitudinal

measurements of fibrocyte concentration in predicting the outcome of autoimmune ILD has not been reported to date.

In the current study, we hypothesized that the blood fibrocyte concentration predicts outcome and treatment responsiveness in autoimmune ILD. To test this hypothesis, we performed a cohort study in which we correlated serial peripheral fibrocyte counts with PFT, response to therapy, and survival over time.

Materials and methods

Subjects and study visits: We recruited healthy subjects and subjects with autoimmune ILD between October 2010 and July 2015. At enrollment, subjects with ILD underwent a thorough history and physical examination, serologic workup for autoimmune disease, and high-resolution chest computerized tomography. Diagnoses of autoimmune diseases were adjudicated by 2 study team members (JO and BM) in accordance with published guidelines for rheumatoid arthritis [18], scleroderma [19], mixed connective tissue disease [20], myositis-related ILD [21], and interstitial pneumonia with autoimmune features [3]. Usual interstitial pneumonia (UIP) was identified based on histology, or when lung biopsies had not been performed, "definite UIP pattern" on high-resolution chest CT [22]. Initiation of immunosuppressive therapy was defined as initiation of azathioprine (≥50 mg daily) or mycophenolate mofetil (≥1000 mg daily) after ≥3 months off these medications, or increase in prednisone by ≥10 mg daily after ≥3 months on a stable dose of immunosuppressive medications. At each visit, PFT were performed per established guidelines [23, 24], and venous blood was collected. PFT variables were expressed as percent of predicted values of the NHANES-III database. PFT decline was defined as either a reduction of forced vital capacity (FVC) by ≥ 10% of predicted value or diffusion constant for carbon monoxide (DL_{CO}) by ≥ 15% predicted value. Study follow-up ended in January 2018, when death or censure were assigned by reviewing electronic medical records and publicly available death records.

Sample processing and flow cytometry: Blood samples were collected in heparinized tubes and immediately placed on ice, then refrigerated overnight before processing for fibrocytes quantification by flow cytometry without ex vivo manipulations such as culture or enrichment,

as previously described [13, 25-27]. See the on-line data supplement (Appendix S1) for detailed methods and gating strategy.

Statistical analyses: Data were analyzed in SAS (version 9.4 for Windows, Cary, NC, USA) or Prism (version 8 for Mac, GraphPad, San Diego, CA, USA). Descriptive data were summarized as median and interquartile range (IQR). Differences in demographics of subjects with stable disease and those with death or PFT decline were assessed using Wilcoxon rank-sum or Fisher's exact tests. Fisher's exact test was also used to assess the association between PFT decline and death. Log-rank test was used to access the association between UIP pattern and death. In cross-sectional comparisons, groups were compared using the Wilcoxon rank-sum test. Univariable and multivariable logistic regression was used to predict combined outcome of death or decline in functional status. For survival outcome classification, Youden's J-index was calculated to define threshold of maximum total fibrocyte count. Chi-squared test was performed for relative risk of death or functional decline above and below threshold of maximum total fibrocyte count. Wilcoxon signed-rank test was performed to access effect of immunosuppression on paired fibrocyte counts. Results considered significant if two-sided p value < 0.05.

Results

We recruited 26 healthy controls and 50 subjects with autoimmune ILD (Table 1). Among subjects with lung disease, 16 died and 5 experienced a decline in PFT during the 6.25 year study period (Figure 1A), but death was not significantly associated with PFT decline (Figure 1B). Among the subjects without PFT decline, the FVC increased by a median of 4% (IQR 0 to 12%) and DL_{CO} did not change (IQR -9 to +10%) over the study period. The subjects with death or PFT decline did not differ from subjects with stable disease in baseline characteristics (Table S1). UIP pattern was associated with death at 40 months of enrollment (Log-rank, $p = 0.01$).

In a cross-sectional comparison, we found the median concentration of circulating fibrocytes from the initial sample obtained from subjects with autoimmune ILD to be 2.5-fold higher in subjects with ILD as compared to healthy controls (Figure 2A). The majority of the

circulating fibrocytes expressed the chemokine receptors CXCR4, with smaller subsets expressing CCR2, CCR5, and CCR7; all these subsets were significantly higher in subjects with autoimmune ILD. Fibrocytes expressing the hematopoietic stem cell marker CD34, while significantly higher in subjects with ILD, constituted a small subset of total circulating fibrocytes (Figure S1).

To compare the activation state of fibrocytes between groups, we quantified the absolute concentration of circulating fibrocytes that express the myofibroblast differentiation marker, alpha-smooth muscle actin, and found this subset to also be expanded in patients with autoimmune ILD (Figure 2B). To assess key fibrotic signaling pathways within circulating fibrocytes, we quantified the fibrocytes that contained the phosphorylated forms of transcription factors involved in TGF-beta receptor signaling (Smad-2 and -3), the PI3K/AKT/mTOR pathway (AKT-1 and P70S6K), and IL4 and IL13 receptor signaling (STAT6). We found the subset of fibrocytes with activation in these signaling pathways to be significantly higher in subjects with autoimmune ILD as compared to healthy controls (Figure 2C-F). The most highly expressed activation markers expressed by fibrocytes from ILD patients were alpha-smooth muscle actin (expressed by a median of 77% of fibrocytes), phosphorylated Smad-2/3 (69%), and phosphorylated AKT (56%), with a minority of fibrocytes expressing phosphorylated P70S6K and STAT-6 (28% and 19%, respectively). We next assessed the longitudinal change in absolute circulating fibrocyte concentrations in 40 subjects with autoimmune ILD and 7 healthy control subjects. Serial samples exhibited episodes of marked elevation of fibrocyte concentration in subjects with autoimmune ILD, but not in healthy control subjects (Figure 3). During the study period, 13 subjects with autoimmune ILD had fibrocyte concentrations measured both before and after initiation or escalation of immunosuppression. In this group, the median interval between pre-and post-treatment fibrocyte analysis was 88 days (IQR 54-121). Immunosuppressive therapy was associated with a 43% decline in median absolute concentration of circulating fibrocytes and a 41% decline in the activated subset of fibrocytes (Figure 4).

In univariable analysis of the entire cohort, the peak fibrocyte concentration of subjects with autoimmune ILD predicted death or PFT decline: each increase in peak fibrocyte concentration of 10^5 cells/mL was associated with a 5% increase in the odds of death or PFT decline (Table 2). UIP pattern and gender-age-physiology score (a predictive index based on sex, age, predicted DL_{CO} and FVC) have each been shown to predict outcomes in autoimmune lung disease [28-30]; we therefore performed multivariable logistic regression models to include these known predictors. Inclusion of these variables in the model did not change the relationship between peak fibrocyte concentration and outcome (Table 2). We identified a threshold peak fibrocyte concentration of 2.6×10^6 fibrocytes/mL to be associated with doubling of the relative risk of death or PFT decline over the study period (95% CI 1.08-4.1; $p = 0.036$).

Discussion

Clinicians detect worsening of ILD only when it is severe enough to distort lung anatomy macroscopically, as detected by high-resolution chest CT, or to impair whole-organ physiology, as detected by PFT. There are 2 limitations to these assessments: first, CT and PFT are insensitive to early, and perhaps reversible, changes in lung inflammation and scarring. Second, these modalities measure the aggregate of damage to the lung by past insults, which may not correlate to future deteriorations of lung disease and death from respiratory insufficiency. Cellular and biochemical biomarkers of the underlying pathophysiology have the potential to overcome these limitations and provide information that is complementary to such traditional clinical measurements.

A number of biomarkers have been investigated for their utility in making a diagnosis and determining the trajectory of autoimmune ILD: the pattern of autoantibodies can categorize patients according to the risk of developing ILD; proteins derived from respiratory epithelium, including the cell surface glycoprotein Mucin-1 (also known as Krebs von den Lugen-6) and surfactant proteins, correlate with the degree of lung injury; and plasma cytokines and acute phase proteins have been used as surrogates of active inflammation [7, 31]. Few studies, however, have focused on biomarkers of fibrogenesis as predictors of

outcome in autoimmune lung diseases and none, to our knowledge, have assessed the predictive value of longitudinal measurements of such biomarkers over time.

Fibrocytes are released from the bone marrow in response to diverse forms of tissue injury. The mechanism by which fibrocytes contribute to physiologic wound healing and pathological scarring of injured organs is controversial in the literature, with some publications supporting differentiation into myofibroblasts and others providing evidence for a paracrine role in promoting fibrosis [11, 32-34]. Regardless of mechanism, the blood concentration of these cells may serve as an easily measurable marker, not of the extent of organ injury, but the fibrogenic response to that injury. This hypothesis was supported by a study in idiopathic pulmonary fibrosis, in which the proportion of fibrocytes in peripheral blood buffy coat leukocytes at a single time point correlated with concurrent acute exacerbations and was predictive of subsequent death [12]. We next performed a longitudinal study in Hermansky-Pudlak syndrome, a rare autosomal recessive disease, that afforded us the opportunity to describe the behaviour of circulating fibrocytes over time in a relatively homogeneous population of patients with ILD [13]. Unexpectedly, many patients in this population exhibited episodic and marked elevations of the absolute concentration of circulating fibrocytes over time, the magnitude of which was predictive of death from respiratory failure, but interestingly did not correlate with PFT decline [13]. We hypothesized that, at least in patients with this form of ILD, episodes of elevation in fibrocytes in the bloodstream reflect abrupt episodes of lung injury, which cumulatively result in lung function deterioration and respiratory failure.

In the current study, we sought to extend the prior findings to the more common, but far more heterogeneous, category of ILD attributable to autoimmune diseases. Prior work has shown fibrocytes to be elevated in the bloodstream of patients with ILD due to rheumatoid arthritis and scleroderma [14, 16]. Fibrocytes are detectable in the lungs of patients with scleroderma-associated ILD [17], and have been mechanistically linked to ILD in this population [15, 35, 36]. The present work adds to this literature by showing that, similar to patients with Hermansky-Pudlak syndrome, subjects with autoimmune ILD develop

episodic marked elevations in circulating fibrocytes that were predictive of death or decline in pulmonary function. Importantly, the predictive value of fibrocytes was cumulative and continuous, conferring a 5% increased risk per 10^5 increase in fibrocyte concentration, and was independent of the gender-age-physiology score and UIP pattern, each of which have previously been linked to worse outcomes in autoimmune ILD [28, 29, 37, 38]. The threshold value of fibrocyte concentration identified in this study is similar to the values we identified in patients with Hermansky-Pudlak syndrome, chronic sickle cell lung disease, and post-ARDS pulmonary fibrosis [13, 27, 39].

The current study also extends the prior literature by assessing several intracellular signaling events relevant to fibrogenesis in circulating fibrocytes. By detecting epitopes that result from specific amino-acid phosphorylations of transcription factors, flow cytometry is a powerful tool for identifying the signaling landscape in rare cell types with minimal ex vivo manipulation [40]. We detected the majority of circulating fibrocytes to contain phosphorylated Smad-2 and -3, evidence for TGF-beta receptor signaling in these cells [41]. In addition, fibrocytes in autoimmune ILD, but not control subjects, expressed phosphorylated forms of AKT1 and P70SK6, indicating activation of the PI3K/AKT/mTOR pathway. The latter is implicated in lung fibrogenesis and, in particular, in fibrocyte activation [11, 42]. Lastly, we identified a subset of fibrocytes that express phosphorylated STAT-6 in the context of autoimmune ILD. Although not previously described in fibrocytes, STAT-6 is an important mechanism in fibrosis and is relevant to fibroblast activation [43, 44], and prior work has documented the response of fibrocytes to IL4 and IL13 [45, 46].

We recognize several limitations in our study. The sample size was relatively small, reducing the study power and increasingly the likelihood of false-negative findings. This study was performed at a single tertiary care center, raising questions about the generalizability of its findings. Although fibrocytes have been predictive of outcome in other ILD, the current study does include a second validation cohort of patients with autoimmune ILD. By studying autoimmune ILD as a single entity, the study was not powered to detect differences in predictive utility of fibrocytes in individual diseases or in response to specific

immunosuppressive drugs. Finally, the data on the response of fibrocytes to immunosuppressive therapy is based on a small number of patient and should be viewed as hypothesis-generating rather than definitive.

The findings of this study suggest several avenues for future research. First, the utility of fibrocytes as biomarker of prognosis and response to therapy in patients with autoimmune ILD should be compared to other biomarkers. In this regard, combinations of serum biomarkers have been associated with better detection of rheumatoid arthritis-associated ILD than any single biomarker [31]; a similar approach may inform biomarkers of prognosis. Second, we report a fall in circulating fibrocytes in the subset of subjects who were started on immunosuppressive therapy during our study period. This observation suggests the hypothesis that fibrocytes may be useful as a biomarker of response to immunosuppression, for example identifying patients who have not responded to therapy, and conversely, guiding de-escalation of immunosuppression in patients with quiescent disease.

Finally, based on the results of the current study and prior studies of fibrocytes in other fibrotic lung diseases, we propose a general model wherein progression of diverse forms of ILD is the consequence of repeated fibrogenic responses to mostly subclinical episodes of alveolar epithelial injury. We propose that these responses include the release of fibrocytes from bone marrow into the bloodstream, which can be detected on scheduled screening blood tests, for example during routine clinic visits. This model is consistent with the reduction in blood fibrocyte concentration after immunosuppressive therapy which, we posit, suppresses epithelial injury and thus the fibrogenic response to that injury. This hypothesis can be tested in longitudinal studies of different subtypes of ILD.

List of abbreviations

AKT, protein kinase-B; GAP, Gender-age-physiology score; ILD, interstitial lung disease; IL4, interleukin-4; IL13, interleukin-13; IQR, Inter-quartile range; mTOR, mechanistic target

of rapamycin; PFT, pulmonary function tests; PI3K, phosphoinositol 3-kinase; TGF-beta, transforming growth factor beta; UIP, Usual interstitial pneumonia.

Declarations

Ethics approval and consent to participate: We obtained informed consent from the subjects in accordance with the Declaration of Helsinki, according to an institutionally approved protocol (University of Virginia IRB-HSR 15299).

Consent for publication: Not applicable

Availability of data and materials: The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Figure legends

Figure 1: Trends in pulmonary function tests. (A) Trends in forced vital capacity (FVC) and diffusion constant for carbon monoxide (DL_{CO}) over time for ILD subjects. Each line represents one subject. Dashed line represents no change. (B) Change between first and last PFT over the study period. Solid and dashed lines represent correlation and 95% confidence interval; dotted lines represent no change.

Figure 2: Cross-sectional comparison of circulating fibrocyte concentration in subjects with autoimmune ILD and healthy controls. (A) Total circulating fibrocytes ($CD45^+$ $Col1^+$ cells); (B) activated fibrocytes ($CD45^+$ $Col1^+$ αSMA^+ cells); (C-F) fibrocytes staining for phosphorylated Smad-2/3, AKT-1, P70SK6, and STAT6, respectively. Each dot represents one subject; bold horizontal lines in the violin plots show the median and light horizontal lines represent the 25th and 75th percentiles.

Figure 3: Change in circulating fibrocyte concentration over study period among healthy controls (A) and subjects with autoimmune ILD (B). Each line represents one subject.

Figure 4: Effect of immunosuppressive therapy on circulating fibrocyte concentration. Total circulating fibrocytes ($CD45^+$ $Col1^+$ cells; panel A) and activated fibrocytes ($CD45^+$ $Col1^+$ αSMA^+ cells; panel B) are shown. Each gray line represents one sample; bold lines represents median values.

Table 1: Summary of demographic and clinical data of the study subjects.

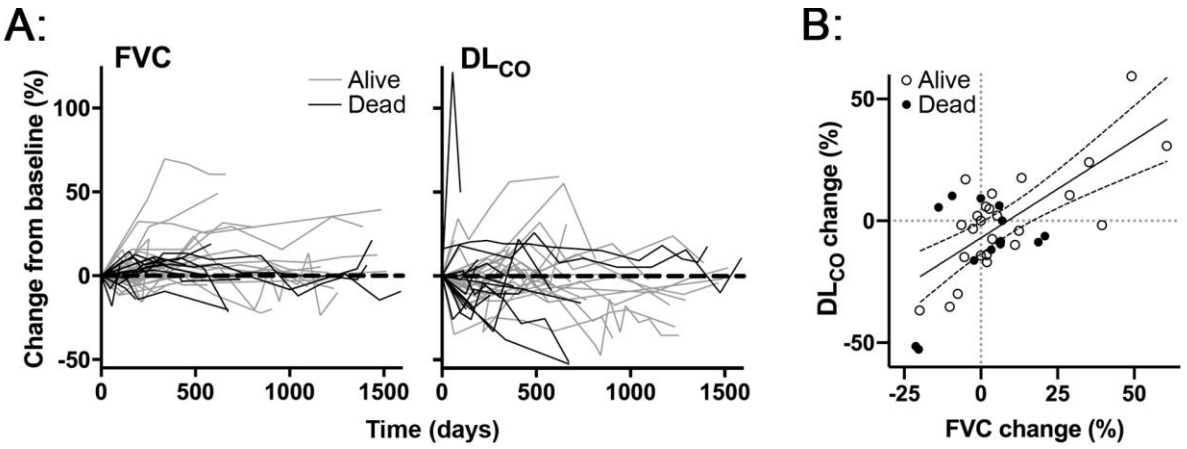
	Healthy controls	ILD subjects	*p* value
Number of subjects	26	50	
Median age (IQR)	55 (48-62)	60 (50 - 69)	0.07
Male sex, n (%)	11 (41)	21 (42)	0.97
Race			
Caucasian, n (%)	14 (54)	37 (74)	
African American, n (%)	7 (27)	13 (26)	0.53
Diagnosis			
IPAF, n (%)	-	18 (36)	
RA, n (%)	-	5 (10)	
MCTD, n (%)	-	3 (6)	
Myositis-related, n (%)	-	13 (26)	
Scleroderma, n (%)	-	11 (22)	
Pulmonary functions tests			
% predicted FVC, median (IQR)	-	61.5 (51, 78)	
% predicted DL_{CO}, median (IQR)	-	42.5 (26, 55)	
UIP pattern, n (% total)	-	11 (22)	
Oxygen use, n (median L/min, IQR)	-	15 (2, 2 - 3)	

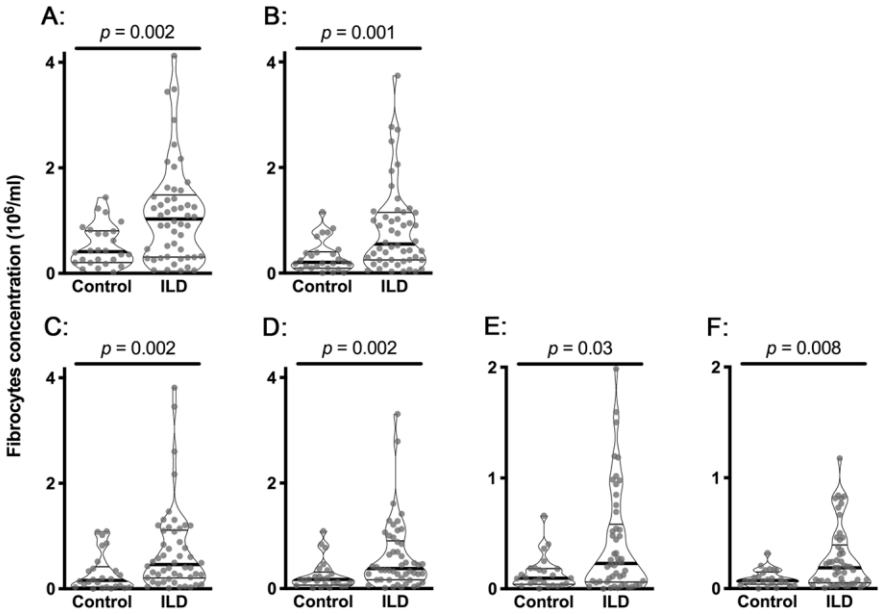
Definitions of abbreviations: DL_{CO}, diffusion capacity for carbon monoxide; FVC, forced vital capacity; IQR, interquartile range; IPAF, interstitial pneumonia with autoimmune feature; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis; UIP, usual interstitial pneumonia.

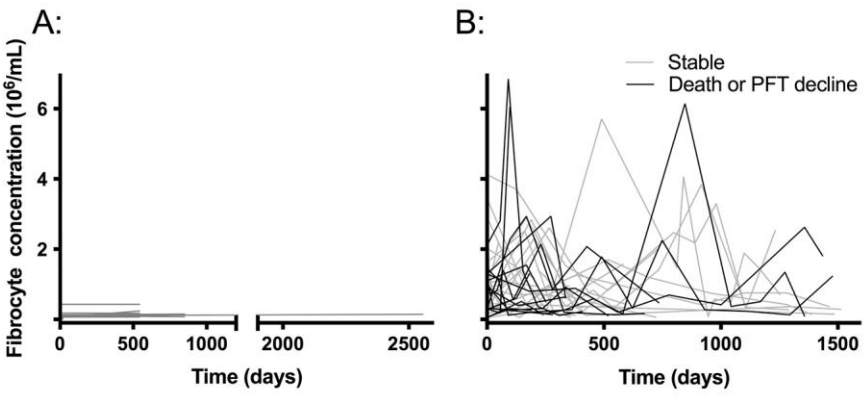
Table 2: Logistic regression to predict death or functional decline

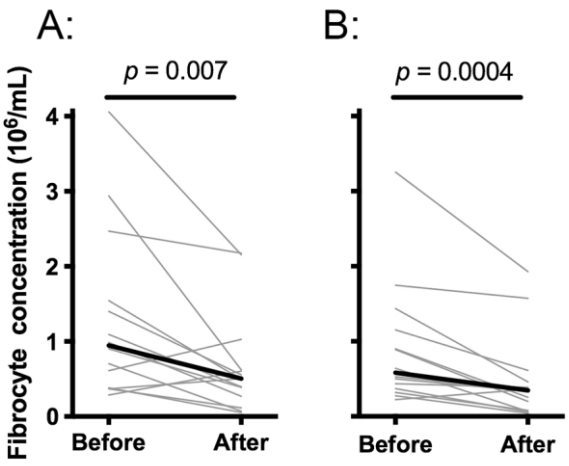
Parameter	Odds ratio estimate (95% CI)		
	Univariate model (n=50)	Multivariate model 1 (n=50)	Multivariate model 2 (n=49)
Peak fibrocyte concentration (per 100,000 increase)	1.044 (1.002, 1.088)	1.050 (1.007, 1.096)	1.050 (1.005, 1.097)
UIP pattern		3.292 (0.783, 13.84)	
GAP stage II or III			2.324 (0.649, 8.319)

Definition of abbreviations: UIP, usual interstitial pneumonia; GAP, gender, age, physiology.


A:
FVC
DLCO
Alive
Dead
Change from baseline (%)
100
50
0
-50
0
500
1000
1500
Time (days)
B:
Alive
Dead
DLCO change (%)
50
0
-50
-25
0
25
50
FVC change (%)


A:
p = 0.002
B:
p = 0.001
C:
p = 0.002
D:
p = 0.002
E:
p = 0.03
F:
p = 0.008
Fibrocytes concentration (10^6/ml)
Control
ILD


A:
B:
Fibrocyte concentration (10^6/mL)
6
4
2
0
500
1000
2000
2500
Time (days)
Stable
Death or PFT decline
0
500
1000
1500
Time (days)


A:
B:
p = 0.007
p = 0.0004
Fibrocyte concentration (10^6/mL)
4
3
2
1
0
Before
After
Before
After

SUPPLEMENTARY INFORMATION

Manuscript Title: Circulating fibrocytes as biomarkers of autoimmune interstitial lung disease: a cohort study

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Appendix S1: Supplemental Materials and Methods

Samples were centrifuged at 135 *g* and 4°C for 10 minutes and the buffy coat layers collected, and incubated in 20 ml of a red cell lysis buffer (150 nM NH_4Cl, 10mM $KHCO_3$, and 0.1 mM Na_2EDTA; pH 7.2) at room temperature for 10 minutes. Cells were then resuspended in phosphate buffered saline containing 0.1% heat-inactivated fetal bovine serum and enumerated under a hemocytometer. Cells were labeled with fluorescent-conjugated antibodies against surface antigens and then washed and permeabilized using a commercial reagent (Cytofix/Cytoperm, BD Biosciences, San Jose, CA, USA) before labeling of intracellular targets. The following antibodies were used (purchased from BD Biosciences, except as noted): anti-CC chemokine receptor-2 peridinin-chlorophyll-protein complex (CCR2, clone 48607; R&D Systems, Minneapolis, MN, USA); anti-CC chemokine receptor-5 allophycocyanin-Cy7 (CCR5, clone 2D7); anti-CC chemokine receptor-7 phycoerythrin-Cy7 (CCR7, clone 3D12); anti-CD34 peridinin-chlorophyll-protein complex (clone 8G12); anti-CD45 V500 (clone H130); anti-CXC chemokine receptor-4 allophycocyanin (CXCR4, clone 12G5); anti-α-smooth muscle actin phycoerythrin (clone 1A4; R&D Systems); anti-collagen-1 (Col-1, Rockland, Gilbertsville, PA, USA); anti-Mothers against decapentaplegic homolog-2 and -3 phosphorylated at serine 433 or 435 (p-Smad-2/3; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-RAC-alpha serine/threonine-protein kinase phosphorylated at leucine 110 allophycocyanin (p-AKT-1; clone C73H10; Cell Signaling Technology, Danvers, MA, USA); anti-ribosomal protein S6 kinase-1 phosphorylated at threonine 389 (p-P70S6K; Cell Signaling Technology); and anti-signal transducer and activator of transcription-6 phosphorylated at tyrosine 641 phycoerythrin (p-STAT6; clone 18). Anti-collagen-1, anti-p-P70S6K anti–p-Smad2/3 and respective control IgG were conjugated to fluorescein isothiocyanate, phycoerythrin, and allophycocyanin, respectively, using Lightning-Link (Novus Biologicals, Centennial, CO, USA) or DyLight antibody conjugation kits (Thermo Fisher Scientific, Waltham, MA, USA), per manufacturers' instructions. Samples were fixed in 2% paraformaldehyde in phosphate buffered saline and data was acquired on a

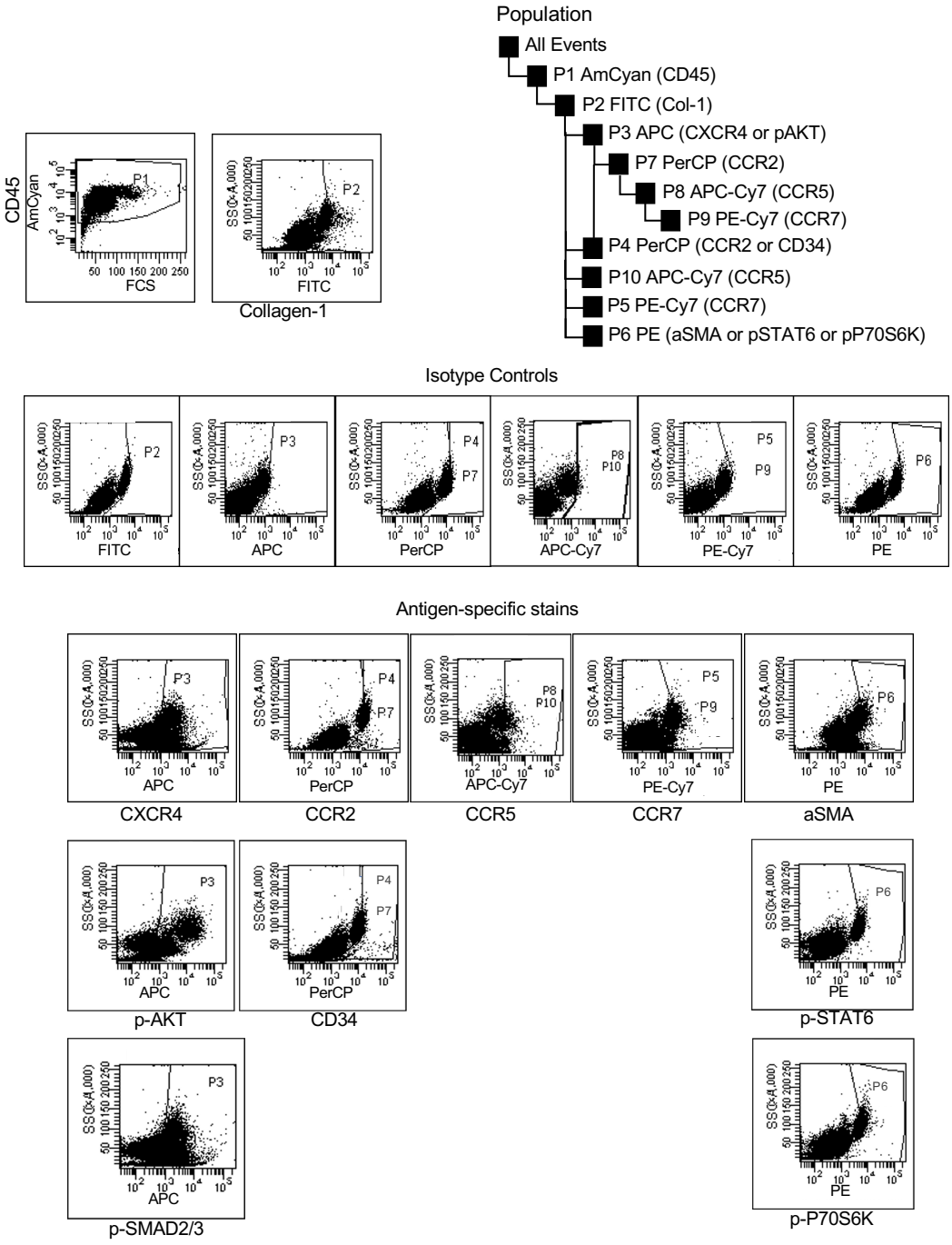
FACSCanto II instrument using BD Diva software (BD Biosciences). Data were analyzed by first gating on $CD45^+$ population and then for $Col\text{-}1^+$ population, using negative control thresholds set at 0.5% using matched IgG control. The $CD45^+$ $Col1^+$ population was then analyzed for staining for other antigens using respective antibody controls, using the gating scheme shown in Figure S1. Absolute concentrations of fibrocytes were calculated as the product of proportion of cell type and the total concentration of leukocytes in the original blood sample.

Table S1:

	Stable disease	Death or PFT decline	*p* value
Number of subjects	31	19	0.78
Median age (IQR)	60 (50 - 67)	61 (46 - 71)	0.77
Male sex, n (%)	11 (35)	10 (53)	0.26
Race			
Caucasian, n (% total)	22 (71)	15 (79)	
Black/African American, n (%)	9 (29)	4 (21)	0.74
Diagnosis			
IPAF, n (%)	12 (39)	6 (32)	
RA, n (%)	3 (10)	2 (11)	
MCTD, n (%)	2 (6)	1 (5)	
Myositis-related, n (%)	9 (29)	4 (21)	
Scleroderma, n (%)	5 (16)	6 (32)	0.81
Smoking status			
active, n (%)	1 (3)	1 (5)	
former, n (%)	16 (52)	11 (58)	
never, n (%)	14 (45)	7 (37)	0.82
Pulmonary functions tests			
% predicted FVC, median (IQR)	61 (51.5, 74.5)	63 (47, 83)	0.54
% predicted DL_{CO}, median (IQR)	48 (27, 61)	39 (23, 49)	0.13
UIP pattern, n (% total)	5 (16)	6 (32)	0.29
Oxygen use, n (median L/min, IQR)	7 (2, 2 - 4)	8 (2, 2 - 3)	0.80
Days of follow-up, median (IQR)	1162 (925 - 1561)	1213 (668 - 1514)	0.34

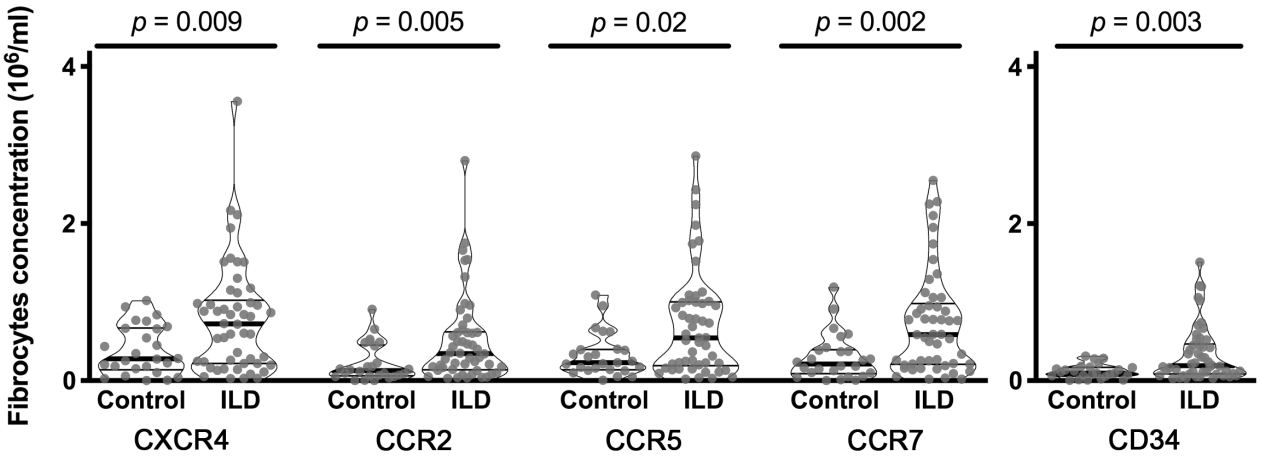
Definitions of abbreviations: DL_{CO}, diffusion capacity for carbon monoxide; FVC, forced vital capacity; IQR, interquartile range; IPAF, interstitial pneumonia with autoimmune feature; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis; PFT, pulmonary function tests; UIP, usual interstitial pneumonia.

Figure S1



Gating strategy.

Figure S2



Cross-sectional comparison of subsets of circulating fibrocyte concentration in subjects with autoimmune ILD and healthy controls. (A) $CD45^+$ $Col1^+$ cells expressing indicated chemokine receptors; (B) $CD45^+$ $Col1^+$ cells expressing CD34. Each dot represents one subject; bold horizontal lines in the violin plots is the median and light horizontal lines represent the 25th and 75th percentiles.