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

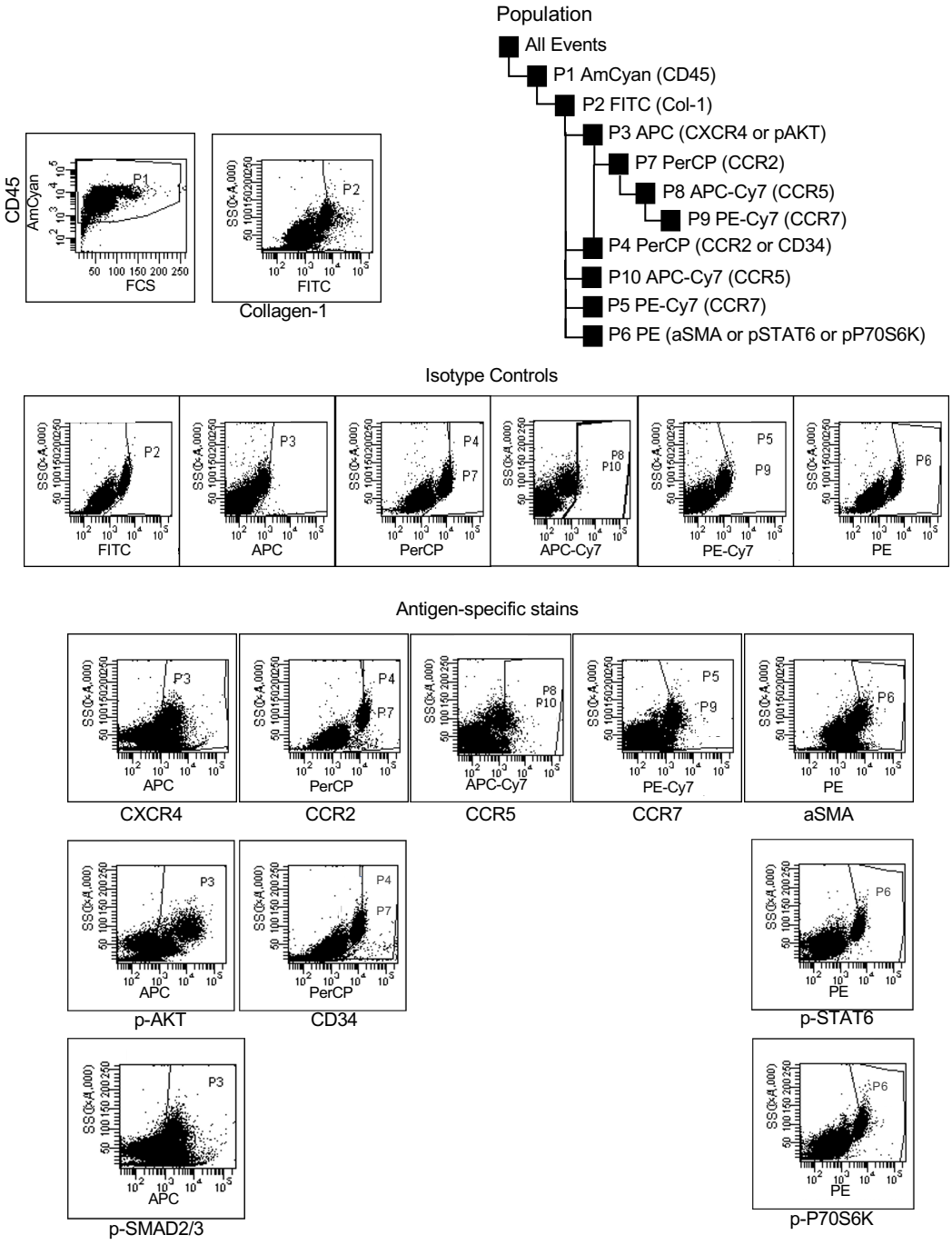
FACSCanto II instrument using BD Diva software (BD Biosciences). Data were analyzed by first gating on $CD45^+$ population and then for $Col\text{-}1^+$ population, using negative control thresholds set at 0.5% using matched IgG control. The $CD45^+$ $Col1^+$ population was then analyzed for staining 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

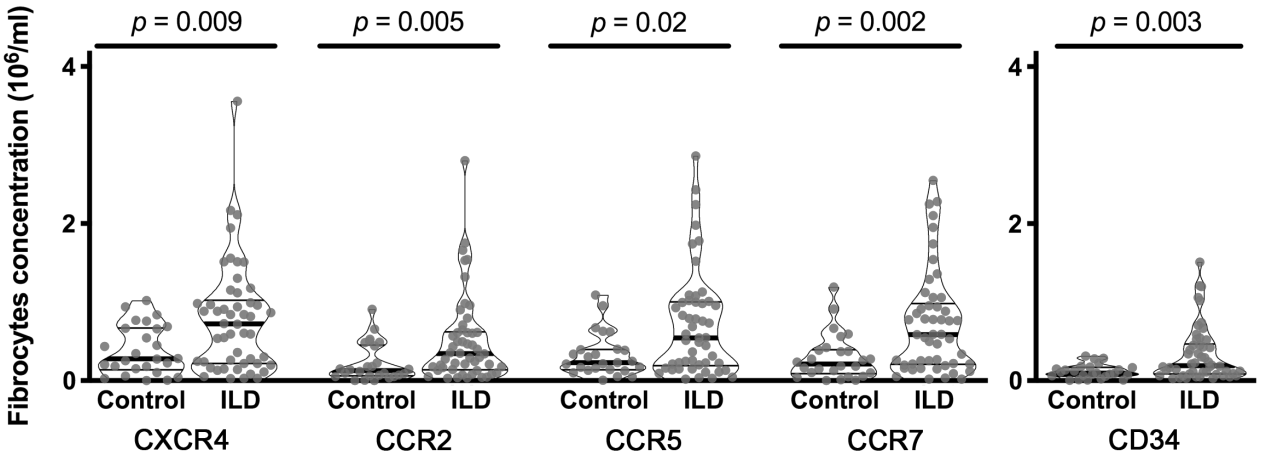
Definitions of abbreviations: DL_{CO}, diffusion capacity for carbon monoxide; FVC, forced vital capacity; IQR, interquartile range; IPAF, interstitial pneumonia with autoimmune feature; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis; PFT, pulmonary function tests; UIP, usual interstitial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