CHEST Online Supplement

Fibulin-1 Predicts Disease Progression in Patients With Idiopathic Pulmonary Fibrosis

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e-Appendix 1.

Detailed methods

Australia: Consecutive patients referred to the Interstitial Lung Disease Clinic at the Royal Prince Alfred Hospital between were recruited and informed consent attained. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=14), probable IPF (n=13), hypersensitivity pneumonitis (HP) (n=11), and sarcoidosis (n=7). Other ILDs included NSIP (n=3), connective tissue ILD (n=22), drug-induced ILD (n=1) and lymphangioleiomyomatosis (LAM) (n=2).

Healthy volunteers with no history of lung disease were also recruited (n=17).Serum was collected at first visit and stored at -80°C until testing. IPF lung tissues were obtained from explanted lung following lung transplantation at St. Vincent's Public Hospital, Sydney (n=5; 2 samples per patient) or from diagnostic biopsies from Perth (n=7; 2-6 samples per patient). Normal human lung tissue was obtained from healthy transplant donor's lungs not suitable for transplantation (n=5). Human ethics approval was given by the Bellberry Human Research Ethics Committee (Perth) #2011-10-497, Ethics Review Committee (RPAH, Sydney) #HREC/10/RPAH/613, Human Ethics Administration (The University of Sydney) #2012/946.

Italy: Consecutive IPF patients referred to the Center for Rare Lung Diseases at the University of Modena and Reggio Emilia were recruited and consent attained. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=28) and other ILDs included LAM (n=2).

Serum was collected at first visit and stored at -80°C until testing. Diagnostic surgical lung biopsies were obtained (n=8; 2-6 samples per patient). Human ethics approval was given by the Comitato Etico Provinciale di Modena #74/08 and 31/12.

San Francisco: Serum was collected at first visit and stored at -80°C until testing. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=17) HP (n=21), and sarcoidosis (n=5). Other ILDs included NSIP (n=1), and connective tissue ILD (n=4).

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Whole lung lysates from explanted lung were stored at -80°C until testing. Human ethics approval was given by the Laurel Heights Panel IRB#10-00198.

Lung Function Measurements: Demographic information including age, gender, body mass index (BMI) and smoking history were collected. Baseline lung function measurements taken included pre-bronchodilator percent predicted forced vital capacity (FVC), percent predicted forced expiratory volume in 1 second (FEV1), percent predicted diffusing capacity of carbon monoxide (DLco) and percent predicted total lung capacity (TLC) (1).The Composite Physiologic Index (CPI) was calculated as previously published (2).

Antibodies

Primary antibodies used for western blot and immunohistological staining on paraffin sections were as follows. Mouse monoclonal antibody (MAb) against human fibulin-1 was obtained from Santa Cruz Biotechnologies (USA; cat#SC25281). MAbs against human anti-glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was obtained from Merck (USA; cat#MAB374). Secondary antibodies for both fibulin-1 and GAPDH were obtained from Dako (Denmark; cat#P0260).

Isotype control antibody for use in immunohistological staining was as follows. Mouse IgG2a antibody from Dako (Denmark; cat#X0943) was used with fibulin-1.

Fibulin-1 positive control

Placental fibulin-1 was prepared as previously published (3) and used as a positive control in western blot experiments for serum. For measurements of whole lung lysates, fibulin-1 transfected HT1080 fibrosarcoma cell lysates (4) were used as a positive control.

Whole Lung Lysates

Whole lung lysates were prepared as follows: Lung tissue was directly snap-frozen in liquid nitrogen immediately after harvest. Samples were stored at –80°C until used for experiments. For immunoblot experiments, frozen lung tissue was pulverized using a stainless steel tissue pulverizer (Fisher Scientific) pre-cooled in liquid nitrogen and was immediately lysed in SDS-PAGE running buffer and analyzed as described below.

Cell Isolation and Culture

Primary parenchymal fibroblasts were isolated from 7 patients diagnosed with non-small cell carcinoma and 8 patients with IPF. Human distal parenchymal fibroblasts were isolated from lung tissue obtained from donors undergoing resection for either thoracotomy or transplantation. Demographic information for donors of tissue is found in Table 2.

Methods for the isolation of the cells are described previously (5). Briefly, tissue from distal parenchymal was minced into 1-2mm pieces and placed into sterile Hanks Buffered Saline Solution (Hanks) and

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centrifuged for 5 minutes at 1000rpm. After aspiration of the supernatant, the tissue pellet was resuspended and plated into tissue culture grade plastic flasks (BD Biosciences, North Ryde, Australia) containing 10% (vol/vol) fetal bovine serum (FBS) with 2% antibiotics in DMEM (Invitrogen). Passages of cells between 3 and 5 were used in all experiments. All cultures tested negative for the presence of mycoplasma before use in experiments.

Primary fibroblasts were seeded in 6-well plates at 1 x 10^4cells/cm^2 in 5%FBS/1% antibiotics/DMEM for 72 hours and then quiesced in 0.1% FBS/1% antibiotics/DMEM for 24 hours. Fresh 0.1% FBS/1% antibiotics/DMEM media is added for a further 72 hours.

From each well, cell culture supernatants are collected and then remaining cells are immediately washed twice in cold sterile phosphate buffered saline (PBS, pH 7.2). On wet ice, total cellular protein extracting buffer is added. Extraction buffer contains 20mM Tris, pH 7.4, 150mM NaCl, 1mM Na2EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail set III (Millipore) and 1mM phenylmethylsulfonyl fluoride (PMSF) (Amresco, Solon, OH, USA). Lysates are then collected and centrifuged at 4°C/14,000g for 5 mins to pellet debris. Cell debris-free fraction is then aspirated and stored.

Supernatants and lysates were stored at -20°C until analysis.

Western Blot

Whole lung lysates, fibroblast cell lysates, fibroblast supernatants and sera were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to a 0.45µm pore size polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked, then probed with the primary antibody to fibulin-1 followed by horseradish peroxidise-conjugated secondary antibodies (Dako, USA). Visualization was carried out using an enhanced chemiluminescence (ECL) western blot detection kit according to the manufacturer's protocol (Millipore, USA).

For whole lung and cell lysates 1.5ug total protein as measured by Bicinchoninic Acid (BCA) assay (Sigma #BCA1) was loaded per lane. The membrane was then stripped and reprobed for GAPDH. Analysis of fibulin-1 level in lysates is the same as for sera, with further additional normalization to GAPDH level.

For measurements of fibulin-1 level in fibroblast supernatants, equal volumes of supernatant were loaded and final fibulin-1 levels are adjusted for the total protein concentration of the respective confluent cell monolayer lysate as measured by BCA and described in the literature (6, 7).

Immunohistochemistry

5µm thick paraffin sections from formalin-fixed tissue were directly stained with for fibulin-1 and in parallel with the isotype control. No antigen retrieval was necessary. After incubation with DakoEnVision

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secondary reagents (Mouse cat#K4006; rabbit cat#K4010), positive staining was visualized using diaminobenzidine (Dako cat#K3467) for 2 minutes. Sections were counterstained in Mayer's hemotoxylin (Sigma cat#MHS1) for 3 minutes.

Serial sections were also stained using standard Masson's trichrome in order to quantify total collagen levels.

Image Capture

Western blot images were captured using a Kodak Image Station 4000mm camera and analysed using Carestream Molecular Imaging software (v. 5.3.3.17476 Carestream Health Inc. 1994-2011).

Immunohistochemisty images were taken at 20X magnification using an Olympus BX60 microscope. 20 consecutive images of each section were taken by a DP71 camera with Kohler illumination. Images were taken over the entire surface of the tissue section in order to obtain a comprehensive picture. Two to six sections from each patient were examined. **Densitometric analysis**

The images of sections stained for fibulin-1 were analysed by the open source software ImageJ (http://rsb.info.nih.gov/ij/)(8). First, each image was colour deconvoluted (9, 10) using an algorithm and the plugin vector H&E DAB (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html) . The resulting brown stain was further investigated and a threshold of positive staining was set manually by examining 5 random images of sections from 5 independent non-diseased control patients. This threshold was then applied to every image. The algorithm then calculated the positively stained area as a numerical value and adjusted for the total positively stained area in the image. The resultant mean value took into account any compression of the tissue during processing as well as the increased tissue mass in fibrotic lesions (11).

Similarly, images of sections stained with Masson's trichrome were colour deconvoluted using an algorithm and the plugin vector Masson trichrome. The resulting green stain was quantified and reported as the percentage area of collagen.

Each of the 20 image values were then averaged to obtain the average staining density for each tissue section.

Finally, the multiple samples from each patient were averaged together to give the final fibulin-1 level or percentage area of collagen per patient as a global estimation.

Representative images and respective densitometric analyses are shown in eFigure 1.

Quality Control and Statistical Analysis

To determine repeatability and the reliability of measurements, the coefficient of repeatability and intraclass correlation coefficient were calculated (12).

Duplicate serum samples from 17 subjects were tested on two separate occasions (data not shown). The coefficient of repeatability was 1.48 units of fold difference. This indicates that the same serum tested the

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first time can be expected to differ by more than 1.48 fold on the second test only 5% of the time. The intraclass correlation coefficient was 0.91 (95%CI 0.77 to 0.97) indicating that there was a high degree (>0.6) of reproducibility of the serum measurements.

Graphs were made using GraphPad Prism 6 Software for Windows (Version 6 GraphPad Software Inc. 1992-2007). Statistical analysis was done using SPSS (Version 21 IBM Corporation 1989-2012). Characteristics of the subjects and spirometric results were summarized with the use of descriptive statistics.

Distributions of serum, parenchyma and fibroblast levels of fibulin-1, and lung function parameters were tested for normality using the Kolmogorov-Smirnov test, Shapiro-Wilk test, and skewness and kurtosis were calculated.

Serum fibulin-1 levels were not normally distributed (n=89; $p<0.0001$). For multivariate linear analyses, serum fibulin-1 levels were transformed to the natural log value to obtain a normal distribution. Between-group differences were assessed by means of one-way analysis of covariance with adjustments for confounders with post-hoc analysis by Tukey's test.

Receiver-operator curves (ROC) was used to model the utility of serum fibulin-1 as a marker of progression. Cox regression, with and without baseline parameters, were used to model the impact of serum fibulin-1 levels on predicting progression. Kaplan-Meier survival curves were used to model the progression-free survival rate of serum fibulin-1 and between group rates were compared using the Mantel-Cox log rank test.

Parenchyma fibulin-1 levels were normally distributed (n=25; $p=0.504$). Between-group differences were assessed by unpaired t-test.

To study the relationship between parenchymal fibulin-1 levels and lung function parameters, diagnostic lung biopsies and corresponding lung function tests no more than 30 days (±1) apart were used. Correlations between tissue fibulin-1 and lung function were analysed using Pearson's product-moment tests.

The sample size for analysis of fibroblast fibulin-1 levels was too small to determine normality (n=15). Between-group differences were assessed by means of unpaired t-test.

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e-Table 1. Characteristics of the patients with IPF from 3 independent populations.

	Cohort 1		Cohort 2		Cohort 3			
	Sydney IPF (n=27)		Modena IPF (n=28)		San Francisco IPF (n=17)		*ANOVA	**Tukey's post-hoc (between group, p value)
	Mean	SD	Mean	SD	Mean	SD	p value	
Age, yr	67.7	7.7	65.9	10.1	71.6	9.7	0.225	n/a
BMI, kg.m2	30.5	6.4	n/a	n/a	n/a	n/a	n/a	n/a
Baseline FEV1%, % predicted	82.7	20.5	80.6	22.0	72.3	14.0	0.248	n/a
Baseline FVC%, % predicted	78.2	19.9	77.7	21.6	62.4	14.3	0.021	(1 vs 3, 0.03) (2 vs 3, 0.04)
Baseline DLco%, % predicted	41.0	16.2	42.3	18.0	38.9	15.6	0.838	n/a
Baseline CPI, units	51.0	12.2	49.0	14.5	57.8	10.0	0.129	n/a
Baseline TLC%, % predicted	66.8	11.2	75.6	12.1	62.0	13.0	0.039	(2 vs 3, 0.03)
First blood draw serum fibulin-1, units	2.0	0.7	2.0	1.1	2.2	1.4	0.800	n/a
	Number	%	Number	%	Number	%		
Male	21	77.80	10	35.70	10	83.30	0.00	(1 vs 2, 0.002) (2 vs 3, 0.008)
History of smoking	17	63.00	17	60.70	11	70.60	0.21	n/a

*One way analysis of variance (ANOVA)
**Significant ANOVA comparisons are then analysed post-hoc for between group differences using Tukey's
IPF idiopathic pulmonary fibrosis, SD standard deviation, n/a not applicable, yr years, FEV1 forced expiratory volume in 1 second, FVC forced vital capacity, DLco diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity

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e-Table 2. Characteristics of the patients with interstitial lung disease, other than IPF

	Sarcoidosis (n=12)		Hypersensitivity Pneumonitis (n=32)		"Other"* ILD (n=35)	
	Mean	SD	Mean	SD	Mean	SD
Age, yr	48	11	61	11	59	14
BMI, kg.m2	31	5	31	5	26	5
Baseline FEV1%, % predicted	79	23	70	19	74	22
Baseline FVC%, % predicted	91	22	68	22	78	24
Baseline DLco%, % predicted	70	23	49	15	48	20
Baseline CPI, units	22	19	46	14	44	18
Baseline TLC%, % predicted	88	20	73	20	76	22
	Number	%	Number	%	Number	%
Male	7	58	10	31	10	29
History of smoking	2	17	16	50	14	40

*"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).

BMI body mass index, FEV1 forced expiratory volume in 1 second, FVC forced vital capacity, DLCO diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, Yr year, IPF idiopathic pulmonary fibrosis

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e-Table 3. Detailed characteristics of patients from whom fibroblasts were obtained for cell culture experiments. .

Donor #	Gender	Age	Diagnosis	Surgery
Non-IPF Control 1	Male	55	NSCCa	Resection
Non-IPF Control 2	Male	66	NSCCa	Resection
Non-IPF Control 3	Female	54	NSCCa	Resection
Non-IPF Control 4	Male	60	NSCCa	Resection
Non-IPF Control 5	Male	57	NSCCa	Resection
Non-IPF Control 6	Male	59	NSCCa	Resection
Non-IPF Control 7	Male	61	NSCCa	Resection
IPF 1	Male	53	Idiopathic pulmonary fibrosis	Transplant
IPF 2	Male	62	Idiopathic pulmonary fibrosis	Transplant
IPF 3	Male	57	Idiopathic pulmonary fibrosis	Transplant
IPF 4	Male	55	Idiopathic pulmonary fibrosis	Transplant
IPF 5	Male	58	Idiopathic pulmonary fibrosis	Transplant
IPF 6	Male	58	Idiopathic pulmonary fibrosis	Transplant
IPF 7	Male	54	Idiopathic pulmonary fibrosis	Transplant
IPF 8	Male	63	Idiopathic pulmonary fibrosis	Transplant

Parenchymal fibroblasts were isolated from lung tissue obtained from donors undergoing resection for either thoracotomy or transplantation. Pulmonary function and smoking data were not available.

IPF idiopathic pulmonary fibrosis, NSCCa nonsmall cell carcinoma

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e-Table 4. Serum fibulin-1 levels differ between patients with IPF and volunteers with no lung disease.

ANCOVA p=0.034	Median serum fibulin-1 level (interquartile range)	Adjusted mean serum fibulin-1 level (units)	95%CI		p value compared to IPF
			lower	upper	
Non-Diseased Control (n=17)	1.03 (0.4)	1.00	0.71	1.43	0.006
Sarcoidosis (n=12)	0.95 (0.54)	1.01	0.63	1.63	0.025
HP (n=32)	1.41 (1.26)	1.48	1.17	1.88	0.135
"Other" *ILD (n=35)	1.49 (0.98)	1.37	1.11	1.69	0.033
IPF (n=72)	1.74 (1.3)	1.83	1.56	2.16	n/a

*"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).

Between group differences were assessed by one way analysis of covariance (ANCOVA) with adjustments for age, gender and smoking history.
CI confidence intervals; IPF idiopathic pulmonary disease, HP hypersensitivity pneumonitis, n/a not applicable

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e-Table 5. Serum fibulin-1 does not correlate with lung function measurements in patients with IPF.

Variable	Spearman's Rho coefficient	p value
Baseline FEV1, % predicted	0.054	0.832
Baseline FVC, % predicted	0.046	0.851
Baseline DLco, % predicted	-0.052	0.842
Baseline CPI, units	0.104	0.714
Baseline TLC, % predicted	-0.181	0.524

Time between lung function measurement and blood draw was no more than 15 days (± 5). Variables were analysed with spearman's rank correlation analysis. Serum fibulin-1 was measured by immunoblot.IPF idiopathic pulmonary fibrosis, FEV1 forced expiratory volume in 1 second, FVC forced vital capacity, DLco diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity.

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e-Table 6. Serum fibulin-1 correlates with lung function parameters in patients with ILDs

Variable	Spearman's Rho coefficient	p value
Baseline FEV1, % predicted	-0.14	0.23
Baseline FVC, % predicted	-0.30	0.01
Baseline DLco, % predicted	-0.25	0.04
Baseline CPI, units	0.34	0.01
Baseline TLC, % predicted	-0.34	0.01

Lung function parameters and demographic information was obtained for 73 patients with interstitial lung disease (ILD). The time between serum collection and lung function measurements was no more than 15 days (±5). Variables were analysed with spearman's rank correlation analysis. Serum fibulin-1 was measured by immunoblot.
FEV1 forced expiratory volume in 1 second, FVC forced vital capacity, DLco diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity

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e-Table 7. Validity of serum fibulin-1 thresholds in patients with IPF to predict disease progression

Representative threshold value	Sensitivity	Specificity
0.697	100%	5%
0.815	100%	14%
1.085	**93%**	**33%**
1.309	89%	38%
1.355	85%	48%
1.417	74%	48%
1.535	70%	62%
1.592	**70%**	**71%**
1.810	52%	71%
1.857	48%	76%
2.076	41%	81%
2.500	37%	86%
2.888	**30%**	**90%**
3.088	26%	90%
3.497	15%	90%

Selected threshold values from the ROC curve (Area under the curve = 0.71, 95% confidence interval 0.57 – 0.86, p=0.012, n=48). Values in bold were used for further analysis
IPF, idiopathic pulmonary fibrosis

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e-Table 8. Measurement of serum fibulin-1 predicts progression in patients with IPF.

	Univariate				Multivariate			
Variable	Hazard Ratio	95% CI		P value	Hazard Ratio	95% CI		P value
Serum fibulin-1, units	1.691	1.225	2.335	0.001*	2.109	1.271	3.500	0.004*
Age, yr	1.019	0.981	1.058	0.332	0.965	0.913	1.019	0.200
History of smoking	0.939	0.411	2.149	0.882	0.879	0.291	2.649	0.818
FVC, % predicted	0.989	0.970	1.009	0.280	0.990	0.965	1.015	0.428
DLco, % predicted	0.971	0.942	1.000	0.050*	0.997	0.960	1.035	0.875

Using univariate analysis, all factors were individually analysed for their relationship to progression. With multivariate analysis, all factors are analysed simultaneously and their independent contribution to the prediction of progression was compared. *p≤0.05
Yr years, FVC forced vital capacity, DLco diffusing capacity of carbon monoxide, CI confidence interval

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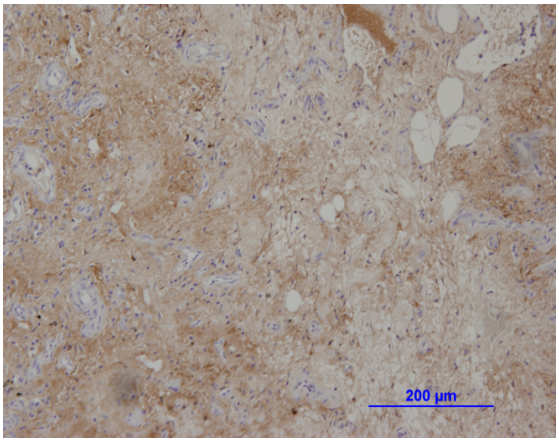
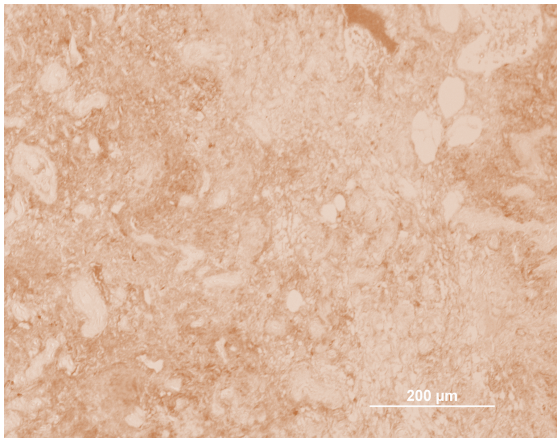
e-Figure 1. Image based protein quantification of immunohistochemical staining of tissue sections.

In order to quantify the amount of fibulin-1 from each patient 2-6 samples of tissue from each patient were stained using monoclonal antibodies to fibulin-1. Each of the samples was imaged 20 times and a computer software programme, Image J, was use to isolate the brown stain (fibulin-1) from the blue stain (hemotoxylin, or cell nuclei). The values are then averaged together. Image A is a tissue section from an IPF patient that has been stained for fibulin-1. Image B is that same image after colour deconvolution. The fibulin-1 level in this image measured 131.1 units. Image C is the isotype control stain of the same section.

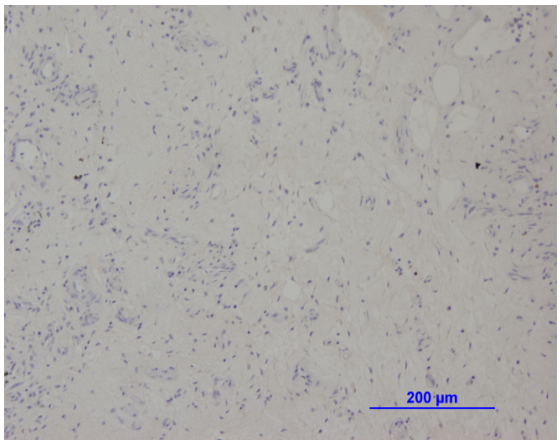
A



B



C



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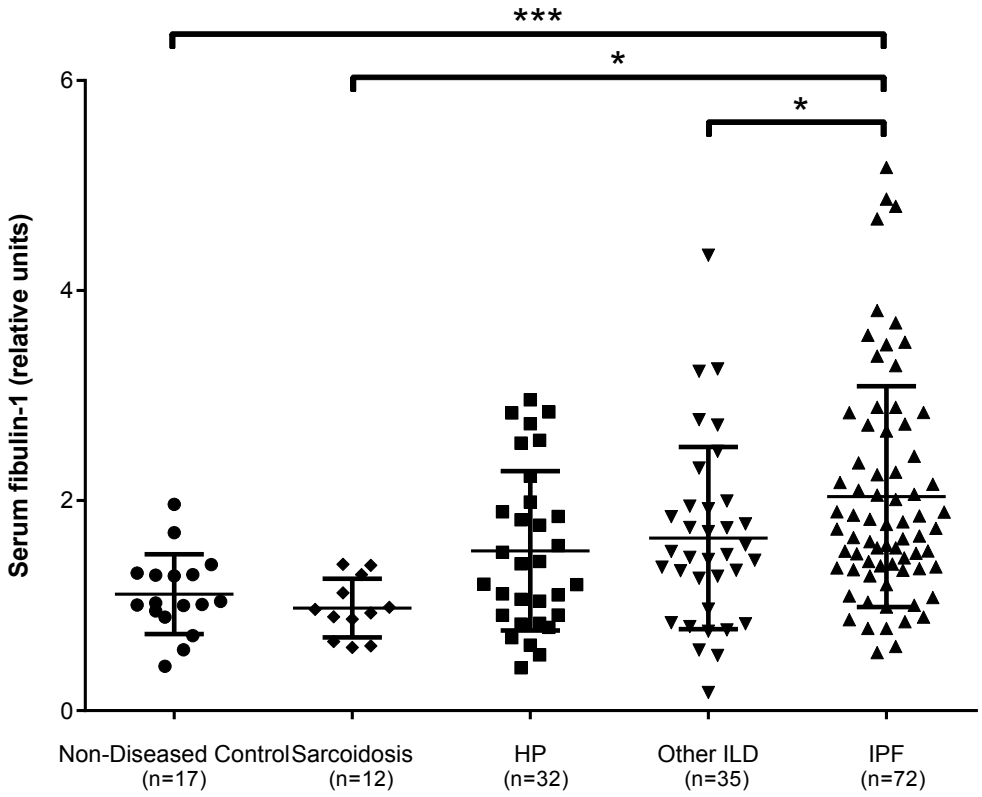
e-Figure 2. Serum fibulin-1 levels are increased in patients with IPF compared to non-diseased controls.



The fibulin-1 level of each serum sample was determined by immunoblotting and reported as relative to a standard serum sample. Densitometric analysis was performed using Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). Serum fibulin-1 levels were adjusted for age, gender and smoking history. (Analysis of covariance, n=168, post-test Tukey's *p<0.05. ***p=0.006, median ± 25th & 75th percentiles).

"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).

HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis

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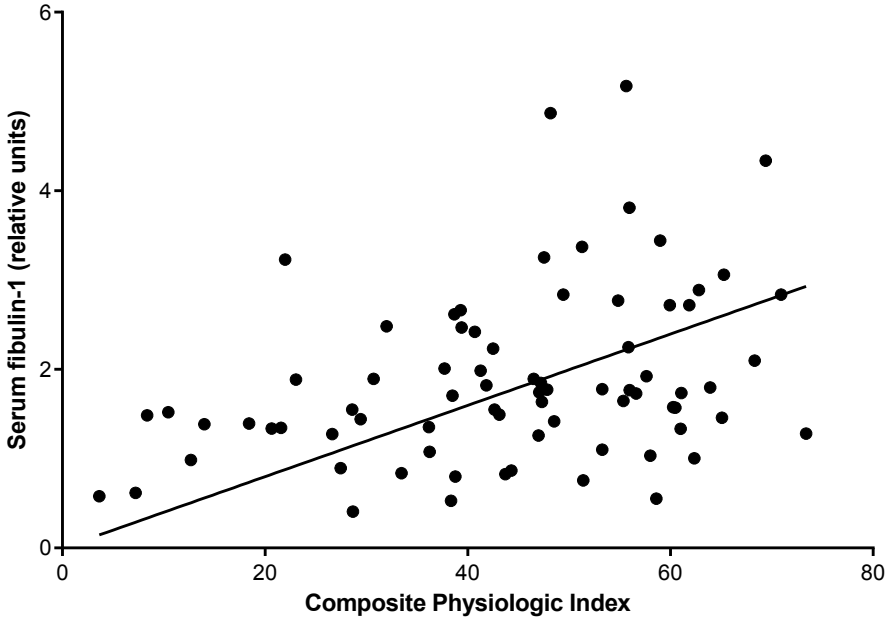
e-Figure 3. Serum fibulin-1 levels correlate with disease severity in patients with fibrotic ILD



The composite physiologic index (CPI) was calculated in 73 patients with interstitial lung disease (ILD).

CPI is derived from lung function measurements taken no more than 15 days (± 5) from blood draw and represents a measurement of disease severity.

The fibulin-1 level of each serum sample was determined by immunoblotting and reported as relative to a standard serum sample. Densitometric analysis was performed using Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). The linear regression line was calculated using Spearman's rank correlation analysis (rho=0.34, p=0.01).

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