




# Tissue and Bronchoalveolar Lavage Biomarkers in Idiopathic Pulmonary Fibrosis Patients on Pirfenidone

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## Abstract

**Background** Pirfenidone is a novel anti-fibrotic agent in idiopathic pulmonary fibrosis with proven clinical benefit. Better human tissue models to demonstrate the immunomodulatory and anti-fibrotic effect of pirfenidone are required.

**Objectives** The purpose of the study was to use transbronchial lung cryobiopsy (TBLC), a novel technique which provides substantial tissue samples, and a large panel of biomarkers to temporally assess disease activity and response to pirfenidone therapy.

**Methods** Thirteen patients with confirmed idiopathic pulmonary fibrosis (IPF) underwent full physiological and radiological assessment at diagnosis and after 6-month pirfenidone therapy. They underwent assessment for a wide range of potential serum and bronchoalveolar lavage biomarkers of disease activity. Finally, they underwent TBLC before and after treatment. Tissue samples were assessed for numbers of fibroblast foci, for Ki-67, a marker of tissue proliferation and caspase-3, a marker of tissue apoptosis.

**Results** All patients completed treatment and investigations without significant incident. There was no significant fall in number of fibroblast foci per unit tissue volume after treatment (pre-treatment: 0.14/mm<sup>2</sup> vs. post-treatment 0.08/mm<sup>2</sup>,  $p=0.1$ ). Likewise, there was no significant change in other markers of tissue proliferation, Ki-67 or Caspase-3 with pirfenidone treatment. We found an increase in three bronchoalveolar lavage angiogenesis cytokines, Placental Growth Factor, Vascular Endothelial Growth Factor-A, and basic Fibroblast Growth Factor, two anti-inflammatory cytokines Interleukin-10 and Interleukin-4 and Surfactant Protein-D.

**Conclusions** TBLC offers a unique opportunity to potentially assess the course of disease activity and response to novel anti-fibrotic activity in IPF.

**Keywords** Pirfenidone · Idiopathic pulmonary fibrosis · Transbronchial lung cryobiopsy

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## Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive inflammatory and fibrotic lung disease characterised by remodelling of the lung parenchyma. The disease follows an unfavourable course frequently resulting in death within

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3 years of diagnosis [1, 2]. Despite promising preliminary studies, there are no validated predictive biomarkers of disease progression or response to novel anti-fibrotic therapies such as Nintedanib and Pirfenidone [3–7].

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) (Roche Pharma, Basel, Switzerland) is a novel anti-inflammatory and anti-fibrotic agent which slows disease progression, as measured by decline in forced vital capacity (FVC) and progression free survival by up to 72 weeks in patients with mild to moderate disease [7–9].

Preclinical animal studies of lung fibrosis have suggested potential anti-fibrotic and anti-inflammatory effects of pirfenidone. These include the downregulation of pro-fibrotic and proinflammatory growth factors and cytokines including transforming growth factor- $\beta$  (TGF- $\beta$ ), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), interleukin-4 (IL-4) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and the upregulation of the anti-inflammatory cytokine interleukin-10 (IL-10). It is thought to modulate vascular remodelling and fibroblast apoptosis [10, 11]. However, there is no validated human tissue model of immunomodulatory effects of pirfenidone.

Transbronchial lung cryobiopsy (TBLC) is a safer alternative to open surgical lung biopsy giving a diagnostic yield of 70% in ILD. Tissue sample size ranges between 15 and 42 mm<sup>2</sup> providing ample tissue for multi-disciplinary team discussion and for immuno-histochemical analysis for potential tissue biomarkers of disease progression or treatment effect [12–15].

This study aims to evaluate the impact of pirfenidone on potential biomarkers of disease progression in plasma and bronchoalveolar lavage (BAL) and lung tissue obtained using TBLC.

## Methods

This was a single site, experimental pre-post study. Patients with a multi-disciplinary team, confirmed diagnosis of IPF based on pathology consistent with UIP and radiology with high probability of IPF, were recruited and provided written consent. Exclusion criteria were as follows: liver or renal failure, echocardiograph evidence of pulmonary hypertension, exacerbation within the previous 3 months or lung function precluding pirfenidone therapy, i.e., FVC < 50% predicted or carbon monoxide gas transfer factor (D<sub>L</sub>CO) < 35% predicted. Patients commenced on pirfenidone at standard doses of 2403 mg per day for 26 weeks [9]. Dosage was titrated to full treatment dose after 2 weeks.

The following procedures were undertaken pre and post 26 weeks of treatment.

1. Measurement of FVC and D<sub>L</sub>CO and 6-min walk distance.
2. High-resolution computed tomography (HRCTs) scored by two thoracic radiologists [16]. Diagnostic criteria included reticulation, traction bronchiectasis and subpleural basal distribution without significant ground glass opacification or radiological evidence of combined pulmonary fibrosis and emphysema.
3. Blood samples were taken immediately prior to bronchoscopy and at least 12 h after the last dose of pirfenidone. Circulating inflammatory mediators in plasma were measured using a MesoScale Discovery (MSD) ELISA multiplex platform [17, 18]. These plates measured a pro-inflammatory panel of cytokines, a chemokine panel, a general human cytokine, an angiogenesis panel and a vascular injury panel. Separate ELISA's were performed for TGF- $\beta$  (R&D systems), MMP-7 (Sigma Aldrich), SP-D (R&D systems) and SDF-1 $\alpha$  (Sigma Aldrich). (Full details in the online supplementary data.) All assays were performed in duplicate and the mean values were analysed.
4. Flexible fiberoptic bronchoscopy and BAL of the right middle lobe. BAL cytokines were measured using an MSD multiplex ELISA [17–19]. Controlled for protein content. All assays were performed in duplicate.
5. TBLC after BAL sampling, TBLC was performed under deep sedation with midazolam and fentanyl, [12, 20]. Cryobiopsies were obtained using a flexible 2.4-mm cryoprobe (ERBE, Tübingen, Germany). The biopsies were reviewed by two thoracic pathologists specialising in ILD with cryobiopsy experience.

Pathologist's determination was made according to criteria established in surgical biopsies [14, 21]. The pathologists assigned a two-tiered (high or low) degree of diagnostic confidence for the presence of UIP pattern or categorised a biopsy as non-diagnostic [12].

The number of fibroblast foci identified was recorded and the surface area of every TBLC tissue fragment was measured using Leica's SlidePath Gateway software. This allowed calculation of the number of FF/mm<sup>2</sup> in pre- and post-treatment biopsies.

Ki-67 analysis was used to evaluate the proliferation index of pneumocytes and alveolar macrophages for each biopsy. This was quantified as an average number of cells showing nuclear expression per 100 pneumocytes or alveolar macrophages counted [22]. Caspase-3 expression in alveolar macrophages and pneumocytes was scored as being present (positive) or absent (negative) (supplementary material). Analysis for fibroblast foci, Ki-67, and caspase was analysed in pooled tissue samples for each patient.

Bronchoscopy, BAL, and TBLC were performed by a single experienced operator (MTH) for uniformity of technique.

## Statistical Methods

Differences in the cytokine distributions over time were evaluated with the Wilcoxon signed-rank test. Exact *p*-values were calculated under the null hypothesis of no difference in the distributions. We have also examined whether they would be considered significant while controlling the false discovery rate at 5% across all tests (either plasma or BAL) using the Benjamini–Hochberg procedure [23].

This study protocol was approved by the Clinical Research Ethics Committee of Cork University Hospital.

## Results

Thirteen patients were recruited, including current (15.4%), former (61.5%), and never (23.1%) smokers (Table 1). One patient reported significant gastrointestinal side effects on treatment and had the dose reduced to 1602 mg per day. The other twelve patients completed the full 6 months on full dose pirfenidone. Drug adherence was confirmed with monthly self-report and prescription table records.

Twenty-six TBLC procedures were carried out in thirteen patients (Table 2). One patient suffered a small 5% asymptomatic pneumothorax. Modest endobronchial bleeding occurred in 6/13 procedures. No patient required overnight admission and no patient suffered an exacerbation of IPF during the 3-month post-procedure. Spirometry after 1 week showed no fall in lung function.

Cytokine and chemokine analysis in BAL is outlined in Table 3. Due to skewness in the cytokine distributions, their values were log transformed to aid interpretation and plotting of values. Log transformed cytokine values at each time point were described by their medians and interquartile ranges (IQR). After controlling for multiple comparisons and false discovery, six cytokines were shown to have increased significantly over that time period.

This is illustrated in the final column of Table 3 and defined by *p*-values based on the Benjamini–Hochberg (BH)

**Table 2** Complications of transbronchial lung cryoprobe biopsy procedure carried out immediately before and after 6-month pirfenidone therapy

Complications post TBLC	Pre pirfenidone biopsy ( <i>n</i> = 13)	Post-pirfenidone biopsy ( <i>n</i> = 13)
Pneumothorax	7.7% (1)	0% (0)
Chest drain insertion	0% (0)	0% (0)
Bleeding	7.7% (1)	38.5% (5)
Treatment of bleeding		
Cold saline	100% (1)	60% (3)
Adrenaline	0% (0)	40% (2)

Values are expressed as percentages of total number of procedures carried out with absolute numbers of events in parenthesis

TBLC transbronchial lung cryobiopsy

procedure There were three cytokines in the angiogenesis panel, PIGF, VEGF-A, and bFGF, two anti-inflammatory cytokines increased, IL-10 and IL-4 and SP-D. The data for the 13 patients for these six cytokines in BAL are illustrated in Fig. 1. In the analysis of plasma, there was no significant signal in any single or panel of cytokines (Table 4).

A total of 56 H&E-stained cryobiopsies were available for analysis. The mean biopsy size was 9.39 mm<sup>2</sup> (range 2.0–32.0 mm<sup>2</sup>) pre-treatment and 15.42 mm<sup>2</sup> (range 1.62–43.01) post-treatment. Mean size for the middle lobe biopsies was 6.5 mm<sup>2</sup> (2.0–18.0 mm<sup>2</sup>) while that for the lower lobe biopsies was 15.18 mm<sup>2</sup> (2.8–32.0 mm<sup>2</sup>). UIP diagnosis was confirmed in all cases with good concordance between the pathologists (kappa value  $\kappa$  = 0.74). Overall biopsies obtained from the lower lobe had a higher degree of diagnostic confidence (71.4% high confidence) than middle lobe lung biopsies (22.2% high confidence) for the identification of UIP features.

A total of 39 fibroblast foci (FF) were identified in the pre-treatment biopsies and 24 FF in the post-treatment biopsies. The average number of FF per unit volume of alveolated lung tissue fell after 6 months of treatment although

**Table 1** Physiological and radiological characteristics of patients before and after 6 months of pirfenidone therapy

Characteristics	Baseline <i>T</i> = 0 ( <i>n</i> = 13)	<i>T</i> = 6 months ( <i>n</i> = 13)	<i>p</i> Value
Median age (range)	69 (44–82)		
Gender (% male)	69.2%		
Median FVC (range) (L)	87% (51.2–104.1)	83.4% (45.8–106)	<i>0.9</i>
Median <i>D</i> <sub>L</sub> CO (range) (ml/min/mmHg)	48.8% (35.7–67%)	47.9% (32–67)	<i>0.8</i>
HRCT global disease fibrosis score	48.5 (25–80)	48.8 (25–80)	<i>0.85</i>
6-min walk distance (m)	346 (278–465)	367 (278–468)	<i>0.98</i>

Statistically significant values (*p* < 0.05) are given in italics

Values are expressed as median with (range)

FVC forced vital capacity, *D*<sub>L</sub>CO diffusion capacity of the lung for carbon monoxide, HRCT high-resolution CT thorax

**Table 3** Distributions of log transformed cytokine values measured in bronchoalveolar lavage fluid at two time points, time 0 immediately before, and T 6 m after 6 months of pirfenidone therapy

Assay	Panel	Obs	Median [IQR] time 0	Median [IQR] time 6 m	Test <i>p</i> -value <sup>†</sup>	< <sup>‡</sup>	BH <i>p</i> -value*
<b>PIGF</b>	<b>Angiogenesis</b>	<b>12</b>	<b>0.03 [−0.13 to 0.20]</b>	<b>0.40 [0.15 to 0.57]</b>	<b>0.000</b>	<b>True</b>	<b>0.00125</b>
<b>VEGF. A</b>	<b>Angiogenesis</b>	<b>12</b>	<b>1.88 [1.56 to 2.03]</b>	<b>2.24 [2.05 to 2.38]</b>	<b>0.000</b>	<b>True</b>	<b>0.0025</b>
<b>IL.10</b>	<b>Proinflammatory</b>	<b>12</b>	<b>−0.80 [−0.94 to −0.72]</b>	<b>−0.46 [−0.60 to −0.34]</b>	<b>0.001</b>	<b>True</b>	<b>0.00375</b>
<b>bFGF</b>	<b>Angiogenesis</b>	<b>11</b>	<b>−0.45 [−0.55 to −0.19]</b>	<b>0.16 [−0.16 to 0.37]</b>	<b>0.005</b>	<b>True</b>	0.005
<b>SP-D</b>	<b>Other</b>	<b>12</b>	<b>1.44 [1.20 to 1.57]</b>	<b>1.64 [1.54 to 1.74]</b>	<b>0.005</b>	<b>True</b>	<b>0.00625</b>
<b>IL.4</b>	<b>Proinflammatory</b>	<b>12</b>	<b>−1.10 [−1.21 to −1.02]</b>	<b>−0.91 [−0.98 to −0.86]</b>	<b>0.007</b>	<b>True</b>	<b>0.0075</b>
IL.5	Cytokine	11	0.85 [0.53 to 0.95]	0.94 [0.88 to 1.02]	0.014	False	0.00875
IL.13	Proinflammatory	12	0.16 [0.07 to 0.31]	0.55 [0.27 to 0.67]	0.016	False	0.01
ICAM.1	Vascular	12	4.64 [4.37 to 4.70]	4.92 [4.75 to 4.95]	0.016	False	0.01125
VEGF.cyto	Cytokine	12	1.80 [1.67 to 2.09]	2.09 [1.94 to 2.29]	0.016	False	0.0125
SAA	Vascular	12	3.76 [3.63 to 3.89]	4.04 [3.92 to 4.09]	0.021	False	0.01375
IL.7	Cytokine	12	−0.02 [−0.16 to 0.21]	0.27 [0.15 to 0.47]	0.027	False	0.015
VCAM.1	Vascular	12	3.07 [2.90 to 3.22]	3.32 [3.13 to 3.42]	0.027	False	0.01625
TNFα	Proinflammatory	12	−0.11 [−0.20 to 0.10]	0.16 [−0.01 to 0.36]	0.027	False	0.0175
IL.15	Cytokine	12	0.11 [−0.15 to 0.20]	0.23 [0.08 to 0.42]	0.042	False	0.01875
IL.8	Proinflammatory	12	2.21 [2.06 to 2.38]	2.47 [2.36 to 2.65]	0.042	False	0.02
MCP.1	Chemokine	12	2.20 [2.08 to 2.31]	2.49 [2.14 to 2.69]	0.042	False	0.02125
Flt.1	Angiogenesis	12	1.02 [0.86 to 1.42]	1.46 [1.30 to 1.53]	0.064	False	0.0225
IL.12/IL.23p40	Cytokine	12	0.39 [0.06 to 0.53]	0.73 [0.22 to 1.15]	0.064	False	0.02375
IL.2	Proinflammatory	12	−0.44 [−0.59 to −0.33]	−0.12 [−0.47 to −0.04]	0.077	False	0.025
Eotaxin	Chemokine	7	1.03 [0.89 to 1.09]	1.67 [1.23 to 1.91]	0.078	False	0.02625
MMP7	Other	6	−0.68 [−0.82 to −0.42]	−0.27 [−0.45 to −0.10]	0.094	False	0.0275
CRP	Vascular	12	3.53 [3.26 to 3.78]	3.74 [3.62 to 3.92]	0.129	False	0.02875
GM.CSF	Cytokine	12	−0.05 [−0.24 to 0.20]	0.22 [−0.16 to 0.29]	0.151	False	0.03
IL.6	Proinflammatory	12	0.46 [0.33 to 0.68]	0.67 [0.43 to 0.91]	0.204	False	0.03125
IP.10	Chemokine	12	1.70 [1.61 to 1.99]	2.11 [1.59 to 2.66]	0.204	False	0.0325
IFN.γ	Proinflammatory	11	0.17 [0.09 to 0.31]	0.31 [0.25 to 0.41]	0.240	False	0.03375
VEGF.D	Angiogenesis	11	1.03 [0.90 to 1.51]	1.33 [0.91 to 1.70]	0.240	False	0.035
IL.16	Cytokine	12	2.50 [2.01 to 2.67]	2.51 [2.44 to 2.62]	0.301	False	0.03625
MDC	Chemokine	12	1.79 [1.71 to 2.00]	1.96 [1.72 to 2.15]	0.301	False	0.0375
MIP.1β	Chemokine	12	1.49 [1.34 to 1.62]	1.73 [1.32 to 1.87]	0.424	False	0.03875
IL.1β	Proinflammatory	12	0.61 [0.52 to 0.77]	0.72 [0.32 to 0.80]	0.470	False	0.04
TARC	Chemokine	12	1.16 [1.10 to 1.23]	1.26 [1.14 to 1.36]	0.519	False	0.04125
TGFβ	Other	7	0.92 [0.59 to 1.24]	1.01 [0.95 to 1.09]	0.688	False	0.0425
MIP.1α	Chemokine	12	1.44 [1.38 to 1.58]	1.47 [1.25 to 1.66]	0.733	False	0.04375
MCP.4	Chemokine	11	1.07 [0.99 to 1.23]	0.98 [0.88 to 1.35]	0.765	False	0.045
Chemokine IL.8	Chemokine	12	2.67 [2.58 to 2.88]	2.68 [2.47 to 2.83]	0.791	False	0.04625
Eotaxin.3	Chemokine	10	0.48 [0.09 to 0.87]	0.43 [0.22 to 0.82]	0.846	False	0.0475
IL.1α	Cytokine	12	0.49 [0.36 to 1.06]	0.57 [0.33 to 0.76]	0.910	False	0.04875
SDF	Other	12	2.71 [2.13 to 3.13]	2.77 [2.48 to 2.98]	1.000	False	0.05

Bold values reflect samples reported as significant while controlling the false discovery rate at 5% across all tests using the Benjamini–Hochberg procedure

Values are expressed as medians with interquartile ranges

*IQR* interquartile range, *Obs* number of paired observations, *BH* Benjamini–Hochberg, *FGF* fibroblast growth factor, *PDGF* platelet-derived growth factor, *MMP-7* matrix metalloproteinase-7, *PIGF* placental growth factor, *VEGF.A* vascular endothelial growth factor A from the angiogenesis ELISA panel, *VEGF cyto* vascular endothelial growth factor from the cytokine ELISA panel measures all isoforms, *VEGF.D* vascular endothelial growth factor D from the angiogenesis ELISA panel, *bFGF* basic fibroblast growth factor, *SP-D* surfactant protein-D, *Tie-2* tyrosine kinase-2, *Flt-1* Fms-like tyrosine kinase-1, *IL-10* human interleukin-10, *IL-4* human interleukin-4, *IL-5* human interleukin-5, *IL-13* human interleukin-13, *IFN-γ* interferon-γ, *IL-1β* interferon-1β, *IL-2* interleukin-2, *IL-6* interleukin-6, *IL-8* interleukin-8, *IL-10* interleukin-10, *IL-12p70* human interleukin-12p70, *IL-13* human interleukin-13, *TNF-α* tumour necrosis factor-α, *GM-CSF* granulocyte macrophage-colony stimulating factor, *IL-1α* human interleukin-1α, *IL-5* human interleukin-5, *IL-7* human interleukin-7, *IL-12/IL-23p40* human interleukin-12/interleukin-

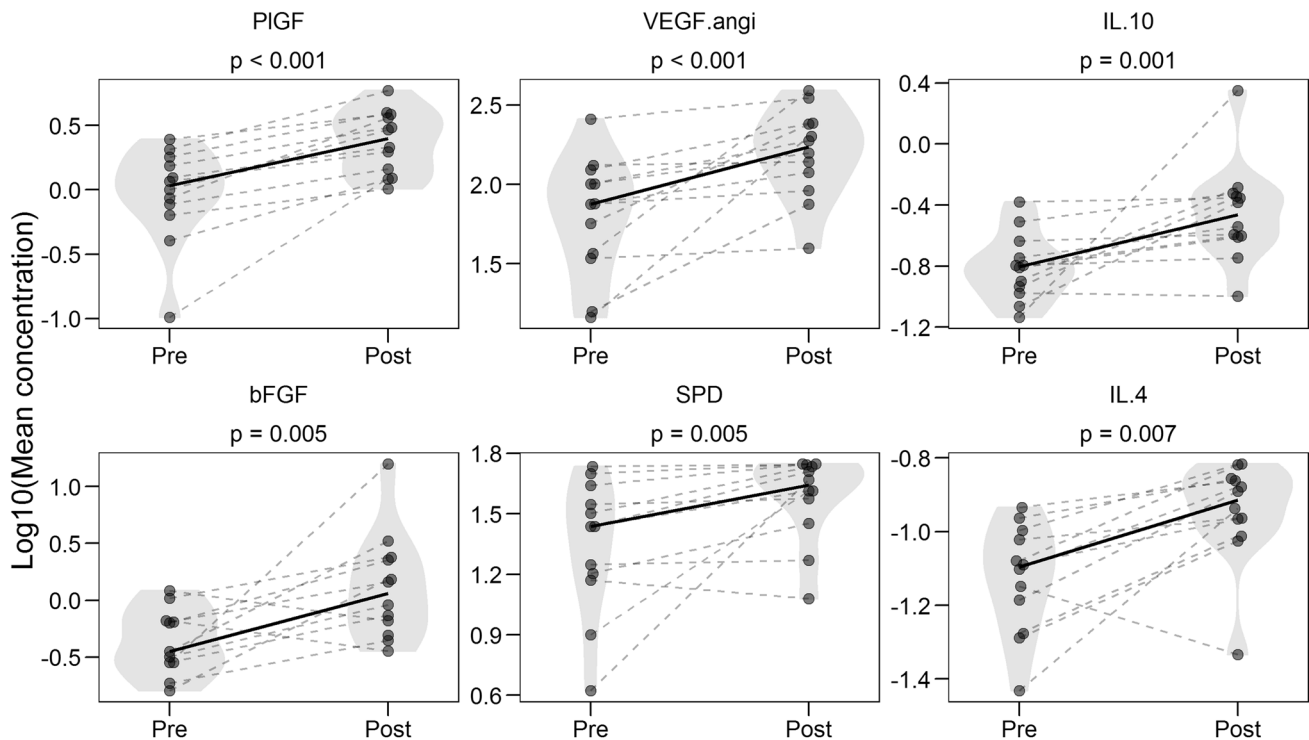
**Table 3** (continued)

23p40, *IL-15* human interleukin-15, *IL-16* human interleukin-16, *IL-17A* human interleukin-17A, *TNF- $\beta$*  tumour necrosis factor- $\beta$ , *SAA* serum amyloid-A, *CRP* c-reactive protein, *VCAM-1* vascular cell adhesion molecule-1, *ICAM-1* intracellular adhesion molecule-1, *SDF-1* stromal cell-derived factor-1, *MIP 1 $\alpha$*  human MIP 1 $\alpha$ , *MIP 1 $\beta$*  human MIP 1 $\beta$ , *MCP-1* human monocyte chemoattractant and activating factor 1, *MCP-4* human monocyte chemoattractant and activating factor 4, *TARC* thymus and activation regulated chemokine, *MDC* macrophage-derived chemokine

\*The *p*-value threshold at that rank given by the Benjamini–Hochberg procedure to control the false discovery rate at 5%

†*p*-Value from a Wilcoxon signed-rank test comparing cytokine values at the two time points

‡Indicator of whether the test *p*-value falls below the threshold at that rank given by the Benjamini–Hochberg procedure



**Fig. 1** Log transformed values of six bronchoalveolar lavage cytokine levels which rose significantly after 6 months of pirfenidone therapy when controlling the false discovery rate at 5% across all tests using the BH procedure. *PIGF* placental growth factor, *VEGF angi* vas-

cular endothelial growth factor from the angiogenesis ELISA panel, *bFGF* basic fibroblast growth factor, *IL-10* human interleukin-10, *SP-D* surfactant protein-D, *IL-4* human interleukin-4

this number was not statistically significant (pre-treatment:  $0.14/\text{mm}^2$  vs. post-treatment  $0.08/\text{mm}^2$ ,  $p = 0.08$ ).

Ki-67 expression, in both pre- and post-treatment biopsy samples, was higher in macrophages than in pneumocytes as demonstrated in previous studies [24]. Pneumocytes overlying FF did not exhibit a higher proliferation index compared to pneumocytes distal to FF. There was no change in Ki-67 proliferation expression pre-treatment to post-treatment in pneumocytes (mean expression 0.77 [SD 0.6] per 100 pneumocytes counted vs. mean expression 1.15 [SD 1.2] per 100 pneumocytes counted,  $p = 0.35$ ) or macrophages (mean expression 5.7 [SD 7.7] per 100 macrophages counted vs. mean expression 5.8 [SD 8.7] per 100 macrophages counted,  $p = 0.9$ ).

There was no change in the proportion of Caspase-3 expression in pneumocytes, (77% expression pre-treatment

vs. 61% post,  $p = 0.5$ ) or macrophages (92% expression pre-treatment vs. 77% post-treatment,  $p = 0.3$ ) after treatment. Fibroblast foci did not express Caspase-3. Overall, there was no relationship between Caspase-3 expression in TBLC samples and change in FVC or  $D_LCO$  over 6 month's treatment. In seven cases, caspase-3 was positive pre-treatment and negative post-treatment, although we were not able to correlate this observation in these patients with change in lung function or HRCT score.

## Discussion

The safety and effectiveness of TBLC in the diagnosis of IPF is now established when performed in specialist centres with appropriate clinical and pathological experience only

**Table 4** Distributions of log transformed cytokine values measured in plasma at two time points, time 0 immediately before, and T 6 m after 6 months of pirfenidone therapy

Assay	Panel	Obs	Median [IQR] time 0	Median [IQR] time 6 m	Test <i>p</i> -value <sup>†</sup>	< <sup>‡</sup>	HC <i>p</i> value*
SDF	Other	7	3.16 [2.13 to 3.24]	3.25 [2.13 to 3.50]	0.031	False	0.00125
VEGF.angi	Angiogenesis	13	1.86 [1.67 to 2.29]	1.81 [1.54 to 1.89]	0.033	False	0.0025
SPD	Other	12	1.03 [0.84 to 1.25]	0.86 [0.68 to 1.06]	0.077	False	0.00375
VEGF.cyto	Cytokine	12	2.04 [1.80 to 2.23]	1.62 [1.37 to 2.21]	0.077	False	0.005
Flt.1	Angiogenesis	13	1.16 [1.07 to 1.50]	1.00 [0.88 to 1.70]	0.127	False	0.00625
Tie.2	Angiogenesis	13	3.00 [2.68 to 3.22]	2.59 [2.47 to 2.79]	0.146	False	0.0075
VEGF.C	Angiogenesis	13	1.77 [1.62 to 1.97]	1.57 [1.47 to 1.83]	0.146	False	0.00875
PIGF	Angiogenesis	13	0.88 [0.82 to 1.09]	0.69 [0.59 to 0.94]	0.168	False	0.01
SAA	Vascular	13	6.22 [6.02 to 6.39]	6.39 [6.02 to 6.83]	0.216	False	0.01125
MDC	Chemokine	13	2.83 [2.68 to 3.01]	2.71 [2.52 to 2.89]	0.273	False	0.0125
sVCAM.1	Vascular	13	5.35 [5.15 to 5.46]	5.39 [5.27 to 5.64]	0.340	False	0.01375
VEGF.D	Angiogenesis	13	2.18 [1.96 to 2.27]	2.00 [1.91 to 2.14]	0.376	False	0.015
IL.7	Cytokine	13	0.63 [0.47 to 0.76]	0.56 [0.40 to 0.80]	0.455	False	0.01625
MIP.1b	Chemokine	13	1.68 [1.56 to 1.92]	1.80 [1.48 to 2.15]	0.455	False	0.0175
TNF.b	Cytokine	12	0.10 [−0.17 to 0.31]	0.13 [0.00 to 0.30]	0.470	False	0.01875
IL.6	Proinflammatory	12	0.23 [0.04 to 0.40]	0.25 [0.09 to 0.41]	0.519	False	0.02
CRP	Vascular	13	6.29 [5.93 to 6.47]	6.36 [6.14 to 6.48]	0.542	False	0.02125
IL.8	Proinflammatory	13	0.83 [0.52 to 0.90]	0.60 [0.53 to 0.91]	0.542	False	0.0225
IL.17A	Cytokine	12	1.12 [0.96 to 1.32]	1.08 [0.90 to 1.27]	0.569	False	0.02375
IP.10	Chemokine	12	2.19 [2.05 to 2.51]	2.55 [2.11 to 2.72]	0.569	False	0.025
IL.1a	Cytokine	11	−0.22 [−0.57 to 0.01]	−0.22 [−0.45 to −0.06]	0.577	False	0.02625
GM.CSF	Cytokine	12	0.28 [−0.04 to 0.45]	0.33 [0.18 to 0.43]	0.622	False	0.0275
IL.12p70	Proinflammatory	10	−0.11 [−0.18 to −0.05]	−0.12 [−0.21 to 0.00]	0.625	False	0.02875
bFGF	Angiogenesis	13	1.47 [1.16 to 1.62]	1.28 [1.07 to 1.51]	0.635	False	0.03
MCP.4	Chemokine	13	1.93 [1.74 to 2.02]	1.84 [1.69 to 2.19]	0.685	False	0.03125
TGFb	Other	12	2.32 [2.08 to 2.41]	2.22 [1.90 to 2.50]	0.733	False	0.0325
IL.12.IL.23p40	Cytokine	13	1.93 [1.61 to 2.03]	1.92 [1.73 to 2.28]	0.735	False	0.03375
sICAM.1	Vascular	13	5.48 [5.29 to 5.69]	5.47 [5.32 to 5.63]	0.735	False	0.035
TNF.a	Proinflammatory	13	0.34 [0.29 to 0.58]	0.45 [0.29 to 0.58]	0.735	False	0.03625
IFN.g	Proinflammatory	11	0.80 [0.51 to 1.09]	0.91 [0.69 to 1.00]	0.765	False	0.0375
MCP.1	Chemokine	13	1.82 [1.68 to 2.28]	1.92 [1.80 to 2.13]	0.787	False	0.03875
IL.5	Cytokine	12	0.63 [0.50 to 0.87]	0.66 [0.53 to 0.93]	0.791	False	0.04
IL.1b	Proinflammatory	7	−0.56 [−0.76 to −0.37]	−0.56 [−1.08 to 0.03]	0.813	False	0.04125
Eotaxin	Chemokine	13	1.99 [1.81 to 2.22]	1.92 [1.81 to 2.24]	0.839	False	0.0425
IL.16	Cytokine	13	2.13 [2.00 to 2.21]	1.86 [1.78 to 2.26]	0.839	False	0.04375
IL.13	Proinflammatory	6	0.33 [0.20 to 0.52]	0.51 [0.36 to 0.57]	0.844	False	0.045
Eotaxin.3	Chemokine	13	0.77 [0.64 to 1.09]	0.79 [0.61 to 1.19]	0.893	False	0.04625
IL.10	Proinflammatory	11	−0.33 [−0.42 to −0.20]	−0.32 [−0.49 to −0.13]	0.898	False	0.0475
IL.15	Cytokine	12	0.17 [0.12 to 0.27]	0.22 [0.07 to 0.26]	0.910	False	0.04875
TARC	Chemokine	13	2.13 [2.01 to 2.22]	2.17 [1.81 to 2.33]	1.000	False	0.05

Values are expressed as medians with interquartile ranges

*IQR* interquartile range, *Obs* number of paired observations, *BH* Benjamini–Hochberg, *FGF* fibroblast growth factor, *PDGF* platelet-derived growth factor, *MMP-7* matrix metalloproteinase-7, *PIGF* placental growth factor, *VEGF* *angio* vascular endothelial growth factor from the angiogenesis ELISA panel, *VEGF* *cyto* vascular endothelial growth factor from the cytokine ELISA panel, *bFGF* basic fibroblast growth factor, *SP-D* surfactant protein-D, *Tie-2* tyrosine kinase-2, *Flt-1* Fms-like tyrosine kinase-1, *IL-10* human interleukin-10, *IL-4* human interleukin-4, *IL-5* human interleukin-5, *IL-13* human interleukin-13, *IFN-γ* interferon-γ, *IL-1β* interferon-1 β, *IL-2* interleukin-2, *IL-6* interleukin-6, *IL-8* interleukin-8, *IL-10* interleukin-10, *IL-12p70* human interleukin-12p70, *IL-13* human interleukin-13, *TNF-α* tumour necrosis factor-α, *GM-CSF* granulocyte macrophage-colony stimulating factor, *IL-1α* human interleukin-1α, *IL-5* human interleukin-5, *IL-7* human interleukin-7, *IL-12/IL-23p40* human interleukin-12/interleukin-23p40, *IL-15* human interleukin-15, *IL-16* human interleukin-16, *IL-17A* human interleukin-17A, *TNF-β* tumour necrosis factor-β, *SAA* serum amyloid-A, *CRP* c-reactive protein, *VCAM-1* vascular cell adhesion molecule-1, *ICAM-1* intracellular adhesion molecule-1, *SDF-1* stromal cell-derived factor-1, *MIP 1α* human MIP 1α, *MIP 1β* human MIP 1β, *MCP-1* human monocyte chemotactic and activating factor 1, *MCP-4* human monocyte chemotactic and activating factor 4, *TARC* thymus and activation regulated chemokine, *MDC* macrophage-derived chemokine

\*The *p*-value threshold at that rank given by the BH procedure to control the false discovery rate at 5%

**Table 4** (continued)

<sup>†</sup>*p*-Value from a Wilcoxon signed-rank test comparing cytokine values at the two time points

<sup>‡</sup>Indicator of whether the test *p*-value falls below the threshold at that rank given by the Benjamini–Hochberg procedure

[12–15]. The principle findings of this study demonstrate that TBLC is a safe and effective clinical tool for longitudinally assessing potential tissue biomarkers of drug effect including pirfenidone. TBLC analysis and BAL biomarkers may complement conventional methods of measuring disease progression or drug effect. This is the first study in a human model that evaluates the effects of Pirfenidone on serum, BAL and tissue markers of disease progression with novel effects not demonstrated in animal models.

Classically in IPF, diagnostic surgical biopsy specimens contain FF. The number of FF correlates well with survival [25, 26]. The bleomycin mouse model of fibrosis has limitations with resolution of interstitial fibrosis at about 28 days which limits its utility in assessing longitudinal change. Diagnostic surgical lung biopsies (SLB) are performed in approximately one-third of patients with IPF and have a 30-day mortality of 2–3% when performed electively and are never likely to be used to monitor disease progression [27]. TBLC has been shown to provide tissue samples that allow histological pattern recognition approaching that of a SLB, with morbidity and mortality rates half those of SLB [12, 15]. Our patients had no serious adverse effects from the procedure. The sample sizes of the TBLC biopsies received ranged from mean 9.39 mm<sup>2</sup> pre-treatment and 15.42 mm<sup>2</sup> post-treatment which compares favourably with other groups [13, 14]. Despite the small cohort size there is a non-significant reduction in fibroblast foci from pre-treatment 0.14/mm<sup>2</sup> to post-treatment 0.08/mm<sup>2</sup>. It may be possible to further analyse TBLC samples using micro-CT scanning recently used for SLB specimens to aid diagnosis, disease progression and response to treatment [28, 29].

Apoptosis may play an important role in lung fibrosis. Maintenance of both epithelial and endothelial cell apoptotic equilibrium is required to clear unwanted cells, and to prevent prolonged inflammation and initiation of many of the mechanisms involved in the fibrotic pathway [30, 31]. Activation of the caspase cascade is one of the principle methods of regulating apoptosis. Upregulated expression of caspase-1 and caspase-3 in airway epithelial cells (AECs) or alveolar macrophages (AMs) has been demonstrated in bleomycin mouse model. The attenuation of this effect with specific caspase inhibitors decreased the number of apoptotic cells and lung fibrosis [32]. Pirfenidone has been shown to ameliorate fibrosis in animal models through its effect on pro-apoptotic genes [33]. The results of our study on human lung tissue after treatment with pirfenidone does not support the hypothesis that it affects apoptosis in either AECs or AMs. Though we were able to identify seven of 13 patients whose

caspase-3 expression seemed to disappear after pirfenidone treatment, we were not able to correlate this observation with disease progression.

Similarly, we postulated pirfenidone might have an effect be on the proliferative activity of AMs or AECs measured using a Ki67 stain on lung tissue from TBLC. Previous work has demonstrated that in IPF, AMs show a high proliferation rate [24, 34], mediated through the production of key pro-fibrotic cytokines. While our data are in support of this work, with Ki-67 expression higher in macrophages than pneumocytes, there was no significant change in markers of tissue proliferation, Ki-67 expression in AECs or AMs post-pirfenidone therapy.

Stratified medicine suggests that biomarkers may define multiple molecular pathways for aberrant wound healing in lung fibrosis [35]. We found no signal from plasma biomarkers of vascular injury, inflammation and chemokine activity studied. However, in BAL, we identified six key biomarkers that seemed to significantly increase during the treatment period. These included three biomarkers of angiogenesis, vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor (bFGF) and placental growth factor (PIGF). Increased VEGF-A expression in early endothelial progenitor cells derived from IPF patients may reflect a compensatory mechanism to maintain endothelial and vascular homeostasis in the IPF lung [36]. Koyama et al. reported decreased levels of VEGF in BAL of IPF patients at single time point [37]. Our longitudinal data suggest that the significant increase of VEGF-A in BAL in IPF patients is a mechanism of action of pirfenidone.

PIGF is a VEGF homolog. PIGF overexpression in transgenic mice causes emphysema. It promotes AEC apoptosis leading to decreased VEGF and may compromise microcirculation [38]. Our data show a rise of PIGF and thereby perhaps indirectly reduce VEGF production. This is counterintuitive if pirfenidone treatment was also associated with increased VEGF as above and emphasises the complexity interaction of cytokines involved in microcirculation and apoptosis. bFGF is a potent mitogenic factor for fibroblasts and myofibroblasts. There are increased levels of bFGF in mast cells of IPF lung tissue and a direct correlation between bFGF and extracellular matrix [39]. Increased levels may reflect a mechanism pirfenidone action or the natural effects of time in an IPF cohort albeit a clinically stable one.

Surfactant protein-D (SP-D) levels in blood have been shown to be elevated in IPF patients and elevated levels are highly predictive of survival [5, 40]. SP-D levels in BAL samples from our series rose significantly. While the

significance of this finding is unclear, we know that Surfactant protein-A in lavage has been shown to be low, even when normalised for the total amount of surface-active material recovered in IPF subjects [41, 42].

Finally, we have demonstrated a rise in the pro-inflammatory cytokines IL-10 and IL-4 in BAL during treatment. The precise role of IL-10 in IPF remains unclear. Both increased levels of IL-10 in AMs from BAL and reduced levels of IL-10 from cell free BAL have been demonstrated [43, 44]. IL-10 is an anti-inflammatory cytokine. In experimental cell lines, pirfenidone inhibits pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6 and increases production of anti-inflammatory IL-10 [45]. The increase we have shown supports a potential anti-inflammatory effect of pirfenidone. IL-10 as has been shown to inhibit bleomycin induced lung fibrosis in mice [46], suggesting an indirect role of pirfenidone as an anti-fibrotic by promoting IL-10 production. While it has been suggested that in animal models pirfenidone limits liver fibrosis by limiting the Th2 response through reducing IL-4 [47], there are no such data in lung fibrosis in human models to date. Increased IL-4 levels in BAL after treatment may also reflect an anti-inflammatory function of pirfenidone.

The strengths of this study include the novelty of using human lung tissue obtained using TBLC to assess the longitudinal effect of treatment with pirfenidone in IPF, in conjunction with a wide panel of serum and BAL biomarkers. The main limitation of this study is modest number of patients recruited to this study and a lack of a control group. A longer, 12-month period of follow up as used in phase three trials [8, 9] might have led to significant clinical or radiological changes and with corresponding more significant histopathological and BAL biomarker changes. To a certain extent, this study is a proof of concept study that TBLC is an effective, safe and acceptable way of monitoring disease progression and treatment effect. We were unable to demonstrate a correlation between lung tissue or BAL biomarkers and lung function change or between BAL biomarkers and pathological changes seen on TBLC. It is possible that with future studies and larger numbers we might see a more significant change in fibroblast foci number or morphology after treatment using conventional histopathology or three-dimensional microCT analysis [28] and perhaps clarify the utility of TBLC in this regard. Future larger studies utilising TBLC might examine the role TGF- $\beta$ , matrix metalloproteinase-7 (MMP-7) or SP-D or the effect of novel anti-fibrotics on these potential biomarkers.

TBLC is safe and effective and its use may compliment established standard physiological and biochemical methods in determining disease progression or drug response in IPF. This study suggests that the Pirfenidone does not act by modifying the number of fibroblastic foci, or through modifying markers of tissue proliferation (Ki-67) and apoptosis

(caspase-3) though larger studies may be needed to confirm these findings.

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## Compliance with Ethical Standards

**Conflict of interest** All authors declare that they have no conflict of interest.

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