Comprehensive gene expression profiling identifies distinct and overlapping transcriptional profiles in non-specific interstitial pneumonia and idiopathic pulmonary fibrosis

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Abstract

Background: The clinical-radiographic distinction between idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP) is challenging. We sought to investigate the gene expression profiles of IPF and NSIP vs. normal controls.

Methods: Gene expression from explanted lungs of patients with IPF (n = 22), NSIP (n = 10) and from normal controls (n = 11) was assessed. Microarray analysis included Significance Analysis of Microarray (SAM), Ingenuity Pathway, Gene-Set Enrichment and unsupervised hierarchical clustering analyses. Immunohistochemistry and serology of proteins of interest were conducted.

Results: NSIP cases were significantly enriched for genes related to mechanisms of immune reaction, such as T-cell response and recruitment of leukocytes into the lung compartment. In IPF, in contrast, these involved senescence, epithelial-to-mesenchymal transition, myofibroblast differentiation and collagen deposition. Unlike the IPF group, NSIP cases exhibited a strikingly homogenous gene signature. Clustering analysis identified a subgroup of IPF patients with intermediate and ambiguous expression of SAM-selected genes, with the interesting upregulation of both NSIP-specific and senescence-related genes. Immunohistochemistry for p16, a senescence marker, on fibroblasts differentiated most IPF cases from NSIP. Serial serum levels of periostin, a senescence effector, predicted clinical progression in a cohort of patients with IPF.

Conclusions: Comprehensive gene expression profiling in explanted lungs identifies distinct transcriptional profiles and differentially expressed genes in IPF and NSIP, supporting the notion of NSIP as a standalone condition. Potential gene and protein markers to discriminate IPF from NSIP were identified, with a prominent role of senescence in IPF. The finding of a subgroup of IPF patients with transcriptional features of both NSIP and senescence raises the hypothesis that “senescent” NSIP may represent a risk factor to develop superimposed IPF.

Keywords: Idiopathic pulmonary fibrosis, Usual interstitial pneumonia, Non-specific interstitial pneumonia, Microarray
Background

Idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP) are the most common forms of idiopathic interstitial pneumonia (IIP) [1]. Better prognosis and response to therapy are reported for NSIP compared to IPF, which is defined by a histologic pattern of usual interstitial pneumonia (UIP) [2]. However, both conditions represent a common indication for lung transplantation (LTx) [3]. While the fibrotic process in IPF is believed to be driven by alveolar injury leading to unresolving wound healing and pro-fibrotic signals [4], the pathogenesis of NSIP is not clear.

The clinical-radiographic distinction is challenging [5], but is particularly important given the differences in prognosis and treatment algorithms. Patients with NSIP will often have a good response to corticosteroids, while IPF can worsen on prednisone [6], and is currently treated with anti-fibrotic agents [7, 8]. This differential response to treatment further highlights the dissimilarities in the molecular basis that defines IPF and NSIP. Despite these differences, the frequent finding of mixed UIP-NSIP patterns on lung biopsies [9] supported the hypothesis that NSIP may represent an early form of IPF [10].

Differential diagnosis between IPF and idiopathic NSIP is based on high-resolution chest CT scan (HRCT) and pathology. Diagnostic radiographic criteria for NSIP are not clearly defined [11], and as many as 25% of patients with IPF present with HRCT features atypical for UIP [12]. Even with biopsies taken from multiple lobes, inter-observer agreement in IIP is only moderate [13]. The finding of fibroblastic foci in cases of NSIP [14] adds more difficulty to the diagnostic process. Currently, multi-disciplinary discussion represents the gold standard approach in interstitial lung disease (ILD) [10], but its accuracy has never been validated. This highlights a clear need for more refined diagnostic tools.

Given the difference in outcomes and response to therapy, we hypothesized that IPF and NSIP exhibit distinct transcriptional profiles, and that specific gene markers may be identified for each condition. As Rosas and Kaminski pointed out, gene expression studies have been highly effective in reclassifying clinically relevant disease phenotypes with similar histologic presentation [15]. The authors also recommended a systems-level, rather than “cherry-picking” approach when analyzing microarray data [15]. In this study, we sought to investigate the gene expression profiles of IPF, NSIP and normal controls. Although we were interested in identifying individual genes and processes that could be candidates as disease markers, we opted for an integrated approach, founded on the analysis of biologically meaningful sets of genes, function and pathways. Using normal controls, we aimed at identifying genes that were specifically increased in each condition, and that could be used in the future in the differential diagnosis process.

Methods

Subjects

Specimens were obtained from the peripheral area of the lower lobe of each lung as soon as the first recipient lung was taken out, snap frozen in liquid nitrogen, and stored at −80 °C. RNA was extracted and hybridized to the Human Gene 1.0 set array (Affymetrix) from explanted lungs (2001–2008) in 22 patients with clinical diagnosis of sporadic IPF, entirely typical UIP HRCT pattern [16] and definite histologic UIP pattern; 10 subjects with clinical diagnosis of idiopathic NSIP and definite histologic pattern of fibrotic NSIP; and 11 normal lung samples (age 52 ± 18 years, 4 females) obtained from the region of normal tissue flanking lung cancer resections in ILD-free patients. Histopathologic diagnoses were based on whole explanted lungs. IPF cases with atypical radiographic features for UIP; and patients with other types of ILD, connective tissue disease or concomitant emphysema were excluded. A separate set of patients who underwent surgical lung biopsies at London Health Science Centre (Western University) (2005–2015) were identified and representative blocks were used for immunohistochemistry (IHC). This set included 23 cases with definite IPF/UIP and 13 with definite NSIP.

The study was approved by the Human Tissue Committees and Research Ethics Boards of the University Health Network (protocol n.11–0932) and Western University (n.105214).

Microarray analysis, immunohistochemistry and serum measurements

RNA was isolated, labeled, and hybridized to the human gene 1.0 set array according to the manufacturer’s protocols (Affymetrix). Data sets for microarray experiments are available at the Gene Expression Omnibus repository, accession n.GSE110147.

Partek software (St.Louis, MO) was used for the preliminary analysis. The q-value (false discovery ratio, used for multiple comparison correction) was used to identify differentially expressed genes and was computed with significance analysis of microarray (SAM). For pathways/networks discovery analysis, Ingenuity Pathway Analysis (IPA; Redwood City, CA) was used. For Gene-set Enrichment Analysis (GSEA), C5 GeneOntology was used as gene-set database [17, 18]. For hierarchic clustering, Cluster 3.0 and Treeview (Eisen's Laboratory, Stanford University) were used.

All IHC slides were reviewed by 2 pathologists. IHC for periostin (POSTN), osteopontin (OPN), p53 and p16, selected ad hoc based on microarray results, was performed using standard techniques.
The serum levels of 6 proteins, a priori selected based on literature review (POSTN, OPN, surfactant protein-A [SP-A], matrix metallopeptidase-9 [MMP-9], Krebs von den Lungen-6 [KL-6], Chemokine-(C-C motif)ligand-18 [CCL-18]) were measured at baseline and at 4-month intervals for 12 months in 34 patients diagnosed with IPF [16] and followed at Western University. The endpoint of the longitudinal study was clinical progression, defined as either: > 10% absolute reduction in forced vital capacity % predicted; > 50 m decline in 6-min walk distance; hospitalization for respiratory causes; LTx assessment; or death.

Details on sample processing, RNA isolation, complementary DNA synthesis, pre-processing on probe-level data, IPA, GSEA, RT-PCR, IHC and serum measurements are provided in the Additional files 1, 2, 3, 4 and 5.

Statistical analysis

The Kolmogorov-Smirnov test assessed variables’ distribution. For comparison of groups, either unpaired t test or the Mann-Whitney test, where indicated, was used. Cox proportional hazards regression analysis was used to identify serum biomarkers significantly predicting clinical progression. Receiver operating characteristic (ROC) analysis was used to determine the accuracy of serum biomarkers in predicting clinical progression (c-statistics). P-values < 0.05 were regarded as significant. Prism-4 software package (GraphPad, La Jolla, CA) was used.

Results

There were no significant differences in pulmonary function tests, exercise capacity or pulmonary artery pressures. NSIP patients were significantly younger than IPF patients (Table 1).

RNA integrity numbers were very similar in the 3 groups: 8.5 ± 0.4 in IPF, 8.8 ± 0.5 in NSIP and 8.6 ± 0.4 in normal controls. Sequential steps in gene expression profiling are shown in Fig. 1. There were no outliers among samples examined in terms of probe intensity (Additional file 1: Figure S1A). The F-ratio (signal-to-noise) ratio (average signal for all genes) was 1.77 (Additional file 1: Figure S1B). Principal Component Analysis (PCA, Additional file 2: Figure S2) showed a sizable, but not major degree of similarity across the genome between IPF and NSIP.

Gene expression profile of IPF

With stringent selection criteria (fold change ≥1.50, q-value< 5%), SAM identified 146 differentially expressed genes, of which 60 upregulated in IPF and 86 upregulated in NSIP. The top 25 upregulated genes in each condition are shown in Tables 2 and 3.

SAM analysis showed that the genes with increased expression in IPF compared to NSIP were involved in epithelial-to-mesenchymal transition (insulin growth factor binding protein-5 [IGFBP-5], Prominin-1), myofibroblast differentiation and proliferation (smooth muscle alpha-actin [ACTA-2], OPN), collagen deposition (OPN, IGFBP-5, POSTN), matrix remodeling (MMP-1, MMP-7, ACTA-2) peripheral blood mononuclear cells (PBMCs) proliferation and infiltration (IGFBP-5, MMP-7) and senescence (IGFBP-5, OPN, MMP-2). Most genes were also

Table 1 Demographic, clinical and functional characteristics of the patients included in the study

| Variable | IPF (n = 22) | NSIP (n = 10) | p value |
|----------|--------------|---------------|---------|
| Male/Female (% males) | 17/5 (77%) | 2/8 (20%) | 0.0051 |
| Age (years) | 62 ± 6 | 45 ± 11 | < 0.0001 |
| BMI (m/Kg²) | 26 ± 5 | 24 ± 3 | n.s. |
| mPAP (mmHg)* | 29 ± 12 | 35 ± 21 | n.s. |
| 6MWD (m) | 293 ± 103 | 292 ± 195 | n.s. |
| FVC (% pred) | 57 ± 19 | 49 ± 18 | n.s. |
| TLC (% pred) | 65 ± 14 | 65 ± 18 | n.s. |
| DLCO (% pred) | 37 ± 10 | 51 ± 18 | 0.0242 |
| Treatment (% of total): | | | |
| Prednisone alone | 7 (31.8%) | 5 (50%) | n.s. |
| Prednisone + Azathioprine | 8 (36.4%) | 2 (20%) | n.s. |
| NAC alone | 1 (4.5%) | 0 (0%) | n.s. |
| No specific treatment | 6 (27.3%) | 3 (30%) | n.s. |

*pooled intraoperative mPAP during lung transplant

IPF idiopathic pulmonary fibrosis, NSIP non-specific interstitial pneumonia, BMI body mass index, mPAP mean pulmonary arterial pressure, 6MW 6-min walk distance, FVC forced vital capacity, TLC total lung capacity, DLCO diffusing lung capacity for carbon monoxide, n.s. not significant

Fig. 1 Outline of microarray analysis. SAM = Significance Analysis of Microarray. RIN = RNA integrity number

Cecchini et al. Respiratory Research (2018) 19:153
markedly upregulated in IPF when compared to normal controls. MMP-1 and OPN were the top 2 upregulated genes in IPF compared to controls (Additional file 3: Table S1). However, several genes previously studied in IPF (POSTN, OPN, MMP-1, MMP-7, Prominin-1) were also significantly upregulated in NSIP, when compared to controls (Fig. 2). In contrast, the increased expression of IGFBP-5 and 6, Mucin-5B, ACTA-2 was specific for IPF (Fig. 2). The expression of surfactant protein-D and heme oxygenase-1 was decreased in IPF compared to both NSIP and controls, while the expression of VEGF-A was reduced in both IPF and NSIP, compared to normal controls. The expression of 3 biologically relevant genes (MMP-7, OPN, IGFBP-5) in IPF vs. NSIP was confirmed by RT-PCR (Additional file 4: Figure S3).

IPA demonstrated the involvement of relevant genes (POSTN, OPN, MMP-1, MMP-7, Prominin-1, and others) in the network “connective tissue disease, organismal injury and abnormality” (Additional file 5: Figure S4A), and other functions related to cell movement and connective tissue disorder (Additional file 3: Table S2).

GSEA analysis (Table 4) demonstrated that numerous gene sets were significantly enriched in IPF vs. NSIP, mostly including biological functions related to cellular movement and proliferation.

**Gene expression profile of NSIP**

Genes with significantly increased expression in NSIP, compared to both IPF and normal controls, were involved in regulatory mechanisms of immune reaction, including the alloreactive T cell response (indoleamine 2–3-dioxygenase-1 [IDO-1]), the humoral arm of innate immunity (long pentraxin-3 [PTX-3], IFN-induced protein 44-like [IFI-44]) and the recruitment of leukocytes into the lung compartment (Endocan, LDL receptor-related protein-2 [LRP-2]). The top 25 genes upregulated in NSIP vs. controls are shown in Additional file 3: Table S3, which notably includes POSTN.

IPA demonstrated the involvement of relevant genes into pathways of interferon signaling, inflammatory response, granulocyte adhesion and anti-microbial response (Additional file 3: Table S4), and in the network

| NCBI Gene Symbol | NCBI Gene name | d | Fold change vs. NSIP group | q value |
|------------------|----------------|---|---------------------------|--------|
| IGFBP5           | insulin like growth factor binding protein 5 | 4.70 | 1.78 | < 0.0001 |
| SLN              | sarcolipin     | 4.69 | 2.57 | < 0.0001 |
| SYNO2            | synaptopodin 2 | 4.61 | 2.08 | < 0.0001 |
| MYH11            | myosin, heavy chain 11, smooth muscle | 4.38 | 1.99 | < 0.0001 |
| DES              | desmin         | 4.33 | 2.05 | < 0.0001 |
| NLGN4Y           | neuroligin 4, Y-linked | 4.24 | 2.55 | < 0.0001 |
| FAM83D           | family with sequence similarity 83, member D | 4.23 | 1.65 | < 0.0001 |
| ACTG2            | actin, gamma 2, smooth muscle, enteric | 4.16 | 2.25 | < 0.0001 |
| TPM2             | tropomyosin 2 (beta) | 4.13 | 1.62 | < 0.0001 |
| CNN1             | calponin 1, basic, smooth muscle | 4.06 | 1.77 | < 0.0001 |
| PRUN2            | prun homolog 2 (Drosophila) | 4.01 | 1.84 | < 0.0001 |
| EIF1AY           | eukaryotic translation initiation factor 1A, Y-linked | 4.00 | 7.99 | < 0.0001 |
| RP54Y1           | ribosomal protein 54, Y-linked 1 | 4.00 | 7.25 | < 0.0001 |
| AHNAK2           | AHNK nucleoprotein 2 | 3.93 | 1.76 | < 0.0001 |
| DDX3Y            | DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked | 3.91 | 11.4 | < 0.0001 |
| KDM5D            | lysine (K)-specific demethylase 5D | 3.89 | 5.23 | < 0.0001 |
| TXLNGY           | taxilin gamma pseudogene, Y-linked | 3.89 | 7.08 | < 0.0001 |
| ATP1A2           | ATPase, Na+/K+ transporting, alpha 2 polypeptide | 3.87 | 1.66 | < 0.0001 |
| ACTA-2           | actin, alpha 2, smooth muscle, aorta | 3.87 | 1.51 | < 0.0001 |
| TXLNGY           | taxilin gamma pseudogene, Y-linked | 3.86 | 5.71 | < 0.0001 |
| ZFY              | zinc finger protein, Y-linked | 3.79 | 4.13 | < 0.0001 |
| PDLIM3           | PDZ and LIM domain 3 | 3.79 | 1.55 | < 0.0001 |
| TAGLN            | transgelin     | 3.77 | 1.60 | < 0.0001 |
| UTY              | ubiquitously transcribed tetratricopeptide repeat | 3.72 | 7.80 | < 0.0001 |
| IGFBP6           | insulin like growth factor binding protein 6 | 3.66 | 1.60 | < 0.0001 |
anti-microbial response, inflammatory response and cancer) (Additional file 5: Figure S4B). Similarly, GSEA demonstrated that gene sets significantly enriched in NSIP vs. IPF involved interferon-gamma-mediated signaling and production, and defense response to virus (Table 5).

Unsupervised clustering analysis identifies an “intermediate” group of IPF patients
Unsupervised hierarchical clustering based on differentially expressed genes demonstrated that a subgroup of 8 IPF subjects did not clearly group in either patient cluster, but rather had intermediate expression of selected genes (Fig. 3). The remaining IPF cases clustered together in a “pure” IPF group. NSIP cases, in contrast, showed a compact, homogenous clustering.

We examined the expression of disease-specific genes in the intermediate group, and found that IGFBP-5 level was similar to that of the “pure” IPF cluster, while in contrast, the expression of Mucin-5B was reduced to the same level observed in NSIP cases. LRP-2 and IFI-44, which are NSIP-specific, were also increased in the intermediate cluster, compared to the “pure” IPF group, to a similar expression level seen in NSIP group (Fig. 3). On the other hand, several senescence-related genes (IGFBP-5, p16) and matrix remodelling gene ACTA-2 were also increased in the intermediate group, compared to NSIP.

The role of senescence
Given the increasingly recognized role of cellular senescence in IPF, we analyzed the expression of senescence effectors, senescence-associated secretory phenotype (SASP) growth factors and SASP matrix remodeling markers. Interestingly, the expression of most genes involved was not significantly increased in IPF (Additional file 3: Table S5). However, IGFBP-5, which is strongly and specifically upregulated in IPF (Fig. 2), plays an important role in the regulation of cellular senescence via a p53-dependent pathway [19]. p16, another key senescence effector, was modestly upregulated in IPF vs. NSIP (fold change 1.15) and, on IHC, 15 out of 23 IPF cases had positive p16 expression on fibroblasts. p16 staining was also diffusely noted on metaplastic epithelium (Fig. 4.

### Table 3 Top 25 up-regulated genes in NSIP vs. IPF (SAM analysis)

| NCBI Gene Symbol | NCBI Gene name | d   | Fold change vs. NSIP group | q value |
|------------------|----------------|-----|---------------------------|---------|
| LRP2             | LDL receptor related protein 2 | 4.49 | 2.12 | < 0.0001 |
| IFI44L           | interferon-induced protein 44-like | 3.99 | 2.67 | < 0.0001 |
| SLC39A8          | solute carrier family 39 (zinc transporter), member 8 | 3.97 | 2.67 | 0.0237 |
| SCN1A            | sodium channel, voltage gated, type I alpha subunit | 3.96 | 1.97 | 0.0237 |
| LNX2             | ligand of numb-protein X 2 | 3.86 | 1.52 | 0.0237 |
| WARS             | tryptophanyl-tRNA synthetase | 3.82 | 1.63 | 0.0237 |
| SLC6A14          | solute carrier family 6 (amino acid transporter), member 14 | 3.76 | 2.54 | 0.0237 |
| FZD5             | frizzled class receptor 5 | 3.76 | 1.61 | 0.0237 |
| PCDH9            | protocadherin 9 | 3.71 | 1.53 | 0.0237 |
| F11              | coagulation factor XI/4q35 | 3.68 | 2.50 | 0.0237 |
| RSAD2            | radical S-adenosyl methionine domain containing 2 | 3.63 | 2.26 | 0.0237 |
| NECA1            | N-terminal EF-hand calcium binding protein 1 | 3.60 | 2.48 | 0.0237 |
| MFS2A            | major facilitator superfamily domain containing 2A | 3.57 | 1.97 | 0.0237 |
| SDR16C5          | short chain dehydrogenase/reductase family 16C, member 5 | 3.50 | 2.23 | 0.0237 |
| MOP-1            | MOP-1 | 3.48 | 1.60 | 0.0237 |
| OAS-2            | 2-O-oligoadenylate synthetase 2 | 3.43 | 1.74 | 0.0237 |
| PHACTR1          | phosphatase and actin regulator 1 | 3.43 | 1.55 | 0.0237 |
| PIGA             | phosphatidylinositol glycan anchor biosynthesis class A | 3.42 | 1.51 | 0.0237 |
| IFIT3            | interferon-induced protein with tetratricopeptide repeats 3 | 3.42 | 1.96 | 0.0237 |
| C1orf162         | chromosome 1 open reading frame 162 | 3.40 | 1.54 | 0.0237 |
| ATP8A1           | ATPase,aminophospholipid transporter (APLT), class I, type 8 | 3.40 | 1.52 | 0.0237 |
| FMO5             | flavin containing monoxygenase 5 | 2.44 | 2.44 | 0.0237 |
| GBP4             | guanylate binding protein 4 | 3.34 | 1.92 | 0.0237 |
| ZNF385B          | zinc finger protein 385B | 3.32 | 2.36 | 0.0237 |
| SLC40A1          | solute carrier organic anion transporter family, member 4C1 | 3.25 | 2.11 | 0.0237 |

d = standardized change in expression (relative difference); q value = false discovery ratio
Additional file 3: Table S6). In NSIP, p16 was focally but variably expressed in metaplastic epithelium, however no staining of fibroblasts was observed in the 13 cases examined (Fig. 4, Additional file 3: Table S6). IHC for POSTN and OPN did not distinguish IPF from NSIP (Additional file 3: Table S6).

Serial periostin serum levels predict the clinical course of IPF

Senescence effector and matrix remodeling protein POSTN was among the top 10 upregulated genes in IPF, compared to normal controls (Additional file 3: Table S1A). Although a similar increase of gene expression was seen in NSIP vs. controls, POSTN serum levels were the only significant predictor of clinical progression in a cohort of patients with IPF, longitudinally followed for a period of 12 months. ROC analysis demonstrated that a POSTN level $\geq$ 338 ng/mL at baseline or its longitudinal increase over 338 ng/mL predicted clinical progression (area under the curve = 0.76, $p = 0.0025$) with 79% sensitivity and 73% specificity. Details on ROC analysis are shown in Additional file 3: Table S7. Univariate regression

![Fig. 2 Gene expression levels determined by oligonucleotide microarray in the IPF (blue), NSIP (green) and normal control (red) groups. Examples of genes upregulated in both IPF and NSIP: a. Matrix Metalloproteinase 7 (MMP-7). b. Osteopontin (also known as Secreted Phosphoprotein 1). Examples of genes specifically upregulated in IPF: c Insulin Growth Factor Binding Protein 5 (IGFBP-5). d. Smooth muscle alpha-2 actin (ACTA-2). Examples of genes upregulated in both NSIP and normal controls: e. Long Pentraxin 3 (PTX-3). f. Indoleamine 2–3-dioxygenase-1 (IDO-1). Examples of genes specifically upregulated in NSIP: g. Interferon-induced protein 44-like (IFN-44). h. LDL receptor-related protein 2 (LRP-2).]

Table 4 Top 5 gene-sets significantly enriched in the IPF group (GSEA)

| Gene Set                                      | NES    | p value   | q value   |
|----------------------------------------------|--------|-----------|-----------|
| Axoneme assembly (biological process)        | –2.65  | < 0.0001  | < 0.0001  |
| Microtubule bundle formation (biological process) | –2.56  | < 0.0001  | < 0.0001  |
| Ciliary plasm (cellular component)           | –2.53  | < 0.0001  | < 0.0001  |
| Cilium movement (biological process)         | –2.44  | < 0.0001  | < 0.0001  |
| Motile cilium (cellular component)           | –2.44  | < 0.0001  | < 0.0001  |

The positive enrichment score indicated a correlation with the IPF group. Ontologies are indicated in brackets.
Table 5: Top 5 gene-sets significantly enriched in the NSIP group (GSEA)

| Gene Set                                      | NES  | p value | q value |
|-----------------------------------------------|------|---------|---------|
| Interferon-gamma-mediated signaling pathway    | 2.48 | < 0.001 | < 0.001 |
| (biological process)                          |      |         |         |
| Defense response to virus (biological process) | 2.42 | < 0.001 | < 0.001 |
| Response to type I Interferon (biological process) | 2.30 | < 0.001 | < 0.001 |
| Regulation of Interferon-gamma production      | 2.27 | < 0.001 | < 0.001 |
| (biological process)                          |      |         |         |
| Regulation of Interleukin-12 production        | 2.26 | < 0.001 | 0.005   |
| (biological process)                          |      |         |         |

NES: Normalized Enrichment Score  
q value: False Discovery Ratio  
The positive enrichment score indicated a correlation with the NSIP group. Ontologies are indicated in brackets.

Table 6: Univariate regression analysis of biomarkers serum levels against 12-month clinical progression in a cohort of 34 patients with IPF

| Biomarker                         | Hazard Ratio (C.I.) | p value      |
|-----------------------------------|---------------------|--------------|
| Periostin (> 338 ng/ml or increase > 338 from baseline) | 1.00 (0.99–1.00) 4.25 (1.53–15.00) | 0.0693 < 0.0001 |
| Osteopontin                       | 1.00 (0.99–1.00)    | n.s.         |
| KL-6                              | 0.98 (0.76–1.25)    | n.s.         |
| MMP-9                             | 1.00 (1.00–1.00)    | n.s.         |
| Surfactant protein A              | 1.00 (1.00–1.00)    | n.s.         |
| CCL-18                            | 1.00 (0.99–1.02)    | n.s.         |

n.s.: not significant, KL-6 Krebs von den Lungen-6, MMP-9 Matrix Metalloproteinase 9, CCL-18 C-C Motif Chemokine Ligand 18
of overlap across the genome between IPF and NSIP, which could be explained by our subsequent clustering analysis findings, pathways and functional analysis revealed a radically different and remarkably homogenous gene signature in NSIP.

The gene signature of IPF has been reassuringly reproducible across several microarray studies [27–29]. Our findings confirm epithelial-to-mesenchymal transition, myofibroblasts proliferation, collagen deposition and PBMCs recruitment and infiltration as leading mechanisms of IPF at the transcriptional level, with the important addition of senescence. However, another novel finding of this study was the complete lack of specificity in the upregulation of POSTN, OPN, MMP-1, MMP-7 and PROM-1, previously and extensively studied in IPF, but not considered in NSIP. Not surprisingly, we found that IHC for POSTN, OPN and, as shown previously by Huh et al. [30], MMP-7 does not distinguish IPF from NSIP. However, by cross-checking upregulated genes against both NSIP and normal controls, we were still able to identify a gene signature truly specific for IPF, which includes MUC5B, ACTA-2 and IGFBP-5. The latter is a critical trigger of senescence [31], which showed a nearly complete separation between IPF and NSIP groups, with differential expression confirmed by RT-PCR.

Based on this finding and on recent literature [32, 33], we specifically looked at senescence as a mechanism considered relevant into the pathogenesis of IPF. Senescence is a state of irreversible replicative arrest induced by pro-ageing stressors, associated with resistance to apoptosis and secretion of SASP [32]. This includes matrix remodelling proteases and growth factors, cytokines and chemokines. Senescence is not always necessarily detrimental, as it can for example protect from cancer by disabling cells accumulating potentially deleterious damage

![Genes upregulated in IPF and NSIP](image)

**Fig. 3** Heat map representing differentially expressed genes (fold change ≥1.50 and q value< 5%), and hierarchic clustering. a Hierarchic clustering based on 146 differentially expressed genes (IPF vs. NSIP groups) in IPF (n = 22), NSIP (n = 10) and IPF-NSIP (n = 5) groups. Each row corresponds to an individual sample, and each column corresponds to an individual gene. Each square on the matrix represents the expression level of an individual gene in each sample, with red and green indicating gene expression levels above or below, respectively, compared with each other. While an IPF and a NSIP cluster are identifiable, an “unpaired” group of 7 IPF subjects and 2 NSIP patients showed intermediate expression and no clear clustering.
and mutations [34]. However, senescence-promoted secretome and the lack of clearance of senescent fibroblasts are indeed fibrogenic (32,33). Although we found relatively few senescence-related and SASP genes to be upregulated in IPF vs. either NSIP or controls, when considering homogenized, whole lung tissue samples, the senescent signal on epithelial cells may be diluted. Furthermore, senescence in IPF occurs prevalently in epithelial cells [35], as confirmed on IHC studies, and these may not account for much of the overall gene expression signal in homogenized samples.

The occurrence of senescence in cells playing a key biological role in the pathogenesis of fibrotic lung disease might be more important that the overall extent of this process in the lung tissue. Among senescence-related genes, p16 is a cyclin-dependent kinase inhibitor that blocks cell cycle progression by antagonizing cyclin-dependent kinases [36]. The CDKN2A gene (which encodes p16) was only modestly upregulated in IPF compared to NSIP, but, importantly, p16 immunostaining on fibroblastic foci differentiated most, although not all, IPF cases from NSIP. Metaplastic bronchial epithelium overlying fibroblastic foci also often expresses high levels of p16. This could be induced in a paracrine fashion by the activity of IGFBP-5 via a p53-dependent mechanism [19]. While we should consider that no single marker may define a cell as senescent, since none of these markers are exclusive to cellular senescence [34], p16 immunostaining as a potential discriminator in IIP will deserve further studies.

Another component of SASP, which may turn senescent fibroblasts into proinflammatory cells [37], is POSTN, a matrix-remodelling protein. Naik et al. previously found
that baseline POSTN levels were predictive of clinical progression [38]. POSTN is one of the most strongly upregulated genes in IPF, although not specifically for this condition. Consistently, Ohta et al. did not find significant differences in POSTN levels between IPF and NSIP subjects [39]. We considered longitudinal changes of POSTN in IPF patients and found that its increase predicts clinical progression better than other biomarkers considered, outperforming a panel of alternative biomarkers selected from literature review. These results will need to be confirmed in a larger population of patients with IIP.

Unsupervised clustering analysis based on differentially expressed genes, further verified against normal controls, provided important information, revealing significant transcriptional heterogeneity of IPF, which is striking, considering the radiographic-pathologic homogeneity of the cohort we selected. In contrast, NSIP appears to be a much more transcriptionally homogenous condition. Alongside a homogenous and compact NSIP group and a “pure” IPF group, an intermediate group of IPF subjects with ambiguous expression did not clearly cluster with either entity. Intriguingly, the intermediate group of patients expressed high levels of 2 NSIP-specific markers (LRP-2 and IFI-44), on one hand, and of a combination of senescence and matrix remodelling-related genes (IGFBP-5, CDKN2A [encoding p16], ACTA-2) on the other. The existence of this “intermediate” cluster of IPF patients, expressing both NSIP-related and SASP-related genes, cannot be attributed to a mixed histologic pattern, as pathologic diagnosis was confirmed on whole lung explants. Furthermore, all IPF cases in this study featured an entirely typical HRCT pattern of UIP.

Considering the interesting observations of radiographic “evolution” of biopsy-proven NSIP cases into an UIP/IFP pattern [40], and the consistently younger age of NSIP patients compared to IPF patients, we advance the hypothesis that “senescent” NSIP itself may represent a risk factor to develop superimposed IPF. Cases with intermediate gene expression pattern, with an upregulation of both NSIP-specific and senescence-related genes, may represent an occurred or still occurring “transition” from NSIP to IPF, possibly driven by senescence. Such hypothesis will need much stronger evidence to be confirmed, but it would help to explain the clinical variability observed not only in IPF, but even in NSIP: while many cases of NSIP respond to immunosuppressive therapy and survive for a long time, some NSIP: while many cases of NSIP respond to immunosuppressive therapy and survive for a long time, some

However, this would leave the clinical variability of NSIP unexplained, given the transcriptional homogeneity we observed in this entity.

Conclusions

Comprehensive gene expression profiling from whole lung explants aligns separate, well-recognizable histopathologic patterns of IIP with distinct transcriptional profiles and differentially expressed genes, and does not support the notion of NSIP as an early manifestation of IPF, but rather point to a standalone, transcriptionally homogenous condition, even in advanced fibrotic cases requiring LTx. LRP-2 and IFI-44 were identified as candidate molecular markers of NSIP, further validated against normal controls. This, however, does not exclude that “senescent” NSIP itself may represent a risk factor for developing IPF, given the remarkable degree of transcriptional overlap observed in a subgroup of IPF cases. Senescence-related genes are indeed prominent in IPF: we identified IGFBP-5 as a candidate molecular marker specific for IPF; p16 as a candidate fibroblast protein marker of IPF; and POSTN serum levels as better predictors of clinical progression, although not specific for IPF. These findings will likely stimulate validation studies in smaller, routinely available biopsy samples, as well as in larger populations.

Additional files
Ethics Boards of the University Health Network (protocol n.11932) and Western University (n.105214). Consent for publication
No personal identifiers of patients are included in the study.

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

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Availability of data and materials
Data sets for microarray experiments are available at the Gene Expression Omnibus repository, accession n.GSE110147.

Authors’ contributions
MC - study design, data collection, analysis and interpretation, manuscript writing; KH - data collection; CH - study design, data interpretation; MJ - study design, data analysis and interpretation, manuscript review; MM - study design, data collection, analysis and interpretation, manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Human Tissue Committees and Research Ethics Boards of the University Health Network (protocol n.11–0932) and Western University (n.105214).

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Abbreviations
ACTA-2: Smooth muscle alpha-actin; CCL-18: Chemokine-(C-C motif) ligand-18; GSEA: Gene set enrichment analysis; HRCT: High-resolution chest CT scan; IDO-1: Indoleamine 2–3-dioxygenase-1; IFN: Induced protein 44-like [IFI-44]; IGFBP-5: Insulin growth factor binding protein-5; IHC: Immunohistochemistry; IIP: Idiopathic interstitial pneumonia; ILD: Interstitial lung disease; IPF: Idiopathic pulmonary fibrosis; KL-6: Krebs von den Lungen-6; LRP-2: LDL receptor-related protein-2; MMP-9: Matrix metalloproteinase 9; NSIP: Non-specific interstitial pneumonia; OGN: Osteopontin; PBMCs: Peripheral blood mononuclear cells; POSTN: Periostin; PTX-3: Long pentraxin 3; SAM: Significance analysis of microarray; SASP: Senescence-associated secretory phenotype; SP-A: Surfactant protein A; UIP: Usual interstitial pneumonia

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