

Original Article

Influence of sex and disease severity on gene expression profiles in individuals with idiopathic pulmonary fibrosis

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Abstract: Epidemiological studies suggest sex-specific trends in the prevalence and mortality of idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) that are distinct for each disease. While the expression of numerous immune and extracellular matrix (ECM) genes in the lung have been well characterized in these diseases, associations elucidating their sex-specific expression patterns by disease type and severity, and the evaluation of hormone-related genes, have not been well studied. Here we performed targeted transcriptional profiling of 48 genes was performed on lung tissue samples from males and females with mild or medium severity IPF or COPD. The genes assessed included those involved in inflammation, ECM remodeling and hormonal processes. Data for 36 lung tissue samples were obtained that were stratified by disease and sex. Expression levels revealed a subset of genes which show differential expression among sexes, disease type, and disease severity. The most significant observations were the increased expression primarily of ECM genes in medium severity IPF (CATHK, COL1A1, COL3, MMP1, MMP7, IL-1RN) compared to mild IPF and COPD. Two genes, CH3L1 and MMP7 showed a tendency of interaction between sex and disease in IPF severity. Surprisingly, there were no significant differences in any of the sex genes measured between the IPF groups; however, ESR1 and AR expression levels were higher and lower, respectively, compared to COPD samples. Overall, this work highlights two genes, CH3L1 and MMP7, that may contribute to gender trends observed for IPF and COPD and are potential targets for future research.

Keywords: Idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, inflammation, steroids, extracellular matrix, gene profiles, chitinase 3-like 1, matrix metalloproteinase 7

Introduction

Idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) are chronic progressive lung disorders with rising mortality rates in the United States. In spite of continued research efforts IPF and COPD have eluded all treatments and remain relentless lethal conditions [1, 2]. Sex-specific trends in the incidence, prevalence and mortality of both diseases have been reported. Multiple studies have suggested that the incidence and prevalence of IPF is higher in males and that females have better survival rates [3-8]. In contrast, the prevalence of COPD is increasing in females and the number of women dying from this dis-

ease now surpasses men in the United States [9-11]. While numerous genetic, environmental, social and behavioral factors are proposed to contribute to these trends, it is also likely that cellular and molecular mechanisms driving disease development and/or progression are not fully conserved between sexes.

The mechanisms underlying the pathogenesis of IPF and COPD are poorly understood, revealing a gap in our knowledge of disease development, progression and management. Historically, inflammation has been touted as a 'driving force' that is central to disease pathology and while it likely plays an important role, other principal events have been recently considered.

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For example, a contemporary hypothesis suggests that injury of the epithelium and subsequent wound repair in the absence of preceding inflammation are fundamental to IPF [12]. Furthermore, immunosuppressive treatments are relatively ineffective suggesting processes in addition to inflammation are critically important. A recent study by Chilosis et al, suggests that despite the disparate clinical and pathological presentation of IPF and COPD some of the cellular and molecular mechanisms driving disease pathology are similar [13]. The authors propose a model of accelerated cell senescence which leads to aberrant cell signalling and consequential ECM remodelling. This aberrant remodelling initiates either airway obstruction in the case of COPD or impaired gas exchange and parenchymal consolidation in IPF. It is further postulated that while accelerated cell senescence is primary to the pathogenesis of both conditions the cell targets are specific to each disease; epithelial cells for IPF and mesenchymal cells for COPD.

Research conducted in animal models to assess the role of sex in IPF has produced conflicting data. For example, female rats exposed to the classic fibrotic agent bleomycin showed more pronounced fibrogenesis compared to their male counterparts [14]. Furthermore, removal of endogenous estrogen by ovariectomizing female mice lessened fibrosis whereas addition of estrogen restored the fibrotic response. Further investigation into the potential hormonal mechanisms has shown that estrogen enhanced fibrotic-related genes including *TGFB1* and *procollagen 1* *in vitro*. However, data more recently published by Haston et. al [15] showed the opposite effect in mice exposed to bleomycin. In these studies, estrogen seemed protective whereas androgens exacerbated the fibrotic response. Similarly, androgens were shown to exacerbate decreased lung function in a related model [16] and Lekgabe et. al [17] has shown that estrogen replacement protected mice against airway fibrosis by reducing the expression of downstream gene targets. These studies suggest the fibrotic response may be species specific.

The influence of sex in the aforementioned mechanisms has not been well studied in relation to IPF and COPD although it has been suggested that sexual dimorphism of the immune response may contribute to sex-specific dis-

ease trends. The immunomodulatory properties of sex steroids have been fairly well established where estrogens tend to enhance the humoral immune response and androgens act to suppress both cell-mediated and humoral responses [18, 19]. More specifically, estrogen receptor 1 (ESR1) levels have been correlated to anti-inflammatory properties in allergic asthma models [20-22] and studies conducted by Degano and colleagues in rats and Leiberman and coworkers in humans both revealed that treatment with ESR1 agonists resulted in a decrease in airway responsiveness to acetylcholine and histamine respectively [20, 23]. Estrogen receptor 2 (ESR2), expressed predominantly in the lung, prostate, and ovarian granulosa cells, does not possess the ability to produce "classical" estrogen responses but has been shown to be beneficial in the treatment of several inflammatory diseases [24-26]. Additional evidence suggests progesterone restricts injury-linked inflammation [27]. Differences in circulating sex hormone levels may underscore stronger resistance to infection and sepsis, reduced incidence of specific tumors, and increased susceptibility to autoimmune diseases between females and males [28, 29]. Sex differences in gene expression have also been observed in these disorders and are proposed to contribute to sex-related incidence and mortality [30-35]. In addition, the presence of enzymes that control circulating steroid levels may have an impact on activation of these receptors by enhancing or repressing the local levels of steroid receptor ligands (estrogen, testosterone, progesterone). Enzymes such as 17 β -HSD and 3 β -HSD contribute to normal lung development and are present in lung epithelial cells [36] but have not been investigated in diseased lung tissues to date.

Modulated expression of a number of genes related to immune regulation and ECM remodeling, both important events involved in IPF and COPD, have been investigated in lung cells and tissues. For example, altered expression of numerous matrix metalloproteinases (MMPs), collagens, fibronectins and a series of cytokines and chemokines are elevated, particularly in IPF. However, few studies have probed the sex differences in their expression profiles.

We are unaware of any clinical studies to date that have assessed the influence of gender in IPF human tissues at the molecular level, but

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Table 1. Genes targeted for expression analysis

Gene Name	Gene Symbol	Gene Type	Gene Name	Gene Symbol	Gene Type
Androgen receptor β	AR β	sex	Matrix metalloproteinase 12	MMP12	Matrix
Estrogen receptor 1	ESR1	sex	Osteopontin	Spp1	Matrix
Estrogen receptor 2	ESR2	sex	Tissue inhibitor of metalloproteinases	TIMP1	Matrix
G-protein coupled estrogen receptor	GPER	sex	Tenascin C	TNC	Matrix
Progesterone receptor	PR	sex	Angiotensinogen	AGT	Immune
5 α reductase	SRD5 α	sex	Chemokine ligand 7	CCL7	Immune
Aromatase	CYP19	sex	Chemokine receptor 3	CCR3	Immune
17 β -hydroxysteroid dehydrogenase	17 β -HSD	sex	Calcium-activated chloride channel regulator 1	Clca1	Immune
3 β -hydroxysteroid dehydrogenase	3 β -HSD	sex	Granulocyte-macrophage colony-stimulating factor	GMCSF	Immune
CD44	CD44	Matrix	C-X-C motif chemokine 12	CXCL12	Immune
Chitinase 3 like 1	CHI3L1	Matrix	Endothelial 1	END1	Immune
Mammalian acidic chitinase	CHIA	Matrix	Hepatocyte inducing factor	HIF1	Immune
Collagen 1 α 1	Col1 α 1	Matrix	Interleukin-4	IL4	Immune
Collagen III	ColIII	Matrix	Interleukin-5	IL5	Immune
Collagen V	ColV	Matrix	Interleukin-6	IL6	Immune
Connective tissue growth factor	CTGF	Matrix	Interleukin-10	IL10	Immune
Cathepsin K	CATK	Matrix	Interleukin-13	IL13	Immune
Epidermal growth factor receptor	EGFR	Matrix	Interleukin 1 receptor antagonist	IL1RN	Immune
Fibrillin 1	FBN1	Matrix	Interferon gamma	INF γ	Immune
Matrix metalloproteinase 1	MMP1	Matrix	Mucin 1	Muc1	Immune
Matrix metalloproteinase 2	MMP2	Matrix	Prostaglandin-endoperoxide synthase 2	Cox2	Immune
Matrix metalloproteinase 3	MMP3	Matrix	RB1-Inducible Coiled-Coil 1	CC1	Immune
Matrix metalloproteinase 7	MMP7	Matrix	Resistin	Flzz1	Immune
Matrix metalloproteinase 9	MMP9	Matrix	Transforming growth factor β -1	TGF β 1	Immune

results from animal and *in vitro* studies suggest hormonal pathways likely play an important role. Furthermore, gender is a prognostic factor in other lung diseases such as lung cancer and most of the studies report that estrogen and related genes (receptors and steroid metabolic enzymes) are associated with decreased risk and increased survival [1]. Using a custom array we simultaneously measured the transcriptional profile of 48 genes related to immune regulation, ECM remodeling and hormonal signaling in lung tissue samples of control individuals and men and women diagnosed with varying degrees of severity of COPD and IPF. Results from this work revealed potential targets important to IPF pathogenesis and the influence of sex-related genes between sexes.

Materials and methods

Custom gene expression array design

To generate gene expression signatures of 48 genes simultaneously in each lung tissue sample, we employed custom-designed micro-fluid-

ic cards manufactured by Applied Biosystems. Each card has 384 wells representing 8 full gene sets where 4 samples are run in duplicate for each gene. The layout and identity of the genes are depicted in **Table 1**. The genes are categorized by functionality and their involvement in hormone (sex genes), immune, and (ECM remodeling processes). Each well contains a pre-validated set of TaqMan® gene expression primers and probes specifically designed to each target. One gene serves as a normalization control (GAPDH).

Lung tissue samples

A total of 36 human lung tissue samples, preserved in RNAlater (Ambion) or flash frozen, were used in this study. Sixteen lung tissue samples from males and females with mild or medium severity IPF and 12 individuals with mild severity COPD were supplied by the Lung Tissue Research Consortium (LTRC) (**Table 2**) with IRB approval. Eight human control lung tissue samples were provided by the National Disease Research Interchange (NDRI). Human

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Table 2. Patient information on lung biopsy tissue samples

Variable	Male	Female
A. Control		
Number of subjects (n)	3	5
Average age (years)	77 (± 7.55)	80.2 (± 6.42)
B. Mild IPF		
Number of subjects (n)	3	3
Average age (years)	66.3 (± 3.39)	66.7 (± 4.92)
Average FVC%	90.7 (± 3.14)	85.3 (± 5.24)
C. Medium IPF		
Number of subjects (n)	6	4
Average age (years)	67.5 (± 3.83)	70.5 (± 5.48)
Average FVC%	64.7 (± 10.48)	69.50 (± 6.44)
D. Mild COPD		
Number of subjects (n)	6	6
Average age (years)	71.5 (± 5.86)	71.0 (± 7.24)
Average FVC%	101.3 (± 14.54)	94.8 (± 17.19)

lung tissue samples were stratified into control or diseased groups. Disease severity was based on the individual's percent forced vital capacity such that 50%-80% represented medium severity and > 80% corresponded to mild severity. Assessing obstructive and restrictive lung disease severity by % forced vital capacity (FVC%) is a procedure that had been shown to be a reliable parameter for this classification [37]. It should also be noted that all samples were obtained from individuals aged 60+ years to prevent complications from hormone replacement therapy.

Lung tissue RNA isolation

Total RNA was isolated from tissues using a commercially available kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Briefly, a portion of lung tissue weighing 15-23 mg was excised and placed in a disposable 50 mL pre-sterilized centrifuge tube containing 594 µl of RLT buffer plus 6 µl 2-Mercaptoethanol (β-ME). The tissue was homogenized in the 50 mL tube containing the buffer with a Fisher Scientific Tissuemiser (cat# 15-338-420). Lysate was removed and placed in a QIAshredder spin column (Qiagen cat# 79654) and centrifuged for 2 minutes. RNA extraction was then carried out using the Qiagen RNeasy® Mini Kit (cat# 74104) as described in the protocol as previously described [33]. The isolated RNA was further treated with DNase I (Qiagen). Extracted RNA was stored at -80°C until further

analysis. Total RNA was quantified using the Thermo Scientific Nanodrop-1000 Spectrophotometer. The samples were also processed on the Allegiant 2100 Bioanalyzer (Agilent, Palo Alto, CA) to assess RNA quality by the RIN number > 8. One microgram of quality validated RNA was reverse transcribed using the Promega Reverse Transcription System (cat# A3500) as previously described [33].

Gene expression measurements

Quantitative real-time PCR was performed on the Applied Biosystems 7900HT Fast Real-Time PCR system. Custom designed micro fluidic cards were removed from storage at 4°C and allowed to reach room temperature while the PCR mix was being prepared.

One hundred nanograms of each sample specific cDNA was added to 50 µl Taqman® Universal PCR Master Mix (Applied Biosystems cat# 4304437) and the appropriate volume of RNase-free water, in an Ambion Rnase-Free 1.5 mL microfuge tube (Ambion cat# AM12450), to obtain a reaction volume of 100 µl per fill reservoir. Once the PCR mix was made and the microfluidic card had reached room temperature, 100 µl of the appropriate sample-specific PCR reaction mix was loaded into each fill reservoir. The card was then centrifuged twice for one minute at 1200 rpm following the manufacturer's instructions. Following centrifugation the plate was sealed using a TaqMan Array sealer as described in the, "Applied Biosystems 7900HT Micro Fluidic Card Getting Started Guide". The fill reservoirs on the card were excised and the plate was loaded into the Applied Biosystems 7900HT Fast Real-Time PCR system. After the run was completed Ct values were generated for each target gene based on a set threshold value. Duplicates were averaged and normalized to the housekeeping gene, GAPDH. Normalized values were used to obtain a ratio of the average normalized expression of the target gene to the average expression of the housekeeping gene using the formula: $(1/2^{\text{average target gene expression}})/(1/2^{\text{average housekeeping gene expression}})$. This method had been previously reported to assess gene expression levels using Ct values [38]. The expression ratios are stratified by gender, disease, and severity and individual

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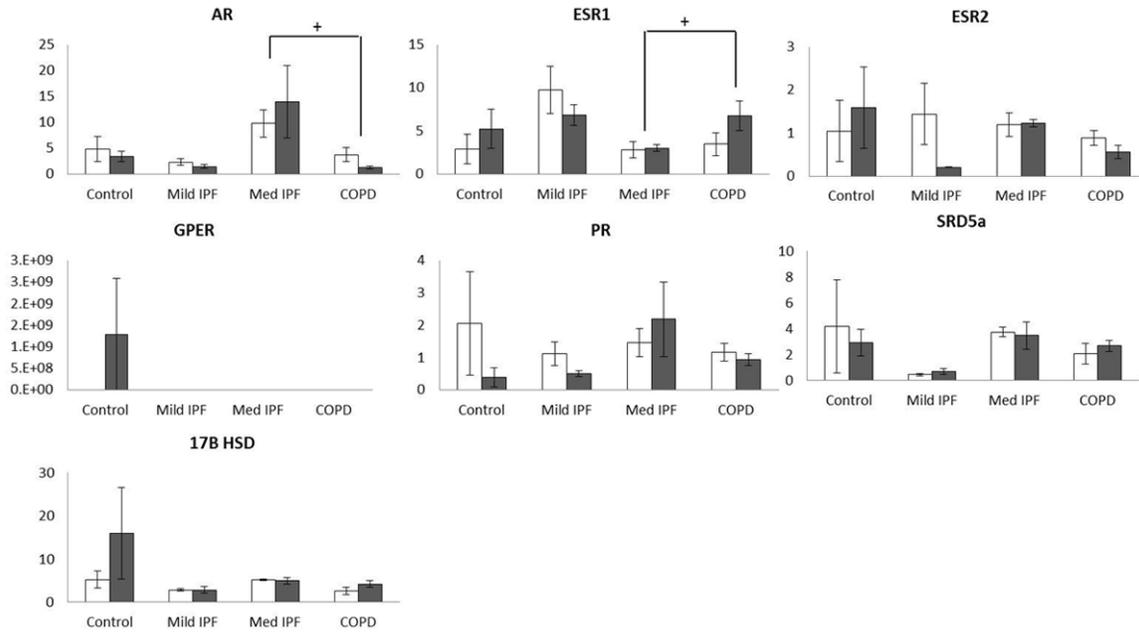


Figure 1. Gene expression levels of sex genes in lung tissue samples from individuals with mild or medium severity IPF or COPD. All values presented as the as mean \pm SE of the ratio of expression for each gene over the housekeeping control gene (GAPDH) where white bars and gray bars represent expression for females and males, respectively. (+) denotes statistically significant differences in expression between IPF and COPD (as listed in **Table 3**). Statistically significant differences in expression analyzed by IPF sex, disease and sex x disease are listed with corresponding *p* values for each graph (from data presented in **Table 4**).

group means and standard errors are calculated. The expression ratios are presented in **Figures 1-3** based on their category of sex, immune or ECM genes, respectively.

Statistical analysis

Gene expression signatures were generated for each patient sample in duplicate and normalized to GAPDH using the ratio method described above. To examine the effects of sex, disease and their interaction on expression levels for IPF, multiple linear regression models were applied. Differences in gene expression levels between mild IPF, medium IPF and COPD groups were tested by one-way analysis of variance. Statistical significant level is set at 0.05 for all tests. Multiple testing is adjusted via controlling of false discovery rate at the level of 0.05.

Results

To elucidate immune-, extracellular matrix (ECM)- and hormonally-related genes that are differentially expressed by sex in human control and diseased lung tissues, we employed custom-designed gene arrays to measure the

transcriptional expression of 48 genes (39 immune- and ECM- relevant genes and 9 sex-related genes) simultaneously by quantitative real-time PCR (qRT-PCR) methodology. The majority of these targets were selected based on previous studies that reveal their altered expression in inflammation or ECM remodeling in animal and/or human studies [2, 12, 33, 39, 40]. The remaining 9 genes were termed sex-genes based on their functional role in hormonal signaling and included hormone receptors and steroid metabolizing enzymes (**Table 1**). The study cohort comprised of 36 lung tissue samples obtained from control individuals and those diagnosed with COPD and mild and medium severity IPF based on reported percent forced vital capacity (FVC%) (**Table 2**). Assessing obstructive and restrictive lung disease severity by FVC% is a procedure that had been shown to be a reliable parameter for this classification [37]. Mild severity IPF is represented by a FVC% > 80, whereas medium severity exists in patients with FVC% values between 50 and 80. Male mild IPF patients had a slightly higher average FVC% (90.7 ± 3.14), than females (85.3 ± 5.24), while female and male medium severity IPF patients had FVC% averages of

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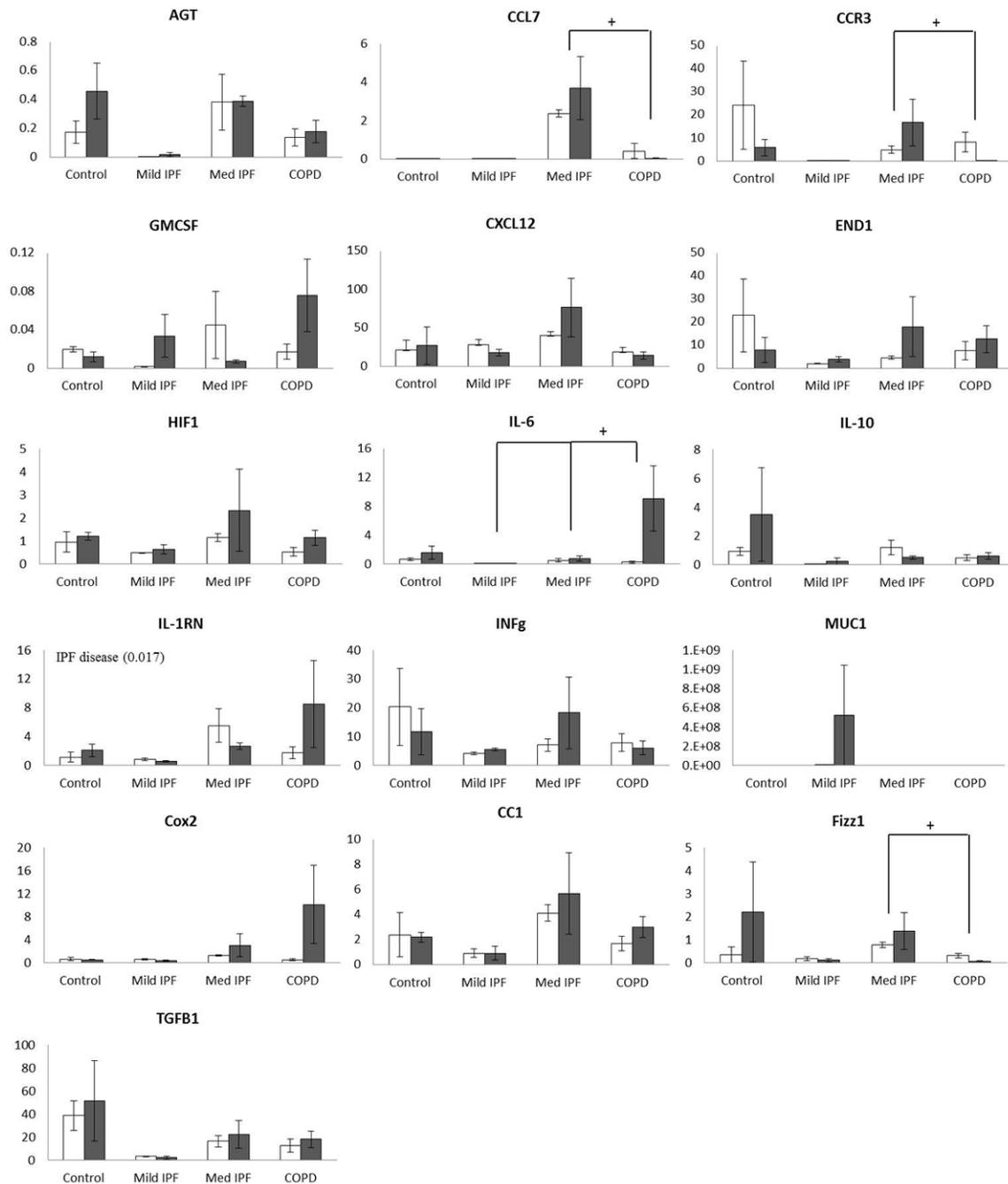


Figure 2. Gene expression levels of immune genes in lung tissue samples from individuals with mild or medium severity IPF or COPD. All values presented as the as mean \pm SE of the ratio of expression for each gene over the housekeeping control gene (GAPDH) where white bars and gray bars represent expression for females and males, respectively. (+) denotes statistically significant differences in expression between IPF and COPD (as listed in **Table 3**). Statistically significant differences in expression analyzed by IPF sex, disease and sex x disease are listed with corresponding p values for each graph (from data presented in **Table 4**).

69.5 \pm 6.44 and 64.7 \pm 10.48, respectively. Individuals with COPD had the highest overall FVC% with an average of 101.3 \pm 14.54 and 94.8 \pm 17.19 for males and females, respec-

tively, which represents mild disease based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria supported by the National Institutes of Health and the World

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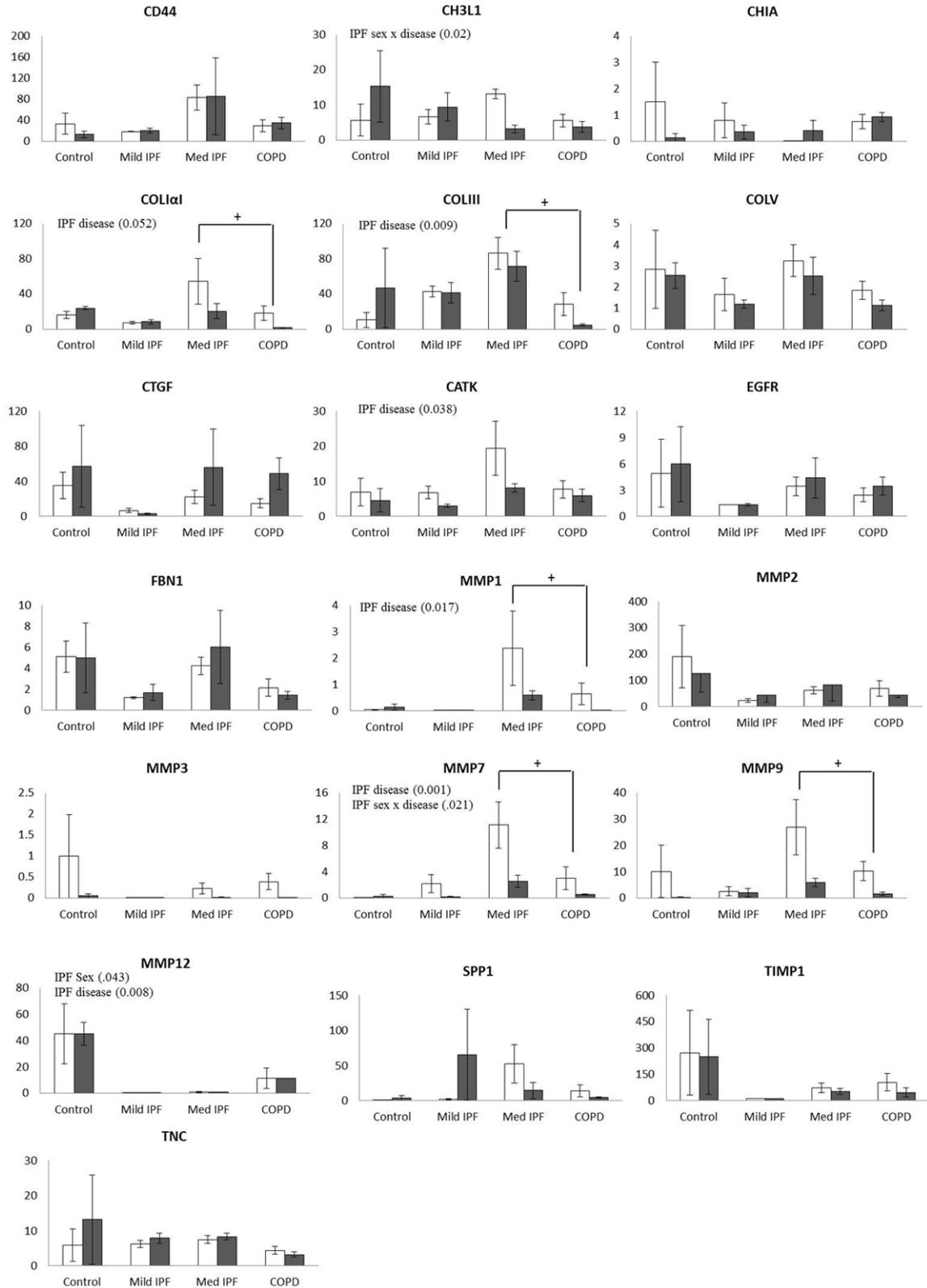


Figure 3. Gene expression levels of ECM genes in lung tissue samples from individuals with mild or medium severity IPF or COPD. All values presented as the as mean \pm SE of the ratio of expression for each gene over the housekeeping control gene (GAPDH) where white bars and gray bars represent expression for females and males, respectively. (+) denotes statistically significant differences in expression between IPF and COPD (as listed in Table 3). Statisti-

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cally significant differences in expression analyzed by IPF sex, disease and sex x disease are listed with corresponding *p* values for each graph (from data presented in **Table 4**).

Table 3. Significant differences in gene expression levels by disease severity and sex in IPF

Gene Name	Gene Symbol	Coefficient (<i>p</i> -value)		
		sex	disease	sex x disease
Cathepsin K	CATK	-1.719 (0.760)	6.252 (0.038)	-4.274 (0.300)
Chitinase 3 like 1	CH3L1	10.459 (0.065)	3.759 (0.180)	-9.847 (0.02)*
Collagen 1a1	COL-1A1	10.312 (0.575)	18.976 (0.052)	-19.716 (0.149)
Collagen III	COL-III	31.858 (0.234)	38.007 (0.009)	-24.517 (0.206)
Interleukin 1 receptor antagonist	IL-1RN	0.984 (0.556)	2.187 (0.017)	-1.774 (0.152)
Matrix metalloproteinase 1	MMP1	0.297 (0.738)	1.166 (0.017)	-0.917 (0.164)
Matrix metalloproteinase 7	MMP7	0.697 (0.770)	5.536 (< 0.001)	-4.292 (0.021)*
Matrix metalloproteinase 12	MMP12	-31.816 (0.043)	-22.074 (0.008)	18.169 (0.103)

Note: *The statistical significance disappeared after adjusting for multiple testing via the controlling of false discovery rate (FDR) of 0.05. To detect top 7 significant interaction effects, the FDR adjusted significance level is 0.0083 based on 48 tests on interaction effects.

Health Organization. The average age of all patients was 70 for males and 72 for females with a range of 66-80 years for the individual disease groups. All patients were greater than 60 years of age to avoid the confounding factor of hormone replacement therapy.

Genes not detected or minimally expressed

Of the entire set of genes investigated, CLCA1 was the only target that was not detected in any of the lung tissue samples examined. The expression of two genes, 3 β -HSD and IL-4, were measurable only in one lung sample. Two genes, IL-5 and IL-13, were detected only in lung samples from COPD patients.

Genes with significantly altered expression by sex and disease severity in IPF

A total of seven genes showed significant expression by severity of IPF (**Table 3**). Six genes we classified as ECM genes (CATHK, COL1A1, COL3, MMP1, MMP7, MMP12, (**Figure 3**) whereas one gene, IL-1RN (**Figure 2**), is from the immune category. These genes were all highly expressed in medium severity IPF for both sexes, except for MMP12.

Genes with a significant sex x disease interaction in IPF

When analyzed for sex and disease interaction, two genes, CHI3L1 and MMP7, appeared as potential candidates (**Table 3, Figure 3**, *p* < 0.05). In this section, multiple testing is adjust-

ed via controlling of false discovery rate of 0.05 for testing all the interaction effects (in total, 48 tests on interaction effects). It should be noted that this association did not reach statistical significance after adjusting for multiple testing, a result most likely due to the small sample size. However; it does highlight these genes a potential targets to investigate further.

Genes with differential expression between IPF and COPD

Overall a large number of genes were commonly expressed in males and females between control and diseased patients (**Figures 1 and 2**). In addition to IL-5 and IL-13 that were expressed only on COPD samples, 11 genes showed differential expression levels between IPF and COPD (**Table 4**, *p* < 0.05) with significant differences for Chemokine ligand 7 and Collagen III after multiple testing (overall FDR of 0.05 controlling for 48 tests for differences). All of these genes primarily showed a difference between COPD and the medium severity IPF group. Among these 11 genes, the expression of IL-6 was also statistically significantly different between COPD and mild severity (*p* = 0.019).

Discussion

Using a custom designed tiling array we were able to quantify the expression of a series of immune, ECM and sex-related genes in lung tis-

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Table 4. Significant differences in gene expression levels between disease severity of IPF and COPD

Gene Name	Gene Symbol	Coefficient (<i>p</i> -value)	
		Mild IPF vs COPD	Med IPF vs COPD
Androgen receptor	AR	0.786 (0.828)	9.387 (0.007)*
Chemokine ligand 7	CCL7	0.111 (0.888)	3.009 (< 0.001)
Chemokine receptor 3	CCR3	0.014 (0.998)	11.517 (0.014)*
Collagen 1a1	COL-1A1	0.0329 (0.998)	27.626 (0.021)*
Collagen III	COL-III	21.885 (0.236)	57.636 (< 0.001)
Estrogen receptor 1	ESR1	1.544 (0.308)	3.850 (0.007)*
Interleukin 6	IL-6	7.811 (0.019)	7.186 (0.015)*
Matrix metalloproteinase 1	MMP1	0.121 (0.839)	1.262 (0.023)*
Matrix metalloproteinase 7	MMP7	0.334 (0.879)	4.855 (0.025)*
Matrix metalloproteinase 9	MMP12	0.0149 (0.977)	13.112 (0.008)*
Resistin	FIZZ1	0.133 (0.771)	1.071 (0.004)*

Note: *The statistical significance disappeared after adjusting for multiple testing via the controlling of false discovery rate (FDR) of 0.05. To detect top 3 significant differences, the FDR adjusted significance level is 0.0031 based on 48 tests on the differences for each type of comparison.

sue samples from individuals with IPF and COPD. Data provided a number of interesting categories of expression profiles which identified genes with; minimal to no expression, disease type and disease severity specific expression, and those that has a potential sex and disease interaction.

A number of genes selected for analysis were not detected or minimally expressed and included CLCA1, 3 β -HSD, IL-4, IL-13, IL-5. CLCA1 is suggested to play a direct role in mucous production and secretion, and two genome scans reveal a linkage between CLCA1 expression and decreased FVC [41-43]. The expression of CLCA1 has also recently been detected in the lung of individuals with COPD and is elevated in smokers [44]. The murine homolog, mCLCA3, has also been shown to be highly up regulated in mice exposed to inhaled asbestos which leads to pulmonary sub-epithelial fibrosis [33]. Further studies have shown CLCA1 is strongly induced in airway epithelium in asthmatic conditions, and is not detected in normal healthy lung [43, 45, 46] and is induced by both allergens and pathogens [47]. Given this information it is surprising that CLCA1 was not detected in any of the patient samples analyzed, particularly in COPD tissue samples where mucoid hyper-secretion is known to be involved in disease pathogenesis [48, 49]. A number of reasons could account for this observation. Many genes involved in pathogen-

esis of IPF and COPD are not likely expressed throughout the course of disease and are transiently expressed during specific stages. CLCA1 may be expressed earlier during the development of disease or is dependent on the cause of disease. A number of factors likely contribute to the development and progression of IPF and COPD including environmental and genetic influences, information that we do not have in this case. The expression of CLCA1 in the lungs of asbestos exposed mice show high expression with continual exposure and

therefore expression may be dependent on the presence of an environmental insult [33]. Also, this same study, and others, showed CLCA1 expression occurs in lung/goblet cells, which may not be the major cell type in the tissue assessed in this study [33, 43, 45, 46] and therefore lack of transcriptional expression may be attributed to the tissue source.

The expression of 3 β -HSD, an enzyme essential for the biosynthesis of all classes of steroid hormones, was only detected in one female diagnosed with mild severity IPF. Reports on the expression of 3 β -HSD in male and female lung tissue has been limited to rats [50] and the lack of expression in human lung tissue may highlight a species difference in expression. Regardless, this implies that local steroid production is low and that influences of hormones, if they exist, occur through production of steroids external to the diseased environment. The expression of IL-4 was also only measurable in one control male tissue sample. IL-4 is a Th2 pro-fibrotic cytokine linked to the expression of several chemokines, including CCL3 and CXCL12, as well as collagen I and collagen III [51, 52]. The expression levels of IL-4 are increased in the bronchoalveolar lavage fluid (BALF) of patients with IPF although the specific cellular source of this cytokine is not clear [53, 54]. IL-4 is produced by a number of cells types including immune and epithelial cells [55]. It is likely that differential cellular

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composition of the tissue, timing or other confounding factors related to the individual patient accounts for this result. IL-13 shares many functional activities with IL-4, including the stimulation of collagen I and III. IL-4 and IL-13 knockout mice reveal that IL-13 is a dominant pro-fibrotic cytokine [51, 52]. Studies assessing the role of IL-5 in airway remodeling show that knockout mice have less fibrosis and collagen III and V expression than wild type mice after exposure to ovalbumin [55, 56]. Through this and other studies, it is proposed that IL-5 is an integral part of lung remodeling and fibrogenesis. It was expected that IL-13 and IL-5 expression would not be detected, or minimally detected, in the control patients, while expression would be augmented in disease. Increased expression of IL-13 and IL-5 was expected in patients diagnosed with COPD as studies have shown elevated protein levels in disease [57]. The lack of IL-13 and IL-5 expression in patients diagnosed with IPF was surprising, but highlights disease stage specificity or cell-specific expression that may contribute to these results. These observations may be an important distinction between IPF and COPD.

A handful of genes showed greatest expression levels by disease stage for IPF. Five ECM genes (CATHK, COL1A1, COL3, MMP1, MMP7, **Figure 3**) and 1 immune gene (IL-1RN, **Figure 2**) showed high expression in medium severity IPF for both sexes whereas one gene, MMP12, was most greatly expressed in controls (**Figure 3**). These results are in line with reports showing up-regulation of these genes in patients and animals models with IPF. Expression trends indicate the increased expression of these genes with disease progression as the greatest levels were observed in the medium severity IPF groups. It is interesting to note that while only 2 genes, CH3L1 and MMP7 has a sex and disease interaction (discussed below), the expression of this entire group of genes was greatest in medium severity females compared to males.

Data revealing hormonal influence on the transcriptional regulation of these genes is minimal and most of the studies performed to date focus on non-pulmonary cells and tissues. For example, estrogen reduced the expression of COL1A1 in select osteosarcoma and mesengial cells [58] and muscle tissues [59, 60] and

lower muscle collagen FSR were observed in hysterectomized/oophorectomized women using ERT compared with nonhysterectomized postmenopausal women [61]. Conversely, androgens have been shown to increase the expression of collagens in rat prostate tissues [62].

IL-1RN acts as an inhibitor of proinflammatory cytokines IL-1 α and IL-1 β , both of which have been associated with increased lung inflammation and ECM remodeling [63, 64]. In fact, it has been suggested that a decreased ratio of IL-1RN/IL-1 β may be an important determinant in IPF pathogenesis [65]. Only a few studies have probed the influence of hormones, particularly estrogen on expression levels of IL-1RN in breast and bone tissues. Expression of IL-1RN is reportedly increased in lung tissue of individuals with COPD and polymorphisms have been linked to an increased risk in developing IPF [66, 67]. A recent study revealed a negative correlation with estradiol in breast tissue and IL-1RN serum levels and tamoxifen, an ESR antagonist, significantly increased expression levels of IL-1Ra [68]. Estrogen was also shown to decrease in IL-1beta and an increase in IL-1ra expression in the ischemic hippocampus suggesting that co-modulation of these genes drives anti-inflammatory effects of estrogen [69]. Conversely, estradiol caused a significant decrease in both IL-1beta and IL-1ra in whole blood cell cultures [70, 71] that was inhibited with an ESR antagonist.

MMP1 is an enzyme that is capable of degrading fibrillar collagens (types I, II and III). It has been previously shown to be highly induced in IPF and it has been suggested by Rosas et al., as a candidate disease biomarker (with MMP7). The promoter of MMP1 contains AP-1 sites that have been shown to be targets of ESR1 inhibition through tethering mechanisms and polymorphisms within these site is associated with risk for IPF [72, 73]. Androgen receptors have also been implicated in negatively regulating MMP1 expression through Ets-related transcription factors [74]. While few studies have explicitly investigated a role for hormones in regulating MMP1 expression (directly or indirectly) these observations support future studies to delineate if these effects are specific to lung cells and tissues and are associated with IPF progression.

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Results for MMP12 showed high expression levels in the controls compared to all diseased groups. Also known as macrophage metallo-elastase, this protein primarily targets and degrades elastin and is induced in tissues and serum of individuals with IPF. Studies investigating the function of MMP12 in IPF are controversial as animal studies using knock out models have shown variable outcomes. For example, MMP12 deficient mice showed no significant difference on bleomycin-induced fibrosis [75] compared to their wildtype counterparts. This is in contrast to TGF- β_1 -stimulated pulmonary fibrosis which was ameliorated in MMP-12-deficient animals [76]. The latter study further revealed that deletion of MMP12 protected mice from Fas-induced pulmonary fibrosis without altering the inflammatory response [77]. These studies highlight that our understanding of the function of MMP12 in IPF is insufficient and variable results may be dependent on the causative agent, genetics, or other factors. In addition, assessing disease severity or sex-specific differences was not a component of these studies. In our previous work with an asbestos model of fibrosis, MMP12 expression levels were maximal in the lung tissue of animals exposed to 9 days compared to 3 and 40 day time-points [33]. It may be that the expression is transient and we did not capture this in the samples used in the current study.

Numerous genes were also differentially expressed between IPF and COPD. Results here suggest that the expression of IL-6 may contribute to the development of COPD in females, possibly through sex-specific regulation of the immune system. An increase in the level of IL-6 has also been described in several chronic lung diseases with inflammatory implications [78]. Interestingly, two sex genes, estrogen and androgen receptors, were both differentially expressed between COPD and medium severity IPF. The expression levels of AR were greater in IPF tissues for males, where ESR1 levels were greater in COPD samples. The other immune and matrix genes including CCL7, CCR3, COL1A1, COL3, MMP1, MMP7, MMP9 and FIZZ1 were more highly expressed in medium severity IPF compared to COPD lung tissues. These results highlight these genes as targets for future studies to investigate their contribution mechanistically to each disease.

A most striking and novel result of this study was the discovery of the tendency of sex and

disease interaction for two genes analyzed, CH3L1 and MMP7. Both genes are involved in regulation of extracellular matrix and have been shown to be elevated in a number of lung diseases including IPF, asthma, COPD. Chitinases are proteolytic enzymes that bind but unexpectedly do not have the ability to actively cleave chitin which makes understanding their function difficult. The function of chitinases are not clear but are suspected to play a role in innate and Th2 immunity [79], ECM remodeling, apoptosis, macrophage activation and angiogenesis [80]. CH3L1 null mice are defective in Th2 inflammation and remodeling driven by Th2 pathways [81]. Interestingly, results from our study reveal that inverse expression profiles were observed for men and women across disease severity for IPF. As disease severity increased expression levels increased for females while they decreased for males. Only a few studies have investigated the levels of one family member, CH3L1, in serum and BALF of individuals with IPF and collectively report enhanced levels in both biological sample types. Korthagen et al., [82] additionally reported increased serum levels of CH3L1 correlated with shorter survival time. In addition to IPF, elevated levels of CH3L1 has also been observed in asthma, COPD and lung cancer [83]. These studies did not investigate a gender association as the majority of the participants were male. We additionally noted an increase in CH3L1 mRNA expression in lung tissues samples from mice exposed to asbestos, however, only male rats were used in this study and no sex association could be observed. Furthermore, the samples were not stratified by disease severity in any of these aforementioned reports. Mechanistically, the transcriptional signals controlling expression of CH3L1 have not been elucidated although repression by androgens in mouse brain tissues has been observed [84]. The promoter is described as having both AP-1 and STAT3 binding sites that contributes to the high levels associated with lung cancer [85]. Whether hormone signaling pathways contribute to this regulation is not known but the opposing sex-specific expression trends suggest steroidal pathways should be further investigated.

MMP7 had higher expression levels in diseased females for both COPD and IPF groups compared to controls of both sexes and diseased males, indicating an interaction of MMP7 with

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sex of the participants. Furthermore, maximal levels are observed in the medium severity IPF group. These results suggest sex-specific mechanisms may be contributing to disease development and progression, controlling processes common to COPD and IPF. MMP7 is considered key regulator of pulmonary fibrosis in mice and humans which is consistent with increased expression with progressive IPF [86]. MMP7 is involved in ECM natural turnover and wound healing and the process of fibrosis is considered ECM remodeling that is unbalanced or dysregulated. MMP7 is typically secreted as an inactive proprotein which is activated when cleaved by extracellular proteinases and therefore mRNA levels may not correspond to active protein. Relevant future studies should include investigations of MMP7 activity between sexes to further investigate the sex-specific role of this protein as it relates to disease pathogenesis.

In conclusion, this is the first study to assess the expression of a targeted set of genes in lung tissues relevant to IPF and COPD including sex-related genes. Clinical studies reveal females have a higher COPD mortality rate than males, while males have a higher IPF incidence, prevalence, and mortality. A number of genes revealed by this work potentially play a role in these observations and warrant further investigations, and it would particularly useful to validate such targets at the protein level in follow-up studies. For all the genes analyzed, only CH3L1 and MMP7 showed an association between sex and disease for IPF. A limitation of the study was the small sample size and the ability to stratify by severity. We originally attempted to acquire samples from severe IPF but could only obtain these from males (results not included). Despite these limitations, this study highlights two very interesting potential targets that should be further studied to elucidate their influence on sex-based differences in fibrotic lung disease.

Uncovering mechanisms whereby lung disease differentially affects men and women will aid in better treatment and management of diseases. Overall, this work highlights genes that may contribute to gender trends observed for lung diseases and are potential targets for future research. These results increase our basic understanding of the molecular mechanisms involved in two distinct lung diseases. Our find-

ings may also aid in the development of future therapeutics as well as enhance our understanding of potential risk factors and predispositions for obstructive and restrictive lung diseases.

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Disclosure of conflict of interest

None.

Abbreviations

IPF, idiopathic pulmonary fibrosis; COPD, chronic obstructive pulmonary disease; ECM, extracellular matrix; LTRC, Lung Tissue Research Consortium; NDRI, National Disease Research Interchange; FVC, forced vital capacity.

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