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## **Anti-fibrotic effects of nintedanib on lung fibroblasts derived from patients with Progressive Fibrosing Interstitial Lung Diseases (PF-ILDs)**

Audrey Joannes, Tom Voisin, Claudie Morzadec, Alice Letellier, Francisco Llamas Gutierrez, Dan Cristian Chiforeanu, Cécile Le Naoures Mear, Stephanie Guillot-Dudoret, Bertrand Richard de Latour, Simon Rouzé, et al.

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**Revised version****Anti-fibrotic effects of nintedanib on lung fibroblasts derived from patients with  
Progressive Fibrosing Interstitial Lung Diseases (PF-ILDs).**

Audrey Joannes<sup>1</sup>, Tom Voisin<sup>1</sup>, Claudie Morzadec<sup>1</sup>, Alice Letellier<sup>1</sup>, Francisco Llamas Gutierrez<sup>2</sup>, Dan Cristian Chiforeanu<sup>2</sup>, Cécile Le Naoures<sup>2</sup>, Stéphanie Guillot<sup>3</sup>, Bertrand Richard De Latour<sup>4</sup>, Simon Rouze<sup>4</sup>, Madeleine Jaillet<sup>5</sup>, Bruno Crestani<sup>5,6</sup>, Lutz Wollin<sup>7</sup>, Stéphane Jouneau<sup>1,8\*</sup> and Laurent Vernhet<sup>1\*</sup>.

<sup>1</sup>Univ Rennes, CHU Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR\_S 1085, F-35000 Rennes, France.

<sup>2</sup>Department of pathology and cytology, Rennes University Hospital, 35033, Rennes, France;

<sup>3</sup>Department of Respiratory Physiology, Rennes University Hospital, 35033, Rennes, France;

<sup>4</sup>Department of Thoracic, cardiac and vascular surgery, Rennes University Hospital, 35033, Rennes, France;

<sup>5</sup>Faculté de Médecine Xavier Bichat, Université de Paris, Institut National de la Santé et de la Recherche Médicale (INSERM), UMR1152, FHU APOLLO, Labex INFLAMEX, Paris, France;

<sup>6</sup>Department of Pulmonology, AP-HP, Hôpital Bichat, FHU APOLLO, Inserm 1152, University of Paris, Paris, France

<sup>7</sup>Boehringer Ingelheim Pharma GmbH & Co, KG, Biberach an der Riss, Germany;

<sup>8</sup>Department of Respiratory Diseases, Competence Center for Rare Pulmonary Disease, Rennes University Hospital, 35033, Rennes, France

\* Equally contributed

26 **Running title:** Anti-fibrotic effects of nintedanib on PPF fibroblasts

27 **Address correspondence to:** Audrey Joannes, Irset - Inserm UMR\_S 1085, 2 avenue du

28 Professeur Léon Bernard, 35043 RENNES CEDEX, France, Email: audrey.joannes@univ-

29 rennes.fr

30 **Nonstandard abbreviations:** ILD: interstitial lung disease, PF-ILD: Progressive fibrosing-

31 ILD, IPF: idiopathic pulmonary fibrosis, HP: hypersensitivity pneumonitis, PPFE:

32 pleuroparenchymal fibroelastosis, PBS: phosphate-buffered saline, HLFs: human lung

33 fibroblasts, TGF- $\beta$ 1: transforming growth factor- $\beta$ 1, PDGF-BB: platelet-derived growth factor

34 BB, COL1: collagen I,  $\alpha$ -SMA:  $\alpha$ -smooth muscle actin, SEM: standard error of the mean,

35 ANOVA: one-way analysis of variance.

36

37 **Abstract**

38 The tyrosine kinase inhibitor nintedanib has been recently approved for the treatment of  
39 Interstitial Lung Diseases (ILDs) that manifest a progressive fibrosis phenotype other than  
40 Idiopathic pulmonary Fibrosis (IPF).

41 Nintedanib reduces the development of lung fibrosis in various animal models resembling  
42 features of PF-ILD and *in vitro*, it inhibits the fibrosing phenotype of human lung fibroblasts  
43 (HLFs) isolated from patients with IPF. To get insight on the cellular and molecular  
44 mechanisms that drive the clinical efficiency of nintedanib in patients with non-IPF PF-ILD,  
45 we investigated its effects on the fibrosing functions of HLFs derived from patients with PF-  
46 hypersensitivity pneumonitis (PF-HP, n=7), PF-sarcoidosis (n=5) and pleuroparenchymal  
47 fibroelastosis (PPFE, n=4).

48 HLFs were treated with nintedanib (10 nM-1  $\mu$ M) and then stimulated with PDGF-BB (25-  
49 50 ng/ml) or TGF- $\beta$ 1 (1 ng/ml) for 24 to 72 hours to assess proliferation and migration or  
50 differentiation.

51 At nanomolar concentrations, nintedanib reduced the levels of PDGF receptor and ERK1/2  
52 phosphorylation, the proliferation and the migration of PF-HP, PF-sarcoidosis and PPFE HLFs  
53 stimulated with PDGF-BB. Moreover, nintedanib also attenuates the myofibroblastic  
54 differentiation driven by TGF- $\beta$ 1 but only when it is used at 1  $\mu$ M. The drug reduced the  
55 phosphorylation of SMAD2/3 and decreased the induction of collagen, fibronectin and  $\alpha$ -  
56 smooth muscle actin expression induced by TGF- $\beta$ 1.

57 In conclusion, our results demonstrate that nintedanib counteracts fundamental fibrosing  
58 functions of lung fibroblasts derived from patients with PF-HP, PF-sarcoidosis and PPFE, at  
59 concentrations previously reported to inhibit control and IPF HLFs. Such effects may contribute  
60 to its clinical benefit in patients suffering from these irreversible ILDs.

61

62

63 **Highlights:**

64 Nintedanib blocks PDGF-BB-induced signalling of PF-ILD HLFs.

65 Nintedanib inhibits PDGF-BB-induced proliferation of PF-ILD HLFs

66 Nintedanib prevents PDGF-BB-induced migration of PF-ILD HLFs

67 Nintedanib diminishes TGF- $\beta$ 1-induced differentiation of PF-ILD HLFs

68 **Keywords (6):** PPF, primary human lung fibroblasts, nintedanib, proliferation, migration,  
69 differentiation.

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Journal Pre-proof

## 72           **1. Introduction**

73           Interstitial lung diseases (ILDs) are defined as a large and heterogeneous group of  
74           parenchymal pulmonary disorders of various etiologies characterized by diffuse damages in the  
75           lung parenchyma [1,2]. Idiopathic pulmonary fibrosis (IPF), the best described ILD, is a  
76           progressive fibrosing ILD (PF-ILD) associated with a high rate of mortality. A proportion of  
77           patients with non-IPF ILD can also develop a progressive fibrosing phenotype that leads to  
78           decline in lung function, quality of life worsening and early mortality [3]. These ILDs notably  
79           include idiopathic non-specific interstitial pneumonia (NSIP), unclassifiable idiopathic  
80           interstitial pneumonia, connective-tissue disease ILDs, chronic sarcoidosis, fibrosing  
81           hypersensitivity pneumonitis (HP), asbestosis, silicosis or pleuroparenchymal fibroelastosis  
82           (PPFE). The estimated prevalence of non-IPF PF-ILD ranges from 6.9 (Europe) to 70.3 (United  
83           States) per 100 000 persons and the estimated incidence from 2.1 (Europe) to 32.6 (United  
84           States) per 100 000 persons per years [4]. It is estimated that around 30% of all ILDs will evolve  
85           toward PF-ILD [5,6].

86           The development of pulmonary fibrosis in non-IPF ILD is probably the consequence of  
87           chronic injuries to lung endothelial cells or epithelial alveolar cells associated with immune  
88           stimulation or autoimmunity. The repeated damages to alveolar tissues lead to the migration  
89           and proliferation of lung fibroblasts and their differentiation into myofibroblasts in fibroblastic  
90           foci [2,7]. Pulmonary tissue then acquires a fibrosis phenotype, characterized by self-  
91           progression and activation loops that contribute to the accumulation of extracellular matrix  
92           (ECM) and end-stage lung remodeling.

93           Nintedanib is a tyrosine kinase inhibitor that has been used for several years in the treatment  
94           of IPF. Recently, nintedanib has also been approved in EU, US and many other countries for  
95           the treatment of patients that develop other PF-ILD [8,9]. The randomized, double-blind  
96           placebo-controlled INBUILD<sup>®</sup> study, which assessed the efficacy and safety of nintedanib,

97 demonstrated that the drug slows the progression of several non-IPF PF-ILDs, established by  
98 the annual rate of decline in forced vital capacity (FVC). Nintedanib likely reduces the  
99 development of IPF by interfering with key functions of lung fibroblasts. Indeed, *in vitro*,  
100 nintedanib inhibits the migration and proliferation of human lung fibroblasts (HLFs) isolated  
101 from patients with IPF by competitively blocking the ATP-binding pocket in various tyrosine  
102 kinase receptors including the platelet-derived growth factor receptor (PDGFR), the fibroblast  
103 growth factor receptor and the vascular endothelial growth factor receptor [10]. However,  
104 besides IPF, the effects of nintedanib on the functions of lung fibroblasts isolated from patients  
105 suffering from other PF-ILD remain largely unknown.

106 In order to demonstrate the pharmacological activity of nintedanib on non-IPF HLFs, we  
107 investigated in the present study the *in vitro* drug effects on the pro-fibrosing functions of HLFs  
108 isolated from ILD patients with PF-HP, PF-sarcoidosis and PPFE.

109

## 110 **2. Materials and Methods**

### 111 **2.1 Primary cultures of HLFs**

112 PF-HP (median age 58 year; range 46.23-66.91 years) (4 females, 3 males), PF-sarcoidosis  
113 (median age 57 year; range 36.57-68.05 years) (2 females, 3 males), PPFE (median age 52.85  
114 year; range 29.5-72.53 years) ( 3 females, 1 male) and IPF (median age 66.94 year; range 56.72-  
115 76.06 years) lung samples were isolated from patients undergoing open lung biopsy using the  
116 explant method [11]. PF-ILD patients were required to meet at least one of the following criteria  
117 for progression of their ILD within 24 months, a relative decline in the FVC of at least 10% of  
118 the predicted value, a relative decline FVC of 5% to less than 10% of the predicted value and  
119 worsening of respiratory symptoms or an increased extent of fibrosis on high-resolution CT, or  
120 worsening of respiratory symptoms and an increased extent of fibrosis [2,3]. IPF (2 females, 7  
121 males) were diagnosed according to the ATS/ERS/JRS/ALAT criteria, including  
122 histopathological features of usual interstitial pneumonia [12,13]. Control samples (median age  
123 64.14 year; range 59.09-71.29 year, males) were from lung cancer patients undergoing  
124 lobectomy or pneumonectomy, away from the tumor. The absence of tumor tissue in control  
125 samples was verified histologically. This study was approved by the local ethics committee  
126 (Ethics Committee CHU Rennes, n° 16.123). Written informed consent was obtained from all  
127 subjects. Cell morphology was checked by phase contrast microscopy. All experiments were  
128 performed in accordance with relevant guidelines and regulations.

### 129 **2.2 Cell culture and treatments**

130 HLFs derived from patients with PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) were  
131 cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Life Technologies,  
132 Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS) (Eurobio scientific, Evry,  
133 France), antibiotic-antimycotic solution (Gibco™, Life Technologies) and L-glutamine used at  
134 passages 5 and 6. Nintedanib was provided by Boehringer Ingelheim Pharma GmbH (Biberach,



135 Germany). To analyze mRNA and protein expressions, HLFs were seeded for 24 h in 6-well  
136 plates, and then starved in FCS-free DMEM for 16 h. To study cell growth, HLFs were serum-  
137 starved for 16 h, cultured in DMEM containing 1 % FCS and 0.01-0.3  $\mu$ M nintedanib (NTD)  
138 for 3 h, and finally stimulated with PDGF-BB (50 ng/ml) (R&D Systems, Bio-Techne, Lille,  
139 France) for a further 72 h. To analyze the TGF- $\beta$ 1-induced myofibroblastic differentiation,  
140 serum-starved HLFs were pretreated with nintedanib (0.01-1  $\mu$ M) for 3 h and then stimulated  
141 with TGF- $\beta$ 1 (1 ng/ml; Preprotech, Neuilly-sur-Seine, France) for 24 h.

### 142 **2.3 Proliferation**

143 After nintedanib and PDGF-BB treatments, cells were dissociated with trypsin, centrifuged  
144 (1500g, 5 min) and resuspended in DMEM containing 10 % FCS. Proliferation was assessed  
145 with the acridine orange/propidium iodide staining (Logos Biosystems, Villeneuve d'Ascq,  
146 France) and an automated fluorescence cell counter (Luna™, Logos Biosystems). The dye  
147 causes viable nucleated cells to fluoresce green and nonviable nucleated cells to fluoresce red.  
148 After cell counting, the relative cell proliferation was calculated by dividing the number of  
149 viable cells exposed to nintedanib +/- PDGF-BB by the number of viable untreated cells  
150 arbitrarily set at 1.

### 151 **2.4 Migration assay**

152 HLF migration was assessed in a modified Boyden chamber assay, using a ThinCert®  
153 permeable support (6.5 mm, 8  $\mu$ m) (Greiner Bio-One, Kremsmünster, Austria) coated with  
154 fibronectin (0.1  $\mu$ g/ml) (Sigma-Aldrich, St. Quentin Fallavier, France). The medium in the  
155 upper compartment (DMEM-0%FCS) contained 0.01-0.3 $\mu$ M nintedanib and the medium in the  
156 lower compartment contained (DMEM-1%FCS) 25 ng/ml PDGF-BB. Cells were incubated for  
157 16 h at 37°C, fixed in 70 % ethanol, and labelled with DAPI. Migration was assessed by  
158 counting the number of cells on the lower surface of the filters. The relative cell migration was

159 defined by dividing the number of migrating cells stimulated with PDGF-BB by the number of  
160 migrating cells left untreated and arbitrarily set at 1.

## 161 **2.5 Protein analysis**

162 HLFs were lysed in ice-cold RIPA buffer containing cOmplete™ Protease Inhibitor Cocktail  
163 (Roche, Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were  
164 separated by electrophoresis on 10 % Tris-glycine SDS polyacrylamide gels and transferred to  
165 nitrocellulose membranes (pore size: 0.2 µm, Invitrogen). Free binding sites were blocked by  
166 incubation in 5 % milk or bovine serum albumin (BSA) (Sigma-Aldrich, St. Quentin Fallavier,  
167 France) for 2 h at room temperature. Membranes were then incubated with the appropriate  
168 primary human antibody (Ab) overnight at 4°C. The primary Abs were anti- $\alpha$ -smooth muscle  
169 actin ( $\alpha$ -SMA) (clone 1A4, Sigma-Aldrich), anti-collagen I (Southern Biotechnology  
170 Associates, Birmingham, AL), anti-GAPDH (mab90009-P, Covalab, Bron, France). Anti-  
171 phospho p44/42 MAPK (197G2), anti-p44/42 MAPK (9101), anti-phospho SMAD2 (3104),  
172 anti-SMAD2 (D43B4), anti-phospho SMAD3 (C25A9), anti-SMAD3 (C67H9), anti-phospho  
173 PDGF receptor (PDGFR)  $\beta$  (3161) and anti-PDGFR (28E1) Abs were from Cell Signaling  
174 Technology (Massachusetts, USA). Digital images were acquired on a gel imaging system  
175 (Chemi doc, BIO-RAD Laboratories, Marnes-La-Coquette, France) equipped with a CCD  
176 camera. Western blots were quantified by densitometry using Image Lab™ software (BIO-  
177 RAD) and normalized to GAPDH. The relative protein expression in HLFs was determined by  
178 arbitrarily setting at 1 the protein expression measured in untreated cells.

## 179 **2.6 RNA isolation and RT-PCR assays**

180 Total RNAs were extracted using the mRNA extraction kit from Macherey Nagel (Hoerd,  
181 France) according to the manufacturer's instructions. RNA concentrations were measured by  
182 spectrofluorimetry using a NanoDrop 1000 (Thermo Fisher scientific, Saint-Herblain, France).

183 Then mRNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription  
184 kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative PCR (polymerase chain  
185 reaction) was performed using the SYBR Green methodology on a CFX384 Real-Time PCR  
186 System (Bio-Rad Laboratories) in duplicates. All the primers were provided by Sigma-Aldrich  
187 (KiCqStart™ SYBR® Green Primers, St. Quentin Fallavier, France). The specificity of gene  
188 amplification was checked at the end of PCR using the comparative cycle threshold method  
189 (CFX Manager Software). The mean Cq values were used to normalize the target mRNA  
190 concentrations to those of the 18S ribosomal protein by the  $2^{-\Delta\Delta Cq}$  method. The mRNA levels  
191 were expressed relatively to those measured in unstimulated HLFs, arbitrarily set at 1.

## 192 **2.7 Statistical analysis**

193 All results are presented as means  $\pm$  standard errors of mean (SEM) of the indicated numbers  
194 of independent biological experiments performed with cell cultures deriving from different  
195 patients. Statistical analyses were performed with Prism 8.0 (GraphPad Software, La Jolla, CA).  
196 Significant differences were determined by ANOVA followed by the Dunnett's Multiple  
197 comparison *t*-test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

198

### 199 3. Results

#### 200 3.1 Nintedanib blocked PDGF-BB-induced signaling of PF-HP, PF-sarcoidosis and 201 PPFE primary HLFs.

202 PDGF-BB modulates cell functions, notably in lung fibroblasts, by stimulating the PDGFR  
203 which, in turn, controls the activation of the ERK1/2 signaling pathway [14]. Our results show  
204 that PDGF-BB increased the level of phosphorylation of the PDGFR and ERK1/2 in PF-HP  
205 (Fig. 1A-B), PF-sarcoidosis (Fig. 1C-D), PPFE (Fig. 1E-F), IPF and control (Supplementary  
206 Fig. 1) HLFs. To explore the effects of nintedanib on the PDGFR signaling pathway in PF-ILD  
207 HLFs, cells were first treated for 3 h with 10 nM-300 nM nintedanib and then stimulated for 10  
208 min with PDGF-BB. The drug decreased in a concentration-dependent manner the PDGF-BB-  
209 induced phosphorylation of PDGFR and ERK in PF-HP (Fig. 1 A-B) and PF-sarcoidosis (Fig.  
210 1 C-D) HLFs, as well as in IPF and control HLFs (Supplementary Fig. 1 A-D). Its effects were  
211 significant from 10-30 nM and maximal at 300 nM. In PPFE HLFs, nintedanid also reduced  
212 PDGFR and ERK phosphorylation but this effect was only significant at 300 nM.

#### 213 3.2 Nintedanib inhibited PDGF-BB-induced proliferation of PF-HP, PF-sarcoidosis 214 and PPFE HLFs.

215 Fibroblast proliferation is an important step of self-sustaining fibrosis that likely promotes  
216 the formation of pulmonary fibroblastic foci [15] in the lungs of patients suffering from IPF or  
217 non-IPF PF-ILD [2,7]. We thus explored the potential of nintedanib to block cell proliferation  
218 induced by PDGF-BB, a potent mitogenic cytokine. HLFs were incubated for 3 h with  
219 nintedanib and then stimulated with PDGF-BB for the next 72 h. As expected, PDGF-BB  
220 increased the proliferation of PF-ILD HLFs (Fig. 2 A, B, C), IPF and control HLFs  
221 (Supplemental Fig. 1 E, G). Nintedanib significantly and dose-dependently reduced the PGDF-  
222 BB-induced proliferation of PF-HP, PF-sarcoidosis and PPFE HLFs. At 100 nM, nintedanib  
223 totally inhibited the proliferation of all PF-ILD HLFs.

224 **3.3 Nintedanib prevented PDGF-BB-induced migration of PF-HP, PF-sarcoidosis and**  
225 **PPFE HLFs.**

226 The migratory cell dynamic is another important function involved in the formation of  
227 fibroblastic foci and self-sustaining fibrosis [15,16]. Besides proliferation, PDGF-BB also  
228 promotes cell migration through its chemoattractant properties. Figure 3 shows that PDGF-BB  
229 significantly increased the migration of the different PF-ILD HLFs. Nintedanib significantly  
230 decreased, in a concentration-dependent manner, the migration of PF-HP, PF-sarcoidosis and  
231 PPFE stimulated with PDGF-BB. Significant inhibition started at 30 nM and reduction close to  
232 baseline was reached at 300 nM. Similarly, the drug strongly reduced the migration of IPF and  
233 control HLFs cultured with PDGF-BB (Supplemental Fig. 1 F, H).

234 **3.4 Nintedanib diminished TGF- $\beta$ 1-induced fibroblast to myofibroblast transition of**  
235 **PF-HP, PF-sarcoidosis and PPFE HLFs.**

236 The canonical TGF- $\beta$  signaling pathway, mediated by the signal transducers and  
237 transcriptional modulators SMAD2 and SMAD3, drives the production of myogenic markers  
238 linked to myofibroblast differentiation. This signaling pathway controls fibrogenesis by  
239 regulating the production of ECM and the development of contractile actin stress fibers. In  
240 order to study the effects of nintedanib on the fibroblast to myofibroblast transition, PF-HP, PF-  
241 sarcoidosis and PPFE HLFs were treated with the drug for 3 h and then stimulated with TGF-  
242  $\beta$ 1 for the next 24 hours. Figure 4 shows that TGF- $\beta$ 1 induced the phosphorylation of SMAD2  
243 and SMAD3 proteins in all three PF-ILD HLFs (Fig. 4). The cytokine also strongly increased  
244 the production of the myofibroblastic markers fibronectin, collagen-1 (COL1) and  $\alpha$ -smooth-  
245 muscle actin ( $\alpha$ -SMA), at both mRNA (Fig. 5) and protein levels (Fig. 6). In these conditions,  
246 nintedanib weakly reduced the phosphorylation of SMAD 2/3 in PF-ILD HLFs. Its effects were  
247 only significant when nintedanib was used at its highest concentration (1  $\mu$ M) (Fig. 4).  
248 Similarly, nintedanib poorly limited the expression of the different myofibroblastic markers, at

249 both mRNA and protein levels in non-IPF HLFs (Figures 5 and 6), as well as in IPF and control  
250 HLFs (Supplementary Fig. 2). Nintedanib significantly reduced the *FIBRONECTIN*, *COL1A1*  
251 and *ACTA2* mRNA levels in PF-HP and PPFE HLFs, mainly at its highest concentration (1  
252  $\mu\text{M}$ ), and it only prevented the *FIBRONECTIN* gene expression in PF-sarcoidosis HLFs. In  
253 addition, 1  $\mu\text{M}$  nintedanib prevented the expression of fibronectin, COL1 and  $\alpha$ -SMA in PF-  
254 HP HLFs (Fig. 6). At this concentration, the drug also significantly reduced  $\alpha$ -SMA protein  
255 levels in PF-sarcoidosis and PPFE HLFs. However, nintedanib had no significant effect on  
256 COL1 levels in these two cell types. As expected, high drug concentrations significantly  
257 prevented protein expression of the three myofibroblastic markers in IPF and control HLFs  
258 (Supplemental Fig. 2 C-D and G-H).

259

#### 260 4. Discussion

261 Our study demonstrates for the first time that the tyrosine kinase inhibitor nintedanib, used  
262 *in vitro* at pharmacologically relevant concentrations, reduced the proliferation, migration and  
263 differentiation of primary HLFs derived from progressive fibrosing (PF)-HP, PF-sarcoidosis  
264 and PPFE HLFs.

265 Several reports have previously shown that nintedanib could modulate different signalling  
266 pathways and pro-fibrotic functions in HLFs isolated from patients with IPF [17–19] or with  
267 systemic sclerosis-associated ILD (SSc-ILD) [20]. The present results show that nintedanib also  
268 exerts potent *in vitro* antifibrotic effects on HLFs deriving from patients with three other ILDs  
269 that could be associated with a progressive fibrosing phenotype, i.e fibrosing HP, sarcoidosis  
270 and PPFE. As described in IPF HLFs, nintedanib strongly inhibited the PDGFR-dependent  
271 signaling pathways in the three types of HLFs [19]. Our results suggest that low drug  
272 concentrations (30-100 nM) significantly and similarly prevented the proliferation and  
273 migration of PF-HP, PF-sarcoidosis and PPFE HLF stimulated with PDGF-BB. Moreover,

274 these concentrations are equivalent to those inhibiting the proliferation and migration of IPF-  
275 HLFs [19] and SSc-ILD HLFs [20]. Consequently, nintedanib seems to be equally active to  
276 block PDGF effects in IPF and non-IPF HLFs.

277 In addition, we demonstrated that nintedanib partially blocked the switch of fibroblasts to  
278 myofibroblasts induced by TGF- $\beta$ 1 in PF-HP, PF-sarcoidosis and PPFE HLFs. During this step,  
279 HLFs gain the expression of contractile and ECM proteins. Specifically, the production of  $\alpha$ -  
280 SMA allows the contraction of myofibroblasts and contributes to the matrix stiffening [21].  
281 Nintedanib significantly reduced the TGF- $\beta$ 1-induced expression of  $\alpha$ -SMA in the three PF-  
282 ILD HLFs, but its effects were more potent in PF-HP HLF. In addition, we show that nintedanib  
283 specifically reduced COL1 expression in PF-HP HLFs. The production and secretion of  
284 collagen is another critical event conferring the stiffness of fibrotic lung tissues. In viable  
285 precision-cut lung slices from bleomycin-treated rats and IPF patients, nintedanib modulates  
286 type III collagen turnover [22]. In IPF HLFs, nintedanib also inhibited COL1 fibril formation  
287 and altered the appearance of collagen fibril bundles [23]. As reported for IPF [17,18] and SSc-  
288 ILD HLFs [20], our results clearly show that the concentrations of nintedanib required to block  
289 the myofibroblast differentiation are higher than those preventing proliferation and migration  
290 of HLFs. Indeed, COL1 and  $\alpha$ -SMA expressions were only significantly reduced in HLFs  
291 treated with 1  $\mu$ M nintedanib. The reasons why different ranges of concentrations of nintedanib  
292 are necessary to prevent the proliferation and migration of HLFs on the one hand and the  
293 differentiation of HLFs on the other hand remain still unclear since the half maximal inhibitory  
294 concentrations of nintedanib for the PDGFR and TGF- $\beta$ 1 receptor tyrosine kinases are very  
295 similar [24,25].

296 It was recently discussed that the fibrotic process controlling the development of non-IPF  
297 PF-ILD may be similar to those involved in the progression of IPF [2,7,26,27]. Different recent  
298 reports are in agreement with this hypothesis. In fibrotic HP, a single-cell transcriptional

299 analysis had identified a subpopulation of fibroblasts with a high expression of *ACTA2* or  
300 *COL1A1* [28]. In fibrotic lung tissues of patients with sarcoidosis, gene and protein expressions  
301 in fibroblastic foci were quite similar to those detected in patients with IPF [29]. PPFE, a very  
302 rare subtype of interstitial pneumonia [30] is also characterized by the development of an elastic  
303 fibre-rich fibrosis in pulmonary parenchyma. The fact that nintedanib similarly inhibited major  
304 fibroblastic functions in IPF and other PF-ILD HLFs also supports a common mechanism of  
305 fibrogenesis in these heterogeneous fibrotic ILDs. Finally, our results suggest that nintedanib  
306 may prevent the decline in lung function in most PF-ILDs, including PPFE, by inhibiting the  
307 main functions of HLFs [31].

## 308 **5. Conclusion**

309 In conclusion, we found that nintedanib, used at pharmacologically relevant concentrations,  
310 counteracts *in vitro* key fibrotic functions of lung fibroblasts isolated from patients with PF-  
311 HP, PF-sarcoidosis and PPFE. These cellular and molecular effects are likely to contribute to  
312 its clinical benefit in patients suffering from these irreversible ILDs.

313

314

315



316 **6. Authorship contributions**

317 *Conceptualization:* AJ, LV, SJ conceived and designed the study.

318 *Performed data analysis:* AJ, CM, TV performed the experiments. AJ analyzed and interpreted  
319 the data.

320 *Resources:* FLG, DCC, CLN, BDLT, SR provided lung biopsy. SG and AL provide clinical  
321 data. MJ and BC provided HLFs.

322 *Writing - review & editing:* AJ, LV, SJ and LW.

323 All authors read and approved the final manuscript.

324

325

Journal Pre-proof

326 **7. References**

- 327 [1] M. Kolb, M. Vašáková, The natural history of progressive fibrosing interstitial lung  
328 diseases, *Respir. Res.* 20 (2019) 57. <https://doi.org/10.1186/s12931-019-1022-1>.
- 329 [2] M. Wijsenbeek, V. Cottin, Spectrum of Fibrotic Lung Diseases, *N. Engl. J. Med.* 383  
330 (2020) 958–968. <https://doi.org/10.1056/NEJMra2005230>.
- 331 [3] V. Cottin, N.A. Hirani, D.L. Hotchkin, A.M. Nambiar, T. Ogura, M. Otaola, D. Skowasch,  
332 J.S. Park, H.K. Poonyagariyagorn, W. Wuyts, A.U. Wells, Presentation, diagnosis and  
333 clinical course of the spectrum of progressive-fibrosing interstitial lung diseases, *Eur.*  
334 *Respir. Rev. Off. J. Eur. Respir. Soc.* 27 (2018). [https://doi.org/10.1183/16000617.0076-](https://doi.org/10.1183/16000617.0076-2018)  
335 2018.
- 336 [4] V. Cottin, R. Teague, L. Nicholson, S. Langham, M. Baldwin, The Burden of Progressive-  
337 Fibrosing Interstitial Lung Diseases, *Front. Med.* 9 (2022) 799912.  
338 <https://doi.org/10.3389/fmed.2022.799912>.
- 339 [5] V. Cottin, C. Valenzuela, Progressive pulmonary fibrosis: all roads lead to Rome (but not  
340 all at the same speed), *Eur. Respir. J.* 60 (2022). [https://doi.org/10.1183/13993003.01449-](https://doi.org/10.1183/13993003.01449-2022)  
341 2022.
- 342 [6] A. Olson, N. Hartmann, P. Patnaik, L. Wallace, R. Schlenker-Herceg, M. Nasser, L.  
343 Richeldi, A.-M. Hoffmann-Vold, V. Cottin, Estimation of the Prevalence of Progressive  
344 Fibrosing Interstitial Lung Diseases: Systematic Literature Review and Data from a  
345 Physician Survey, *Adv. Ther.* 38 (2021) 854–867. [https://doi.org/10.1007/s12325-020-](https://doi.org/10.1007/s12325-020-01578-6)  
346 01578-6.
- 347 [7] M. Selman, A. Pardo, When things go wrong: Exploring possible mechanisms driving the  
348 progressive fibrosis phenotype in interstitial lung diseases, *Eur. Respir. J.* (2021).  
349 <https://doi.org/10.1183/13993003.04507-2020>.
- 350 [8] K.R. Flaherty, A.U. Wells, V. Cottin, A. Devaraj, S.L.F. Walsh, Y. Inoue, L. Richeldi, M.  
351 Kolb, K. Tetzlaff, S. Stowasser, C. Coeck, E. Clerisme-Beaty, B. Rosenstock, M.  
352 Quaresma, T. Haeufel, R.-G. Goeldner, R. Schlenker-Herceg, K.K. Brown, INBUILD  
353 Trial Investigators, Nintedanib in Progressive Fibrosing Interstitial Lung Diseases, *N.*  
354 *Engl. J. Med.* 381 (2019) 1718–1727. <https://doi.org/10.1056/NEJMoa1908681>.
- 355 [9] A.U. Wells, K.R. Flaherty, K.K. Brown, Y. Inoue, A. Devaraj, L. Richeldi, T. Moua, B.  
356 Crestani, W.A. Wuyts, S. Stowasser, M. Quaresma, R.-G. Goeldner, R. Schlenker-Herceg,  
357 M. Kolb, INBUILD trial investigators, Nintedanib in patients with progressive fibrosing  
358 interstitial lung diseases-subgroup analyses by interstitial lung disease diagnosis in the  
359 INBUILD trial: a randomised, double-blind, placebo-controlled, parallel-group trial,  
360 *Lancet Respir. Med.* 8 (2020) 453–460. [https://doi.org/10.1016/S2213-2600\(20\)30036-9](https://doi.org/10.1016/S2213-2600(20)30036-9).
- 361 [10] L. Wollin, E. Wex, A. Pautsch, G. Schnapp, K.E. Hostettler, S. Stowasser, M. Kolb, Mode  
362 of action of nintedanib in the treatment of idiopathic pulmonary fibrosis, *Eur. Respir. J.*  
363 45 (2015) 1434–1445. <https://doi.org/10.1183/09031936.00174914>.
- 364 [11] S. Marchand-Adam, J. Marchal, M. Cohen, P. Soler, B. Gerard, Y. Castier, G. Lesèche,  
365 D. Valeyre, H. Mal, M. Aubier, M. Dehoux, B. Crestani, Defect of hepatocyte growth  
366 factor secretion by fibroblasts in idiopathic pulmonary fibrosis, *Am. J. Respir. Crit. Care*  
367 *Med.* 168 (2003) 1156–1161. <https://doi.org/10.1164/rccm.200212-1514OC>.
- 368 [12] G. Raghu, M. Remy-Jardin, L. Richeldi, C.C. Thomson, Y. Inoue, T. Johkoh, M. Kreuter,  
369 D.A. Lynch, T.M. Maher, F.J. Martinez, M. Molina-Molina, J.L. Myers, A.G. Nicholson,  
370 C.J. Ryerson, M.E. Streck, L.K. Troy, M. Wijsenbeek, M.J. Mammen, T. Hossain, B.D.  
371 Bissell, D.D. Herman, S.M. Hon, F. Kheir, Y.H. Khor, M. Macrea, K.M. Antoniou, D.  
372 Bourros, I. Buendia-Roldan, F. Caro, B. Crestani, L. Ho, J. Morisset, A.L. Olson, A.  
373 Podolanczuk, V. Poletti, M. Selman, T. Ewing, S. Jones, S.L. Knight, M. Ghazipura, K.C.  
374 Wilson, Idiopathic Pulmonary Fibrosis (an Update) and Progressive Pulmonary Fibrosis

- 375 in Adults: An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline, *Am. J. Respir.*  
 376 *Crit. Care Med.* 205 (2022) e18–e47. <https://doi.org/10.1164/rccm.202202-0399ST>.
- 377 [13] G. Raghu, M. Remy-Jardin, J.L. Myers, L. Richeldi, C.J. Ryerson, D.J. Lederer, J. Behr,  
 378 V. Cottin, S.K. Danoff, F. Morell, K.R. Flaherty, A. Wells, F.J. Martinez, A. Azuma, T.J.  
 379 Bice, D. Bouros, K.K. Brown, H.R. Collard, A. Duggal, L. Galvin, Y. Inoue, R.G. Jenkins,  
 380 T. Johkoh, E.A. Kazerooni, M. Kitaichi, S.L. Knight, G. Mansour, A.G. Nicholson, S.N.J.  
 381 Pipavath, I. Buendía-Roldán, M. Selman, W.D. Travis, S. Walsh, K.C. Wilson, American  
 382 Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin  
 383 American Thoracic Society, Diagnosis of Idiopathic Pulmonary Fibrosis. An Official  
 384 ATS/ERS/JRS/ALAT Clinical Practice Guideline, *Am. J. Respir. Crit. Care Med.* 198  
 385 (2018) e44–e68. <https://doi.org/10.1164/rccm.201807-1255ST>.
- 386 [14] M. Hetzel, M. Bachem, D. Anders, G. Trischler, M. Faehling, Different Effects of Growth  
 387 Factors on Proliferation and Matrix Production of Normal and Fibrotic Human Lung  
 388 Fibroblasts, *Lung.* 183 (2005) 225–237. <https://doi.org/10.1007/s00408-004-2534-z>.
- 389 [15] T.E. King, A. Pardo, M. Selman, Idiopathic pulmonary fibrosis, *Lancet Lond. Engl.* 378  
 390 (2011) 1949–1961. [https://doi.org/10.1016/S0140-6736\(11\)60052-4](https://doi.org/10.1016/S0140-6736(11)60052-4).
- 391 [16] M. Chilosi, A. Zamò, C. Doglioni, D. Reghellin, M. Lestani, L. Montagna, S. Pedron,  
 392 M.G. Ennas, A. Cancellieri, B. Murer, V. Poletti, Migratory marker expression in  
 393 fibroblast foci of idiopathic pulmonary fibrosis, *Respir. Res.* 7 (2006) 95.  
 394 <https://doi.org/10.1186/1465-9921-7-95>.
- 395 [17] K.E. Hostettler, J. Zhong, E. Papakonstantinou, G. Karakiulakis, M. Tamm, P. Seidel, Q.  
 396 Sun, J. Mandal, D. Lardinois, C. Lambers, M. Roth, Anti-fibrotic effects of nintedanib in  
 397 lung fibroblasts derived from patients with idiopathic pulmonary fibrosis, *Respir. Res.* 15  
 398 (2014) 157. <https://doi.org/10.1186/s12931-014-0157-3>.
- 399 [18] S. Rangarajan, A. Kurundkar, D. Kurundkar, K. Bernard, Y.Y. Sanders, Q. Ding, V.B.  
 400 Antony, J. Zhang, J. Zmijewski, V.J. Thannickal, Novel Mechanisms for the Antifibrotic  
 401 Action of Nintedanib, *Am. J. Respir. Cell Mol. Biol.* 54 (2016) 51–59.  
 402 <https://doi.org/10.1165/rcmb.2014-0445OC>.
- 403 [19] L. Wollin, I. Maillet, V. Quesniaux, A. Holweg, B. Ryffel, Antifibrotic and anti-  
 404 inflammatory activity of the tyrosine kinase inhibitor nintedanib in experimental models  
 405 of lung fibrosis, *J. Pharmacol. Exp. Ther.* 349 (2014) 209–220.  
 406 <https://doi.org/10.1124/jpet.113.208223>.
- 407 [20] I. Atanelishvili, T. Akter, A. Noguchi, O. Vuyiv, L. Wollin, R.M. Silver, G.S.  
 408 Bogatkevich, Antifibrotic efficacy of nintedanib in a cellular model of systemic sclerosis-  
 409 associated interstitial lung disease, *Clin. Exp. Rheumatol.* 37 Suppl 119 (2019) 115–124.
- 410 [21] S. Asano, S. Ito, K. Takahashi, K. Furuya, M. Kondo, M. Sokabe, Y. Hasegawa, Matrix  
 411 stiffness regulates migration of human lung fibroblasts, *Physiol. Rep.* 5 (2017) e13281.  
 412 <https://doi.org/10.14814/phy2.13281>.
- 413 [22] C. Hesse, V. Beneke, S. Konzok, C. Diefenbach, J.M. Bülow Sand, S.R. Rønnow, M.A.  
 414 Karsdal, D. Jonigk, K. Sewald, A. Braun, D.J. Leeming, L. Wollin, Nintedanib modulates  
 415 type III collagen turnover in viable precision-cut lung slices from bleomycin-treated rats  
 416 and patients with pulmonary fibrosis, *Respir. Res.* 23 (2022) 201.  
 417 <https://doi.org/10.1186/s12931-022-02116-4>.
- 418 [23] L. Knüppel, Y. Ishikawa, M. Aichler, K. Heinzelmann, R. Hatz, J. Behr, A. Walch, H.P.  
 419 Bächinger, O. Eickelberg, C.A. Staab-Weijnitz, A Novel Antifibrotic Mechanism of  
 420 Nintedanib and Pirfenidone. Inhibition of Collagen Fibril Assembly, *Am. J. Respir. Cell*  
 421 *Mol. Biol.* 57 (2017) 77–90. <https://doi.org/10.1165/rcmb.2016-0217OC>.
- 422 [24] F. Hilberg, G.J. Roth, M. Krssak, S. Kautschitsch, W. Sommergruber, U. Tontsch-Grunt,  
 423 P. Garin-Chesa, G. Bader, A. Zoepfel, J. Quant, A. Heckel, W.J. Rettig, BIBF 1120: triple

- 424 angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy,  
425 *Cancer Res.* 68 (2008) 4774–4782. <https://doi.org/10.1158/0008-5472.CAN-07-6307>.
- 426 [25] F. Hilberg, U. Tontsch-Grunt, A. Baum, A.T. Le, R.C. Doebele, S. Lieb, D. Gianni, T.  
427 Voss, P. Garin-Chesa, C. Haslinger, N. Kraut, Triple Angiokinase Inhibitor Nintedanib  
428 Directly Inhibits Tumor Cell Growth and Induces Tumor Shrinkage via Blocking  
429 Oncogenic Receptor Tyrosine Kinases, *J. Pharmacol. Exp. Ther.* 364 (2018) 494–503.  
430 <https://doi.org/10.1124/jpet.117.244129>.
- 431 [26] K.K. Brown, F.J. Martinez, S.L.F. Walsh, V.J. Thannickal, A. Prasse, R. Schlenker-  
432 Herceg, R.-G. Goeldner, E. Clerisme-Beaty, K. Tetzlaff, V. Cottin, A.U. Wells, The  
433 natural history of progressive fibrosing interstitial lung diseases, *Eur. Respir. J.* 55 (2020).  
434 <https://doi.org/10.1183/13993003.00085-2020>.
- 435 [27] V. Cottin, L. Wollin, A. Fischer, M. Quaresma, S. Stowasser, S. Harari, Fibrosing  
436 interstitial lung diseases: knowns and unknowns, *Eur. Respir. Rev. Off. J. Eur. Respir.*  
437 *Soc.* 28 (2019). <https://doi.org/10.1183/16000617.0100-2018>.
- 438 [28] J. Wang, L. Zhang, L. Luo, P. He, A. Xiong, M. Jiang, Y. Liu, S. Liu, Q. Ran, D. Wu, Y.  
439 Xiong, X. He, G. Li, Characterizing cellular heterogeneity in fibrotic hypersensitivity  
440 pneumonitis by single-cell transcriptional analysis, *Cell Death Discov.* 8 (2022) 38.  
441 <https://doi.org/10.1038/s41420-022-00831-x>.
- 442 [29] J.C. Kamp, L. Neubert, H. Stark, J.B. Hinrichs, C. Boekhoff, A.D. Seidel, F. Ius, A.  
443 Haverich, J. Gottlieb, T. Welte, P. Braubach, F. Laenger, M.M. Hoepfer, M.P. Kuehnel,  
444 D.D. Jonigk, Comparative Analysis of Gene Expression in Fibroblastic Foci in Patients  
445 with Idiopathic Pulmonary Fibrosis and Pulmonary Sarcoidosis, *Cells.* 11 (2022) 664.  
446 <https://doi.org/10.3390/cells11040664>.
- 447 [30] V. Cottin, S. Si-Mohamed, R. Diesler, P. Bonniaud, C. Valenzuela, Pleuroparenchymal  
448 fibroelastosis, *Curr. Opin. Pulm. Med.* 28 (2022) 432–440.  
449 <https://doi.org/10.1097/MCP.0000000000000907>.
- 450 [31] M. Nasser, S. Si-Mohamed, S. Turquier, J. Traclet, K. Ahmad, F. Philit, P. Bonniaud, L.  
451 Chalabreysse, F. Thivolet-Béjui, V. Cottin, Nintedanib in idiopathic and secondary  
452 pleuroparenchymal fibroelastosis, *Orphanet J. Rare Dis.* 16 (2021) 419.  
453 <https://doi.org/10.1186/s13023-021-02043-5>.
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455

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**462 9. Competing interests**

463 SJ has received fees, funding or reimbursement for national and international conferences,  
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465 Astra Zeneca, Bellorophon Therapeutics, Biogen, BMS, Boehringer Ingelheim, Chiesi,  
466 Fibrogen, Galecto Biotech, Genzyme, Gilead, GSK, LVL, Mundipharma, Novartis, Olam  
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469 that they have no competing interests.

470

471

**472 10. Figure Legends****473 Figure 1. Nintedanib blocked the PDGFR signalling pathway in PF-HP, PF-sarcoidosis**  
**474 and PPFE HLFs.**

475 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-0.3  $\mu$ M  
476 nintedanib for 3 h and then stimulated with 25 ng/ml PDGF-BB for 10 min. **A,C,E.**  
477 Representative Western blots showing the phosphorylation of PDGF receptor and ERK. **B, D,**  
478 **F.** Proteins were quantified by densitometry and then normalized to GAPDH. The fold change  
479 in the levels of phosphorylated proteins found in stimulated cells was compared to the levels of  
480 phosphorylated proteins measured in untreated cells, arbitrarily set at 1. Results are expressed  
481 as means  $\pm$  SEM of 4-7 independent experiments. Significant differences were determined by  
482 ANOVA followed by the Dunnett's multiple comparison *t*-test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  
483  $p < 0.001$ ). NTD: nintedanib

**484 Figure 2. Nintedanib blocked the proliferation of PF-HP, PF-sarcoidosis and PPFE HLFs**  
**485 stimulated with PDGF-BB.**

486 PF-HP (n=7) (**A**), PF- sarcoidosis (n=5) (**B**) and PPFE (n=4) (**C**) HLFs were treated with  
487 0.01-0.3  $\mu$ M nintedanib for 3 h and then stimulated with 50 ng/ml PDGF-BB for 72 h. Cell  
488 proliferation was determined by the fluorescent acridine orange/propidium iodide staining and  
489 automated fluorescence cell counting. The relative cell proliferation was calculated by dividing  
490 the number of viable cells treated with nintedanib +/- PDGF-BB by the number of viable  
491 untreated cells, which was arbitrarily set at 1. All results are expressed as means  $\pm$  SEM of 4-7  
492 independent experiments. Significant differences were determined by ANOVA followed by the  
493 Dunnett's multiple comparison *t*-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). NTD: nintedanib

494

**495 Figure 3. Nintedanib decreased the PDGF-BB-induced migration of PF HP, sarcoidosis**  
**496 and PPFE HLFs.**

497 PF-HP (n=7) (A), PF-sarcoidosis (n=5) (B) and PPFE (n=4) (C) HLFs were treated with 0.01-  
498 0.3  $\mu$ M nintedanib for 24 h along with 25 ng/ml PDGF-BB, used as a chemoattractant to induce  
499 cell migration. The relative cell migration was calculated by dividing the number of migrating  
500 cells treated with nintedanib +/- PDGF-BB by the number of untreated migrating cells,  
501 arbitrarily set at 1. Values are expressed as means  $\pm$  SEM of 4-7 independent experiments.  
502 Significant differences were determined by ANOVA followed by the Dunnett's multiple  
503 comparison *t*-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

504 **Figure 4. Nintedanib attenuated the phosphorylation of SMAD2/3 induced by TGF- $\beta$ 1 in**  
505 **PF-HP, PF-sarcoidosis and PPFE HLFs.**

506 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-1  $\mu$ M  
507 nintedanib for 3 h and then stimulated with 1 ng/ml TGF- $\beta$ 1 for 30 min. **A, C, E.** Representative  
508 Western blots showing the phosphorylation of SMAD2 and SMAD3. **B, D, F.** Proteins were  
509 quantified by densitometry and then normalized to the GAPDH. The fold change in the levels  
510 of phosphorylated proteins found in stimulated cells was determined by arbitrarily setting at 1  
511 the levels of phosphorylated proteins measured in untreated cells. Results are expressed as  
512 means  $\pm$  SEM of 4-7 independent experiments. Significant differences were determined by  
513 ANOVA followed by Dunnett's multiple comparison *t*-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*  $p < 0.001$ ).  
514 NTD: nintedanib

515 **Figure 5. Nintedanib decreased the mRNA levels of myofibroblastic markers in PF-HP,**  
516 **PF-sarcoidosis and PPFE HLFs stimulated with TGF- $\beta$ 1.**

517 PF-HP (n=7), PF-sarcoidosis (n=5) and PPFE (n=4) HLFs were treated with 0.01-1  $\mu$ M  
518 nintedanib for 3 h and then stimulated with 1 ng/ml TGF- $\beta$ 1 for 24 h. **A, B, C.** Relative  
519 *FIBRONECTIN*, *COL1A1* and *ACTA2* mRNA levels were measured by quantitative RT-PCR  
520 and normalized to endogenous ribosomal *18S* mRNA levels. Data are expressed relatively to  
521 the mRNA levels founds in unstimulated HLFs, arbitrarily set at 1. Results are expressed as

522 means  $\pm$  SEM of 4-7 independent experiments. Significant differences were determined by  
523 ANOVA followed by Dunnett's Multiple comparison *t*-test (\*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  
524  $p<0.001$ ). NTD: nintedanib

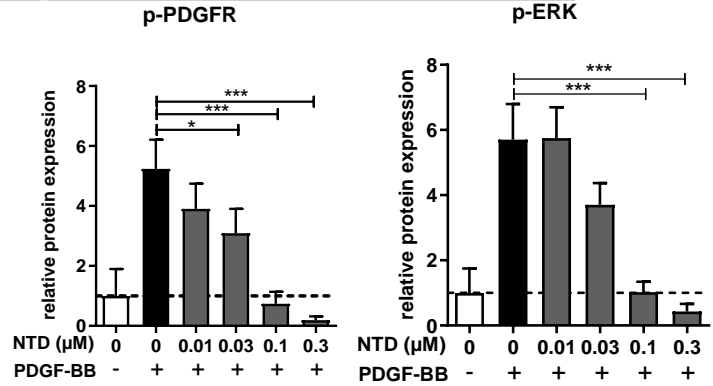
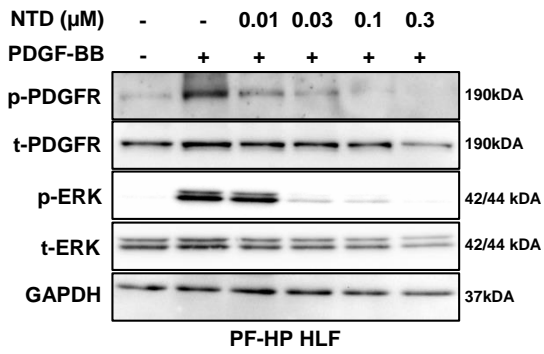
525 **Figure 6. Nintedanib decreased the TGF- $\beta$ 1-induced differentiation of PF-HP, PF-**  
526 **sarcoidosis and PPFE HLFs.**

527 PF-HP (n=7), PF-sarcoidosis (n=6) and PPFE (n=4) HLFs were treated with 0.01-1  $\mu$ M NTD  
528 for 3 h and then stimulated with 1 ng/ml TGF- $\beta$ 1 for 24 h. **A, C, E.** Representative Western  
529 blots of Fibronectin (FN), collagen-1 (COL1) and  $\alpha$ -SMA. **B, D, F.** Protein levels were  
530 quantified by densitometry and normalized to the GAPDH levels. The fold change in protein  
531 expression in stimulated cells was determined by arbitrarily setting at 1 the protein expression  
532 measured in untreated cells. Results are expressed as means  $\pm$  SEM of 4-7 independent  
533 experiments. Significant differences were determined by ANOVA followed by the Dunnett's  
534 multiple comparison *t*-test (\*  $p<0.05$ ; \*\*  $p<0.01$ ). NTD: nintedanib

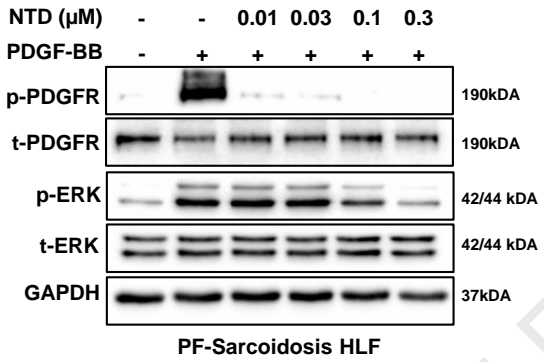


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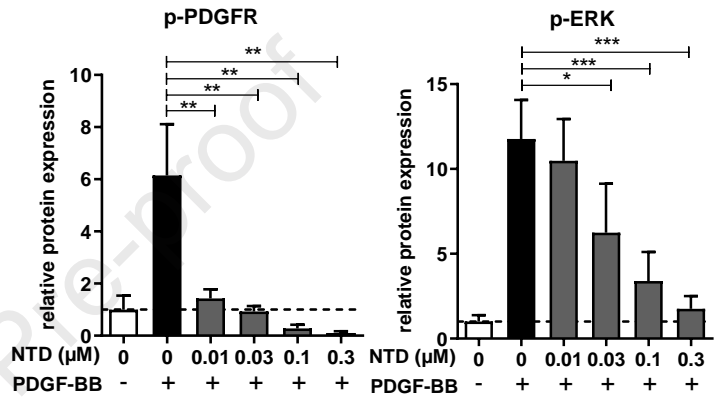
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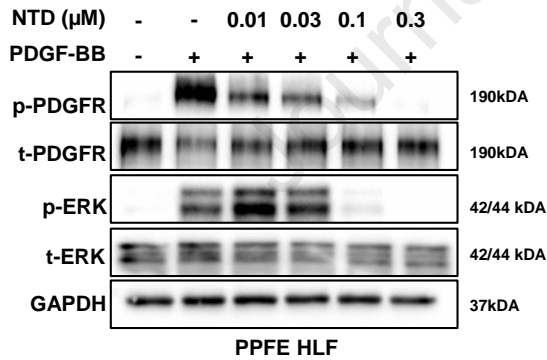
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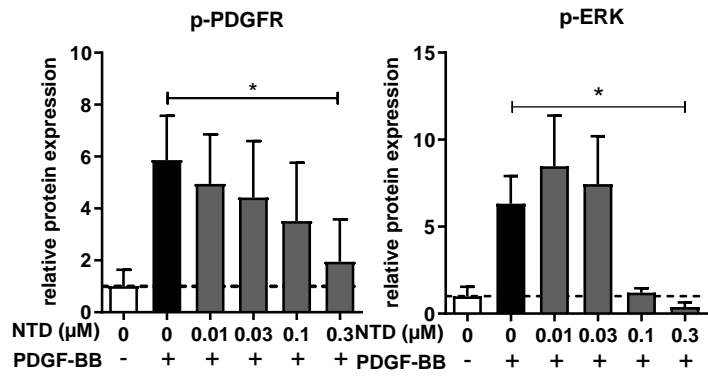
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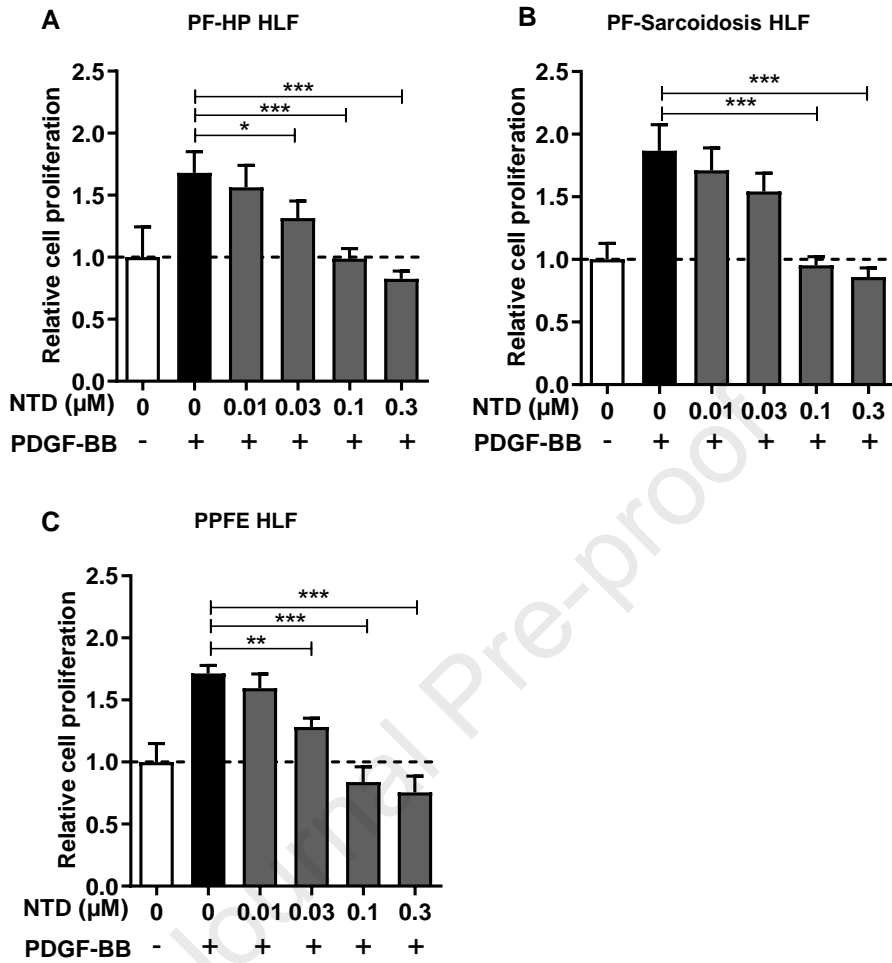


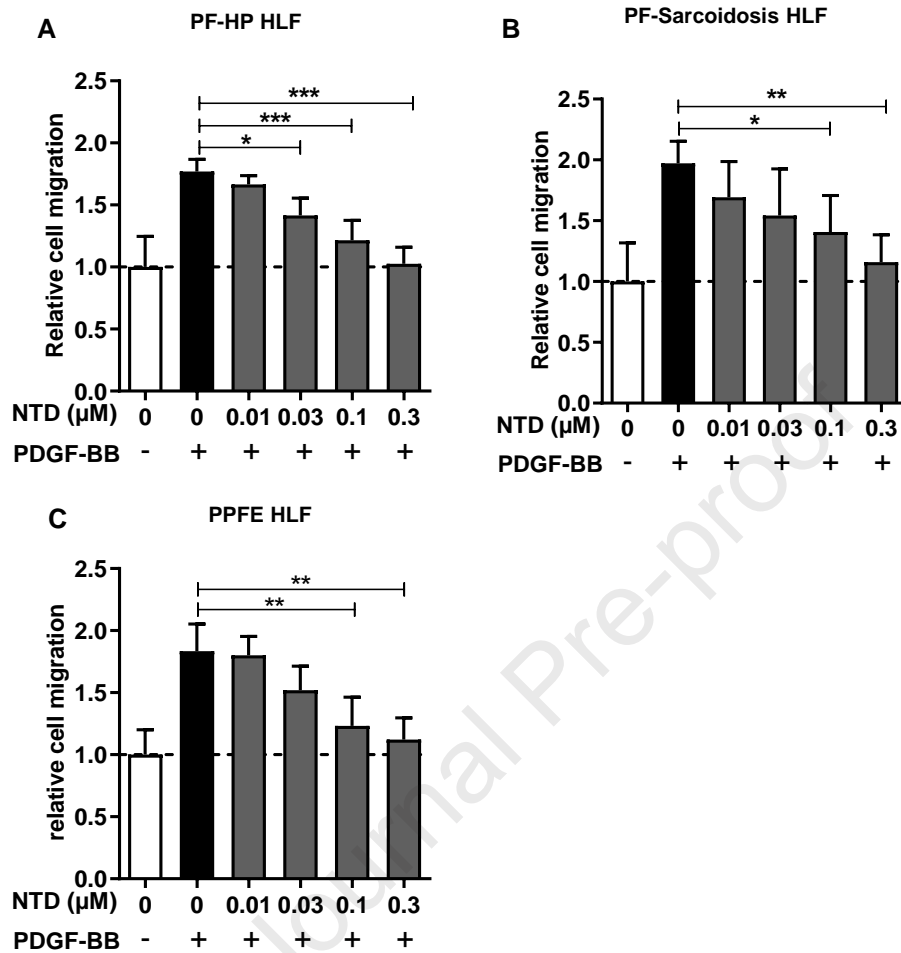
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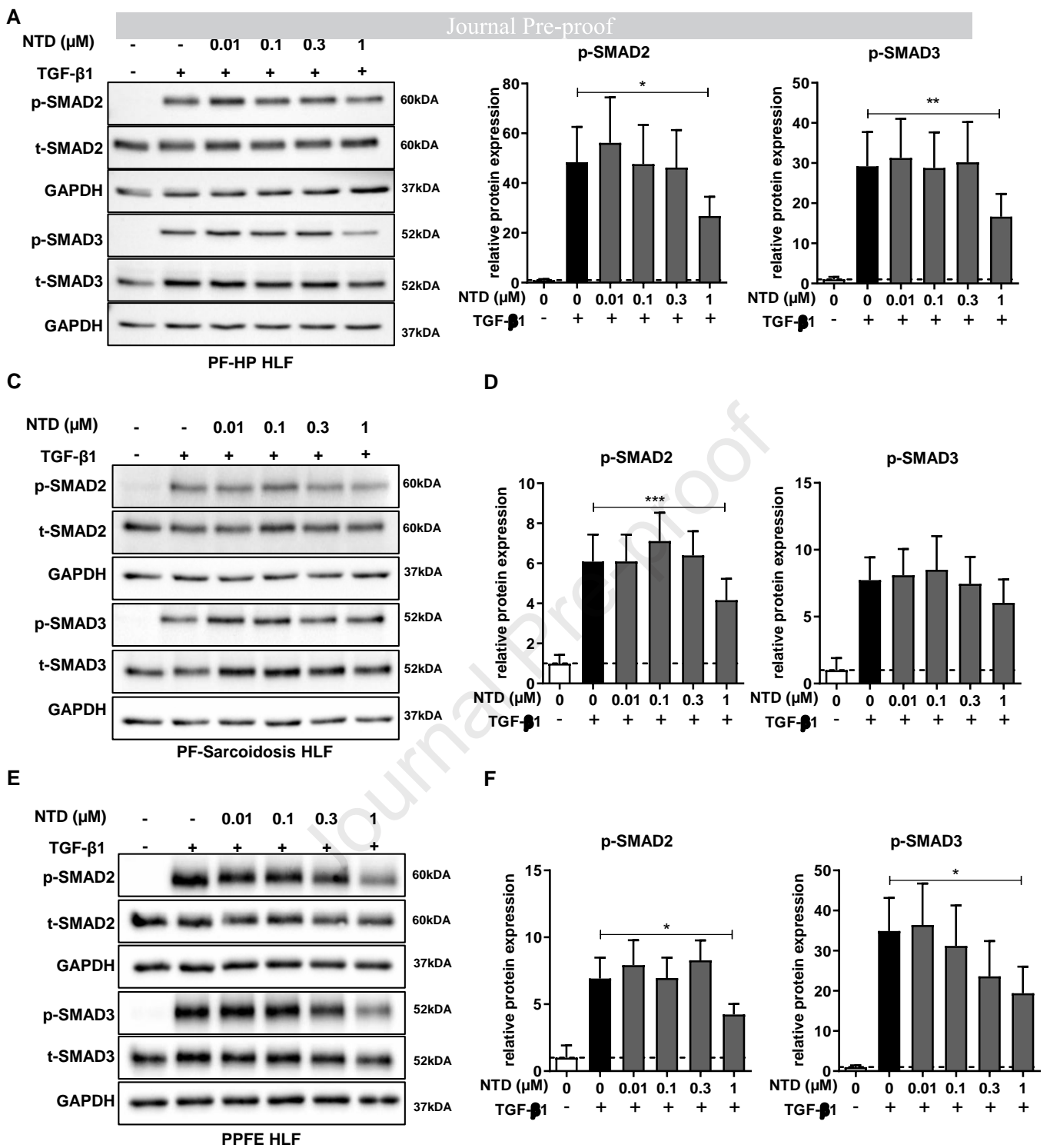


**F**





**Figure 3**

**Figure 4**

**Figure 5**

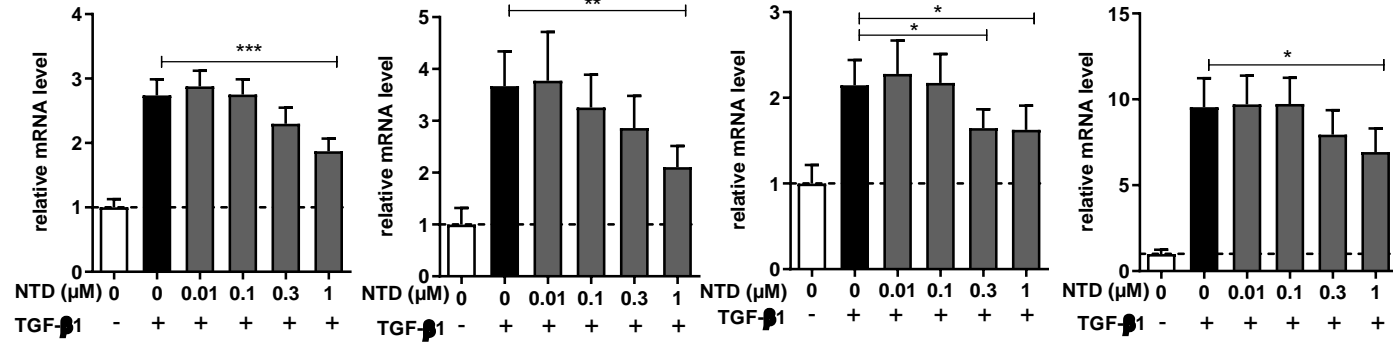
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*COL1A1*

*COL1A2*

*ACTA2*

**A**



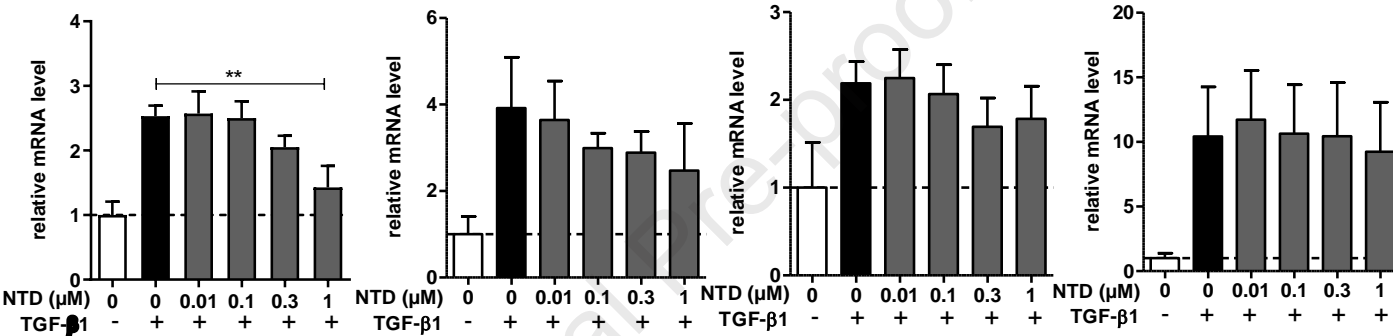
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*FIBRONECTIN*

*COL1A1*

*COL1A2*

*ACTA2*



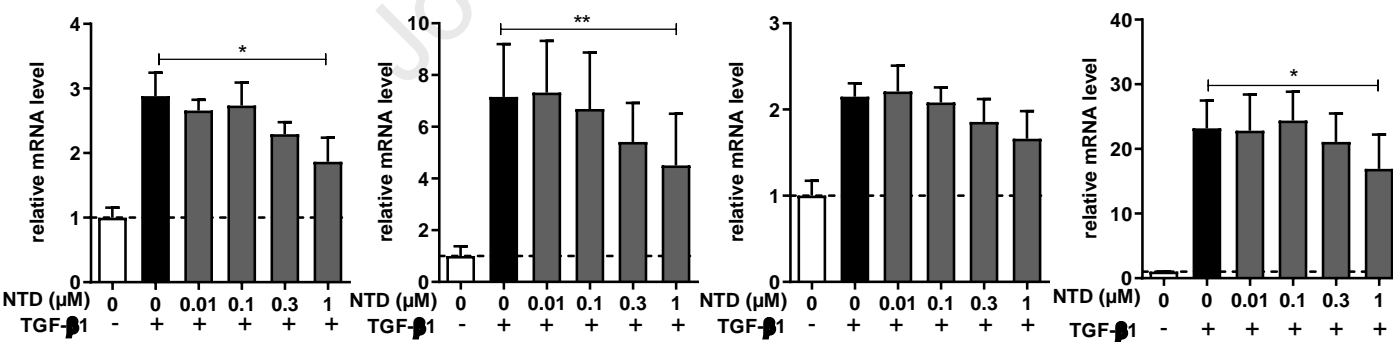
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*COL1A1*

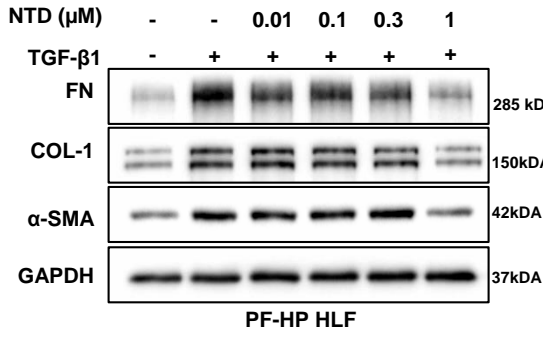
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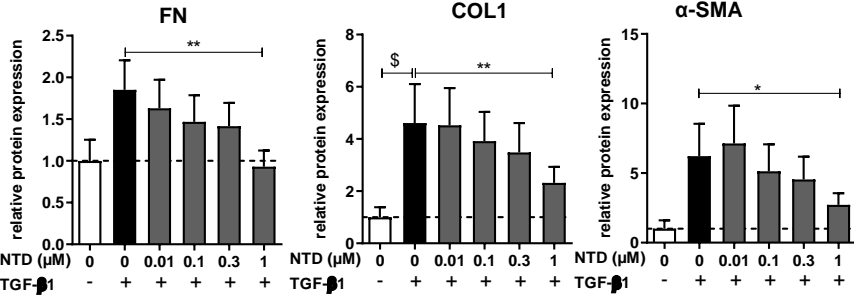


**Figure 6**

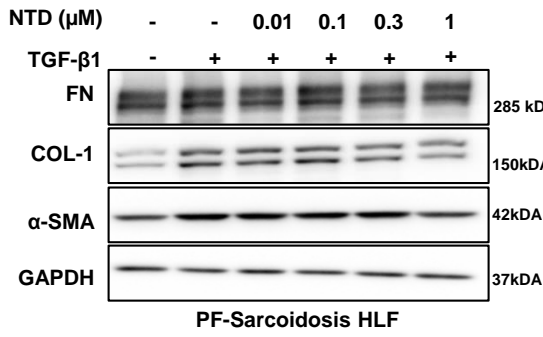
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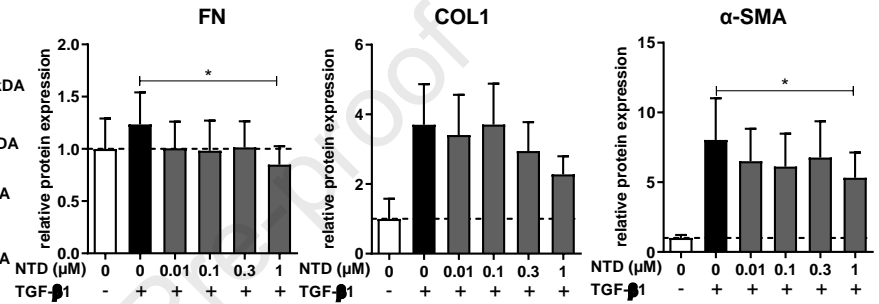
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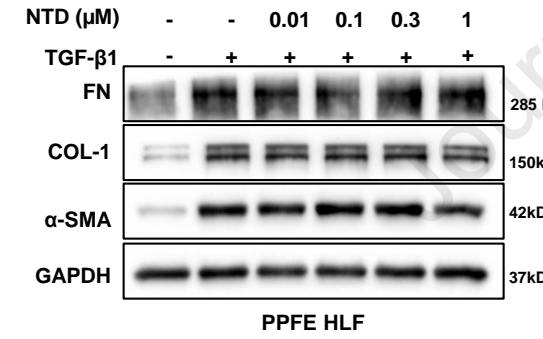
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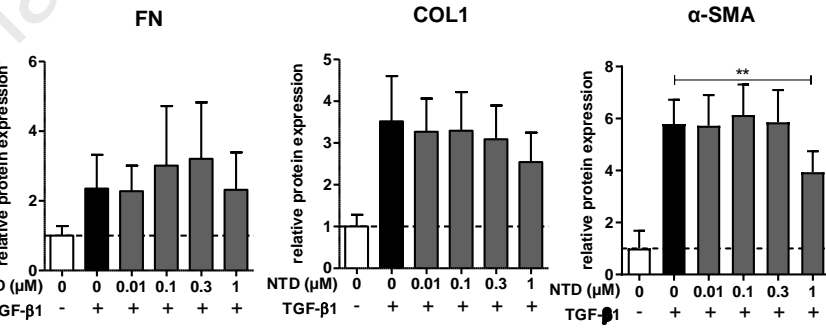
**D**



**E**



**F**



**Authors statement**

*Conceptualization:* AJ, LV, SJ conceived and designed the study.

*Performed data analysis:* AJ, CM, TV performed the experiments. AJ analyzed and interpreted the data.

*Resources:* FLG, DCC, CLN, BDLT, SR provided lung biopsy. SG and AL provide clinical data. MJ and BC provided HLFs.

*Writing - review & editing:* AJ, LV, SJ and LW.

All authors read and approved the final manuscript.

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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