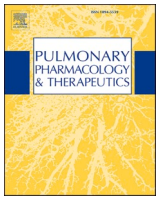
Pulmonary Pharmacology & Therapeutics 83 (2023) 102267

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt



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

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ARTICLE INFO

Keywords 6):
PPF
Primary human lung fibroblasts
Nintedanib
Proliferation
Migration
Differentiation

ABSTRACT

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

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

HLFs were treated with nintedanib (10 nM-1 μM) and then stimulated with PDGF-BB (25–50 ng/ml) or TGF-β1 (1 ng/ml) for 24–72 h to assess proliferation and migration or differentiation.

At nanomolar concentrations, nintedanib reduced the levels of PDGF receptor and ERK1/2 phosphorylation, the proliferation and the migration of PF-HP, PF-sarcoidosis and PPFE HLFs stimulated with PDGF-BB. Moreover, nintedanib also attenuates the myofibroblastic differentiation driven by TGF-β1 but only when it is used at 1 μM. The drug reduced the phosphorylation of SMAD2/3 and decreased the induction of collagen, fibronectin and α-smooth muscle actin expression induced by TGF-β1.

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

Abbreviations: ILD, interstitial lung disease; PF-ILD, Progressive fibrosing-ILD; IPF, idiopathic pulmonary fibrosis; HP, hypersensitivity pneumonitis; PPFE, pleuroparenchymal fibroelastosis; PBS, phosphate-buffered saline; HLFs, human lung fibroblasts; TGF-β1, transforming growth factor-β1; PDGF-BB, platelet-derived growth factor BB; COL1, collagen I; α-SMA, α-smooth muscle actin; SEM, standard error of the mean; ANOVA, one-way analysis of variance.

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https://doi.org/10.1016/j.pupt.2023.102267
Received 5 July 2023; Received in revised form 22 September 2023; Accepted 9 November 2023
Available online 15 November 2023

1. Introduction

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

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

Nintedanib is a tyrosine kinase inhibitor that has been used for several years in the treatment of IPF. Recently, nintedanib has also been approved in EU, US and many other countries for the treatment of patients that develop other PF-ILD [8,9]. The randomized, double-blind placebo-controlled INBUILD® study, which assessed the efficacy and safety of nintedanib, demonstrated that the drug slows the progression of several non-IPF PF-ILDs, established by the annual rate of decline in forced vital capacity (FVC). Nintedanib likely reduces the development of IPF by interfering with key functions of lung fibroblasts. Indeed, *in vitro*, nintedanib inhibits the migration and proliferation of human lung fibroblasts (HLFs) isolated from patients with IPF by competitively blocking the ATP-binding pocket in various tyrosine kinase receptors including the platelet-derived growth factor receptor (PDGFR), the fibroblast growth factor receptor and the vascular endothelial growth factor receptor [10]. However, besides IPF, the effects of nintedanib on the functions of lung fibroblasts isolated from patients suffering from other PF-ILD remain largely unknown.

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

2. Materials and methods

2.1. Primary cultures of HLFs

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

2.2. Cell culture and treatments

HLFs derived from patients with PF-HP (n = 7), PF-sarcoidosis (n = 5) and PPFE (n = 4) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, Life Technologies, Courtaboeuf, France) supplemented with 10 % fetal calf serum (FCS) (Eurobio scientific, Evry, France), antibiotic-antimycotic solution (Gibco™, Life Technologies) and L-glutamine used at passages 5 and 6. Nintedanib was provided by Boehringer Ingelheim Pharma GmbH (Biberach, Germany). To analyze mRNA and protein expressions, HLFs were seeded for 24 h in 6-well plates, and then starved in FCS-free DMEM for 16 h. To study cell growth, HLFs were serum-starved for 16 h, cultured in DMEM containing 1 % FCS and 0.01–0.3 μM nintedanib (NTD) for 3 h, and finally stimulated with PDGF-BB (50 ng/ml) (R&D Systems, Bio-Techne, Lille, France) for a further 72 h. To analyze the TGF-β1-induced myofibroblastic differentiation, serum-starved HLFs were pretreated with nintedanib (0.01–1 μM) for 3 h and then stimulated with TGF-β1 (1 ng/ml; Preprotech, Neuilly-sur-Seine, France) for 24 h.

2.3. Proliferation

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

2.4. Migration assay

HLF migration was assessed in a modified Boyden chamber assay, using a ThinCert® permeable support (6.5 mm, 8 μm) (Greiner Bio-One, Kremsmünster, Austria) coated with fibronectin (0.1 μg/ml) (Sigma-Aldrich, St. Quentin Fallavier, France). The medium in the upper compartment (DMEM-0%FCS) contained 0.01–0.3 μM nintedanib and the medium in the lower compartment contained (DMEM-1%FCS) 25 ng/ml PDGF-BB. Cells were incubated for 16 h at 37 °C, fixed in 70 % ethanol, and labelled with DAPI. Migration was assessed by counting the number of cells on the lower surface of the filters. The relative cell migration was defined by dividing the number of migrating cells stimulated with PDGF-BB by the number of migrating cells left untreated and arbitrarily set at 1.

2.5. Protein analysis

HLFs were lysed in ice-cold RIPA buffer containing cOmplete™ Protease Inhibitor Cocktail (Roche, Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were separated by electrophoresis on 10 % Tris-glycine SDS polyacrylamide gels and transferred to nitrocellulose membranes (pore size: 0.2 μm, Invitrogen). Free binding sites were blocked by incubation in 5 % milk or bovine serum

albumin (BSA) (Sigma-Aldrich, St. Quentin Fallavier, France) for 2 h at room temperature. Membranes were then incubated with the appropriate primary human antibody (Ab) overnight at 4 °C. The primary Abs were anti-α-smooth muscle actin (α-SMA) (clone 1A4, Sigma-Aldrich), anti-collagen I (Southern Biotechnology Associates, Birmingham, AL), anti-GAPDH (mab90009-P, Covalab, Bron, France). Anti-phospho p44/42 MAPK (197G2), anti-p44/42 MAPK (9101), anti-phospho SMAD2 (3104), anti-SMAD2 (D43B4), anti-phospho SMAD3 (C25A9), anti-SMAD3 (C67H9), anti-phospho PDGF receptor (PDGFR) β (3161) and anti-PDGFR (28E1) Abs were from Cell Signaling Technology (Massachusetts, USA). Digital images were acquired on a gel imaging system (Chemi doc, BIO-RAD Laboratories, Marnes-La-Coquette, France) equipped with a CCD camera. Western blots were quantified by densitometry using Image Lab™ software (BIO-RAD) and normalized to GAPDH. The relative protein expression in HLFs was determined by arbitrarily setting at 1 the protein expression measured in untreated cells.

2.6. RNA isolation and RT-PCR assays

Total RNAs were extracted using the mRNA extraction kit from Macherey Nagel (Hoerdt, France) according to the manufacturer's instructions. RNA concentrations were measured by spectrofluorimetry using a NanoDrop 1000 (Thermo Fisher scientific, Saint-Herblain, France). Then mRNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative PCR (polymerase chain reaction) was performed using the SYBR Green methodology on a CFX384 Real-Time PCR System (Bio-Rad Laboratories) in duplicates. All the primers were provided by Sigma-Aldrich (KiCqStart™ SYBR® Green Primers, St. Quentin Fallavier, France). The specificity of gene amplification was checked at the end of PCR using the comparative cycle threshold method (CFX Manager Software). The mean Cq values were used to normalize the target mRNA concentrations to those of the 18S ribosomal protein by the $2^{(-\Delta\Delta Cq)}$ method. The mRNA levels were expressed relatively to those measured in unstimulated HLFs, arbitrarily set at 1.

2.7. Statistical analysis

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

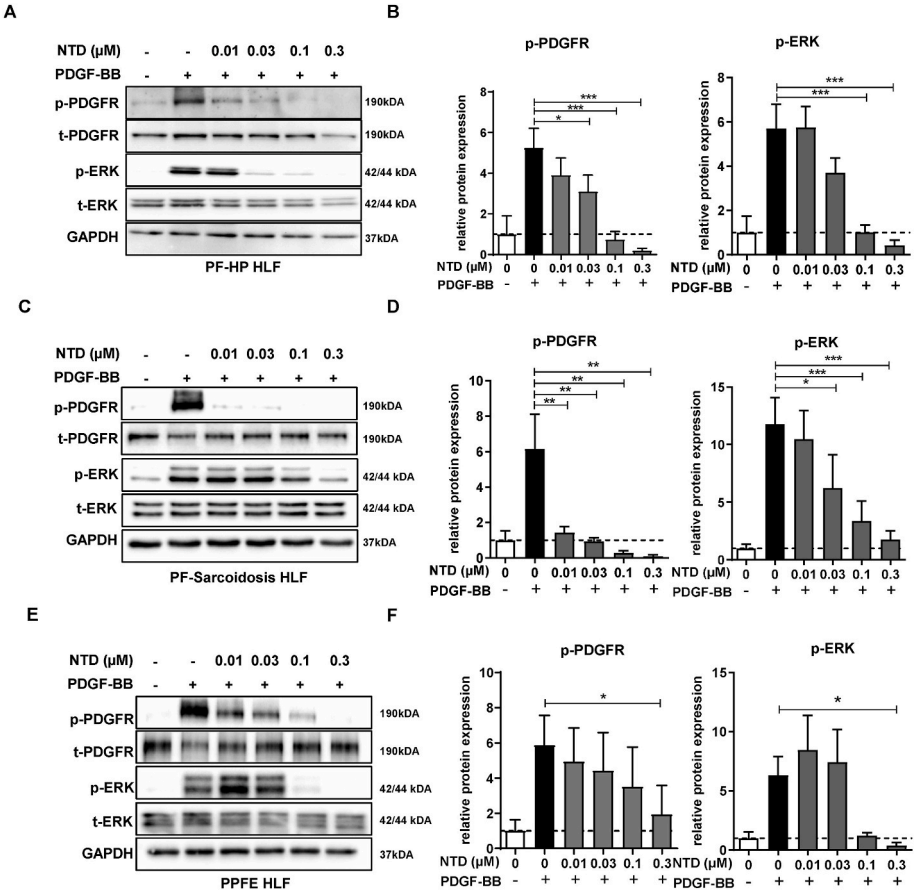

Fig. 1. Nintedanib blocked the PDGFR signalling pathway in PF-HP, PF-sarcoidosis and PPFE HLFs. PF-HP (n = 7), PF-sarcoidosis (n = 5) and PPFE (n = 4) HLFs were treated with 0.01–0.3 μM nintedanib for 3 h and then stimulated with 25 ng/ml PDGF-BB for 10 min. **A,C,E.** Representative Western blots showing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

3. Results

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

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

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

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

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

The migratory cell dynamic is another important function involved in the formation of fibroblastic foci and self-sustaining fibrosis [15,16]. Besides proliferation, PDGF-BB also promotes cell migration through its chemoattractant properties. Fig. 3 shows that PDGF-BB significantly increased the migration of the different PF-ILD HLFs. Nintedanib

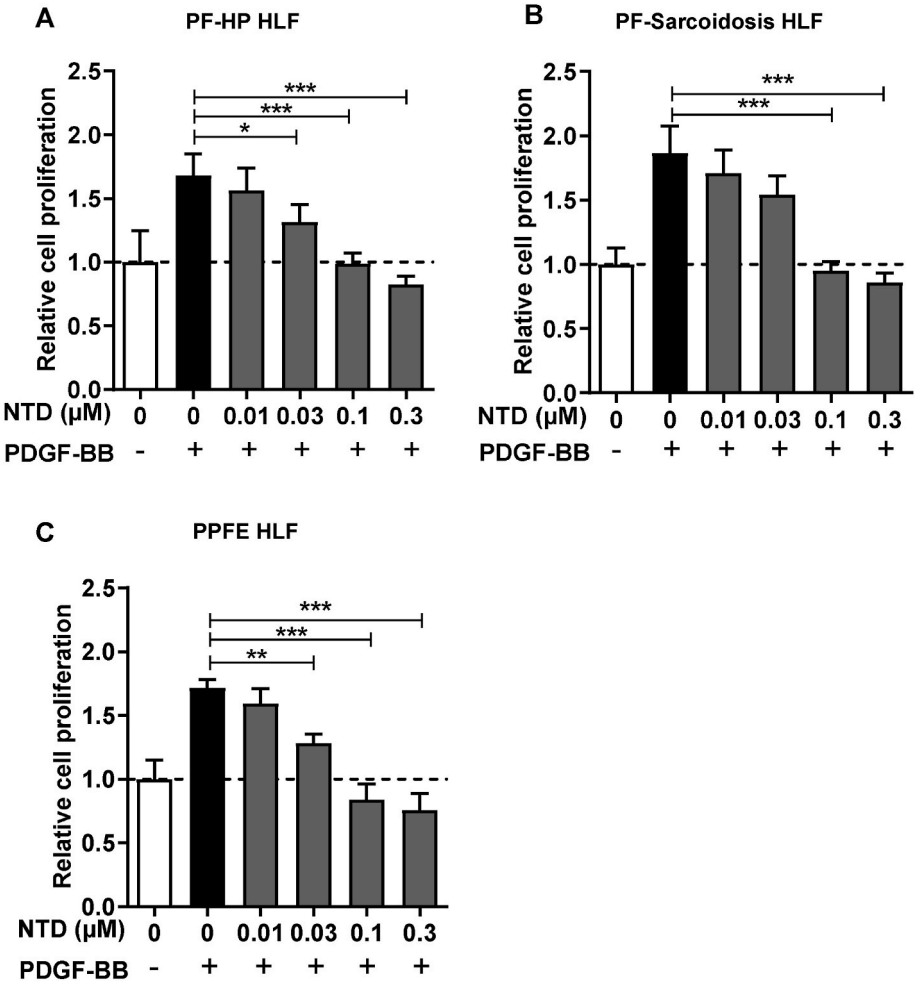

Fig. 2. Nintedanib blocked the proliferation of PF-HP, PF-sarcoidosis and PPFE HLFs stimulated with PDGF-BB. PF-HP (n = 7) **(A)**, PF- sarcoidosis (n = 5) **(B)** and PPFE (n = 4) **(C)** HLFs were treated with 0.01–0.3 μM nintedanib for 3 h and then stimulated with 50 ng/ml PDGF-BB for 72 h. Cell proliferation was determined by the fluorescent acridine orange/propidium iodide staining and automated fluorescence cell counting. The relative cell proliferation was calculated by dividing the number of viable cells treated with nintedanib ± PDGF-BB by the number of viable untreated cells, which was arbitrarily set at 1. All results are expressed as means ± SEM of 4–7 independent experiments. Significant differences were determined by ANOVA followed by the Dunnett's multiple comparison *t*-test (*$p < 0.05$,**$p < 0.01$,***$p < 0.001$). NTD: nintedanib.

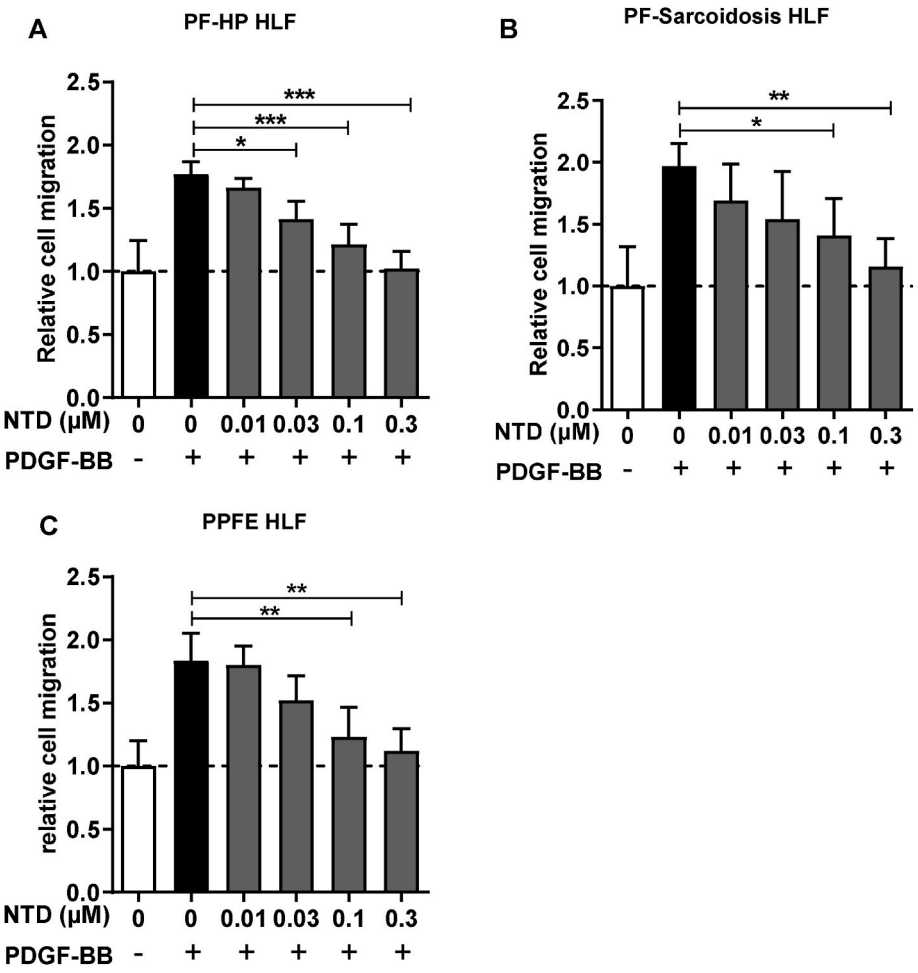

Fig. 3. Nintedanib decreased the PDGF-BB-induced migration of PF HP, sarcoidosis and PPFE HLFs. PF-HP (n = 7) **(A)**, PF-sarcoidosis (n = 5) **(B)** and PPFE (n = 4) **(C)** HLFs were treated with 0.01–0.3 μM nintedanib for 24 h along with 25 ng/ml PDGF-BB, used as a chemoattractant to induce cell migration. The relative cell migration was calculated by dividing the number of migrating cells treated with nintedanib ± PDGF-BB by the number of untreated migrating cells, arbitrarily set at 1. Values are expressed as means ± SEM of 4–7 independent experiments. Significant differences were determined by ANOVA followed by the Dunnett's multiple comparison *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001).

significantly decreased, in a concentration-dependent manner, the migration of PF-HP, PF-sarcoidosis and PPFE stimulated with PDGF-BB. Significant inhibition started at 30 nM and reduction close to baseline was reached at 300 nM. Similarly, the drug strongly reduced the migration of IPF and control HLFs cultured with PDGF-BB (Supplemental Fig. 1 F, H).

3.4. Nintedanib diminished TGF-β1-induced fibroblast to myofibroblast transition of PF-HP, PF-sarcoidosis and PPFE HLFs

The canonical TGF-β signaling pathway, mediated by the signal transducers and transcriptional modulators SMAD2 and SMAD3, drives the production of myogenic markers linked to myofibroblast differentiation. This signaling pathway controls fibrogenesis by regulating the production of ECM and the development of contractile actin stress fibers. In order to study the effects of nintedanib on the fibroblast to myofibroblast transition, PF-HP, PF-sarcoidosis and PPFE HLFs were treated with the drug for 3 h and then stimulated with TGF-β1 for the next 24 h. Fig. 4 shows that TGF-β1 induced the phosphorylation of SMAD2 and SMAD3 proteins in all three PF-ILD HLFs (Fig. 4). The cytokine also strongly increased the production of the myofibroblastic markers fibronectin, collagen-1 (COL1) and α−smooth-muscle actin (α-SMA), at both mRNA (Fig. 5) and protein levels (Fig. 6). In these conditions, nintedanib weakly reduced the phosphorylation of SMAD 2/3 in PF-ILD HLFs. Its effects were only significant when nintedanib was used at its highest concentration (1 μM) (Fig. 4). Similarly, nintedanib poorly limited the expression of the different myofibroblastic markers, at both mRNA and protein levels in non-IPF HLFs (Figs. 5 and 6), as well as in IPF and control HLFs (Supplementary Fig. 2). Nintedanib significantly reduced the *FIBRONECTIN*, *COL1A1* and *ACTA2* mRNA levels in PF-HP and PPFE HLFs, mainly at its highest concentration (1 μM), and it only prevented the *FIBRONECTIN* gene expression in PF-sarcoidosis HLFs. In addition, 1 μM nintedanib prevented the expression of fibronectin, COL1 and α-SMA in PF-HP HLFs (Fig. 6). At this concentration, the drug also significantly reduced α-SMA protein levels in PF-sarcoidosis and PPFE HLFs. However, nintedanib had no significant effect on COL1 levels in these two cell types. As expected, high drug concentrations significantly prevented protein expression of the three myofibroblastic markers in IPF and control HLFs (Supplemental Fig. 2 C-D and G-H).

4. Discussion

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

Several reports have previously shown that nintedanib could

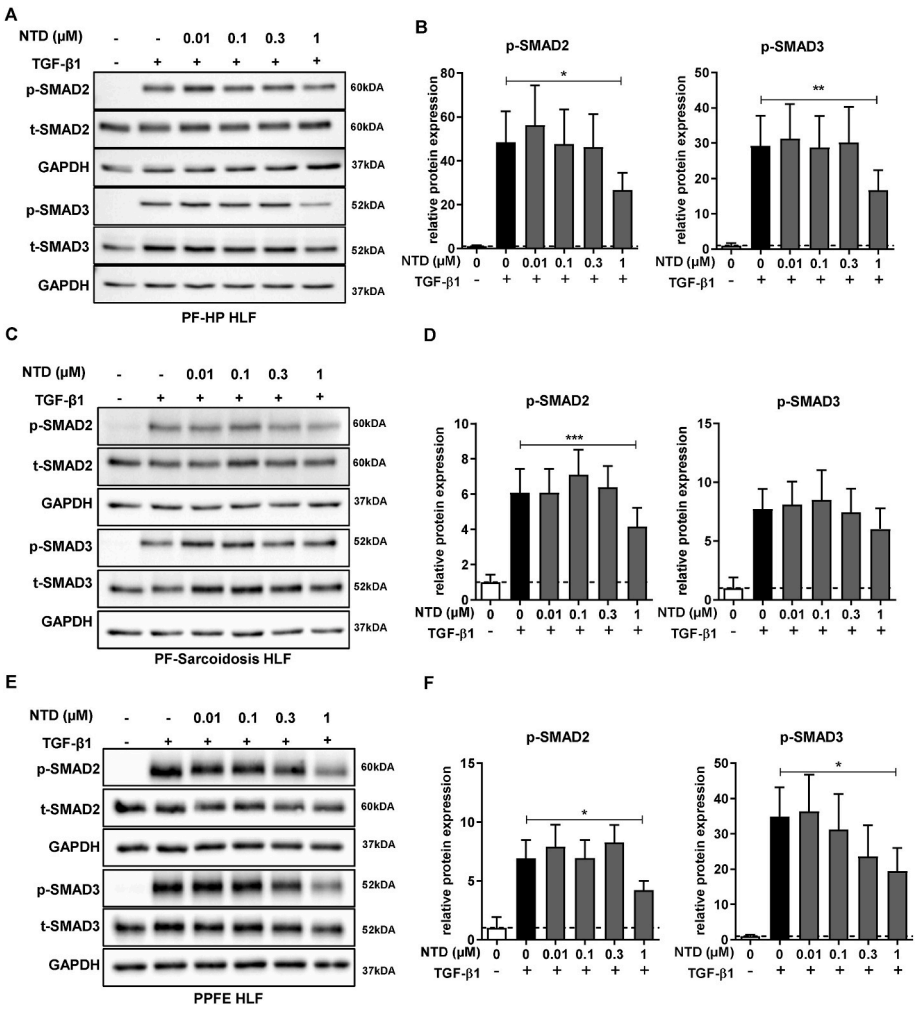

Fig. 4. Nintedanib attenuated the phosphorylation of SMAD2/3 induced by TGF-β1 in PF-HP, PF-sarcoidosis and PPFE HLFs. PF-HP (n = 7), PF-sarcoidosis (n = 5) and PPFE (n = 4) HLFs were treated with 0.01–1 μM nintedanib for 3 h and then stimulated with 1 ng/ml TGF-β1 for 30 min. **A, C, E.** Representative Western blots showing the phosphorylation of SMAD2 and SMAD3. **B, D, F.** Proteins were quantified by densitometry and then normalized to the GAPDH. The fold change in the levels of phosphorylated proteins found in stimulated cells was determined by arbitrarily setting at 1 the levels of phosphorylated proteins measured in untreated cells. Results are expressed as means ± SEM of 4–7 independent experiments. Significant differences were determined by ANOVA followed by Dunnett's multiple comparison *t*-test (*p < 0.05, **p < 0.01, *p < 0.001). NTD: nintedanib.

modulate different signalling pathways and pro-fibrotic functions in HLFs isolated from patients with IPF [17–19] or with systemic sclerosis-associated ILD (SSc-ILD) [20]. The present results show that nintedanib also exerts potent *in vitro* antifibrotic effects on HLFs deriving from patients with three other ILDs that could be associated with a progressive fibrosing phenotype, i.e fibrosing HP, sarcoidosis and PPFE. As described in IPF HLFs, nintedanib strongly inhibited the PDGFR-dependent signaling pathways in the three types of HLFs [19]. Our results suggest that low drug concentrations (30–100 nM) significantly and similarly prevented the proliferation and migration of PF-HP, PF-sarcoidosis and PPFE HLF stimulated with PDGF-BB. Moreover, these concentrations are equivalent to those inhibiting the proliferation and migration of IPF-HLFs [19] and SSc-ILD HLFs [20]. Consequently, nintedanib seems to be equally active to block PDGF effects in IPF and non-IPF HLFs.

In addition, we demonstrated that nintedanib partially blocked the switch of fibroblasts to myofibroblasts induced by TGF-β1 in PF-HP, PF-sarcoidosis and PPFE HLFs. During this step, HLFs gain the expression of contractile and ECM proteins. Specifically, the production of α-SMA allows the contraction of myofibroblasts and contributes to the matrix stiffening [21]. Nintedanib significantly reduced the TGF-β1-induced expression of α-SMA in the three PF-ILD HLFs, but its effects were more potent in PF-HP HLF. In addition, we show that nintedanib specifically reduced COL1 expression in PF-HP HLFs. The production and secretion of collagen is another critical event conferring the stiffness of fibrotic lung tissues. In viable precision-cut lung slices from bleomycin-treated rats and IPF patients, nintedanib modulates type III collagen turnover [22]. In IPF HLFs, nintedanib also inhibited COL1 fibril formation and altered the appearance of collagen fibril bundles [23]. As reported for IPF [17,18] and SSc-ILD HLFs [20], our results clearly show that the concentrations of nintedanib required to block the myofibroblast differentiation are higher than those preventing proliferation and migration of HLFs. Indeed, COL1 and α-SMA expressions were only significantly reduced in HLFs treated with 1 μM nintedanib. The reasons why different ranges of concentrations of nintedanib are necessary to prevent the proliferation and migration of HLFs on the one hand and the differentiation of HLFs on the other hand remain still unclear since the half maximal inhibitory concentrations of nintedanib for the PDGFR and TGF-β1 receptor tyrosine kinases are very similar [24,25].

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

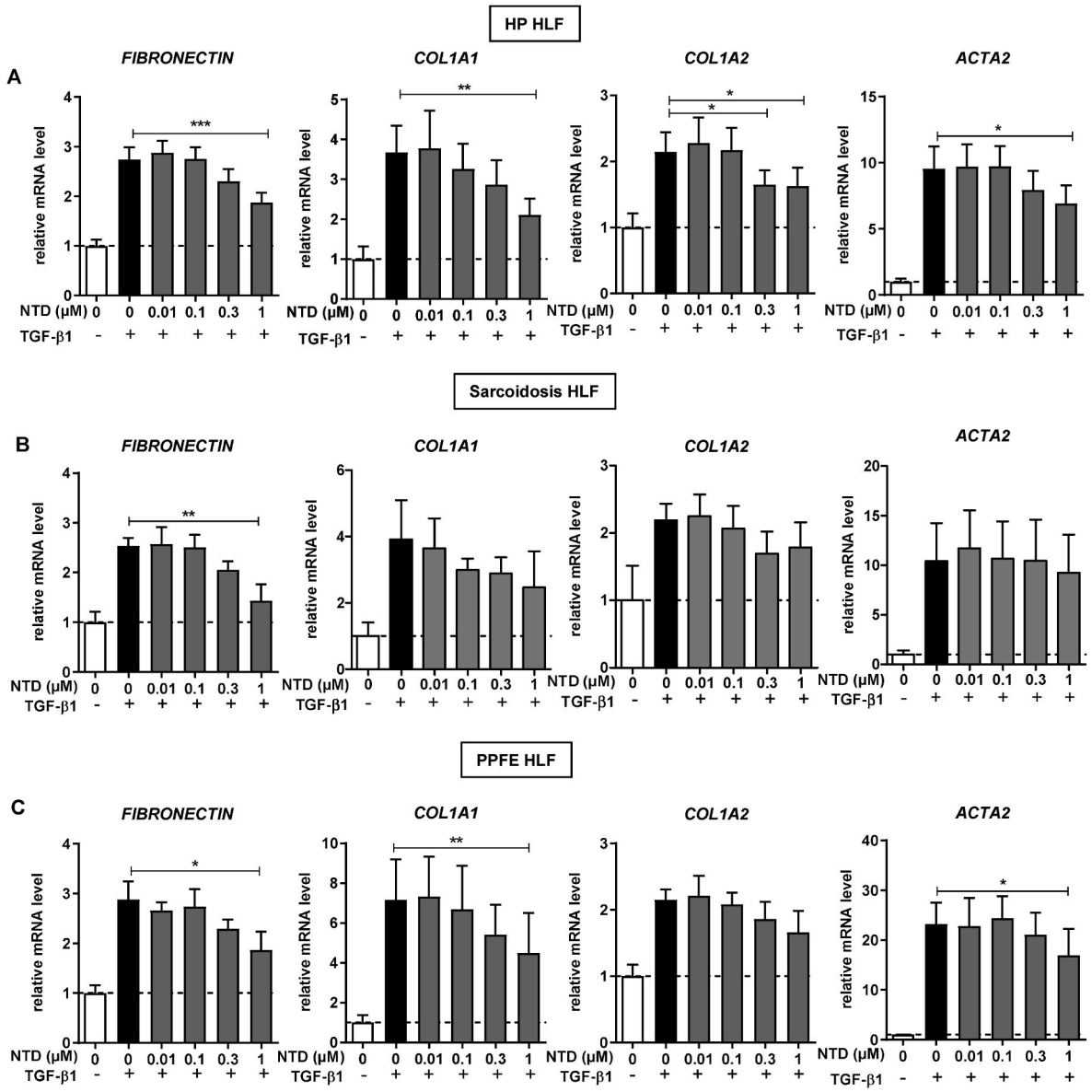

Fig. 5. Nintedanib decreased the mRNA levels of myofibroblastic markers in PF-HP, PF-sarcoidosis and PPFE HLFs stimulated with TGF-β1. PF-HP (n = 7), PF-sarcoidosis (n = 5) and PPFE (n = 4) HLFs were treated with 0.01–1 μM nintedanib for 3 h and then stimulated with 1 ng/ml TGF-β1 for 24 h. **A, B, C.** Relative *FIBRONECTIN*, *COL1A1* and *ACTA2* mRNA levels were measured by quantitative RT-PCR and normalized to endogenous ribosomal *18S* mRNA levels. Data are expressed relatively to the mRNA levels founds in unstimulated HLFs, arbitrarily set at 1. Results are expressed as means ± SEM of 4–7 independent experiments. Significant differences were determined by ANOVA followed by Dunnett's Multiple comparison *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001). NTD: nintedanib.

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

5. Conclusion

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

Funding

This work was supported by The Institut National de la Santé et de la Recherche Médicale (INSERM), the Université de Rennes (Univ Rennes) and Boehringer Ingelheim (BI). BI had no role in the design, analysis or interpretation of the results in this study. BI was given the opportunity to review the manuscript for medical and scientific accuracy as it relates to BI substances, as well as intellectual property considerations.

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

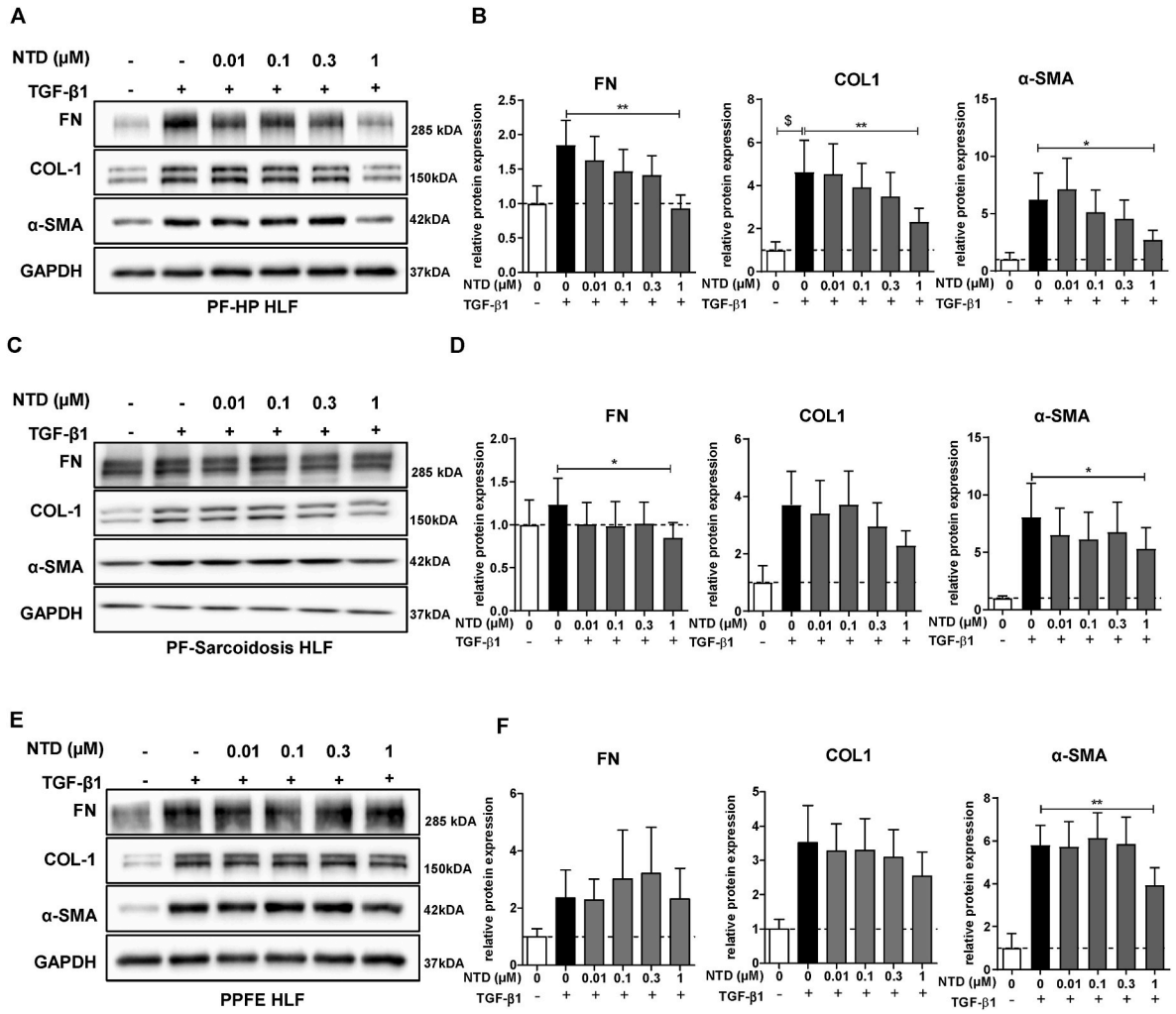

Fig. 6. Nintedanib decreased the TGF-β1-induced differentiation of PF-HP, PF-sarcoidosis and PPFE HLFs. PF-HP (n = 7), PF-sarcoidosis (n = 6) and PPFE (n = 4) HLFs were treated with 0.01–1 μM NTD for 3 h and then stimulated with 1 ng/ml TGF-β1 for 24 h. **A, C, E.** Representative Western blots of Fibronectin (FN), collagen-1 (COL1) and α-SMA. **B, D, F**. Protein levels were quantified by densitometry and normalized to the GAPDH levels. The fold change in protein expression in stimulated cells was determined by arbitrarily setting at 1 the protein expression measured in untreated cells. Results are expressed as means ± SEM of 4–7 independent experiments. Significant differences were determined by ANOVA followed by the Dunnett's multiple comparison *t*-test (*p < 0.05; **p < 0.01). NTD: nintedanib.

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.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Laurent Vernhet reports financial support was provided by Boehringer Ingelheim Pharma GmbH & Co KG Biberach.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pupt.2023.102267.

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