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RESEARCH ARTICLE



Transcriptomic analysis reveals lipid metabolism and macrophage involvement associated with nintedanib treatment in a rat bleomycin model

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Abstract

Background and Purpose: Idiopathic pulmonary fibrosis is a progressive lung disease with high clinical need. In this context, nintedanib significantly reduces acute exacerbation risk, however its comprehensive effect on fibrosis remains unclear. This study aims to evaluate nintedanib transcriptomic impact on lung fibrosis to uncover molecular signatures for new therapeutics.

Experimental Approach: Lung fibrosis was induced by two intratracheal bleomycin administrations. Nintedanib was administered orally daily for 3 weeks. Histological and transcriptome data were obtained using automated fibrosis quantification, Ashcroft Score and RNA sequencing, respectively. Correlation network analysis (WGCNA) modules were identified and examined by cell and pathway enrichment analysis. Lipid peroxidation was tested using malondialdehyde.

Key Results: Nintedanib reduced fibrotic lesions by ~15%, decreased severe Ashcroft scores. WGCNA identified two clusters correlated with histological parameters, where nintedanib counter-regulates gene expression. One cluster was associated with mesenchymal cells (fibroblast and smooth muscle; $-\log p\text{-val} > 4.45$), energy production, cellular metabolism and extracellular matrix-related pathways ($-\log q\text{-val} > 11$). The second cluster, enriched predominantly in resident macrophages ($-\log p\text{-val} > 12.36$), was related to lysosomal activity and lipid metabolism ($-\log q\text{-val} > 21$). Nintedanib inhibited bleomycin elevated malondialdehyde levels.

Conclusions and Implications: The study highlighted two distinct protective effects of nintedanib on lung fibrosis. One well-known on mesenchymal cells and matrix-related components. Plus, one related to the involvement of the pneumocyte-macrophage paracrine lipid axis, where oxidized lipids alter macrophage activities,

Abbreviations: BLM, group name, bleomycin-treated animals without nintedanib; IPF, idiopathic pulmonary fibrosis; MDA, malondialdehyde; ME, module eigengene; NINT, group name, bleomycin-treated animals with nintedanib; SAL, group name, control group; WGCNA, weighted correlation network analysis.

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leading to the formation of lipid-filled macrophages. This may open new research directions.

KEYWORDS

bleomycin, fibrosis, idiopathic pulmonary fibrosis (IPF), lipid, macrophages, metabolism, nintedanib

1 | INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a deadly interstitial lung disease with unclear pathogenic clues, characterized by damaged lung parenchyma architecture, progressive restrictive-ventilatory limitation, hypoxia, dyspnoea and cough (Spagnolo et al., 2018). Its development is believed to be influenced by both genetic and environmental factors, and leads to its distinctive histopathologic and radiologic pattern defined usual interstitial pneumonia (Raghu et al., 2011). Its prevalence varies widely due to different factors, with recent estimates indicating an incidence of 0.09–1.30 and a prevalence of 0.33–4.51 per 10,000 individuals (Maher et al., 2021). However, significant geographic gaps in the epidemiology, diagnosis and study methodologies of IPF, coupled with limited pharmacological options, which only slow disease progression, underscore the high and urgent medical need (Maher et al., 2021; Spagnolo et al., 2018). Indeed, the only two approved drugs for IPF, **pirfenidone** and **nintedanib**, cannot completely cure or halt the disease, and their side effects, which can be very severe in some cases, may become more threatening than the disease itself, leading to treatment discontinuation (Chianese et al., 2024).

Nintedanib is a small molecule that acts at the intracellular level and inhibits a variety of multiple tyrosine kinase receptors, with the following being the most significantly impacted: **vascular endothelial growth factor receptor (VEGFR)**, **platelet-derived growth factor receptor (PDGFR)** and **fibroblast growth factor receptor (FGFR)** (Raghu et al., 2015). Nintedanib gained approval for treating IPF thanks to its efficacy in reducing acute exacerbations, minimizing the risk of disease progression and slowing the decline of forced vital capacity in patients with lung fibrosis (Saito et al., 2019; Spagnolo et al., 2018). Like other tyrosine kinase inhibitors, it plays a regulatory role in essential processes such as differentiation, growth, metabolism and apoptosis (Grimminger et al., 2010). Furthermore, it is proposed that various co-ordinated canonical and not-canonical mechanisms which regulate the profibrotic response of key effector cells in fibrosis, may contribute to the clinical benefits of this drug in IPF (Rangarajan et al., 2016).

Animal models that resemble pulmonary fibrosis could contribute to the discovery of novel potential therapeutic approaches for complex lung diseases, even though there is no adequate and relevant pre-clinical model recapitulating IPF (Jenkins et al., 2017). However, it is widely accepted and pointed out in guidelines that the bleomycin (blm) murine model stands out as the best-characterized and extensively studied IPF animal model for preclinical testing of active

What is already known

- Nintedanib exhibits effectiveness in enhancing idiopathic pulmonary fibrosis and diminishing lung fibrosis in animal models.
- Nintedanib interferes with key processes in lung fibrosis, including fibroblast-to-myofibroblast transition and extracellular matrix deposition.

What does this study add

- Nintedanib exhibits varying degrees of counter-regulation on different gene clusters impacted by bleomycin-induced lung fibrosis.
- One of these identified gene clusters is associated with macrophages activity and lipid metabolism.

What is the clinical significance

- Further investigation may uncover therapeutic strategies targeting lipid metabolism in fibrotic lung diseases.
- Nintedanib's impact on lipid metabolism and macrophage activity suggests a novel, therapeutically exploitable mechanism.

compounds on IPF (Jenkins et al., 2017; Tashiro et al., 2017). Transcriptome profiling and advanced molecular biology techniques, such as single-cell omics, offered critical insights into cellular dysfunctions in complex pathologies, and recent successes in applying these methods to lung fibrosis models and IPF-related experiments provided valuable translational perspectives (Adams et al., 2020; Bonatti et al., 2023; Morse et al., 2019; Peng et al., 2013; Sheu et al., 2019).

In IPF and in fibrotic models, nintedanib is reported to interfere with a number of biological processes critical for the disease development and progression, including the fibroblast-to-myofibroblast transition and the excessive production and deposition of extracellular matrix components such as collagen (Spagnolo et al., 2018). Particularly, its effect on fibroblasts is widely documented (Rangarajan et al., 2016) and Shue et al. reported a well-defined set of significantly dysregulated genes associated with nintedanib treatment in IPF

fibroblasts (Sheu et al., 2019). Nevertheless, the understanding of the full spectrum of nintedanib effects remains incomplete. It is acknowledged that nonreceptor tyrosine kinases, as well as unidentified targets and cellular processes, may play pivotal roles in mediating antifibrotic effects *in vivo* (Rangarajan et al., 2016; Wollin et al., 2014). In this study, we present the first comprehensive transcriptome and histomorphometric analysis of a rat double-hit bleomycin model of lung fibrosis treated with nintedanib. By applying network-based cluster analysis, we aim to identify gene expression patterns that reveal diverse molecular effects of nintedanib on lung fibrosis. This approach seeks to uncover mechanisms underlying nintedanib's benefits beyond its broad-spectrum effects, potentially leading to the identification of new targetable therapeutic pathways.

2 | METHODS

2.1 | Experimental design

All the procedures involving animals were reviewed, approved and authorized by (1), the Italian Public Health System for Animal Health and Food Safety within the Italian Ministry of Health (authorization number 246/2021-PR) and by (2), the Chiesi Body for the Protection of Animals committee. Experiments were also performed in full compliance with the European ethics standards in conformity with directive 2010/63/EU, Italian D. Lgs 26/2014, the revised 'Guide for the Care and Use of Laboratory Animal' (Guide for the Care and Use of Laboratory Animals, 1996) and are outlined in agreement with the ARRIVE guidelines (Percie du Sert et al., 2020) and the recommendations provided by the British Journal of Pharmacology (Lilley et al., 2020). Moreover, the animal study of this paper is conformed to National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals. NIH publication No. 86-23. Revised 1985. U.S. Government Printing Office, Washington, D.C. 20402-9325).

Male Sprague–Dawley rats, weighing 200–250 g at arrival (6–8 weeks of age), were supplied by Charles River Italia, Lecco (Italy). Before any use, animals followed an acclimatization period of at least 5 days. No prophylactic or therapeutic treatment was administered to any animals during the acclimatization period. Animals were housed two/three per cage and lighting in the holding room was controlled (light 7.00–19.00, dark 19.00–7.00). The animals (Charles River, Italy) were intra-tracheally injected with bleomycin ($1 \text{ U} \cdot \text{kg}^{-1} \cdot 0.5 \text{ ml}^{-1}$) at day 0 and after 4 days under *sevoflurane* (4% in oxygen) anaesthesia, using a Penn Century Microsprayer (Penn-Century Inc., Philadelphia, USA) to induce pulmonary fibrosis. Following the experimental procedure, animals were returned to their cages and monitored for recovery. In case of delayed anaesthesia recovery or signs of distress, they were placed on a heating pad. The same procedure was performed on control animals using a saline solution. More information regarding the model was published before in Bonatti et al. (2023). Seven days after the first bleomycin dose, the animals were randomly divided into two groups, one of which received nintedanib $100 \text{ mg} \cdot \text{kg}^{-1}$ (Group name: NINT) and the other received its vehicle (methylcellulose 0.5%

in water; Group name: - BLM). Nintedanib and the vehicle were administered daily by oral gavage (per os, PO) for 3 weeks. During the weeks of treatment, the animals treated with saline received the vehicle only (Group name: SAL). A graphical representation of the experimental design and of the different groups is reported in Figure 1a. Throughout the entire duration of the experiment, including the acclimatization period and oral treatment phase, animals had *ad libitum* access to controlled tap water and a standard certified laboratory rat diet (VRF1, Special Diets Service, UK, supplied to Charles River Italia). Moreover, body weight was monitored twice weekly over the course of the experiment and the trends are shown in Figure 1b. Each of the three groups contained six animals. All the animals were anaesthetized with thiopental ($200 \text{ mg} \cdot \text{kg}^{-1} \cdot 10 \text{ ml}^{-1}$) and sacrificed by exsanguination 28 days after the first bleomycin induction. Following completion of the experiment each sample was assigned a code and all subsequent analyses were conducted accordingly to ensure blinded conditions.

2.2 | Histopathology

Left lung lobes were formalin-fixed and paraffin-embedded to obtain longitudinal $5 \mu\text{m}$ thick Masson trichrome-stained sections for histopathological examination. The presence of lipid-laden alveolar macrophages as pathological findings was evaluated in each group. The sections were scanned with NanoZoomer S60 (Hamamatsu Photonics K.K., Shizuoka, Japan) and imported into the Visiopharm Integrator System (VIS; version 2017.2.4.3387) for automated analysis of lung fibrosis using a VIS Analysis Protocol Package). The sections were graded based on the Ashcroft scale (grades 0–8), as described by Ashcroft et al. (1988) and modified by Hubner et al. (2008).

2.3 | Molecular experimental methods, data analysis and statistical procedures

2.3.1 | RNA extraction and sequencing

Four animals per group were randomly selected from the main experiment (details in "Experimental design") before being killed, as described above, for transcriptomic analysis. Fresh right lungs of each animal were rinsed and immediately collected in RNeasy Lysis Reagent (Qiagen, Germany) and immediately collected in RNeasy Lysis Reagent (Sigma, USA) for RNA preservation, and then they were homogenized in QIAzol lysis reagent (QIAGEN, Netherlands) with gentleMACS Dissociator (Miltenyi Biotec, Germany). RNA was extracted from the lung homogenates using the QIAcube robotic workstation with the miRNeasy Mini Kit with an in-column DNase digestion protocol (QIAGEN, Netherlands). The RNA quality was measured using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). The QuantSeq FWD (Lexogen) kit was used to prepare libraries, which were then sequenced on an Illumina NextSeq500 platform, producing at least 20 million reads/sample (75 bp Single End). The dataset is available with accession number GSE278200. Reads were aligned to

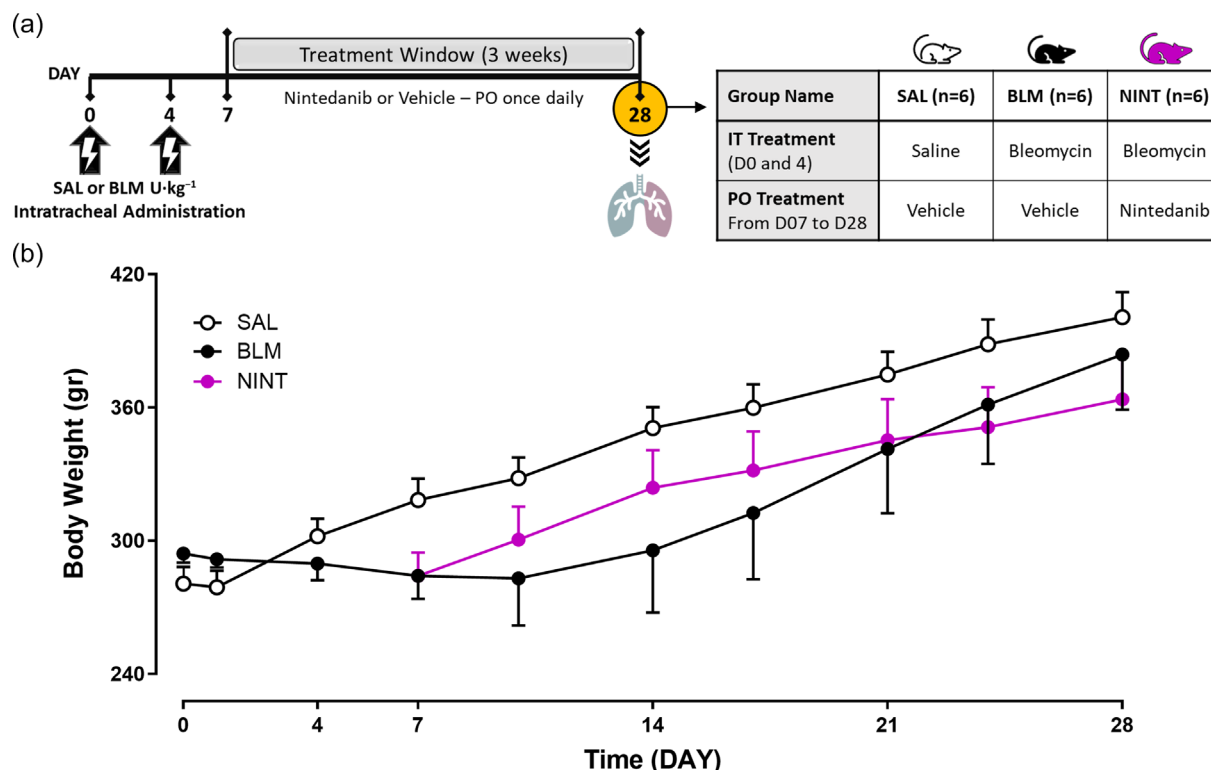


FIGURE 1 Overview of the rat model. (a) Graphical description of the rat model and the experimental set-up, indicating the workflow and timeline of the different interventions. (b) The body weight trend of the animals during the experiment. For each group of treatment, the values are expressed as a mean $\pm$ SEM. Bleomycin-treated animals without nintedanib (BLM) and bleomycin-treated animals with nintedanib (NINT) growth curves were separated after animal randomization on day 7.

the *Rattus norvegicus* genome (v. 6) using STAR (Dobin et al., 2013) and counts were calculated using the HTSeq Python package (Anders et al., 2015) and Ensembl annotation (v. 101). ClustVis (Metsalu & Vilo, 2015) was used to perform Principal Component Analysis on the normalized read counts and the heatmaps were generated using the Morpheus software (developed by the Broad Institute, <https://software.broadinstitute.org/morpheus>)

2.3.2 | Transcriptomic analysis and associated statistical methods

Differentially Expressed Genes (DEGs) were identified with the Limma-voom tool (Ritchie et al., 2015) using SAL as control group. Only genes with a minimum of 10 counts in at least three samples were considered. Modules of co-expressed genes were identified using the 'WeiGhted Correlation Network Analysis (WGCNA)' package in R (Langfelder & Horvath, 2008). WGCNA was conducted on all 12 samples treated as independent observations, as this unsupervised method analyses gene co-expression patterns across the full dataset without group-based stratification. Only protein coding genes with at least 10 counts in all the samples and 50 counts in eight out of 12 samples were used for module construction. Modules were identified using the blockwise-modules function set at default, except for the following parameters: soft thresholding power =

9, networkType = 'signed', corType = 'pearson', TOMType = 'signed', minimum module size = 30, mergeCutHeight = 0.25, minKMEtoStay = 0.5. The correlation between gene expression module profiles and phenotypic traits (Ashcroft score and % of Fibrosis) was evaluated using Pearson correlation. Metascape was used to identify significantly enriched pathways and processes in modules (Zhou et al., 2019). Enrichment of cell-type specific signatures within modules was assessed through gene set overrepresentation analysis. Specifically, an enrichment score was calculated that reflects the degree to which cell type marker genes are present more than would be expected (overrepresented) among the genes assigned to a WGCNA module. The score was calculated by the ratio of the number of marker genes found in a WGCNA module divided by the total number of genes assigned to that module, to the total number of genes in the marker gene set divided by the total number of genes in the rat genome. The statistical significance of the enrichment score was estimated using Fisher's exact test. We considered significantly enriched gene sets with an enrichment score ≥ 4 and a Fisher's exact P value ≤ 0.001 . Cell-type specific markers were obtained from Mouse Cell Atlas (Fei et al., 2022; Han et al., 2018; Wang et al., 2023), from Morse et al. (Morse et al., 2019) and from Hong et al (Hong et al., 2021). Only genes with module membership ≥ 0.8 were retained in each module for this analysis. We used the same analysis to evaluate the enrichment of specific signatures from our previous work (Bonatti et al., 2023). Specifically, we compared the module-specific signature

of our previous time course investigation with our current study. Once again, only genes with module membership ≥ 0.8 were retained in each module for this analysis. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2007) was employed to identify the enrichment for 'nintedanib-sensitive' gene sets within the WGCNA modules. These gene sets originated from a series of relevant publications that analysed the gene expression effects of nintedanib in different types of interstitial lung disease *in vitro* and *in vivo* models (Mikami et al., 2022; Roach et al., 2021; Sheu et al., 2019; Wilson et al., 2022).

2.3.3 | Assessment of lipid peroxidation by malondialdehyde (MDA)

For biological validation of lipid peroxidation, parallel groups of animals, derived from an independent experiment conducted in a different experimental session were used. These animals, subjected to the same treatments and procedures of the main experiment described in "Experimental design" were included for the MDA analysis on lung homogenates subdivided in SAL ($n = 6$), bleomycin ($n = 9$) and NINT ($n = 7$). Frozen right lobes were homogenized in 10 ml of ice-cold 1X PBS per g of tissue, with gentleMACS™ Dissociator. Lung homogenate was frozen/thawed three times and centrifuged (5000 g for 10 minutes) to obtain a supernatant cell lysate. The amount of MDA was determined with the Lipid Peroxidation Colorimetric Assay Kit (Abcam AB233471, UK) according to the manufacturer's protocol.

2.3.4 | Sample size, graphs and statistical analysis

Data and statistical analyses complied with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2025). The number of animals assigned to each group in the main experiment is shown in Figure 1a. The number of animals of the parallel independent experiment used to analyse lipid peroxidation by malondialdehyde are reported in Figure 5. Based on our expertise with the model, a group size of six/eight animals is sufficient to detect statistically significant increases in fibrosis parameters through histological assessments using non-parametric tests. This was previously demonstrated in our publication describing the model (Bonatti et al., 2023). For RNA-seq analysis, four animals per group were randomly selected from the original cohort. Although this sample size was chosen considering published RNA-seq guidelines, which indicate that even a small number of replicates can provide sufficient statistical power when effect sizes are large and sequencing depth is adequate (Conesa et al., 2016) and is further supported by our prior bleomycin model experiment (Bonatti et al., 2023). However, it does not comply with the recommendations by the British Journal of Pharmacology (Curtis et al., 2025). Accordingly, the results of the DEG analysis, which was based considering four animals per group, should be interpreted as exploratory and reflective of overall gene expression trends. WGCNA,

along with downstream analyses based on its results, was performed on all 12 samples treated as independent observations. This unsupervised method analyzes gene co-expression patterns across the full dataset without group stratification. Venn-diagrams were performed using Venny 2.0 (Oliveros, 2007). Histograms and statistical analysis were performed with Graphpad Prism v. 10.1.0 (GraphPad Software, San Diego, California, USA). The statistical analysis of histological examination and MDA measurement was conducted using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. All the statistical analysis related to the transcriptomic data are outlined in the dedicated method section 'Transcriptomic data analysis and associated statistical methods'.

2.4 | Materials

Bleomycin sulfate was obtained from Baxter Oncology GmbH (Halle, Germany), nintedanib free base was obtained from Carbosynth (Compton, UK), while pentothal sodium from MSD Animal Health Srl (Segrate, Italy). Methylcellulose was obtained from Sigma-Aldrich, USA (400 cP) and PBS from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). All solutions were prepared following the manufacturer instructions.

Details of other materials and suppliers are provided in specific sections.

2.5 | Nomenclature of targets and ligands

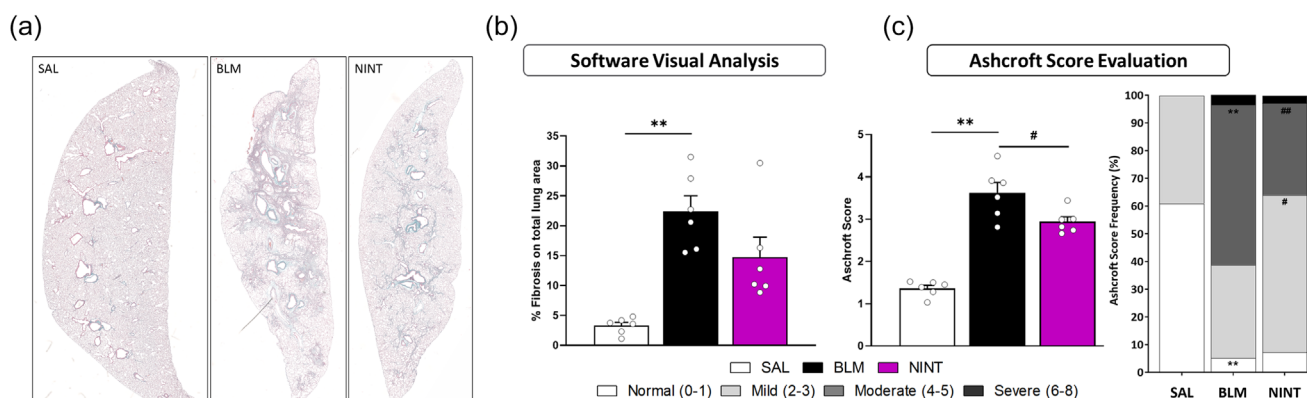
Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2025 (Alexander, Fabbro, et al., 2025; Alexander, Gibb et al., 2025).

3 | RESULTS

3.1 | Therapeutic effect of nintedanib on bleomycin-induced lung fibrosis

Bleomycin administration caused initial weight loss, reaching a minimum around day 7. Thereafter, bleomycin-treated animals gradually regained weight. Nintedanib-treated rats recovered weight more rapidly during the first week, but from day 21 onward their weight gain slowed and their average weight fell below that of the bleomycin-only group. Qualitative evaluation at day 28 showed that bleomycin treatment resulted in lung fibrosis, marked by dense collagen agglomerates that replaced normal lung parenchyma. Nintedanib-treated lungs exhibited a reduction in terms of density of fibrotic area and collagen deposition (Figure 2a). Automated quantification registered approximately 25% fibrotic tissue coverage in the BLM group, while in nintedanib-treated animals showed only around 15% (Figure 2b). The

Left lung lobe - HISTOLOGICAL ANALYSIS



Right lung lobes - GENE EXPRESSION ANALYSIS

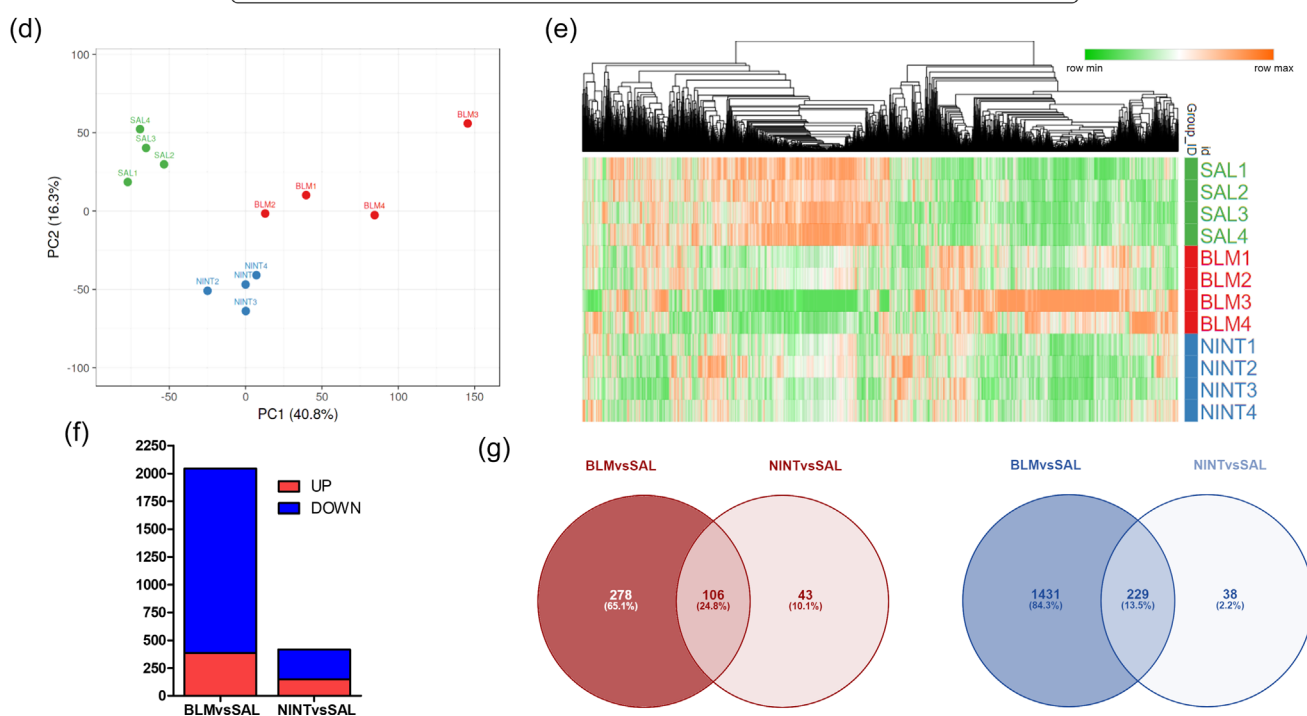


FIGURE 2 Comprehensive overview of the key findings. (a) Representative Masson's trichrome-stained lung tissue sections at the end of the experiment, 28 days after the first bleomycin instillation. (b) Quantification of pulmonary fibrosis using the automated software visual analysis and (c) Ashcroft score evaluation, expressed as mean values or percentages of the frequency across different score ranges. ** $P \leq 0.01$ versus SAL, ## $P \leq 0.01$ versus BLM and # $P \leq 0.05$ versus BLM. The histological evaluations were performed on all the animals in the experiment ($n = 18$). (d) Graphical representation of the sample variability using the principal component analysis. The normalized counts of all genes were utilized for this purpose. (e) The heatmap displays the normalized counts of all animals that performed transcriptome analysis. Only genes that exhibited a trend of de-regulation in at least one of the comparisons were chosen for the heatmap. The columns were hierarchically clustered using a Euclidean distance metric. (f) Bar graphs depicting the number of genes that showed a trend toward up- or down- in the two groups, bleomycin-treated animals without nintedanib (BLM) and bleomycin-treated animals with nintedanib (NINT), in comparison to the control group (SAL). (g) Venn-diagrams showing genes that exhibited a trend toward up- or down-regulation among the different groups, presented in red and blue, respectively.

Ashcroft score analysis revealed a significant median 19% reduction compared with the BLM group (Figure 2c). Moreover, the frequency of moderate (Chianese et al., 2024; Raghu et al., 2015) scores decreased in favour of mild ones (Maher et al., 2021; Raghu et al., 2011). As expected, the BLM group showed high variability,

with certain animals displaying more severe and widespread lesions than others. Nevertheless, despite these notable disparities, the variability did not impact the statistical significance of histologic parameters. The mean values of these parameters were comparable to other studies on a similar rat model.

3.2 | Bleomycin induced the production of lipid-laden alveolar macrophages

The majority of the analysed sections from the animals treated with bleomycin exhibited clusters of larger and atypical alveolar macrophages with a foamy appearance resembling lipid-laden alveolar macrophages, prevalently localized in areas of thickened tissue. These structures are absent in the severely injured fibrotic regions where the alveoli are no longer visible. A detail of these histologic findings is reported in Figure 5a. These structures are not present in any of the control animals.

3.3 | Overall impact of nintedanib on bleomycin-altered lung transcriptome

The principal component analysis performed on the RNAseq data revealed a clear separation among the analysed groups (Figure 2d). The NINT group (light blue), like the controls (SAL, green), showed less intra-group variability than the BLM group (red).

This intra-group variability of BLM is also evident in the transcriptome profiles (Figure 2e). In detail, compared with control group, BLM1

and BLM2 exhibit relatively moderate alterations in contrast to BLM3 and BLM4 that showed substantial variations. The number of genes with trends toward up- and down-regulation ranged from 416 (NINT vs. SAL) to 2044 (BLM vs. SAL) (Figure 2f). The number of genes exhibiting a down-regulation trend significantly exceeded those showing an up-regulation trend, as previously observed for this model at this time point (Bonatti et al., 2023). The number of genes with trends toward up- and down-regulation affected by bleomycin but unaffected by nintedanib ranged respectively from 106 to 229 (Figure 2G). In contrast, genes exhibiting trends toward up- and down-regulation modified by bleomycin alone ranged respectively from 278 to 1431. Additionally, the number of genes with trends indicating responsiveness to nintedanib independently from bleomycin ranged respectively from 43 to 38.

3.4 | Identification of specific gene expression signatures associated with nintedanib effect

Based on the observed gene expression trends indicating a nintedanib effect, we employed a network-based approach to identify distinct transcriptional profiles affected by nintedanib. The WGCNA identified

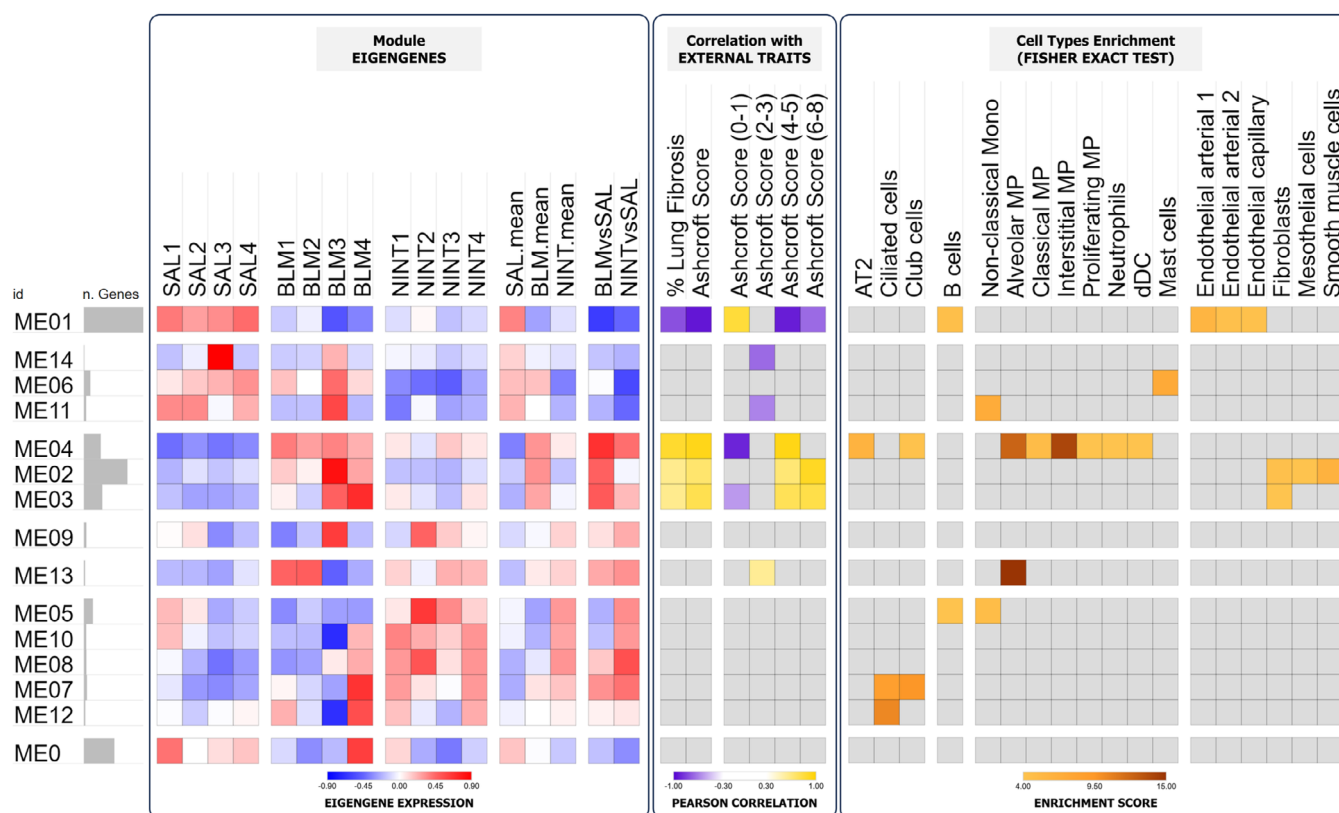


FIGURE 3 Gene modules identified using signed weighted correlation network analysis (WGCNA). Representative heatmaps of each WGCNA module. The first heatmap (Module EIGENGENES) is coloured based on the module eigengenes obtained for each animal. Additionally, the mean of each group and the ratio of the mean values for the bleomycin-treated animals without nintedanib (BLM) and bleomycin-treated animals with nintedanib (NINT) groups against the control group (BLM-NINT vs. SAL) are reported. The second heatmap (Correlation with EXTERNAL TRAITS) depicts Pearson correlations obtained during WGCNA analysis with the indicated external traits. The third heatmap (Cell Types Enrichment) displays the enrichment of cell-type-specific gene lists for genes with module membership ≥ 0.8 (cell-types are grouped based on the lineage, i.e., epithelial, lymphoid, myeloid and stromal). In these last two heatmaps, non-statistically significant values are represented in grey.

14 modules of co-regulated genes. All the modules with their correspondent module eigengenes (MEs, representative of the expression profile), correlation with lung fibrosis and specific cell types are graphically reported in Figure 3. The first four detected modules accounted for 81.25% of all genes, with Module 1 alone accounting for roughly half of them.

ME01 is characterized by elevated expression in the control group and reduced levels in BLM and NINT groups, with variable down-regulation in BLM. ME02 exhibited increased expression in all BLM animals, with notable intra-group variation, particularly in BLM3, while NINT expression levels were low and comparable to those of the SAL group. In ME03, a similar pattern emerged, although NINT animals appeared closer to BLM1 and BLM2 than the SAL group in terms of expression levels. ME04 was unique, with uniform expression across the entire BLM group, higher than those in the SAL group, and an intermediate expression profile of the NINT group, positioned between SAL and BLM. The first four MEs significantly correlated, with varied intensities and orientations, with the values of the Ashcroft Score and percentage of fibrotic tissue (Figure 3).

The enrichment analysis of cell-type-specific signatures unveiled the specific cell types associated with the main modules of interest (Figure 3). Module 1 was enriched in vascular endothelial and B cells (score = 4.93 and 4.68, respectively), while Module 2 showed a significant enrichment for genes linked to cell types crucial for the fibrotic process, such as smooth muscle cells and fibroblasts (score = 6.31 and

4.45). Module 3 also showed a slight association with fibroblast signature (score = 4.05). Interestingly, genes within Module 4 were predominantly enriched for those associated with various types of resident macrophages (score = 12.36 for alveolar macrophages and score = 14 for interstitial macrophages) and alveolar type 2 cells (alveolar type 2; score = 6.14), as well as other immune-inflammatory cells (dendritic cells and neutrophils, scores = 4.16 and 4.12, respectively).

3.5 | Pathway analysis of the weighted correlation network analysis (WGCNA) modules

The pathway analysis revealed numerous signalling pathways and biological processes enriched with high statistical significance (Figure 4), the complete list is reported in supplementary material (Table S1). Module 1 was enriched in pathways related to gene regulatory processes, with very high statistically significant enrichment scores (q-val. $<10^{-30}$) for terms such as chromatin organization, mRNA metabolic processes, histone binding and transcription coregulator activity, with the highest enrichment for chromatin activities (q-val. $<10^{-61}$). This module reached very high statistical significance (q-val. $<10^{-25}$) also for terms related to cytoskeletal dynamics, such as cell projection assembly, microtubule cytoskeleton organization and the RHO GTPases cycle.

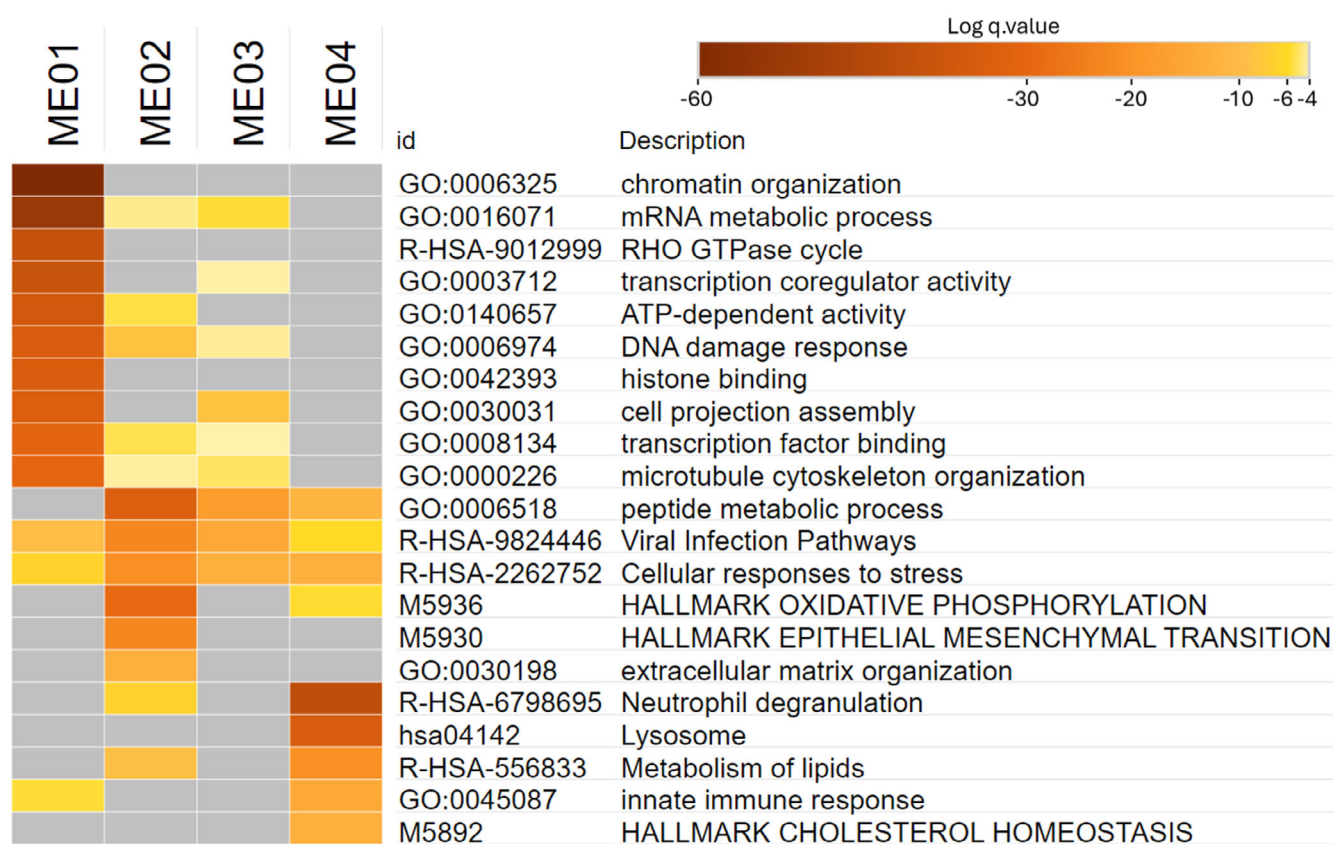


FIGURE 4 Pathway enrichment analysis of WGCNA modules. The heatmap displays Log q values derived from pathway analysis conducted using Metascape. The most noteworthy pathways and terms were selected from the original comprehensive list, starting with the most significant ones. These selections are either uniquely associated with a specific module or commonly enriched among a group of modules.

Modules 2, 3 and 4 displayed significant similarities regarding enriched pathways. These common pathways highlighted processes and mechanisms related to cellular and viral translational control, ranging from ribosomal subunit formation and activity to protein synthesis and modification processes, including peptide metabolic and biosynthetic processes, translation and amide biosynthesis. Some of these terms were also enriched in Module 1. The common line of Modules 2, 3 and 4 focused on events related to translation and protein synthesis, whereas the one shared with Module 1 delved into the dominance of mRNA metabolism and associated cellular regulation.

In addition to these common pathways, Modules 2 and 4 had uniquely enriched terms. Module 2 was enriched for pathways related to cellular respiration and energy metabolism, such as oxidative phosphorylation and respiratory electron transport of mitochondria. Moreover, there was a strong enrichment for pathways linked to the fibrotic process, like epithelial-mesenchymal transition and extracellular matrix organization. Module 4 was enriched in mechanisms related to the innate immune response, with very high statistical significance ($q\text{-val.} < 10^{-30}$) for neutrophil degranulation and lysosomal activity, and pathways governing lipid metabolism, including cholesterol homeostasis and lipid biosynthesis, alongside processes like monocarboxylic acid metabolism and adipogenesis.

The results related to these two modules are summarized in the graphical abstract.

3.6 | Comparison with other relevant studies

A gene set overrepresentation analysis provided statistically significant enrichment scores for Modules 1, 2 and 4 when compared with our previous time course study (Bonatti et al., 2023), where we investigated the temporal fluctuations in gene expression during the progression of lung fibrosis in the same rat model. Specifically, Module

1 is enriched in genes belonging to the late response phase, while Modules 2 and 4 are enriched in genes belonging to the 'long-lasting' one. A complete list is reported in supplementary materials (Table S2).

With the gene set enrichment analysis, we obtained a statistically significant enrichment score for an *in vitro* experiment published by Sheu et al. (Sheu et al., 2019), where gene expression changes associated with nintedanib treatment in IPF fibroblasts were assessed. Specifically, the statistical significance was achieved by comparing Module 2 with the list of down-regulated genes after nintedanib treatment. The associated gene set enrichment analysis leading-edge analysis provided a list of 40 genes for this enrichment. All the results of the gene set enrichment analysis and leading-edge analysis are reported in supplementary materials (Tables S3 and S4).

The gene set overrepresentation analysis, employing cell-type-specific markers from an IPF single-cell RNA-sequencing study (Morse et al., 2019), revealed robust and statistically significant enrichment scores for Modules 2 and 4 for some specific IPF-related cell types. Specifically, Module 2 exhibited enrichment for genes associated with fibroblasts and proliferating cells, while Module 4 demonstrated significance for specific signatures of alveolar type 1 cells/club, alveolar type 2 cells, low-quality basal and two distinct types of macrophages (SPP1^{hi} and FABP4^{hi}, with 18.91 and 8.62 as enrichment score respectively). We also obtained other statistically significant enrichments for minor modules; the complete list is reported in the Supporting Information (Table S5).

3.7 | Nintedanib attenuates bleomycin-induced lipid peroxidation

To evaluate lipid peroxidation in our model we analysed the concentration of MDA, a widely used marker of oxidative damage. As

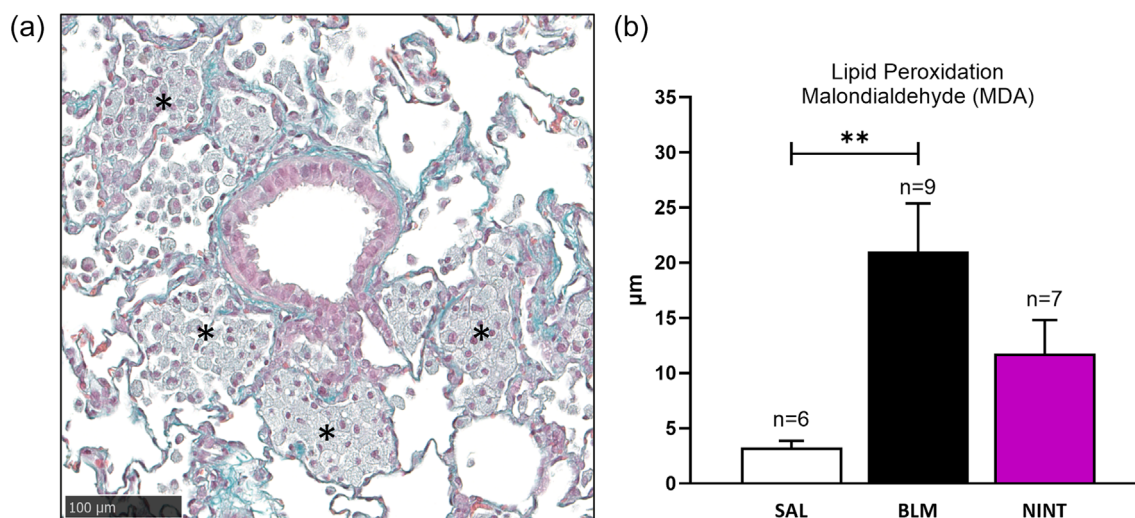


FIGURE 5 Effect of nintedanib on lipid associated lung damage induced by bleomycin. (a) Clusters of lipid-laden alveolar macrophages (* foam cells) are illustrated on Masson's trichrome-stained lung tissue sections from BLM (bleomycin-treated animals without nintedanib) group. Original magnification $\times 20$. (b) Bar graphs showing malondialdehyde (MDA) concentration in lung homogenates from all the groups. Data reported as mean $\pm$ SEM, $**P \leq 0.01$ versus SAL.

reported in Figure 5b, a statistically significant increased MDA concentration was observed in BLM group compared with SAL. The NINT group also displayed an increased MDA concentration compared with SAL, although to a lesser extent compared with BLM, indicating that nintedanib partially attenuates lipid peroxidation and consequently oxidative damage.

4 | DISCUSSION

Despite substantial advances in understanding the biological mechanisms of chronic lung diseases, provided also by the development of different animal models (Miles et al., 2020; Naikawadi et al., 2016; Nureki et al., 2018; Pierce et al., 2007; Tashiro et al., 2017; Trujillo et al., 2010; Valque et al., 2019), progress in identifying effective treatments for idiopathic pulmonary fibrosis (IPF) has been unsatisfactory. The main molecular targets of nintedanib, are well-documented (Rangarajan et al., 2016) and many published studies established its effects on lung fibroblasts, the key effector cells in the pathogenesis of fibrosis (Hostettler et al., 2014; Sheu et al., 2019; Zhou et al., 2020). However, an in-depth biomolecular analysis of its effects on lung fibrosis *in vivo* is lacking, which, given its positive clinical outcomes, may uncover additional antifibrotic mechanisms crucial for the development of future therapeutic approaches.

Therefore, to explore the complete molecular mechanisms associated with nintedanib treatment in IPF, we employed a transcriptomic approach to analyse mRNA alterations in a bleomycin-induced lung fibrosis rat model. A similar approach was performed by Sheu et al. (Sheu et al., 2019), which evaluated changes in mRNA and miRNA profiles on IPF fibroblasts exposed to two doses of nintedanib. However, in our study, the use of an animal model can provide a more comprehensive and translational understanding of the impact of nintedanib compared with a cellular model.

First, our research revealed significant variations in the impact of bleomycin on lung injury severity, as evidenced in histological and transcriptomic data. Indeed, BLM1 and BLM2 showed moderate histological alterations, with expression profiles similar to the control group. Conversely, BLM3 and BLM4 exhibited more severely damaged lungs and a substantially altered transcriptomic profile. The variability in fibrosis severity induced by bleomycin in rats has been already reported and examined by Persson et al. (Persson et al., 2020), where they reported the observation of two animal subgroups, low and high bleomycin-responders. However, the heterogeneity of bleomycin effect seen in our study was critical in identifying diverse gene expression profiles related to nintedanib's activity. If the model had a uniform response, some gene expression signatures would not have emerged.

In detail, two module eigengenes (MEs) highlighted a counter-regulatory effect of nintedanib on the genes affected by the fibrotic process induced by bleomycin. The ME02 with a strong counter-regulating effect and characterized by a high variability of expression inside the BLM group, suggested that nintedanib effectively normalizes gene expression levels regardless of the initial severity of lung

injury. The ME04 with a mild yet discernible counter-regulating effect and characterized by an uniform pattern of gene expression in animals treated with bleomycin, suggesting that this group of genes were not influenced by the varying levels of severity to bleomycin. In contrast, in ME01, the less severe animals of the BLM group (BLM1 and BLM2) exhibit gene levels comparable to those of all the NINT (group name, bleomycin-treated animals with nintedanib) animals. Therefore, conclusive evidence regarding the counter-regulatory effect of nintedanib on these genes cannot be provided in the absence of knowledge about the initial severity of NINT animals.

The effect highlighted by Module 2 is arguably the most anticipated one. Indeed, Module 2 is enriched in genes associated with cell types pathogenically involved in IPF, including fibroblasts and smooth muscle cells, which are generally targeted by anti-fibrotic drugs. Moreover, this module is enriched in genes reported to be modified by nintedanib in the fibroblast model of Sheu et al. (Sheu et al., 2019). Not surprisingly, this module features pathways related to extracellular matrix organization and epithelial-to-mesenchymal transition, processes reported to be affected by nintedanib in a fibrotic context (Rangarajan et al., 2016). Regarding other terms enriched in this module, especially those related to mitochondrial involvement and oxidative phosphorylation, there is also evidence indicating their contribution to lung fibrosis (Bueno et al., 2020; Rangarajan et al., 2017; Siekacz et al., 2021). However, the effect of nintedanib on these processes remains to be elucidated.

Module 4 is significantly enriched in alveolar macrophages and alveolar type 2 cells associated gene signatures and shows, among others, enrichment in pathways related with lipid metabolism and cholesterol homeostasis. These findings collectively hint at a potential dysregulation of lipid-related pathways in the model, particularly during the interaction between alveolar macrophages and alveolar type 2 cells (Romero et al., 2015) and notably, this mechanism is independent of lesion severity and is affected by nintedanib.

This hypothesis is also supported by the presence of agglomerates of lipid-laden alveolar macrophages in bleomycin-treated animals (Figure 5a). These cells, also known as 'foam cells', are established pathogenic features and have been shown to be extremely relevant in animal models of lung fibrosis (Azuma et al., 2005; Hou et al., 2019; Romero et al., 2015; Yasuda et al., 1994; Zhu et al., 2024) and in different contexts of human lung fibrosis (Basset-Leobon et al., 2010; Hayes et al., 2013; Hou et al., 2019). Increasing evidence suggests that pulmonary fibrosis is caused by lipid metabolism issues, such as anomalies in LDL metabolism and alterations in the oxidative status of various lipids (Hou et al., 2019; Romero et al., 2015; Shi et al., 2024). Furthermore, pulmonary fibrosis shares lipid risk factors with disorders characterized by abnormalities in lipid metabolism, such as atherosclerosis and obesity (Shi et al., 2024). In addition, other significant lipid-related cell types (such as alveolar type 2 cells) and highly specific IPF-related macrophages are being investigated as potential initiators and contributors to the pathogenic mechanism of lung fibrosis (Adams et al., 2020; Morse et al., 2019; Shi et al., 2024).

In the context of lung fibrosis this mechanism has been elucidated by Romero and colleagues under the name of pneumocyte-

macrophage paracrine lipid axis (Romero et al., 2015). Data of the present investigation strongly indicate the involvement of this axis in our model, specifically pertaining to Module 4. According to this view, lipids produced by alveolar type 2 cells for the generation of the surfactant are oxidized in the pathologic oxidant environment. The high concentration of oxidized lipids alters macrophage activities, causing up-regulation of lipid receptors and subsequent lipid internalization and accumulation, that lead to the formation of lipid-filled macrophages (Romero et al., 2015). Once internalized by macrophages, oxidized lipids (such as ox-LDL) can also accumulate in the lysosome and converted into free-cholesterol (Bobryshev et al., 2016). Once taken up by macrophages, cholesterol and other lipids have been shown to activate multiple pro-fibrotic pathways and particularly to induce **TGFβ1** signalling (Romero et al., 2015).

The presence of an oxidative environment and related oxidized lipid is in our model is highlighted by the dysregulation of various oxidative stress-related pathways and confirmed by the elevated MDA levels measured in bleomycin-treated rats. Macrophage scavenger receptors able to recognize and internalize oxidized lipids, such as *Orl1* (oxidized low density lipoprotein receptor 1) and *Scarb2* (scavenger receptor class B, member 2) (Bobryshev et al., 2016), are member of Module 4 and showed a trend toward upregulation. The same observations were reported in a model of silica dust, where the authors demonstrated elevated levels of intracellular lipids and high transcript levels for the lipid scavenger receptor (Hou et al., 2019), suggesting that the process is caused by the fibrosis and not by the agent used to induce it. Moreover, lysosome is the second most significant enriched term in Module 4, with no statistically significant enrichment in any other module. Considering that all these findings are related to Module 4 where nintedanib has a counter-regulatory effect and the lower level of MDA registered in the NINT group, we can speculate that this molecule could exert a protective effect on lipid oxidation associated with pulmonary fibrosis, supported by already published data. Indeed, Li and colleagues (Li et al., 2022) demonstrated that two progressive doses of nintedanib can reverse the increased production of cholesterol and pro-inflammatory cytokines that were induced in ox-LDL-treated HUVECs. Moreover, the increase in reactive oxygen species and MDA, and the declining activity of glutathione peroxidase in ox-LDL-treated HUVECs, were significantly abolished by the two nintedanib concentrations (Li et al., 2022). The assertion that nintedanib positively affects pathways related to lysosome and lipid metabolism is supported also by a separate study that evaluated the effects of nintedanib on gastric cancer cells (Dong et al., 2024).

These findings and references pertain more to general pulmonary fibrosis and related models; nevertheless, the importance of different types of macrophages is gaining increasing attention in IPF. In this context, Module 4 is enriched in the two IPF-specific macrophages populations identified by Morse et al. (Morse et al., 2019), SPP1^{hi}, from the interstitial compartment and FABP4^{hi} from the alveoli. In our study *Spp1* and *Fabp4* genes belong to Module 4 and showed a trend toward upregulation. Interestingly, after nintedanib treatment, *Spp1* expression is reduced, whereas **Fabp4** increases slightly. According to

Morse et al., the overall percentage of FABP4^{hi} macrophages decreases in the most diseased IPF lower lobes, suggesting that these cells either die or transition to SPP1^{hi} macrophages (Morse et al., 2019). Therefore, our findings may indicate that the impact of nintedanib may help to reduce or slow down the transition of FABP4^{hi} macrophages into SPP1^{hi} macrophages.

5 | CONCLUSIONS

In our rat model of bleomycin-induced lung fibrosis, we unravel a specific molecular therapeutic property of nintedanib by a network-based method applied to transcriptomic data and integrated with histomorphometric findings. Indeed, other than the usual pathways known to be affected by nintedanib, we identified a gene expression signature linked to macrophages and alveolar type 2 cells that affects lipid metabolism and oxidation. To our knowledge, this is the first evidence of the mechanistic effect of nintedanib on lipid metabolism in the context of pulmonary fibrosis. We acknowledge that our study has some limitations and that further research is needed to explore this topic in more depth. However, our findings provide additional support for the potential of targeting the lipid metabolism and macrophages as a therapeutic strategy for lung fibrosis.

AUTHOR CONTRIBUTIONS

Martina Bonatti: Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); visualization (equal); writing—original draft (lead); writing—review and editing (equal). **Vanessa Pitozzi:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); visualization (equal); writing—review and editing (equal). **Paola Caruso:** Formal analysis (equal); methodology (equal); writing—review and editing (equal). **Silvia Pontis:** Data curation (equal); methodology (equal); writing—review and editing (equal). **Maria Gloria Pittelli:** Methodology (equal); writing—review and editing (equal). **Caterina Frati:** Data curation (equal); methodology (equal); writing—review and editing (equal). **Denise Madeddu:** Data curation (equal); methodology (equal); writing—review and editing (equal). **Federico Quaini:** Writing—review and editing (equal). **Costanza Anna Maria Lagrasta:** Methodology (equal); writing—review and editing (equal). **Ilaria Minato:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); visualization (equal); writing—review and editing (equal). **Emanuela Bocchi:** Data curation (equal); formal analysis (equal); writing—review and editing (equal). **Maurizio Civelli:** Writing—review and editing (equal). **Gino Villetti:** Writing—review and editing (equal). **Marcello Trevisani:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); writing—review and editing (equal). **Barbara Montanini:** Conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (lead);

resources (equal); supervision (equal); visualization (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

VP, PC, SP, MGP, MC, GV and MT are employees of Chiesi Farmaceutici S.p.A. FQ was engaged as a consultant by Chiesi Farmaceutici S.p.A. All the remaining authors have no actual or perceived conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions. The dataset is available with accession number GSE278200.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the British Journal of Pharmacology, guidelines for [Design and Analysis](#) and [Animal Experimentation](#) and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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REFERENCES

- Adams, T. S., Schupp, J. C., Poli, S., Ayaub, E. A., Neumark, N., Ahangari, F., Chu, S. G., Raby, B. A., Deluliis, G., Januszyk, M., Duan, Q., Arnett, H. A., Siddiqui, A., Washko, G. R., Homer, R., Yan, X., Rosas, I. O., & Kaminski, N. (2020). Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis. *Science Advances*, 6(28), eaba1983. <https://doi.org/10.1126/sciadv.aba1983>
- Alexander, S. P. H., Fabbro, D., Peach, C. J., Gibb, A. J., Kelly, E., Mathie, A. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Southan, C., Davies, J. A., Beuve, A., Brouckaert, P., Bryant, C., Burnett, J. C., Farndale, R. W., Friebe, A., Garthwaite, J., ... Waldman, S. A. (2025). The Concise Guide to PHARMACOLOGY 2025/26: Catalytic receptors. *British Journal of Pharmacology*, 182(Suppl 1), S259–S306. <https://doi.org/10.1111/bph.70233>
- Alexander, S. P. H., Gibb, A. J., Kelly, E., Mathie, A. A., Peach, C. J., Veale, E. L., Cidlowski, J. A., Davenport, A. P., Fabbro, D., Spedding, M., Striessnig, J., Armstrong, J. F., Buneman, O. P., Faccenda, E., Harding, S. D., Southan, C., Davies, J. A., Ahlers-Dannen, K. E., Alqinyah, M., ... Zolghadri, Y. (2025). The Concise Guide to PHARMACOLOGY 2025/26: Introduction and Other Protein Targets. *British Journal of Pharmacology*, 182(Suppl 1), S1–S23. <https://doi.org/10.1111/bph.70229>
- Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics*, 31(2), 166–169. <https://doi.org/10.1093/bioinformatics/btu638>
- Ashcroft, T., Simpson, J. M., & Timbrell, V. (1988). Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *Journal of Clinical Pathology*, 41(4), 467–470. <https://doi.org/10.1136/jcp.41.4.467>
- Azuma, A., Li, Y. J., Abe, S., Usuki, J., Matsuda, K., Henmi, S., Miyauchi, Y., Ueda, K., Izawa, A., Sone, S., Hashimoto, S., & Kudoh, S. (2005). Interferon-beta inhibits bleomycin-induced lung fibrosis by decreasing transforming growth factor-beta and thrombospondin. *American Journal of Respiratory Cell and Molecular Biology*, 32(2), 93–98. <https://doi.org/10.1165/rcmb.2003-0374OC>
- Basset-Leobon, C., Lacoste-Collin, L., Aziza, J., Bes, J. C., Joza, S., & Courtade-Saidi, M. (2010). Cut-off values and significance of Oil Red O-positive cells in bronchoalveolar lavage fluid. *Cytopathology*, 21(4), 245–250. <https://doi.org/10.1111/j.1365-2303.2009.00677.x>
- Bobryshev, Y. V., Ivanova, E. A., Chistiakov, D. A., Nikiforov, N. G., & Orekhov, A. N. (2016). Macrophages and their role in atherosclerosis: Pathophysiology and transcriptome analysis. *BioMed Research International*, 2016, 9582430. <https://doi.org/10.1155/2016/9582430>
- Bonatti, M., Pitozzi, V., Caruso, P., Pontis, S., Pittelli, M. G., Frati, C., Mangiaracina, C., Lagrasta, C. A. M., Quaini, F., Cantarella, S., Ottonello, S., Villetti, G., Civelli, M., Montanini, B., & Trevisani, M. (2023). Time-course transcriptome analysis of a double challenge bleomycin-induced lung fibrosis rat model uncovers ECM homeostasis-related translationally relevant genes. *BMJ Open Respiratory Research*, 10(1), e001476. <https://doi.org/10.1136/bmjresp-2022-001476>
- Bueno, M., Calyeca, J., Rojas, M., & Mora, A. L. (2020). Mitochondria dysfunction and metabolic reprogramming as drivers of idiopathic pulmonary fibrosis. *Redox Biology*, 33, 101509. <https://doi.org/10.1016/j.redox.2020.101509>
- Chianese, M., Screm, G., Salton, F., Confalonieri, P., Trotta, L., Barbieri, M., Ruggero, L., Mari, M., Reccardini, N., Geri, P., Hughes, M., Lerda, S., Confalonieri, M., Mondini, L., & Ruaro, B. (2024). Pirfenidone and nintedanib in pulmonary fibrosis: Lights and shadows. *Pharmaceuticals (Basel)*, 17(6), 709. <https://doi.org/10.3390/ph17060709>
- Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Szczesniak, M. W., Gaffney, D. J., Elo, L. L., Zhang, X., & Mortazavi, A. (2016). A survey of best practices for RNA-seq data analysis. *Genome Biology*, 17, 13. <https://doi.org/10.1186/s13059-016-0881-8>
- Curtis, M. J., Alexander, S. P. H., Cortese-Krott, M., Kendall, D. A., Martemyanov, K. A., Mauro, C., Panettieri, R. A. Jr., Papapetropoulos, A., Patel, H. H., Santo, E. E., Schulz, R., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., Wang, X., & Ferdinandy, P. (2025). Guidance on the planning and reporting of experimental design and analysis. *British Journal of Pharmacology*, 182, 1413–1415. <https://doi.org/10.1111/bph.17441>

- Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15–21. <https://doi.org/10.1093/bioinformatics/bts635>
- Dong, X., Wang, L., Wang, D., Yu, M., Yang, X. J., & Cai, H. (2024). Proteomic study on nintedanib in gastric cancer cells. *PeerJ*, 12, e16771. <https://doi.org/10.7717/peerj.16771>
- Fei, L., Chen, H., Ma, L., E, W., Wang, R., Fang, X., Zhou, Z., Sun, H., Wang, J., Jiang, M., Wang, X., Yu, C., Mei, Y., Jia, D., Zhang, T., Han, X., & Guo, G. (2022). Systematic identification of cell-fate regulatory programs using a single-cell atlas of mouse development. *Nature Genetics*, 54(7), 1051–1061. <https://doi.org/10.1038/s41588-022-01118-8>
- Grimminger, F., Schermuly, R. T., & Ghofrani, H. A. (2010). Targeting non-malignant disorders with tyrosine kinase inhibitors. *Nature Reviews. Drug Discovery*, 9(12), 956–970. <https://doi.org/10.1038/nrd3297>
- Han, X., Wang, R., Zhou, Y., Fei, L., Sun, H., Lai, S., Saadatpour, A., Zhou, Z., Chen, H., Ye, F., & Huang, D. (2018). Mapping the mouse cell atlas by Microwell-seq. *Cell*, 172(5), 1091–1107.
- Hayes, D. Jr., Kirkby, S., K, S. M., Mansour, H. M., Khosravi, M., Strawbridge, H., & Tobias, J. D. (2013). Reduction of lipid-laden macrophage index after laparoscopic Nissen fundoplication in cystic fibrosis patients after lung transplantation. *Clinical Transplantation*, 27(1), 121–125.
- Hong, J., Arneson, D., Umar, S., Ruffenach, G., Cunningham, C. M., Ahn, I. S., Diamante, G., Bhetraratana, M., Park, J. F., Said, E., Huynh, C., Le, T., Medzikovic, L., Humbert, M., Soubrier, F., Montani, D., Girerd, B., Tréguët, D. A., Channick, R., ... Yang, X. (2021). Single-cell study of two rat models of pulmonary arterial hypertension reveals connections to human pathobiology and drug repositioning. *American Journal of Respiratory and Critical Care Medicine*, 203(8), 1006–1022. <https://doi.org/10.1164/rccm.202006-2169OC>
- Hostettler, K. E., Zhong, J., Papakonstantinou, E., Karakiulakis, G., Tamm, M., Seidel, P., Sun, Q., Mandal, J., Lardinois, D., Lambers, C., & Roth, M. (2014). Anti-fibrotic effects of nintedanib in lung fibroblasts derived from patients with idiopathic pulmonary fibrosis. *Respiratory Research*, 15(1), 157. <https://doi.org/10.1186/s12931-014-0157-3>
- Hou, X., Summer, R., Chen, Z., Tian, Y., Ma, J., Cui, J., Hao, X., Guo, L., Xu, H., Wang, H., & Liu, H. (2019). Lipid uptake by alveolar macrophages drives fibrotic responses to silica dust. *Scientific Reports*, 9(1), 399. <https://doi.org/10.1038/s41598-018-36875-2>
- Hubner, R. H., Gitter, W., El Mokhtari, N. E., Mathiak, M., Both, M., Bolte, H., Freitag-Wolf, S., & Bewig, B. (2008). Standardized quantification of pulmonary fibrosis in histological samples. *BioTechniques*, 44(4), 507–511.
- Jenkins, R. G., Moore, B. B., Chambers, R. C., Eickelberg, O., Konigshoff, M., Kolb, M., Laurent, G. J., Nanthakumar, C. B., Oltman, M. A., Pardo, A., & Selman, M. (2017). An official American Thoracic Society workshop report: Use of animal models for the pre-clinical assessment of potential therapies for pulmonary fibrosis. *American Journal of Respiratory Cell and Molecular Biology*, 56(5), 667–679. <https://doi.org/10.1165/rcmb.2017-0096ST>
- Langfelder, P., & Horvath, S. (2008). WGCNA: An R package for weighted correlation network analysis. *BMC Bioinformatics*, 9, 559. <https://doi.org/10.1186/1471-2105-9-559>
- Li, L., Chen, Y., & Shi, C. (2022). Nintedanib ameliorates oxidized low-density lipoprotein-induced inflammation and cellular senescence in vascular endothelial cells. *Bioengineered*, 13(3), 6196–6207. <https://doi.org/10.1080/21655979.2022.2036913>
- Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, 177(16), 3611–3616. <https://doi.org/10.1111/bph.15178>
- Maier, T. M., Bendstrup, E., Dron, L., Langley, J., Smith, G., Khalid, J. M., Patel, H., & Kreuter, M. (2021). Global incidence and prevalence of idiopathic pulmonary fibrosis. *Respiratory Research*, 22(1), 197. <https://doi.org/10.1186/s12931-021-01791-z>
- Metsalu, T., & Vilo, J. (2015). ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Research*, 43(W1), W566–W570. <https://doi.org/10.1093/nar/gkv468>
- Mikami, S., Miura, Y., Kondo, S., Sakai, K., Nishimura, H., Kyoyama, H., Moriyama, G., Koyama, N., Noguchi, H., Ohkubo, H., Kanazawa, S., & Uematsu, K. (2022). Nintedanib induces gene expression changes in the lung of induced-rheumatoid arthritis-associated interstitial lung disease mice. *PLoS ONE*, 17(6), e0270056. <https://doi.org/10.1371/journal.pone.0270056>
- Miles, T., Hoyne, G. F., Knight, D. A., Fear, M. W., Mutsaers, S. E., & Prele, C. M. (2020). The contribution of animal models to understanding the role of the immune system in human idiopathic pulmonary fibrosis. *Clinical & Translational Immunology*, 9(7), e1153. <https://doi.org/10.1002/cti2.1153>
- Morse, C., Tabib, T., Sembrat, J., Buschur, K. L., Bittar, H. T., Valenzi, E., Jiang, Y., Kass, D. J., Gibson, K., Chen, W., Mora, A., Benos, P. V., Rojas, M., & Lafyatis, R. (2019). Proliferating SPP1/MERTK-expressing macrophages in idiopathic pulmonary fibrosis. *The European Respiratory Journal*, 54(2), 1802441. <https://doi.org/10.1183/13993003.02441-2018>
- Naikawadi, R. P., Disayabutr, S., Mallavia, B., Donne, M. L., Green, G., La, J. L., Rock, J. R., Looney, M. R., & Wolters, P. J. (2016). Telomere dysfunction in alveolar epithelial cells causes lung remodeling and fibrosis. *JCI Insight*, 1(14), e86704. <https://doi.org/10.1172/jci.insight.86704>
- Nureki, S. I., Tomer, Y., Venosa, A., Katzen, J., Russo, S. J., Jamil, S., Barrett, M., Nguyen, V., Kopp, M., Mulugeta, S., & Beers, M. F. (2018). Expression of mutant *Sftpc* in murine alveolar epithelia drives spontaneous lung fibrosis. *The Journal of Clinical Investigation*, 128(9), 4008–4024. <https://doi.org/10.1172/JCI99287>
- Oliveros, J. C. (2007). Venny. An interactive tool for comparing lists with Venn's diagrams. (2007–2015) 2.0: Available from: <https://bioinfogp.cnb.csic.es/tools/venny/index.html>
- Peng, R., Sridhar, S., Tyagi, G., Phillips, J. E., Garrido, R., Harris, P., Burns, L., Renteria, L., Woods, J., Chen, L., Allard, J., Ravindran, P., Bitter, H., Liang, Z., Hogaboam, C. M., Kitson, C., Budd, D. C., Fine, J. S., Bauer, C. M. T., & Stevenson, C. S. (2013). Bleomycin induces molecular changes directly relevant to idiopathic pulmonary fibrosis: A model for “active” disease. *PLoS ONE*, 8(4), e59348. <https://doi.org/10.1371/journal.pone.0059348>
- Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., ... Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Journal of Cerebral Blood Flow and Metabolism*, 40(9), 1769–1777. <https://doi.org/10.1177/0271678X20943823>
- Persson, I. M., Hakansson, H. F., Orborn, A., Liu, J., von Wachenfeldt, K., Olsson, L. E., Mahmutovic Persson, I., & Falk Häkansson, H. (2020). Imaging biomarkers and pathobiological profiling in a rat model of drug-induced interstitial lung disease induced by bleomycin. *Frontiers in Physiology*, 11, 584. <https://doi.org/10.3389/fphys.2020.00584>
- Pierce, E. M., Carpenter, K., Jakubzick, C., Kunkel, S. L., Flaherty, K. R., Martinez, F. J., & Hogaboam, C. M. (2007). Therapeutic targeting of CC ligand 21 or CC chemokine receptor 7 abrogates pulmonary fibrosis induced by the adoptive transfer of human pulmonary fibroblasts to immunodeficient mice. *The American Journal of Pathology*, 170(4), 1152–1164. <https://doi.org/10.2353/ajpath.2007.060649>
- Raghu, G., Collard, H. R., Egan, J. J., Martinez, F. J., Behr, J., Brown, K. K., Colby, T. V., Cordier, J. F., Flaherty, K. R., Lasky, J. A., Lynch, D. A., Ryu, J. H., Swigris, J. J., Wells, A. U., Ancochea, J., Bouras, D.,

- Carvalho, C., Costabel, U., Ebina, M., ... ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis. (2011). An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *American Journal of Respiratory and Critical Care Medicine*, 183(6), 788–824. <https://doi.org/10.1164/rccm.2009-040GL>
- Raghu, G., Rochwerf, B., Zhang, Y., Garcia, C. A., Azuma, A., Behr, J., Garcia, C. A. C., Brozek, J. L., Collard, H. R., Cunningham, W., Homma, S., Johkoh, T., Martinez, F. J., Myers, J., Protzko, S. L., Richeldi, L., Rind, D., Selman, M., Theodore, A., ... Schünemann, H. J. (2015). An official ATS/ERS/JRS/ALAT clinical practice guideline: Treatment of idiopathic pulmonary fibrosis. An update of the 2011 clinical practice guideline. *American Journal of Respiratory and Critical Care Medicine*, 192(2), e3–e19. <https://doi.org/10.1164/rccm.201506-1063ST>
- Rangarajan, S., Bernard, K., & Thannickal, V. J. (2017). Mitochondrial dysfunction in pulmonary fibrosis. *Annals of the American Thoracic Society*, 14(Supplement_5), S383–S388. <https://doi.org/10.1513/AnnalsATS.201705-370AW>
- Rangarajan, S., Kurundkar, A., Kurundkar, D., Bernard, K., Sanders, Y. Y., Ding, Q., Antony, V. B., Zhang, J., Zmijewski, J., & Thannickal, V. J. (2016). Novel mechanisms for the antifibrotic action of nintedanib. *American Journal of Respiratory Cell and Molecular Biology*, 54(1), 51–59. <https://doi.org/10.1165/rccm.2014-0445OC>
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, 43(7), e47. <https://doi.org/10.1093/nar/gkv007>
- Roach, K. M., Castells, E., Dixon, K., Mason, S., Elliott, G., Marshall, H., Poblocka, M. A., Macip, S., Richardson, M., Khalfaoui, L., & Bradding, P. (2021). Evaluation of pirfenidone and nintedanib in a human lung model of fibrogenesis. *Frontiers in Pharmacology*, 12, 679388. <https://doi.org/10.3389/fphar.2021.679388>
- Romero, F., Shah, D., Duong, M., Penn, R. B., Fessler, M. B., Madenspacher, J., Stafstrom, W., Kavuru, M., Lu, B., Kallen, C. B., Walsh, K., & Summer, R. (2015). A pneumocyte-macrophage paracrine lipid axis drives the lung toward fibrosis. *American Journal of Respiratory Cell and Molecular Biology*, 53(1), 74–86. <https://doi.org/10.1165/rccm.2014-0343OC>
- Saito, S., Alkhatib, A., Kolls, J. K., Kondoh, Y., & Lasky, J. A. (2019). Pharmacotherapy and adjunctive treatment for idiopathic pulmonary fibrosis (IPF). *Journal of Thoracic Disease*, 11(Suppl 14), S1740–S1754. <https://doi.org/10.21037/jtd.2019.04.62>
- Sheu, C. C., Chang, W. A., Tsai, M. J., Liao, S. H., Chong, I. W., & Kuo, P. L. (2019). Gene expression changes associated with nintedanib treatment in idiopathic pulmonary fibrosis fibroblasts: A next-generation sequencing and bioinformatics study. *Journal of Clinical Medicine*, 8(3), 308. <https://doi.org/10.3390/jcm8030308>
- Shi, X., Chen, Y., Shi, M., Gao, F., Huang, L., Wang, W., Wei, D., Shi, C., Yu, Y., Xia, X., Song, N., Chen, X., Distler, J. H. W., Lu, C., Chen, J., & Wang, J. (2024). The novel molecular mechanism of pulmonary fibrosis: Insight into lipid metabolism from reanalysis of single-cell RNA-seq databases. *Lipids in Health and Disease*, 23(1), 98. <https://doi.org/10.1186/s12944-024-02062-8>
- Siekacz, K., Piotrowski, W. J., Iwanski, M. A., Gorski, P., & Bialas, A. J. (2021). The role of interaction between mitochondria and the extracellular matrix in the development of idiopathic pulmonary fibrosis. *Oxidative Medicine and Cellular Longevity*, 2021, 9932442. <https://doi.org/10.1155/2021/9932442>
- Spagnolo, P., Tzouveleakis, A., & Bonella, F. (2018). The management of patients with idiopathic pulmonary fibrosis. *Frontiers in Medicine (Lausanne)*, 5, 148. <https://doi.org/10.3389/fmed.2018.00148>
- Subramanian, A., Kuehn, H., Gould, J., Tamayo, P., & Mesirov, J. P. (2007). GSEA-P: A desktop application for gene set enrichment analysis. *Bioinformatics*, 23(23), 3251–3253. <https://doi.org/10.1093/bioinformatics/btm369>
- Tashiro, J., Rubio, G. A., Limper, A. H., Williams, K., Elliot, S. J., Ninou, I., Aidinis, V., Tzouveleakis, A., & Glassberg, M. K. (2017). Exploring animal models that resemble idiopathic pulmonary fibrosis. *Frontiers in Medicine (Lausanne)*, 4, 118. <https://doi.org/10.3389/fmed.2017.00118>
- Trujillo, G., Meneghin, A., Flaherty, K. R., Sholl, L. M., Myers, J. L., Kazerooni, E. A., Gross, B. H., Oak, S. R., Coelho, A. L., Evanoff, H., Day, E., Toews, G. B., Joshi, A. D., Schaller, M. A., Waters, B., Jarai, G., Westwick, J., Kunkel, S. L., Martinez, F. J., & Hogaboam, C. M. (2010). TLR9 differentiates rapidly from slowly progressing forms of idiopathic pulmonary fibrosis. *Science Translational Medicine*, 2(57), 57ra82. <https://doi.org/10.1126/scitranslmed.3001510>
- Valque, H., Gouyer, V., Duez, C., Leboeuf, C., Marquillies, P., Le Bert, M., Plet, S., Ryffel, B., Janin, A., Gottrand, F., & Desseyn, J. L. (2019). Muc5b-deficient mice develop early histological lung abnormalities. *Biology Open*, 8(11), bio046359. <https://doi.org/10.1242/bio.046359>
- Wang, R., Zhang, P., Wang, J., Ma, L., E, W., Suo, S., Jiang, M., Li, J., Chen, H., Sun, H., Fei, L., Zhou, Z., Zhou, Y., Chen, Y., Zhang, W., Wang, X., Mei, Y., Sun, Z., Yu, C., ... Han, X. (2023). Construction of a cross-species cell landscape at single-cell level. *Nucleic Acids Research*, 51(2), 501–516. <https://doi.org/10.1093/nar/gkac633>
- Wilson, A. C., Chiles, J., Ashish, S., Chanda, D., Kumar, P. L., Mobley, J. A., et al. (2022). Integrated bioinformatics analysis identifies established and novel TGFβ1-regulated genes modulated by anti-fibrotic drugs. *Scientific Reports*, 12(1), 3080.
- Wollin, L., Maillet, I., Quesniaux, V., Holweg, A., & Ryffel, B. (2014). Antifibrotic and anti-inflammatory activity of the tyrosine kinase inhibitor nintedanib in experimental models of lung fibrosis. *The Journal of Pharmacology and Experimental Therapeutics*, 349(2), 209–220. <https://doi.org/10.1124/jpet.113.208223>
- Yasuda, K., Sato, A., Nishimura, K., Chida, K., & Hayakawa, H. (1994). Phospholipid analysis of alveolar macrophages and bronchoalveolar lavage fluid following bleomycin administration to rabbits. *Lung*, 172(2), 91–102. <https://doi.org/10.1007/BF00185080>
- Zhou, B. Y., Wang, W. B., Wu, X. L., Zhang, W. J., Zhou, G. D., Gao, Z., & Liu, W. (2020). Nintedanib inhibits keloid fibroblast functions by blocking the phosphorylation of multiple kinases and enhancing receptor internalization. *Acta Pharmacologica Sinica*, 41(9), 1234–1245. <https://doi.org/10.1038/s41401-020-0381-y>
- Zhou, Y. Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., Benner, C., & Chanda, S. K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature Communications*, 10, 1523. <https://doi.org/10.1038/s41467-019-09234-6>
- Zhu, Y., Choi, D., Somanath, P. R., & Zhang, D. (2024). Lipid-laden macrophages in pulmonary diseases. *Cells*, 13(11), 889. <https://doi.org/10.3390/cells13110889>

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