CHEST

# Molecular Endotyping of Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a devastating and incurable progressive fibrotic lung condition associated with a significant disease burden. In recent years there has been an exponential increase in the number of preclinical and clinical studies performed in IPF. IPF is defined according to rigid diagnostic criteria; hence, a significant subset of patients with unclassifiable disease has been excluded from these studies. The traditional diagnostic classification of all progressive fibrotic lung diseases uses specific clinical, radiological, and histopathological features to define each condition. However, the considerable heterogeneity within each form of pulmonary fibrosis has raised the possibility of distinct pathophysiological mechanisms culminating in a common phenotype. Thus, the classification of fibrotic lung diseases according to the driving molecular mechanisms rather than specific user-defined histopathological and radiological features could improve several aspects of clinical care. Discoveries from basic science research have defined multiple complex molecular pathways involved in the pathogenesis of pulmonary fibrosis that may provide markers for the molecular endotyping of this disease. In addition, these molecular pathways have revealed potential therapeutic targets. Reclassifying progressive fibrotic lung diseases according to molecular endotypes may allow for more accurate assessment of prognosis and individualized treatment. Furthermore, recent developments that have been applied to a narrow group of patients with IPF may be applicable to those with other progressive fibrotic lung diseases. This review presents the latest developments from translational research in this area and explains how molecular endotyping could revolutionize the diagnosis, stratification, and treatment of pulmonary fibrosis.

CHEST 2016; 149(1):228-237

**KEY WORDS:** biomarkers; gene; idiopathic pulmonary fibrosis; interstitial lung disease; molecular biology

Idiopathic pulmonary fibrosis (IPF) is a progressive and incurable condition with a rising incidence and a median survival of just 3 years.[1,2] Like other progressive fibrotic lung diseases, IPF is diagnosed using clinical, radiological, and histopathological criteria.[2] According to current guidelines, a lung biopsy is required to confirm IPF if high-resolution CT (HRCT) scanning does not show a typical usual interstitial pneumonia (UIP) pattern.[2] However, lung biopsies carry a risk and may not provide a definitive diagnosis.[3] Therefore, a significant number of patients have unclassifiable but

**ABBREVIATIONS:** AEC = alveolar epithelial cell; ECM = extracellular matrix; FPF = familial pulmonary fibrosis; HRCT = high-resolution CT; ILD = interstitial lung disease; IPF = idiopathic pulmonary fibrosis; miRNA = micro RNA; MMP = matrix metalloproteinase; TGFβ = transforming growth factor-β; Th = T-helper; UIP = usual interstitial pneumonia

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**DOI:** http://dx.doi.org/10.1378/chest.15-1511

progressive pulmonary fibrosis and are ineligible for inclusion into clinical trials and for new therapies.[4]

The pathophysiology of pulmonary fibrosis is complex and involves numerous molecular pathways and cell types. The current model for IPF pathogenesis suggests that abnormal alveolar epithelial wound healing occurs as a result of insults including cigarette smoke, gastroesophageal reflux, and infection.[5,6] Subsequent activation of inflammatory cells, vascular leak, and release of profibrotic cytokines create an environment supportive of exaggerated fibroblast and myofibroblast activity.[6] These cells deposit excessive extracellular matrix (ECM) within the lung parenchyma, and the resulting architectural distortion impairs gas exchange (Fig 1).[6]

Although recent research has focused on IPF, it is likely that common molecular pathways underlie IPF and other progressive fibrotic lung diseases. Increased understanding of these shared pathways could broaden the application of recent discoveries in IPF to all progressive pulmonary fibrosis, including unclassifiable disease. This review presents the latest developments in this field and describes how they could transform clinical practice.

## Integrating Basic Mechanisms of Pulmonary Fibrosis and Improved Management of Disease

Progressive fibrotic lung diseases are heterogeneous and unpredictable. Some patients with IPF decline rapidly whereas others follow a more indolent course[2]; similarly, there is considerable variability in the natural history of other progressive fibrotic lung diseases.[4,7] Thus, it is feasible that distinct biological processes drive rapidly and slowly progressing pulmonary fibrosis regardless of cause. In addition, there may be conserved pathological

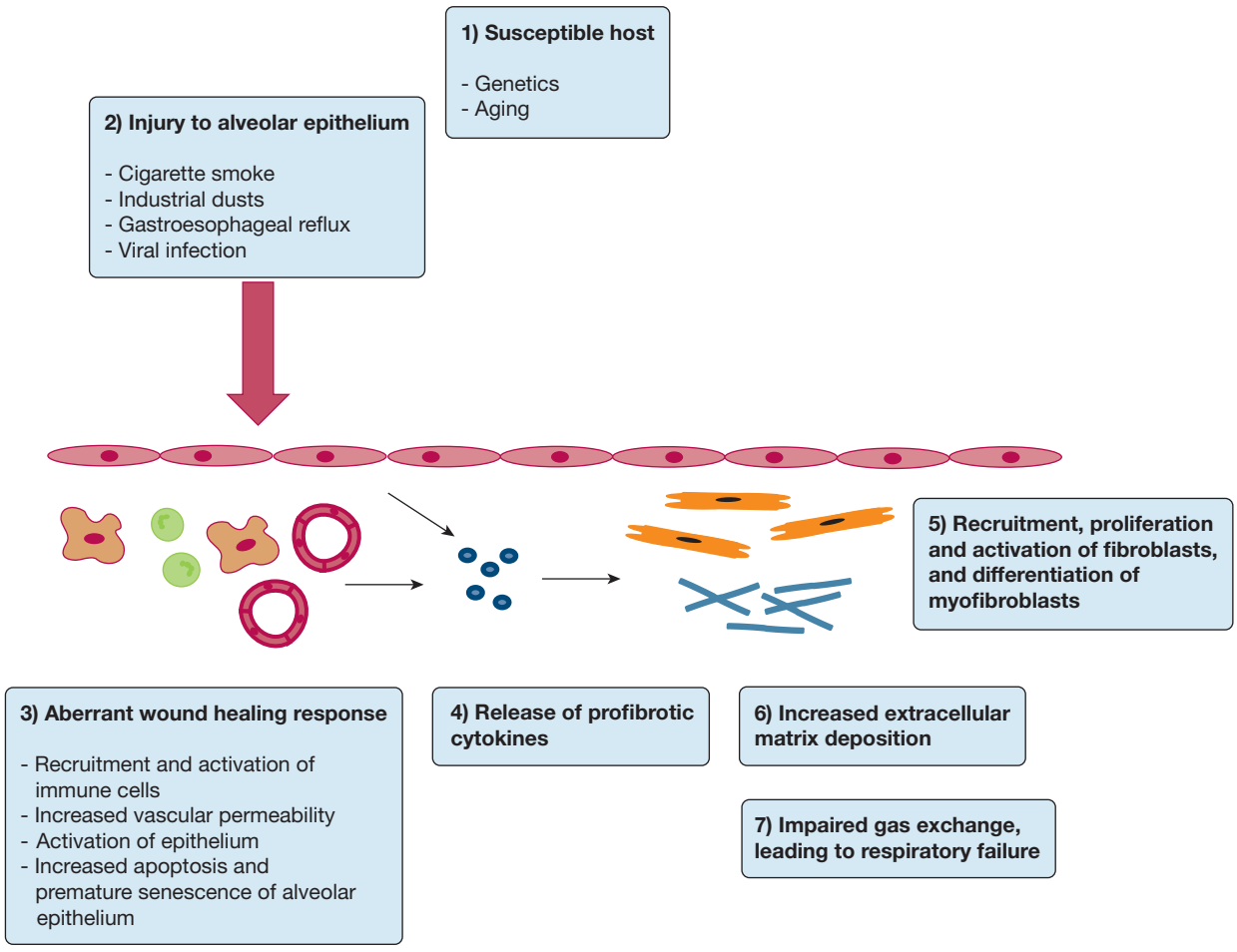


Figure 1 – *The pathogenesis of pulmonary fibrosis: the idiopathic pulmonary fibrosis model. Idiopathic pulmonary fibrosis is the result of a complex abnormal wound-healing response involving a multitude of cell types (1-3). The subsequent release of profibrotic mediators (4) results in the recruitment, proliferation, and activation of fibroblasts and differentiation of myofibroblasts (5). The activity of these cells generates the excessive extracellular matrix characteristic of fibrosis (6). The resulting architectural distortion of the lung parenchyma causes impaired gas exchange and respiratory failure (7). Arrows used to demonstrate sequence of events.*

mechanisms across fibrotic lung diseases with different clinical phenotypes. This hypothesis challenges the traditional classification of pulmonary fibrosis and proposes defining disease according to the driving molecular mechanisms. We propose that management of these conditions could be revolutionized by focusing on the molecular pathogenesis of disease rather than the disease phenotype or cause.

Biomarkers are important in characterizing pulmonary fibrosis. There are four broad categories of biomarker: diagnostic, prognostic, endotypic, and theragnostic. Diagnosis of pulmonary fibrosis can sometimes be problematic, particularly when patients are not fit for invasive testing or when asymptomatic patients exhibit very early disease. In these cases, noninvasive diagnostic biomarkers will be useful adjuncts to radiological tests. Lung function measured at diagnosis can be used to estimate prognosis,[8,9] and serum matrix metalloproteinase (MMP) 7 has been identified as a prognostic biomarker.[10,11] Theragnostic biomarkers, so called because they determine whether a disease has responded to therapy,[8] are required in pulmonary fibrosis when objective measures such as lung function continue to deteriorate even when IPF responds to therapy.[12,13] Finally, endotypic biomarkers reflect the distinct molecular phenotype of diseased cells or tissue and may provide prognostic information and therapeutic targets that cross several disease-specific clinical phenotypes. This review focuses on disease mechanisms that are shared across clinical pulmonary fibrosis phenotypes, and how these may reveal distinct endotypes of pulmonary fibrosis and facilitate the expansion of new therapeutic strategies to all forms of progressive fibrotic lung disease.

## Genetic Basis of Pulmonary Fibrosis

Inhaled insults capable of initiating pulmonary fibrosis are common, yet disease develops in only a minority of individuals. Hence, those who develop pulmonary fibrosis are considered more susceptible to injury, and interactions between genetic and environmental factors are thought to be critical.[14] Up to 20% of idiopathic interstitial pneumonias are familial, and familial pulmonary fibrosis (FPF) appears to be inherited in an autosomal dominant pattern with variable penetrance.[14]

Sporadic fibrosis and FPF are thought to reflect a continuum of genetic risk rather than exist as distinct entities, as rare variants in FPF-associated genes have been found in sporadic IPF.[14] In addition, common molecular pathways have been identified in both inherited and sporadic pulmonary fibrosis.[14] Implicated genes in FPF include those involved with surfactant proteins, telomere biology, inflammation and immunity, cell-cell adhesion, and cell cycle progression.[14-17] One genetic abnormality will not generate the same histopathological or radiological appearance consistently, which suggests that common pathophysiological mechanisms can generate different disease phenotypes.[14] This has important implications for the disease classification of both sporadic fibrosis and FPF.

No single genetic defect is responsible for all FPF, and most cases of FPF result from unknown genetic variants.[14] Therefore, currently genetic screening in pulmonary fibrosis is not routine in clinical practice. However, screening for single-nucleotide polymorphisms in the *Toll-interacting protein* gene and the promoter of the mucin gene *MUC5B* may be useful for patient stratification, particularly for clinical trials. Both of these single-nucleotide polymorphisms carry an increased risk of IPF but a better prognosis when present,[18-20] and therefore could be used to categorize clinical trial participants according to their risk of progression.[14] In addition, the *MUC5B* polymorphism, along with shorter telomere length, has been found in asymptomatic patients with a high risk of pulmonary fibrosis.[21] Further study is required to assess how genetic variants may interact with other risk factors for disease and influence therapeutic response, particularly as more drugs become available.

## Altered Gene Expression Promotes Pulmonary Fibrosis

In addition to germline genetic variants, differences in gene expression through epigenetic changes have been linked to pulmonary fibrosis. External factors such as cigarette smoke alter transcriptional profiles via epigenetic mechanisms.[22] Modified genes identified in IPF influence numerous biological functions including ECM turnover and signaling, epithelial-to-mesenchymal transition, cell growth, immunity, and developmental pathways.[22]

The evaluation of gene expression has several potential applications. Gene expression signatures measured from peripheral blood can discriminate between IPF and non-IPF progressive fibrotic lung diseases,[23] which could be a useful diagnostic adjunct in very early disease, although this method is unlikely to supersede HRCT in established cases. Furthermore, transcriptome pathway analysis may identify molecular drivers of fibrosis and so may have applications for personalized care in the future.

Micro RNAs (miRNAs) bind to messenger RNA and influence posttranscriptional processes, thus regulating protein expression. These molecules are important to developmental pathways, many of which are integral to fibrogenesis.[24] Some miRNAs are differentially expressed in the serum of patients with IPF,[24] although longitudinal analyses of miRNA expression have not been performed. Downregulation of some miRNAs such as the miR-200 and miR-29 families and the upregulation of others such as miR-21 have been found in animal models of pulmonary fibrosis and the lungs of patients with IPF.[25-28] In addition, restoration of normal miRNA levels has ameliorated pulmonary fibrosis in animal models.[25-28] Therefore, miRNAs warrant further investigation as biomarkers and therapeutic targets.

## Dysfunctional Alveolar Epithelial Repair

Abnormal epithelial wound healing, apoptosis, and senescence are central to fibrogenesis, and several epithelium-related molecules may have diagnostic, prognostic, and therapeutic applications.

The αvβ6 integrin is a cell surface protein that is necessary to the activation of the profibrotic cytokine, transforming growth factor-β (TGFβ).[29] Pulmonary αvβ6 expression is increased in IPF,[29] and higher expression is associated with increased mortality.[30] Therefore, αvβ6 is a promising prognostic marker. A novel nano single-photon emission CT imaging technique has been used to measure αvβ6 expression in a mouse model of pulmonary fibrosis, and this correlated well with other markers of fibrosis and histological αvβ6 expression.[31] This technique can quantify whole-lung αvβ6 expression in a repeatable manner and therefore could have a major impact on the diagnosis and stratification of pulmonary fibrosis if validated in humans. Furthermore, αvβ6 is an attractive therapeutic target, as it is epithelially restricted and temporally and spatially linked to the development of fibrosis.[29] The currently available antifibrotics, pirfenidone and nintedanib, do not influence αvβ6-mediated TGFβ activation,[32] which leaves this important disease pathway unexploited. STX-100, a novel humanized anti-αvβ6 monoclonal antibody, is currently undergoing investigation in a phase II clinical trial.[33]

Surfactant proteins are produced by type II alveolar epithelial cells (AECs), and raised serum levels in pulmonary fibrosis may reflect type II AEC hyperplasia or damage.[34] Serum concentrations of surfactant protein-A and surfactant protein-D have good specificity and sensitivity for interstitial lung disease (ILD) but cannot distinguish among ILD subtypes.[10,34,35] This limits their diagnostic use, although they may be useful in the early assessment of high-risk patients.[21] In addition, some mutant surfactant proteins enhance pulmonary fibrosis by inducing endoplasmic reticulum stress,[14] and treatment with 4-phenylbutyric acid has been shown to correct the processes preceding this in vitro with one form of mutant prosurfactant protein C.[36] This may represent a therapeutic approach for some genetic surfactant protein abnormalities, but it remains in the early developmental stages.

Finally, premature AEC senescence and apoptosis are key features in pulmonary fibrosis. Telomere shortening limits epithelial renewal, and short leukocyte telomere length is common and is associated with poor survival in IPF.[14,37] In addition, telomerase expression in IPF lungs is attenuated,[38] and telomerase gene mutations have been described in sporadic IPF and FPF.[16,39,40] These findings indicate a potential role for telomere length as a biomarker. No therapeutic agent reverses telomere shortening but some groups have postulated that stem cells could restore the alveolar epithelium.[41] Two small clinical trials have suggested that this approach may be feasible and safe in IPF,[41,42] but further investigation is required to clarify the role of stem cells in pulmonary fibrosis.

## Fibroblast Proliferation and ECM Remodeling

Excessive deposition and cross-linking of ECM proteins is fundamental to fibrogenesis, and molecules involved in ECM homeostasis have potential clinical applications in pulmonary fibrosis. Matrix metalloproteinases degrade ECM components and have been extensively studied in this area. Peripheral blood MMP concentrations, as well as neoepitopes generated by MMP activity, are elevated in IPF.[8,34] Therefore, MMP levels could be integrated into diagnostic models for pulmonary fibrosis, particularly for the early assessment of high-risk populations.[21]

Blood MMP7 and BAL MMP9 concentrations have been associated, with poor survival and rapidly progressing IPF, respectively.[10,43] Furthermore, higher serum concentrations of MMP-generated neoepitopes were associated with disease progression and worse survival in IPF by the PROFILE investigators.[8] These findings highlight the importance of MMP-mediated matrix degradation in IPF and the potential role of matrix

neoepitopes as prognostic markers.[8] The PROFILE study was the first to assess serial biomarker concentrations in pulmonary fibrosis and relate dynamic changes to disease progression.[8] If applied to all progressive fibrotic lung diseases, this approach may reveal useful prognostic assays for clinical practice.

Extracellular matrix deposition is the final common pathological event in pulmonary fibrosis, and targeting matrix production, or deposition, is an attractive, albeit challenging, therapeutic approach. Numerous ongoing, early-stage clinical trials of novel therapeutics are targeting these pathways in IPF[6,44] (Table 1),[33,45-59] and rational decision making, both clinically and preclinically, to determine the most effective agents in a given situation will be facilitated by the identification of biomarkers that predict therapeutic response. If effective, these agents and their companion biomarkers could be beneficial in treating other progressive pulmonary fibrotic lung diseases.

**TABLE 1** ] Novel Targeted Agents Under Investigation in Idiopathic Pulmonary Fibrosis

| Targeted Disease Pathway | Compound Name | Mechanism | Developmental Phase | ClinicalTrials.gov Study |
|---|---|---|---|---|
| Fibroblast proliferation and ECM remodeling | Lebrikizumab | IL-13 inhibitor | II | NCT01872689[45] (recruiting) |
| | Tralokinumab | IL-13 inhibitor | II | NCT01629667[46] (ongoing) |
| | SAR156597 | IL-4 and IL-13 inhibitor | II | NCT02345070[47] (recruiting) |
| | Simtuzumab | Lysyl oxidase homolog 2 inhibitor | II | NCT01769196[48] (ongoing) |
| | IW001 | Modulates immune response to type V collagen | I | NCT01199887[49] (completed) |
| | Omipalisib | Phosphatidylinositide 3-kinase and mechanistic target of rapamycin inhibitor | I | NCT01725139[50] (recruiting) |
| | Vismodegib | Hedgehog pathway inhibitor | II | NCT02168530[51] (suspended) |
| | Sirolimus | mechanistic target of rapamycin inhibitor | II | NCT01462006[52] (recruiting) |
| Fibroblast proliferation, ECM remodeling, and immune dysregulation | TD139 | Galectin-3 inhibitor | I/II | NCT02257177[53] (recruiting) |
| Alveolar epithelial cell dysfunction | STX-100 | αvβ6 integrin inhibitor | II | NCT01371305[33] (recruiting) |
| Fibroblast proliferation and alveolar epithelial dysfunction | BMS-986020 | Lysophosphatidic acid receptor antagonist | II | NCT01766817[54] (recruiting) |
| | FG-3019 | Connective tissue growth factor inhibitor | II | NCT01890265[55] (recruiting) |
| Immune dysregulation | PRM151 | Recombinant pentraxin-2 | I | NCT01254409[56] (completed) |
| | Rituximab | Anti-CD20 antibody | II | NCT01969409[57] (ongoing) |
| Infection | Cotrimoxazole | Antimicrobial | III | NCT01777737[58] (recruiting)<br>EME-TIPAC study[59] (EudraCT reference: 2014-004058-32; recruiting) |

ECM = extracellular matrix; IL = interleukin. Ongoing clinical trial information from Clinicaltrials.gov, correct as of July 2015. For a more complete review of compounds that have been assessed in clinical trials of idiopathic pulmonary fibrosis, see the reviews by Ahluwalia et al[6] and Wuyts et al.[44]

## Inflammation and Immune Dysregulation

The innate and the adaptive immune responses are integral to fibrogenesis. Epithelial injury and cell death activate the innate immune system, and the resulting macrophage population can secrete profibrotic mediators, including TGFβ and galectin-3.[6] The role of adaptive immunity in fibrosis is complex; T-helper 2 (Th2) and Th17-type responses are profibrotic, whereas Th1 and regulatory-type T cells are thought to be reparative.[6] Recent research has enhanced our understanding of these complex responses in pulmonary fibrosis.

No immune markers have diagnostic roles in pulmonary fibrosis. However, serum levels of the cytokines CCL18 and CXCL13, which influence B-cell migration and macrophage activation, respectively, are associated with increased mortality in IPF.[60,61] In addition, increased expression of some T-cell and monocyte subsets at baseline has been correlated with IPF disease progression,[62] although this finding was inconsistent. Further evidence is required before immune markers can be used routinely in clinical practice.

Whereas modulation of the immune response could be therapeutic in pulmonary fibrosis, immunosuppression has deleterious effects in IPF.[63] Other approaches being assessed in early clinical trials include PRM151, a recombinant pentraxin-2 that regulates monocyte differentiation states,[64] and TD139, a galectin inhibitor.[53,56]

## Infection

Infection may precipitate and perpetuate pulmonary fibrosis in susceptible hosts. Influenza induces αvβ6 integrin-mediated TGFβ activation, epithelial cell death, and collagen deposition in vivo.[5] In addition, increased rates of infection with several herpesviruses have been reported in pulmonary fibrosis[65,66] and increased herpesvirus DNA expression in BAL fluid has been found in asymptomatic subjects at high risk of FPF.[21] Although these studies have not established causation, herpes simplex virus-1 infection of lung macrophages can upregulate the expression of profibrotic mediators in vitro,[65] which suggests a role for viruses in fibrogenesis.

Bacterial infections may also contribute to pulmonary fibrosis. *Streptococcus* infection can exacerbate pulmonary fibrosis in mice through the expression of pneumolysin,[67] and disease progression in IPF has been associated with some *Streptococcus* and *Staphylococcus* species.[68] Furthermore, the bacterial burden in BAL fluid has been shown to be predictive of lung function decline and death in IPF.[69] These findings imply that screening for infection may have a role in predicting clinical outcomes from pulmonary fibrosis.

Although antibiotics reduced fibrosis in mice after *Streptococcus pneumoniae* infection,[67] the therapeutic role of antibiotics in pulmonary fibrosis is unclear. One trial found that the addition of cotrimoxazole to triple therapy improved survival, but it did not slow lung function decline in IPF.[70] Whether cotrimoxazole will alter disease progression in patients not receiving triple therapy, which is known to promote infection,[63] is currently under investigation (EudraCT reference: 2014-004058-32).[58,59]

## Endotyping Pulmonary Fibrosis

Although some endotypes may reflect distinct disease phenotypes,[71] the most useful endotypes will define outcomes regardless of phenotype or cause, integrating patients with different traditionally defined forms of pulmonary fibrosis into new disease groups. Endotypes depend on the samples obtained,[30,72] and each endotyping method, including lung tissue histology, BAL, and interpretation of pulmonary signals in serum, has advantages and disadvantages. Ultimately, the best method of molecular endotyping will reflect the molecular characteristics of diseased tissue, predict outcomes, be minimally invasive, and be repeatable, as endotypes are likely to change over time. This makes minimally invasive endotyping strategies, such as serum sampling and molecular imaging[31], crucial and suggests that histological endotyping will be of limited value.

The changing endotype could be vital for determining therapeutic strategies for progressive pulmonary fibrosis, especially if an endotype can be identified by a companion biomarker for one of the many therapeutic agents currently undergoing evaluation (Table 1). Figure 2 illustrates how molecular endotyping could be applied to clinical practice and support treatment decisions.

## Redefining Fibrotic Lung Diseases

The pathophysiology of progressive pulmonary fibrosis is complex, involves multiple molecular pathways, and is affected by a number of environmental factors. Individuals with the same condition according to traditional criteria may have disease resulting from distinct pathological pathways, resulting in a variable and unpredictable clinical course.[7] In addition, the same disease mechanisms can generate different clinical phenotypes, as seen in FPF in which patients with the same mutation can present with different pathologies.[7,14] Alternatively, some patients with rheumatoid arthritis-associated ILD develop a clinical

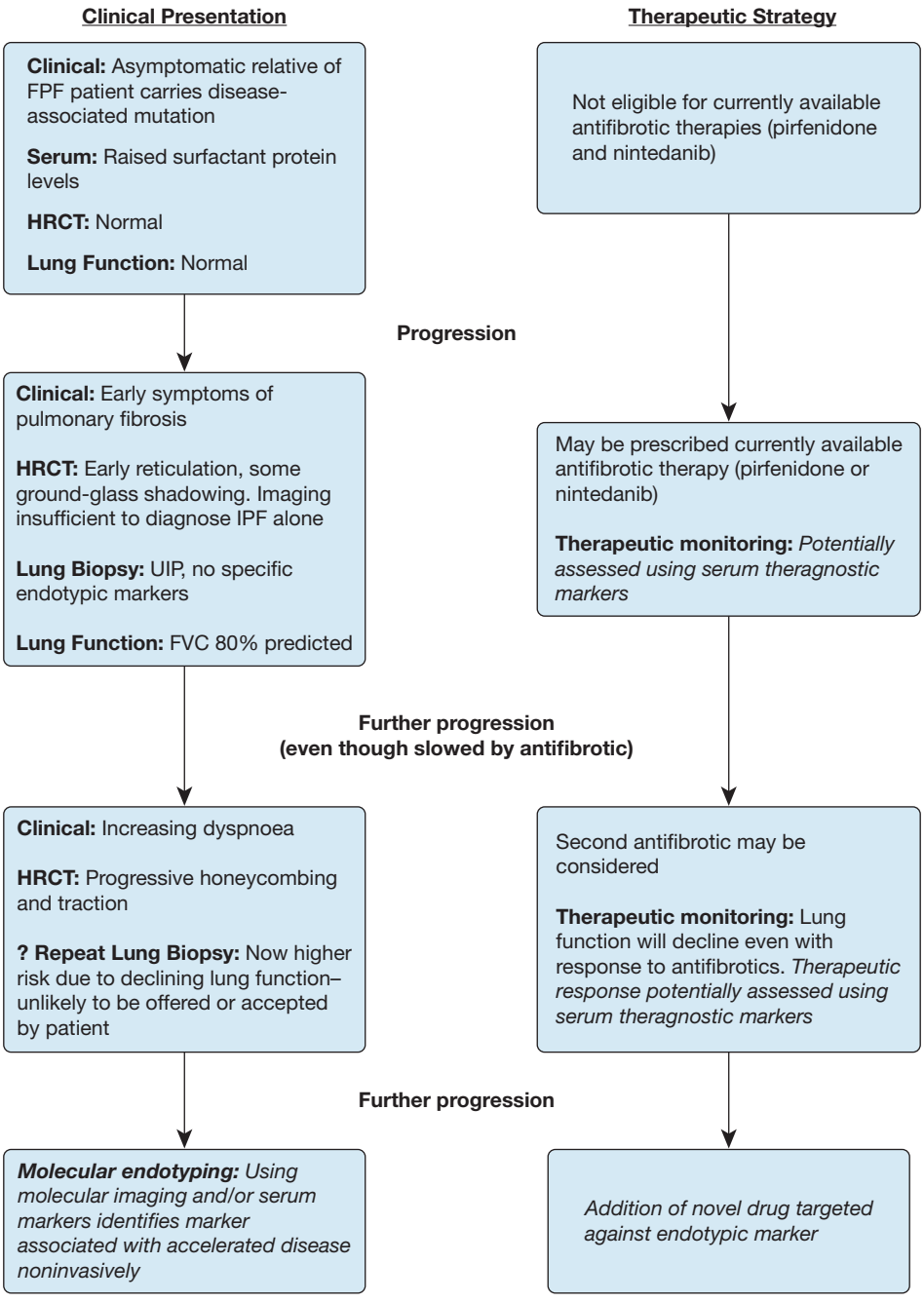


Figure 2 – *An example of an approach to endotyping in clinical practice. In this hypothetical scenario, an asymptomatic relative of a patient with FPF carries a disease-associated mutation. Molecular endotyping is used at each stage of the patient's clinical course to assess for early disease, therapeutic monitoring, and treatment selection, and to guide decisions about therapy. FPF = familial pulmonary fibrosis; FVC = forced vital capacity; HRCT = high-resolution CT scan; UIP = usual interstitial pneumonia.*

picture similar to that of IPF, with classical radiological appearances of UIP, rapid deterioration, and ultimately death.[73,74] We propose that the pathological mechanisms driving fibrosis, as determined by the molecular endotype,[30] may be better able to predict disease outcomes and reflect disease activity than currently used methods. Therefore, integration of endotyping into the diagnostic guidelines could improve assessment of these patients.

This approach is exemplified by the work of DePianto et al,[72] who identified two gene clusters with increased expression in IPF lung tissue relative to that of the control subjects but variable expression within the IPF population. Concentrations of two serum biomarkers, MMP3 and CXCL13, were related to the bronchiolar and lymphoid clusters of genes, respectively, and correlated with symptoms, lung function, and

survival.[72] These findings support the hypothesis that distinct pathological processes underlie the same condition, and may explain the heterogeneity of pulmonary fibrosis. Furthermore, this study identified a noninvasive method of assessing molecular signals within lung tissue, which is attractive for clinical practice.

Defining disease according to the molecular pathways involved will enable the stratification of patients according to the predominant disease mechanisms. This strategy may lead to more accurate predictions of prognosis and will promote the prudent use of novel targeted therapies (Table 1). Furthermore, this approach could address major questions in pulmonary fibrosis, such as which antifibrotic should be used first, how to determine disease response, and the role of combination therapies.

Molecular endotypes should be integral to the protocols of clinical trials. Genetic and protein biomarkers may be used to stratify patients, which could reveal methods of predicting therapeutic response. In addition, patients with currently unclassifiable pulmonary fibrosis could be included on the basis of their molecular endotype rather than their clinical phenotype. This would allow those with non-IPF pulmonary fibrosis to benefit from innovations emerging from IPF research.

## Conclusion

Our recent understanding of the pathophysiology of pulmonary fibrosis has increased dramatically, predominantly owing to research in IPF. These advances have generated novel techniques for the diagnosis, stratification, and treatment of fibrotic disease. However, as the mechanisms underlying these conditions become clearer, it appears that the current diagnostic criteria for progressive pulmonary fibrosis are inadequate and molecular endotyping should be integrated into diagnostic models.

## Acknowledgments

**Financial/nonfinancial disclosures:** The authors have reported to *CHEST* the following: G. J. has Sponsored Research Agreements with GlaxoSmithKline, Novartis, and Biogen Idec; he has performed consultancy for Intermune, MedImmune, Boehringer Ingelheim, Biogen Idec, and Pulmatrix; and he has received lecture fees from Intermune, MedImmune, and Boehringer Ingelheim. None declared (A. T. G.).

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