PULMONARY FIBROSIS

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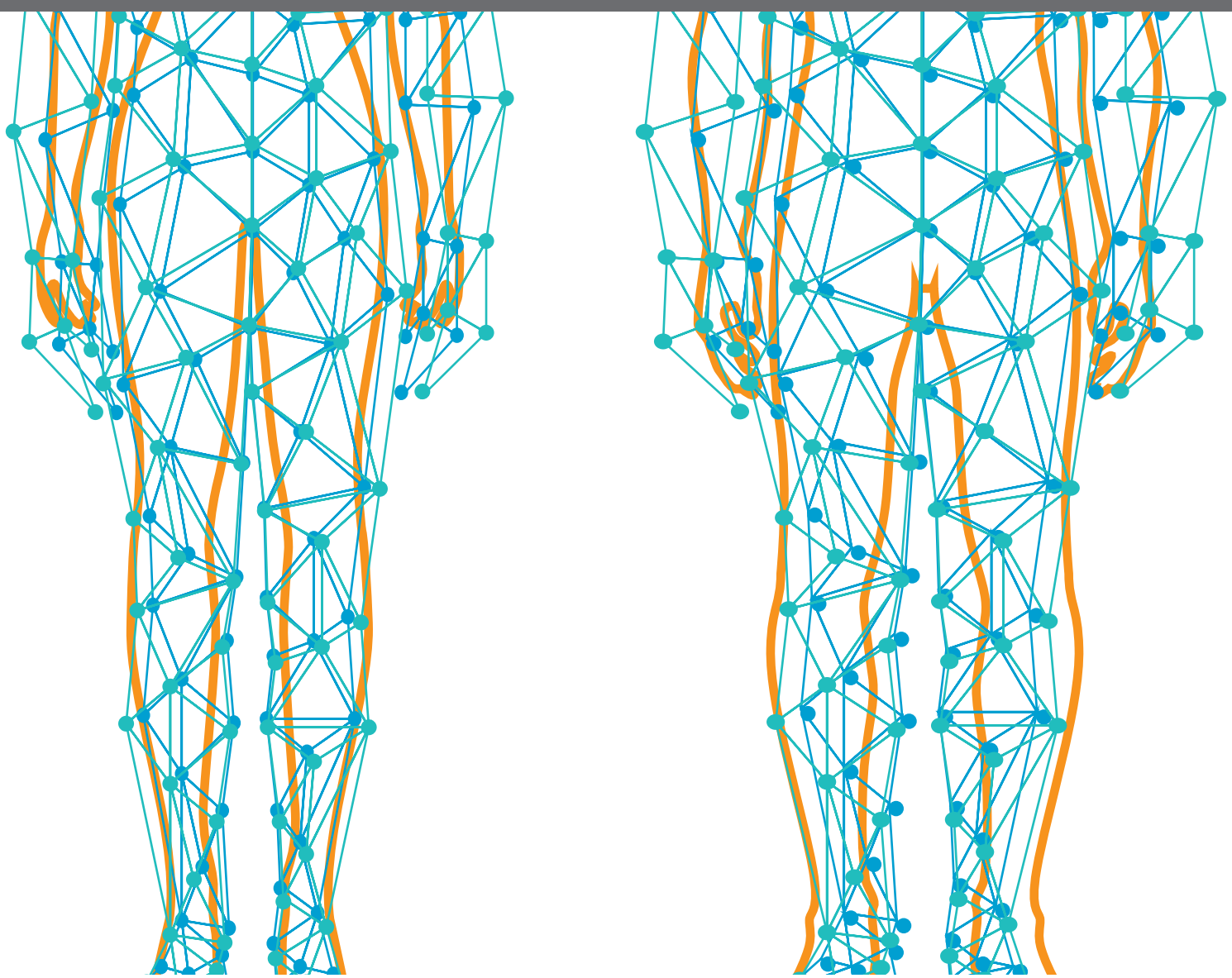

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PULMONARY FIBROSIS

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REVIEW
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Exploring Animal Models That Resemble Idiopathic Pulmonary Fibrosis

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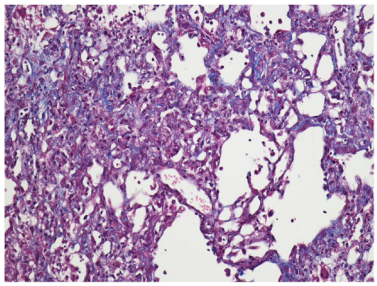
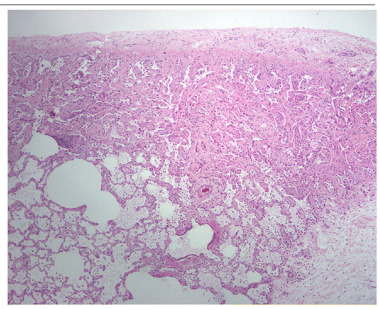
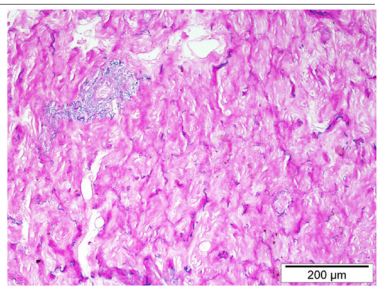
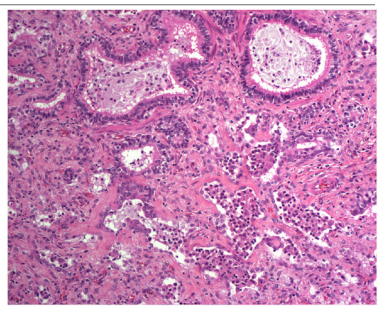
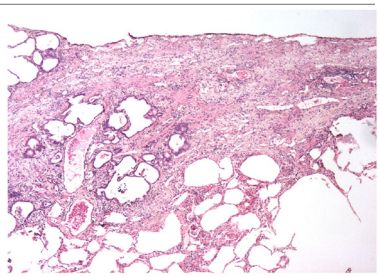
Large multicenter clinical trials have led to two recently approved drugs for patients with idiopathic pulmonary fibrosis (IPF); yet, both of these therapies only slow disease progression and do not provide a definitive cure. Traditionally, preclinical trials have utilized mouse models of bleomycin (BLM)-induced pulmonary fibrosis—though several limitations prevent direct translation to human IPF. Spontaneous pulmonary fibrosis occurs in other animal species, including dogs, horses, donkeys, and cats. While the fibrotic lungs of these animals share many characteristics with lungs of patients with IPF, current veterinary classifications of fibrotic lung disease are not entirely equivalent. Additional studies that profile these examples of spontaneous fibroses in animals for similarities to human IPF should prove useful for both human and animal investigators. In the meantime, studies of BLM-induced fibrosis in aged male mice remain the most clinically relevant model for preclinical study for human IPF. Addressing issues such as time course of treatment, animal size and characteristics, clinically irrelevant treatment endpoints, and reproducibility of therapeutic outcomes will improve the current status of preclinical studies. Elucidating the mechanisms responsible for the development of fibrosis and disrepair associated with aging through a collaborative approach between researchers will promote the development of models that more accurately represent the realm of interstitial lung diseases in humans.

Keywords: bleomycin, idiopathic pulmonary fibrosis, murine model, asbestosis, aged mice

BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is a devastating chronic lung disease, primarily affecting middle aged and older adults (1, 2). Lung function decline is gradual, with the potential for intermittent, unpredictable, acute exacerbations and the development of associated pulmonary hypertension (3). Disease diagnosis is primarily based on a typical radiology pattern (high-resolution computed tomography—HRCT) of usual interstitial pneumonia (UIP) characterized by reticulation and honeycomb cysts of subpleural and bibasilar distribution coupled with exclusion of other known causes of lung fibrosis as assessed by absence of exposures (occupational, environmental, drug), a negative immunologic profile and compatible bronchoalveolar lavage fluid (BALF) findings (i.e., absence

TABLE 1 | Selected pulmonary fibrosis conditions in animal species.

Selected pulmonary fibrosis conditions in animal species			
Species	**Model/disease**	**Features**	**Histology**
Mouse (C57BL/6J)	Bleomycin (BLM) (experimental)	Increased collagen deposition Patchy fibrosis associated with inflammatory infiltrates Resolution in young mice starting at 3 weeks	
Dog (West Highland Terrier)	Interstitial lung disease (ILD)	Septal widening and collagen deposition Normal alveolar cells	
Donkey	Chronic pleuropulmonary fibrosis	Associated with asinine herpesvirus 5 infection Pleural, subpleural, and septal fibrosis extending to interstitium Intra-alveolar fibrosis and alveolar septal elastosis	
Horses	Equine multinodular pulmonary fibrosis	Associated with equine herpesvirus 5 infection Multifocal coalescing nodules within parenchyma, centered on alveoli	
Cats	Idiopathic pulmonary fibrosis	Temporally heterogeneous fibrosis without inflammation Patchy remodeling leading to honeycomb lung in late disease	

A variety of animal species exhibit pulmonary fibrosis conditions with characteristics similar to idiopathic pulmonary fibrosis (IPF) in humans. ILD has been studied as fibrosis occurs spontaneously in many animals, including West Highland Terriers, donkeys, cats, and horses. Mice have been used as experimental models with BLM-induced pulmonary fibrosis. Histologic images are provided at 10–20× magnification on Trichrome stain to display representative characteristics and similarities. Reproduced with permission, from Dr. Paul Mercer. Clin Sci (2015) 128:235–256, © The Biochemical Society (97).

TABLE 2 | Pros/cons of animal models for studying pulmonary fibrosis.

Murine models	Pros	Cons
Bleomycin	Early molecular signature most similar to accelerated acute phase of IPF in humans	Patchy, young mice resolve spontaneously unless repeatedly doses
Silica	Good model of lung injury in humans and persistence of fibrotic lesions	Lack of reproducibility, difficult delivery, prolonged time to fibrosis, absence of usual interstitial pneumonia (UIP)-like lesions
Asbestosis	Recapitulates asbestos exposure in human lung fibrosis	Inhalation model requires at least a month for fibrosis to develop. Single intratracheal dose leads to central fibrosis rather than subpleural, unevenly distributed between lungs
Cytokine overexpressing	Ability to dissect downstream signaling events relevant to specific fibrotic-inducing cytokines	Models limited to dissecting specific pathways, rather than recapitulating the complexity of human disease
Fluorescent isothiocyanate	Relatively reproducible and persistent fibrotic phenotypes	Lack representative UIP and inflammatory infiltrates preceding fibrosis
Radiation induced	Results in fibrosis, not pneumonitis if B6 mice are used	Need to wait a long time for development of fibrosis
Familial models	Gave insight on telomere and telomerase gene involvement in IPF	May produce a susceptible phenotype, requiring a second hit
Humanized (NOD/SCID mice)	Can afford insight into role of different fibroblast populations, dissects the contribution of epithelial-fibroblast crosstalk in the absence of immune cells	May not be representative of human disease where immune cells play a role. Expensive and requires specialized housing
Domestic animals	**Pros**	**Cons**
Dogs	Usually present in middle to old age. IPF in Westies shares some features of human disease; foci with severe lesions, histological criteria more typical for UIP may be present. Spontaneously develop ILD	The diffuse interstitial lesion, present in all affected Westies, histologically resembles fibrotic NSIP in man
Cats	Anatomy of distal lung similar to humans. UIP-like disease. Spontaneously develop ILD	Strain-dependent
Donkeys	Spontaneously develop ILD	Majority of cases of APF share key pathological features with human pleuroparenchymal fibroelastosis not IPF
Horses	Spontaneously develop ILD. Overlapping features of pulmonary fibrosis including weight loss and characteristic radiologic findings	Pathology not the same as IPF

of lymphocytosis). Hallmark features of UIP include epithelial cell hyperplasia, basement membrane denudation, honeycomb cysts, and accumulation of myofibroblasts foci in a pattern with regional and temporal heterogeneity (4).

Disease pathogenesis still remains elusive and controversial. Currently, the prevailing hypothesis assumes an ineffective wound healing response to alveolar epithelial cell injury (5, 6). Injury magnitude and susceptibility appears to be related to aging and genetic predisposition, with subsequent innate immune system and fibroblast activation (3, 5, 7). The overall prognosis of patients with IPF is highly unpredictable and poor with median survival after diagnosis being approximately 3.8 years (3, 8, 9). Attempts to understand disease pathogenesis, identify prognosticators, and unravel novel therapeutic targets (10–14) have relied on animal models. Unfortunately, no animal model fully recapitulates the histologic pattern of UIP or exhibits features of progressive disease. This, however, should not underestimate the fact that animal models are essential prerequisites for the subsequent application of prognostic tests and therapeutic interventions. Numerous clinical trials have been completed based on preclinical studies in animals and have led to the FDA approval of two drugs, pirfenidone and nintedanib (15, 16). Although these drugs slow disease progression, they do not cure IPF (5, 17), thus at the best case leaving patients with significant pulmonary disability. Therefore, further studies in animal models that more closely mimic human IPF are needed to investigate potentially curative therapies. Although it is recognized that the spontaneous development of lung fibrosis in domestic animals (cats, dogs, etc.) can be informative, the most indispensable models for studies of pathogenesis and preclinical therapeutic assessment involve rodents. Many traditional and newly developed experimental models have provided us with valuable insights into disease pathogenesis and helped us to identify novel therapeutic targets to assess and validate in clinical trials (18–20). For a tabular representation of these models, see **Table 1**. This review aims to summarize current state of knowledge on animal modeling of lung fibrosis, mainly focusing on rodents, including environmental and genetic models, highlight limitations, and suggest future potentials.

MURINE MODELS

Bleomycin (BLM)

The model of BLM-induced lung fibrosis represents the most commonly applied experimental model. BLM is a chemotherapeutic antibiotic that has been identified as a pro-fibrotic agent when lymphoma patients developed pulmonary fibrosis after intravenous administration of BLM. It has been used in multiple species including mice, rats, guinea pigs, hamsters, dogs, and primates; yet, mice are most common (21). A sheep model is also currently under development (22). A recent ATS workshop report

confirmed that there is a consensus view of the intratracheal BLM model as "the best-characterized animal model available for preclinical testing" (23).

Mechanism of Action and Kinetics of Injury

It is believed that BLM acts by causing single and double-strand DNA breaks in tumor cells and thereby interrupting cell cycle leading to apoptosis. BLM hydrolase, a BLM-inactivating enzyme, majorly affects drug effects on a tissue-specific basis. The lungs maintain low levels of the enzyme, as compared to liver, and therefore are more susceptible to BLM-induced injury. An overproduction of reactive oxygen species, due to chelation of metal ions and reaction of the formed pseudoenzyme with oxygen, leads to epithelial cell death (days 1–3), excessive inflammatory infiltrates (days 3–9, neutrophils found in the BALF at day 3 and lymphocytes at day 6), and ultimately to fibroblasts activation, extracellular matrix deposition, and development of fibrosis (days 10–21 with a peak around day 14), at the molecular (24–26) and histologic (21, 24, 25, 27) levels. Relative to IPF, it has been shown that the early molecular signature in mice is most similar to the accelerated acute phase of IPF in humans (28). Measurements of alveolar septal thickening, intra-alveolar fibrosis, increases in alveolar macrophages, and dilation of bronchioles and alveolar ducts demonstrated a rather uniform fibrotic state in a large sample size (29). Nevertheless, BLM-induced lung fibrosis has been severely criticized for not being representative of IPF due to the rapidity of its development, inflammation preceding fibrosis, and self-resolution nature usually after 21–28 days following BLM challenge.

Strains, Gender, and Age

C57BL/6J mice have been the predominant animal model, as this particular strain is highly susceptible to lung injury following intratracheal BLM administration (30, 31). Conversely, the BALB/c or SV129 strains confer resistance to BLM-induced pulmonary fibrosis, presumably due to alterations in transforming growth factor (TGF)-β expression (31). This phenomenon parallels the experience in humans regarding genetic susceptibility and other potential risk factors for development of fibrosis in end organs following exposure to BLM. The majority of studies investigating BLM-induced pulmonary fibrosis to date have used young male mouse models, aged 8–12 weeks (28, 29, 32). Young mice, however, have been shown to undergo spontaneous resolution of BLM-induced pulmonary fibrosis, a phenomenon not observed in aged mice (24, 33, 34). Whether sex differences in mice parallel human IPF, which exhibits a tendency toward male predominance has not been fully determined. However, the use of aged male mice may provide a more clinically relevant model of IPF (33).

Route of Delivery and Dose Regimens

So far, BLM has been delivered by multiple methods including intratracheal, intraperitoneal, subcutaneous, intravenous, and inhalational. However, intratracheal is the most commonly route of administration (21, 24, 26, 28, 29, 32, 35–39). It is believed that intratracheal administration better recapitulates the human phenotype that is limited to the lungs. However, it requires a surgical incision at the level of the trachea, and thus, it is associated with considerable peri-operative mortality. To this end, investigators are now applying the orotracheal route of delivery that exhibits similar kinetics of injury as intratracheal administration with significantly less side effects.

Another issue identified in studies using the BLM mouse model is the wide range of dosing regimens used (40). In mouse studies, weight-based dosing is most common, beginning at 1.25 U/kg (39) and up to a maximum of 4 U/kg (35, 36). This dose is usually suspended in 50–100 μL of phosphate-buffered saline for intratracheal instillation. Peng et al. performed BLM dose-escalation experiments with mortality rates of 19% with 3 U/kg and 50% with 5 U/kg (28). A slightly lower dose of 2.0–2.5 U/kg appears to provide the most effective model of lung fibrosis, while reducing sample loss due to high mortality. With regard to frequency of dosing, Degryse and colleagues directly addressed the issue of single versus repetitive dosing to model IPF using BLM-induced pulmonary fibrosis (24). Results from their investigation in young mice found that repetitive dosing of BLM promoted persistent fibrosis, evidenced by measures of hydroxyproline content and inflammatory cell infiltrates, in contrast to single dose experiments that demonstrated spontaneous resolution in young mice (24, 34). Most studies evaluating therapeutic interventions have not used repetitive dosing; rather, the use of a single dose of intratracheal BLM is usually followed shortly by administration of the therapy under investigation (35–38). Potential therapies are usually administered within 1–7 days following BLM exposure, leading to the conclusion that the therapeutic measures may provide benefit primarily through prevention of the inflammatory cascade rather than reversal of fibrosis, thus limiting their applicability to human IPF (40). More recent studies have begun to explore administration of drugs after 7 days (41, 42). To our knowledge, only two studies to date have evaluated repetitive BLM injury (43, 44). Lee et al. administered intratracheal BLM (0.04 U) biweekly for a total of 4 months in young mice (43). They reported that in response to repeated BLM administration, mice developed hyperplasia of Club cells (Clara cells) and cuboidal alveolar epithelial cells, infiltration of the perialveolar ducts by inflammatory cells, septal thickening, enlarged alveoli, and extensive fibrosis (43).

Silica

Silica administration into murine lungs leads to the development of fibrotic nodules that resemble lesions that develop in humans following exposure to mineral fibers and particulate aerosols (45). Silica delivery presents with many variations including aerosolization, intratracheal, or orotracheal instillation (46–50). The fibrotic response is strain dependent with C57BL/6 mice found to be more susceptible than CBA/J mice after intratracheal delivery of silica fibers. Nodules develop around silica deposits and silica fibers are easily identified both by histology and polarization microscopy (47). The fibrotic response is associated with limited inflammation and enhanced fibrotic lesions mediated by increased production of pro-fibrotic growth factors and cytokines including PDGF, TGF-β, TNFa, and IL-10 (51–53). Kinetics of injury is highly heterogeneous and dependent upon route of administration, dose regimens, and formulations of

silica particles (45). In particular, intratracheal models are easier, shorter (fibrosis develops within 14–28 days), and cost-efficient, while aerosolized route of delivery takes longer to produce fibrotic lesions (40–120 days) (45). Heating preparation procedures before instillation are mandatory in order to inactivate any trace endotoxin (45). The greatest advantage of the silica model of lung fibrosis is the persistence of fibrotic lesions due to diminished clearance of silica particles from the lungs (51, 52, 54). However, the model presents with major caveats including problematic and highly expensive equipment for aerosolized delivery, prolonged waiting periods until development of fibrosis (4–16 weeks), lack of reproducibility of fibrotic pattern, and absence of characteristic UIP-like lesions such as fibroblastic foci, regional heterogeneity, and hyperplastic epithelium. These have severely limited its widespread applicability in the preclinical setting (20).

Asbestosis

Another model that recapitulates an important form of human lung fibrosis is that of asbestos exposure. Asbestos-induced model of lung fibrosis is clearly distinguished from IPF by several histologic findings including asbestos bodies embedded within the fibrous tissue, fewer myofibroblasts foci and bronchial wall fibrosis. In some cases, the pattern of UIP can be also present (55, 56). Some of these features are recapitulated in inhalation models in animals and have helped us understand the pathogenesis of both asbestosis and IPF (57–61). A single intratracheal administration of asbestos fibers mediates development of fibrosis; however, the model presents with several caveats since fibrosis that tends to be central rather than subpleural and is quite often unevenly distributed between lungs. Inhalation models develop a more peripheral pattern; yet, disease development can be prolonged, especially if using chrysotile fibers. The intratracheal animal models with amphibole fibers follow the kinetics of BLM models, with fibrosis development at day 7 and peak at day 14. Inhalation models may take up to a month for establishment of fibrotic injuries. Mechanistically, the deposition of asbestos fibers triggers fibrosis through alveolar epithelial cell apoptosis, M2 polarization of macrophages, and overproduction of pro-fibrotic cytokines by activated T lymphocytes, all events leading to myofibroblast differentiation and extracellular matrix production (57–61).

Age-Related Models

IPF is an age-related disease paradigm, and more recently, it has been proposed that many of the hallmarks of aging including genomic instability, telomere attrition, epigenetic alterations, deregulated cellular bioenergetics, and cellular senescence, can be considered characteristics of the fibrotic lung (62–64). Studies have shown that older mice are more susceptible than younger mice to pro-fibrotic stimuli including BLM (26). This is of particular interest given that IPF is predominant in older individuals. Transgenic deletion of senescence-related genes including RAGE, and relaxin has been associated with spontaneous age-dependent development of lung fibrosis indicating the cardinal role of aging in disease susceptibility (65–67). On the other hand, the role of "virome" as a pro-fibrotic mediator has been further dissected in the context of aged-related development of lung fibrosis by demonstrating that only aged mice (>15 months) develop γ-herpesvirus-68-induced lung fibrosis through a mechanism that involved alveolar epithelial cell reprogramming to produce pro-fibrotic factors and enhanced TGF-β signaling in lung fibroblasts (68). In addition, Torres-Gonzalez et al. (69) reported that aging mice receiving gamma herpesvirus responded with endoplasmic reticulum stress, apoptosis of type II lung epithelial cells, and activation of profibrotic pathways.

This evidence could be reminiscent to the presence of herpes viral genomes within IPF lungs and the epidemiological association between viral infections and IPF acute exacerbations (70).

Cytokine Overexpression

During the past two decades, more sophisticated and advanced methods have been widely used to study the features of lung fibrosis on an experimental setting. Both gene transfer *via* adenoviral or lentiviral vectors and transgenic approaches have been used to overexpress pro-fibrotic cytokines including TGF-β, TNF-α, IL-1β, and IL-13 and promote fibrotic phenotypes by dissecting downstream signaling pathways that are highly relevant to human lung fibrosis (71–73). The overexpression of TGF-β can be produced via adenoviral intranasal delivery or doxycycline-induced transgenic overexpression in CC-10-positive lung epithelial cells. Both models are strain dependent with C57BL/6 mice being more susceptible that BALB/c. In the doxycycline-inducible Clara cell (CC10)-promoter driven model of TGF-β-induced lung fibrosis, addition of doxycycline to the water of animals leads to release of the tetracycline-controlled transcriptional suppressor allowing the reverse tetracycline transactivator to bind to the transgene (TGF-β) and promote its acute expression even 12 h after treatment with doxycycline (74). That leads to alveolar epithelial apoptosis and myofibroblast accumulation leading to airway and parenchymal fibrotic response starting at day 7 and peaking at days 14–21. Fibrosis may persist and progress over the duration of doxycycline exposure for up to 2 months (74). Similar kinetics are also observed with adenoviral delivery of TGF-β through the intranasal route leading to epithelial cell apoptosis (day 1), mononuclear cell infiltration (days 3–7), and fibrotic scarring that tends to be more persistent than those produced by BLM exposure and thus tend to mimic better human disease features (71). Nevertheless, both models quite often produce highly variable and heterogeneous kinetics of injury with regards to severity and extent of lesions and lack of major reproducibility. A similar concept has been also applied for adenoviral-mediated gene transfer of IL-1β (75) and TNF-α (76, 77) or lung-specific transgenic overexpression of IL-13 (78), thereby resulting in an early inflammatory response and later collagen deposition through activation of TGF-β signaling pathway. Nonetheless, these models are not well established and thus can only be used to dissect relevant pathogenic pathways and not on a general basis to recapitulate the complexity of human disease.

Other Models

Fluorescent isothiocyanate (FITC) is another chemical compound used to induce experimental lung fibrosis (79). Fluorescein acts as a hapten and binds to airway proteins, thus

acting as a depot for prolonged exposure to the injurious stimulus leading to fibrotic responses within 2–4 weeks that persist up to 24 weeks (79, 80). The model produces relatively reproducible and persistent fibrotic phenotypes in both BALB/c and C57BL/6 mice. Disadvantages include absence of representative UIP findings and predominant inflammatory infiltrates that precede fibrosis (80). The model is mostly dependent on Th2 cytokines (IL-13) and was seminally discovered to explore the relationship between the chemokine signaling receptor 2 (CCR2) and its ligand CCL12 for recruitment of fibrocytes during progression of fibrosis (81). It offers the advantage of easily trackable fluorescence-labeled fibrotic tissues. Nevertheless, model robustness is largely dependent on technical issues that are highly variable including the batch of FITC and the size of the particles formulated through sonication (20). Smaller particle sizes due to prolonged sonication may lead to acute toxicity and death (20). Finally and most important, FITC is an artificial chemical compound with limited human relevance since this type of injurious stimulus has never been described in humans (20).

Radiation-induced fibrosis represents a human relevant injury that leads to development of fibrosis which is strain dependent (C57BL/6 are the most susceptible) and can be local or systemic if other organs are not shielded (82–87). It is a relatively slow procedure that results in mature fibrosis after 24 weeks; yet, fibrosis is majorly dependent on inflammation and free-radical-mediated DNA damage and less on TGF-β (84).

Familial models of IPF have been also used to study the contribution of the disease genetic background that significantly altered our perspective regarding disease pathogenesis and treatment response. Mice with targeted deletion of shelterin, a six-protein complex that binds and preserves telomeric repeats, from type alveolar epithelial cells, have been shown to develop spontaneous fibrosis (88, 89).

Mutations in the telomere and telomerase genes have been associated with familial IPF (90). Telomere dysfunction results in alveolar epithelial stem cell senescence, which is sufficient to drive lung remodeling and recruit inflammation. Telomerase reverse transcriptase has been reported to be transiently increased in BLM, hypoxic, or silica-induced lung injury (91–93). On the other hand, telomerase-deficient mice, despite significant telomere shortening, did not present with enhanced BLM-induced fibrotic responses (94).

Although mutations resulting in SP-C deficiencies are linked to a small subset of spontaneous and familial cases of interstitial lung disease (ILD) and interstitial pulmonary fibrosis (95, 96), SP-C-deficient mice do not fully recapitulate familial interstitial pulmonary fibrosis (97) as they develop mild ILD and an emphysematous phenotype. It is more than evident that these mutations may generate a susceptible phenotype; yet, a second hit of environmental origin is needful to partially recapitulate human phenotype.

Finally, humanized models of lung fibrosis involving the intravenous instillation of human IPF lung fibroblasts into immunodeficient non-obese diabetic mice (NOD/SCID) have recently garnered much attention (98–100). This model allows for cell trafficking during different stages of fibrosis development and progression, offers unique insights into different fibroblast populations that reflect IPF heterogeneity, and dissects the contribution of epithelial–fibroblast crosstalk into the disease pathogenesis considering the absence of immune cells (99). Nevertheless, the latter is not representative of human disease where immune cells appear to play cardinal role. Another major disadvantage that limits its widespread applicability is the high cost and the specialized housing that is required (101). The use of animals with humanized immune system may also provide unique insights and fully recapitulate features of IPF (101).

DOMESTIC ANIMALS

The field of comparative oncology has set the stage for collaborations that utilize spontaneous models of progressive fibrotic lung diseases of mutual interest to veterinary and human medicine. The results of these kinds of studies promise to enhance the understanding of common factors important to disease development in a variety of species and to refine treatments for both humans and animals. Moreover, they may provide insights into unanswered questions involving naturally occurring models of pulmonary fibrosis.

In contrast to the six million dogs and cats that develop cancers, the incidence and prevalence of pulmonary fibrosis in animals is not known (102). West Highland Terriers (Westies), cats, donkeys, and horses develop ILD (102–107). There is limited information on the spectrum of clinical parameters (e.g., radiology) and pathology of these lung diseases leading to the classification of "idiopathic pulmonary fibrosis" being applied to such cases, without using the same strict clinical criteria that have been developed for human IPF. Recent evidence suggests that in contrast to IPF in humans, applying the term "idiopathic" in animals may be premature because of more non-specific features in lung interstitial disease in animals. The American Thoracic Society/European Respiratory Society definition of IPF incorporates histology, radiographic, and clinical course in the definition and the exclusion of other known causes of ILD, including environmental exposures, connective tissue disease, and drug toxicity (108). Further studies using multidisciplinary classification of veterinary lung disease to better characterize the disease in animals will help to define their relation to human disease and their potential role as models to develop treatments for both human and veterinary medical practice.

A study on Westies (109) found that the majority of dogs with IPF showed multifocal areas of accentuated subpleural and peribronchiolar fibrosis with occasional "honeycombing" and profound alveolar epithelial changes, reminiscent of human UIP and not commonly seen in NSIP. Interstitial fibroblastic foci, characteristic of UIP, were not seen in WHWTs with IPF. Progressive fibrosis, with intra-alveolar organizing fibrosis alongside interstitial mature collagen deposition, was present within the more severely affected areas of lung in WHWTs with IPF. Severe pulmonary lesions were seen more commonly in the caudal than in the cranial lung lobes.

A more recent study correlating CT scans and course of disease in Westies found a generalized ground-glass pattern was determined to be a sign of a mild form of canine idiopathic pulmonary fibrosis, whereas mosaic ground-glass and mild

honeycombing patterns was identified in moderate and severe forms of the disease (110).

The ubiquitous gammaherpesvirus equine herpesvirus 5 (EHV 5) has been detected in lung tissue from horses that develop progressive pulmonary fibrosis and is now considered to be the likely cause of this disease in these animals (111). The pathology of this disease is distinct from human IPF, demonstrating multiple nodules and is therefore termed equine multinodular pulmonary fibrosis (111). Although the pathology is not the same as IPF, there are striking overlapping features including weight loss and gradual exercise intolerance, accompanied by characteristic radiologic features (111). Temporal heterogeneity or fibroblast foci, hallmarks of human disease, are not present in the disease in horses, though these characteristics have been described in feline pulmonary fibrosis (111, 112). Similar to EBV in humans, which has been associated with IPF, EHV 5 is a ubiquitous subclinical gammaherpesviral infection in horses (113). Considered largely non-pathogenic in the natural host, some strains of EHV5 appear to be pathogenic and capable of inducing lung fibrosis (103). While EHV5 was isolated from horses with spontaneous disease, the virus was not isolated from dead inoculated horses that developed lung fibrosis (111). This model raises interesting questions regarding induction of lung fibrosis by EHV 5 during viral latency versus lytic infection.

For a tabular representation of the overall advantages and disadvantages of each model, see **Table 2**.

Read-Out Assays for Assessment of Fibrotic Injury

Each experimental model presents with its own kinetics of fibrotic injury; however, investigators have applied standard operating procedures for reproducible evaluations of lung fibrosis. In view of the pathologic hallmarks of IPF, appropriate read-out assays include assessment of collagen deposition, alveolar epithelial cell apoptosis, and BALF complemented by survival analysis and respiratory mechanics. These are achieved with the following modalities: (1) histological analysis with Masson trichrome and H&E staining coupled with Aschroft score that quantifies extent of fibrotic changes, (2) hydroxyproline or total collagen content for quantification of lung collagen deposition, (3) TUNEL assay for the identification of apoptotic cells, (4) BALF analysis to assess changes in differential cell count and levels of inflammatory and fibrotic markers, (5) survival analysis with Kaplan–Meier plots, (6) *in vivo* lung function measurements (elastance and compliance) using the Flexi-vent ventilator, and (7) micro-CT imaging which provides state-of-the art multidimensional imaging of the injured lung that is reminiscent of HRCT applied for IPF diagnosis (19).

Limitations

The past 35 years more than 500 experimental studies have been performed describing therapeutic efficacy of novel compounds in the BLM model. Unfortunately, less than 5% have applied a therapeutic protocol indicated by drug administration at >7 days following BLM challenge (18, 114). Even day 7 in most of the experimental models represents a stage of inflammation or early fibrosis, evidence that comes in contrast to the clinical situation in which treatment is initiated after onset of symptoms and when fibrosis has already been established. Intriguingly, pirfenidone and nintedanib received approval to proceed to clinical trials based on preventive protocols or even therapeutic protocols targeting the inflammatory or the early-fibrotic phase of the BLM model (115–117). In addition, most of the therapeutic compounds have been preclinically tested in young animals while it has been clearly shown that aged mice are more susceptible to fibrotic injury (26), which is in accordance with patients with IPF. Importantly, preclinical efficacy of the majority of anti-fibrotic agents was tested in a single model, majorly the BLM-induced model, and based on histologic end points, such as collagen deposition that are not clinically relevant, at least to the extent of lung function tests or survival analysis. Moreover, many of the therapeutic outcomes were subject to evaluation bias considering that most of the preclinical studies were not blinded and the investigator was aware of the animals' treatment. Reproducibility issues arising from different experimental settings between different labs could also account for discrepancies in treatment effects and lack of generalizable results. Finally, it is worth mentioning that animal size needs to be balanced with the statistical power needed to generate robust data and that insufficient reporting of experimental animal data or unpublished negative therapeutic results severely hamper the validity of experimental studies.

Conclusion and Personal View

An animal model is a simple representation of a complex biology system. Critics focusing on the reasons why an animal model cannot fully reproduce human disease are not helpful and do not elicit a solution. The role of an animal model is to recapitulate specific aspects of a disease. Consequently, animal models should be carefully selected, designed, and conducted in order to bridge translational gaps between bench and bedside. Currently, the BLM model of lung fibrosis represents the cheapest, easiest, fastest, most reproducible, and thus most extensively used animal model of IPF; advantages that overcome the handicap of minimal representation of human disease. So far, it has provided us with invaluable insights into IPF pathogenesis, prognosis, and treatment. We recommend the use of the male aged BLM mouse model as the first-line animal model to test safety and efficacy of a therapeutic compound administered during the stage of established fibrosis. Nevertheless, efficacy preclinical studies should be enriched with two or even three animal models including clinically relevant end points such as lung function mechanics, survival analysis as well as biomarkers (28). Collaboration between veterinary and human clinical researchers must be encouraged in order to establish and solidify a common language, and common diagnostic criteria and nomenclature, thus strengthening the opportunity for advancements toward a cure for lung fibrosis in both animal and humans. Humanization of animal models, spontaneous fibroses animals and application of high-throughput "omics" tools may help us improve the clinical translation in the near future.

AUTHOR CONTRIBUTIONS

Conception and study oversight: MG, AL, VA, and AT. Drafting manuscript: JT, GR, KW, SJ, and IN. Critical revision of manuscript: MG, AL, VA, and AT. Final approval: all authors.

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REVIEW
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Comorbid Conditions in Idiopathic Pulmonary Fibrosis: Recognition and Management

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Idiopathic pulmonary fibrosis (IPF), a fibrosing interstitial pneumonia of unknown etiology, primarily affects older adults and leads to a progressive decline in lung function and quality of life. With a median survival of 3–5 years, IPF is the most common and deadly of the idiopathic interstitial pneumonias. Despite the poor survivorship, there exists substantial variation in disease progression, making accurate prognostication difficult. Lung transplantation remains the sole curative intervention in IPF, but two anti-fibrotic therapies were recently shown to slow pulmonary function decline and are now approved for the treatment of IPF in many countries around the world. While the approval of these therapies represents an important first step in combatting of this devastating disease, a comprehensive approach to diagnosing and treating patients with IPF remains critically important. Included in this comprehensive assessment is the recognition and appropriate management of comorbid conditions. Though IPF is characterized by single organ involvement, many comorbid conditions occur within other organ systems. Common cardiovascular processes include coronary artery disease and pulmonary hypertension (PH), while gastroesophageal reflux and hiatal hernia are the most commonly encountered gastrointestinal disorders. Hematologic abnormalities appear to place patients with IPF at increased risk of venous thromboembolism, while diabetes mellitus (DM) and hypothyroidism are prevalent metabolic disorders. Several pulmonary comorbidities have also been linked to IPF, and include emphysema, lung cancer, and obstructive sleep apnea. While the treatment of some comorbid conditions, such as CAD, DM, and hypothyroidism is recommended irrespective of IPF, the benefit of treating others, such as gastroesophageal reflux and PH, remains unclear. In this review, we highlight common comorbid conditions encountered in IPF, discuss disease-specific diagnostic modalities, and review the current state of treatment data for several key comorbidities.

Keywords: idiopathic pulmonary fibrosis, idiopathic interstitial pneumonia, interstitial lung disease, pulmonary fibrosis, co-morbidity

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INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF), a fibrosing interstitial lung disease (ILD) of unknown etiology, primarily affects older adults and leads to a progressive decline in lung function and quality of life (1–4). With an estimated prevalence of 18–63 cases per 100,000 and median survival of 3–5 years (5, 6), IPF remains the most common and deadly of the idiopathic interstitial pneumonias (IIPs).

Despite poor survivorship, there exists substantial variability in disease progression, whereby most patients experience a steady clinical decline, some remain stable over many years and others die from rapidly progressive disease (3, 7, 8). Lung transplantation remains the sole curative intervention for IPF, but two anti-fibrotic therapies were recently shown to slow pulmonary function decline in phase III clinical trials (9–11). *Post hoc* analyses of clinical trial datasets also suggest that anti-fibrotic therapy may reduce the risk of acute exacerbations and improve overall survival in those with IPF (12–14).

While the identification of therapies that effectively slow IPF progression represents a monumental step forward in the care of patients with IPF, pharmacotherapy is but one component of the multi-pronged approach necessary to optimally manage patients with IPF. Other equally important pieces include evidence-based prognostication (15) improvement of functional status with formal pulmonary rehabilitation and supplemental oxygen (where appropriate), patient education with regard to IPF pathobiology, natural history, and clinical trial availability, and management of common comorbidities (4, 16). In this review, we highlight common comorbidities encountered in IPF, discuss diagnostic and screening modalities, and review the current state of treatment data for such conditions.

PULMONARY COMORBIDITIES

Emphysema

Roughly 70–80% of individuals with IPF endorse a history of cigarette smoking, which has long been an established IPF risk factor (17–20). Not surprisingly, about 30% of IPF patients have concurrent pulmonary emphysema (21, 22), including 8–27% with ≥10% emphysematous involvement throughout the lungs (**Figure 1**) (22, 23). The syndrome of combined pulmonary fibrosis and emphysema (CPFE) has recently gained recognition as a potentially unique IPF phenotype (24). Individuals with CPFE tend to be males with an extensive smoking history and increased oxygen requirement (21–24). Pulmonary function testing in these individuals often shows relatively preserved total lung capacity and forced vital capacity (FVC), with a disproportionate reduction in diffusion capacity (DLCO) (**Figure 2**) (21, 22, 24). These physiologic hallmarks of CPFE likely reflect the opposing impact of parenchymal fibrosis and parenchymal destruction on airflow and lung volumes, along with additive impact on gas exchange.

High-resolution computed tomography (HRCT) is part of the routine diagnostic evaluation of all patients with suspected IPF (4), and routine semi-quantitative assessment of emphysematous involvement may help readily identify those with CPFE once a diagnostic consensus is established. The recognition of CPFE has potential management implications. Some studies suggest that CPFE is associated with reduced survival (21, 23, 25), but others have not replicated this observation (22, 26). Paradoxically, patients with CPFE appear to have a slower rate of FVC decline, perhaps due to the impact of emphysema on the manner in which FVC reflects progressive fibrosis (27). An increased prevalence of pulmonary hypertension (PH) has also been demonstrated among those with CPFE (22, 23), which may also impact survival (23, 24). Treatment of patients with IPF and CPFE with inhaled long-acting anti-cholinergic, inhaled long-acting beta-agonist and/or inhaled corticosteroids is of unclear benefit (21, 24). We believe that clinicians should consider the addition of these therapies according to chronic obstructive pulmonary disease consensus guidelines (28).

Lung Cancer

Compared to those in the general population, individuals with IPF have a nearly 5-fold increased risk of developing lung cancer, with 3–22% of patients affected and an estimated incidence of 11 cases per 100,000 person-years (29–32). The annual lung cancer risk also appears to rise in the years following IPF diagnosis (32), which was supported by an autopsy study that identified concurrent pulmonary malignancy in nearly 50% of cases with histologic UIP (33). The strong link between IPF and cigarette smoking history (17) likely explains a portion of the increased lung cancer risk, as the overwhelming majority of patients with IPF who develop lung cancer endorse such a history (32–34). The higher prevalence of lung cancer among those with CPFE compared to lone IPF also supports this observation (34–36).

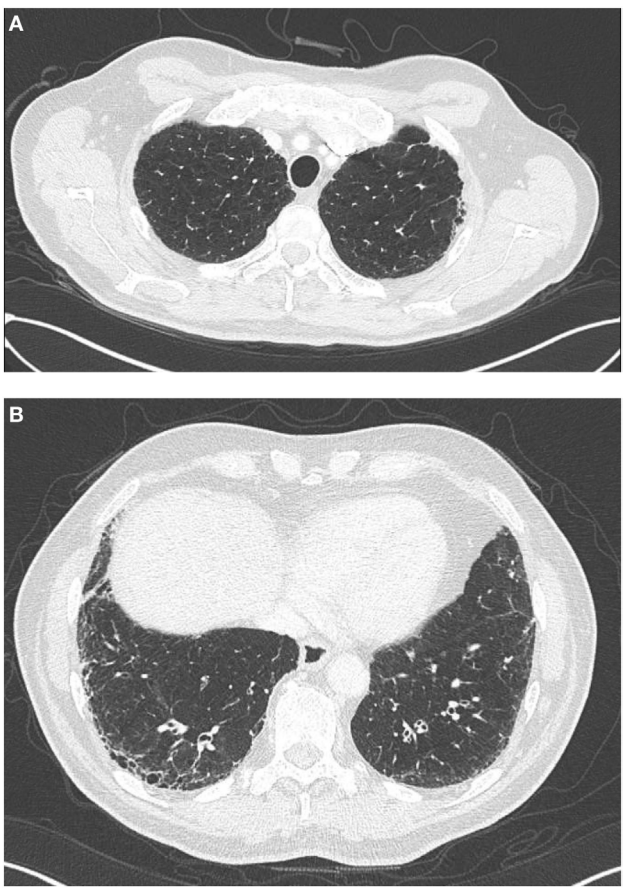
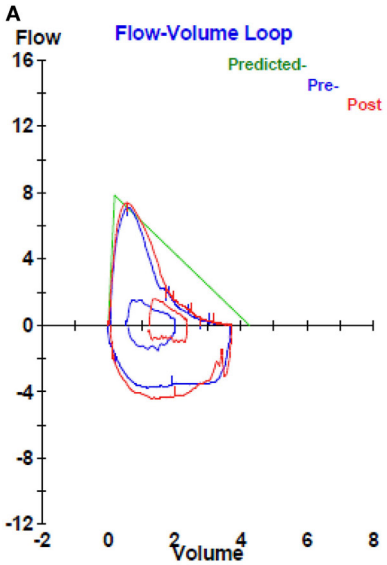


FIGURE 1 | High-resolution computed tomography findings in a patient, with combined pulmonary fibrosis and emphysema. Centrilobular emphysema is observed on apical views **(A)** and basilar predominant sub-pleural reticulation and honeycombing characteristic of UIP is observed on basilar views **(B)**.



B

		Ref	C.I. (95)	Pre	% Ref	Post	% Ref
Spirometry							
FVC	Liters	4.27	(3.3 - 5.2)	3.70	87	3.74	87
FEV1	Liters	3.09	(2.3 - 3.9)	2.44	79	2.57	83
FEV1/FVC	%	72	(62.7 - 82.0)	66		69	
FEV3	Liters			3.07		3.19	
FEV3/FVC	%			83		85	
FEV6	Liters	4.01	(3.1 - 4.9)	3.41	85	3.52	88
FEV1/FEV6	%	77	(67.9 - 85.8)	72		73	
FEF25-75%	L/sec	2.22	(0.6 - 3.8)	1.16	52	1.42	64
PEF	L/sec	7.82	(5.5 - 10.2)	7.13	91	7.46	95
FET100%	Sec			12.30		11.16	
FIVC	Liters			3.71		3.67	
PIF	L/sec			3.75		4.40	
Lung Volumes							
TLC	Liters	7.14	(5.5 - 8.7)	5.77	81		
VC	Liters	4.27	(3.3 - 5.2)	3.73	87		
IC	Liters	2.95	(2.2 - 3.7)	2.35	80		
FRC PL	Liters	3.84	(2.4 - 5.3)	3.42	89		
ERV	Liters	1.47	(1.1 - 1.8)	1.38	94		
RV	Liters	2.60	(1.8 - 3.4)	2.04	78		
RV/TLC	%	38	(28.7 - 46.3)	35			
Diffusing Capacity							
DLCO	mL/mmHg/min	26.4	(19.5 - 33.3)			4.9	18
DL Adj	mL/mmHg/min	26.4	(19.5 - 33.4)			4.9	18
DL/VA Adj	mL/mHg/min/L	3.66				1.03	28
VA	Liters	7.22	(6.1 - 8.4)			4.75	66

FIGURE 2 | Pulmonary function testing in a patient with combined pulmonary fibrosis and emphysema. Flow volume loop **(A)** shows mild obstructive morphology, but normal spirometry **(B)**. Lung volumes **(B)** are normal. Diffusion capacity **(B)** is markedly reduced.

Most studies show squamous cell carcinoma to slightly predominate over adenocarcinoma (37), while a recent investigation of IPF-related adenocarcinomas demonstrated a high frequency of bronchiole-associated markers in IPF cases compared to non-IPF controls, suggesting that these tumors may arise from abnormally proliferating bronchioles in areas of honeycomb cyst (38).

Survival among those with IPF and comorbid lung cancer is poor (34, 39) and often stems from malignancy-related clinical deterioration, as similar rates of pulmonary function decline have been demonstrated in those with and without comorbid lung cancer (34). While surgical resection of early stage lung cancer may be curative, IPF severity and disease trajectory must be taken into consideration, given an increased risk of postoperative morbidity and mortality (40, 41). Surgical resection of lung cancer appears to increase the risk of acute exacerbation in patients with IPF, which has been reported in 7–32% of patients (42–45). Acute exacerbation and clinical deterioration have also been described in patients with IPF undergoing treatment with chemotherapy (46, 47) and radiation therapy (48), underscoring the importance of patient education and risk/benefit analysis in those with inoperable lung cancer. Recent studies suggest that the anti-proliferative effects of pirfenidone and nintedanib may synergize with current chemotherapeutic regimens, but additional research is needed (37, 49, 50).

As for emphysema, HRCT serves as a reasonable modality for lung cancer screening, but many HRCT protocols still perform non-contiguous imaging, which can miss early nodules and other local changes; contiguous imaging is required (4). Most lung cancers identified are incidental, with a large minority having a potential surgical cure (34, 39). As such, we believe clinicians should consider annual lung cancer screening with low-dose computed tomography (51) in high-risk patients with IPF, including those with CPFE and/or longstanding smoking history. Clinicians should also maintain a low threshold for repeat imaging in those who experience clinical worsening despite stable pulmonary function or develop symptoms atypical for IPF.

Obstructive Sleep Apnea (OSA)

Preliminary studies suggest that OSA may be an underappreciated comorbid condition in those with IPF. Three investigations have shown OSA to be present in 58–88% of patients with IPF, with up to 68% having moderate-to-severe OSA based on an apnea–hypopnea index ≥15 events/hour (52–54). In addition, severe sleep apnea was recently shown to be strongly associated with ischemic heart disease in patients with IPF (54). Despite this potential high prevalence, few IPF patients are evaluated for OSA. A retrospective investigation of IPF patients showed that less than 3% of patients were referred for polysomnography (55). Untreated OSA can result in nocturnal hypoxemia, the presence of which was recently shown to predict worsened survival in patients with IPF (56). Nocturnal hypoxemia strongly correlates with an increased right ventricular systolic pressure (RVSP) (56), which may reflect PH (57).

As with the general population, moderate-to-severe OSA is generally treated with continuous positive airway pressure (CPAP). CPAP initiation has been shown to improve quality of life measures and sleep instruments in those with IPF and comorbid moderate-to-severe OSA, though CPAP non-adherence was

common (58). The optimal tool for OSA screening in patients with IPF has yet to be determined, as commonly utilized OSA screening tools, including the Epworth sleepiness scale and Sleep Apnea Scale for Sleep Disorders Questionnaire, did not differentiate IPF patients with and without OSA in a recent prospective investigation (52). Until an effective screening tool is established, clinicians should maintain a low threshold for polysomnography referral in patients with IPF.

CARDIOVASCULAR COMORBIDITIES

Coronary Artery Disease (CAD)

Idiopathic pulmonary fibrosis and CAD share several risk factors, including increasing age, male gender, and smoking history. Among the largest retrospective studies conducted to date, the estimated prevalence of CAD in those with IPF ranges from 4 to 25% (59–63). A prevalence of up to 68% was described in a cohort of 73 IPF patients who underwent cardiac catheterization as part of a lung transplant work-up (64). Among these individuals, 18% of patients had significant CAD, defined as >50% stenosis of a major coronary vessel on cardiac catheterization or need for percutaneous coronary intervention. Longitudinal analyses have suggested that 7% of patients will develop CAD in the years following IPF diagnosis (63) and that such patients have a 3-fold higher risk of experiencing acute coronary syndrome compared to non-IPF control subjects (61).

The U.S. Preventative Services Task Force concluded that there was insufficient evidence to recommend for or against routine CAD screening in asymptomatic, high-risk individuals in the general population (65). Among symptomatic patients, the American Heart Association suggests that cardiac CT may be a reasonable modality for CAD screening, as the presence of coronary calcifications is a reliable predictor of CAD (66). Because HRCT is recommended for all patients with IPF, the assessment of coronary artery calcification may provide a reliable tool for non-invasive CAD screening in this high-risk population. A study of 57 patients with IPF who underwent cardiac catheterization showed that the presence of moderate-to-severe coronary calcifications had a sensitivity and specificity of >80% for detecting CAD (67). Because significant CAD has been associated with worse survival in patients with IPF (64), clinicians should consider a cardiology referral in patients with angina or moderate-to-severe coronary calcifications on HRCT.

Pulmonary Hypertension

Pulmonary hypertension defined as mean pulmonary artery pressure (mPAP) ≥25 mm Hg (68), commonly complicates IPF, especially as the disease progresses. The true prevalence of PH in those with IPF is difficult to ascertain, as estimates vary widely based on case finding methodology and the IPF population under consideration. A PH prevalence as low as 3% has been reported in patients with IPF using insurance claims data (62) and as high as 84% using transthoracic echocardiogram (TTE) (69). A prevalence of 29–46% has been reported in studies utilizing right heart catheterization, which remains the gold standard for PH detection (60, 68, 70–75). However, these studies may overestimate the true prevalence, as the majority of patients included in these studies underwent right heart catheterization as part of a lung transplant evaluation, suggesting that many had advanced disease.

Pulmonary hypertension should be suspected in patients with dyspnea or oxygen desaturation out of proportion to pulmonary function, disproportionately low DLCO, evidence of right heart failure on physical exam, or evidence of pulmonary artery enlargement and/or right ventricular hypertrophy on imaging studies. TTE, which estimates RVSP as a surrogate for mPAP, is perhaps the most commonly utilized modality to screen for PH. Although TTE-estimated RVSP has been shown to correlate poorly with mPAP determined by right heart catheterization (73, 76), an RVSP >35 mm Hg has been shown to have a sensitivity of >85% for detecting PH in patients with IPF (73). Unfortunately, the specificity of this RVSP cut-off is only 29%, so clinicians should expect a large number of false positives if using this threshold for triggering right heart catheterization. The decision to refer a patient for cardiac catheterization when TTE suggests the presence of PH should be made on a case-by-case basis.

There are currently no approved therapies for the treatment of PH in patients with IPF and the last decade has seen a disappointing number of negative clinical trials using vasodilator therapies. Several studies have investigated the use of PH therapies for IPF in general (regardless of the presence of PH) and failed to demonstrate efficacy in slowing IPF progression (77–79), and did not alter cardiovascular hemodynamics in those with concurrent PH (80, 81). A small trial of ambrisentan in patients with IPF and right heart catheterization-proven PH (NCT00879229) was stopped after a parent trial of ambrisentan showed no benefit in the subgroup of IPF patients with known PH (78). A similar trial of riociguat (NCT02138825), a soluble guanylate cyclase stimulator, was also terminated after interim analysis showed that those in the intervention arm had an increased risk of death and other serious adverse events.

The phosphodiesterase-5 inhibitor sildenafil was studied in patients with advanced IPF (defined by a baseline DLCO of less than 35%). While it did not significantly alter the primary functional endpoint of walk distance, it did show improvements in dyspnea score, oxygenation, and quality of life (82). A *post hoc* subgroup analysis of patients with evidence of PH by TTE showed that sildenafil therapy did improve walk distance as well (83). Based on these data, clinical trials investigating sildenafil in combination with anti-fibrotic therapy for patients with IPF-associated PH (NCT02951429, NCT02802345) are currently enrolling. The most recent evidence-based guidelines conditionally recommend against the routine use of sildenafil in patients with IPF until randomized controlled trials provide more definitive data (16).

Pulmonary Embolism (PE)/Venous Thromboembolism (VTE)

Relatively few studies have assessed the epidemiology and clinical consequences of PE and more broadly VTE in patients with IPF. A study utilizing U.S. insurance claims data suggested that 2.7% of individuals with a diagnosis code for IPF also carried a diagnosis of PE (62). Another U.S. insurance claims-based investigation

showed that among decedents with a diagnosis of IPF, 1.7% had concurrent VTE (84). These estimates were supported by a case–control analysis conducted in the U.K., which reported a VTE prevalence of 2% in their IPF population, which was 2-fold higher than that observed among non-IPF control subjects (61). Danish investigators showed that individuals previously diagnosed with a VTE were at increased risk of developing incident IIP, suggesting that VTE may be a risk factor for IPF and other IIPs (85).

Longitudinal analyses of patients with IPF suggest that the risk of incident VTE is 3–6 times higher among patients with IPF compared to control subjects, with an estimated 6–9 new events per 1,000 person-years (61, 86). As such, clinicians should maintain a low threshold for PE evaluation in patients with progressive symptoms in the setting of stable pulmonary function metrics. PE should also be excluded in patients with acute or subacute clinical worsening, as this often indicates an acute exacerbation (87). A multi-phase HRCT with and without contrast enhancement should be considered to optimally assess the pulmonary vasculature and parenchyma. Lower extremity duplex ultrasound can be considered for patients with a contrast allergy and in those too unstable to undergo HRCT.

The treatment of PE/VTE requires prolonged blockade of the coagulation cascade, which facilitates clot resolution. The American College of Chest Physicians recommend 3 months of anticoagulant therapy in those with VTE provoked by surgery or other known VTE risk factor. These guidelines also recommend at least 3 months of anticoagulant therapy in patients with a first-time unprovoked VTE, after which time the risk–benefit ratio for extended therapy should be considered (88). This assessment is of particular importance in patients with IPF, as warfarin therapy was shown to increase the risk of death in a general population of IPF patients (excluding those who required anticoagulation for a non-IPF indication) (89). Warfarin therapy has also been linked to worse outcomes in uncontrolled studies, including a recent *post hoc* analysis of IPF clinical trial datasets (90, 91). These findings raise the question of whether warfarin therapy should be used in patients with IPF who require anticoagulation for comorbid diseases (e.g. PE, atrial fibrillation). More research is needed to determine the optimal therapy and duration of therapy for patients with IPF with an indication for anticoagulation.

GASTROINTESTINAL COMORBIDITIES

Gastro-Esophageal Reflux (GER)

Gastro-esophageal reflux is another common comorbid condition in patients with IPF (92), but the true prevalence of GER in patients with IPF is difficult to ascertain. Several large epidemiological studies have suggested a prevalence of 30–50% (19, 93, 94), but studies that utilized esophageal pH monitoring suggest that GER may affect over 80% of individuals with IPF (95, 96). Complicating estimates further is the fact that some individuals have silent GER (95) and other primarily non-acid GER (97).

The ideal modality for diagnosing GER remains unclear. While all patients should be screened for GER-associated symptoms, including heartburn, choking, and regurgitation, symptom-based screening has a low sensitivity for detecting pathologic GER (95, 98). Fluoroscopic barium swallow testing can detect GER and microaspiration, but this also suffers from poor sensitivity (99, 100). Gastro-esophageal scintigraphy can detect GER with 80% sensitivity but is not a widely available (101). Esophageal pH monitoring remains the gold standard for diagnosing acid GER, with a reported sensitivity and specificity of over 80% (102, 103). Recent studies suggest that multi-channel intraluminal esophageal impedance testing may be a superior modality for detecting both acid and non-acid GER, but this modality is not widely available at present (103, 104).

It has been hypothesized that GER may contribute to the progression of IPF in some patients. Several studies, with mixed results, have explored the influence of anti-acid therapy on IPF disease course. GER therapy was associated with improved survival in a retrospective, multi-center cohort analysis (19) and less pulmonary function decline in a *post hoc* analysis of three clinical trial datasets (105). These findings were not replicated in a recent *post hoc* analysis of a separate clinical trial dataset (106). Additional data evaluating the efficacy of both medical and surgical GER therapy in patients with IPF are expected in the near future as two phase II clinical trials are underway (NC02085018; NCT01982968).

Hiatal Hernia (HH)

A likely contributor to the high prevalence of GER in patients with IPF is HH, which has been described in 40–53% of patients with IPF (107, 108). While an increasing degree of HH is likely to result in GER symptoms, mild HH can be asymptomatic (108). HH can be identified on HRCT mediastinal views and does not require dedicated imaging. HH treatment largely focuses on GER-associated symptom control, but surgical correction should be considered in patients with refractory symptoms. A retrospective investigation of patients awaiting lung transplantation showed that Nissen fundoplication was well tolerated and was associated with stabilization of oxygen levels in this patient population (109).

ENDOCRINE/METABOLIC COMORBIDITIES

Hypothyroidism

While 1–2% of men and 5–9% of women in the general population carry a diagnosis of hypothyroidism (110–112), a recent case–control analysis demonstrated a substantially higher prevalence among individuals with IPF, with 13% of men and 28% of women affected (20). Furthermore, those with combined IPF and hypothyroidism had reduced survival compared to those with IPF alone. The biology underpinning an increased prevalence of hypothyroidism in IPF remains unclear, but because autoimmune thyroiditis is the most common cause of hypothyroidism in developed nations (110–112), aberrant immune activation in IPF may play a role.

Diabetes Mellitus (DM)

Case–control analyses performed in Japan, Mexico, and the U.K. estimated the prevalence of DM type 2 to be 10–33%, among

TABLE 1 | Common comorbidities in patients with idiopathic pulmonary fibrosis.

Comorbidity	Prevalence (%)	Outcome association
Emphysema	8–34	Mixed results; may increase mortality risk Increased prevalence of pulmonary hypertension (PH)
Lung cancer	3–22	Increased mortality risk Surgical resection may increase mortality and acute exacerbation risk Chemotherapy may increase acute exacerbation risk
Obstructive sleep apnea	58–88	May increase mortality risk through worsening nocturnal hypoxemia
Coronary artery disease	4–68	May increase mortality risk
PH	3–84	Increases mortality risk
Pulmonary embolism/venous thromboembolism	2–3	Treatment with anti-coagulation may increase mortality risk
Gastro-esophageal reflux	30–80	Mixed results; treatment with antacid therapy may improve survival and reduce disease progression
Hiatal hernia	40–53	Surgical correction may improve survival and stabilize oxygenation in patients awaiting transplant
Hypothyroidism	13–28	May increase mortality risk
Diabetes mellitus type 2	10–33	Unknown
Depression	12–49	Unknown
Anxiety	10	Unknown

individuals with IPF, which was significantly higher than that of matched control populations (18, 93, 113). These findings persisted after exclusion of individuals treated with systemic corticosteroid therapy, which is known to alter glucose levels (18, 93). Outcome data were not reported in these studies, so it remains unclear whether the presence of DM influences survival in patients with IPF.

MENTAL HEALTH COMORBIDITIES

Depression and Anxiety

Idiopathic pulmonary fibrosis progression commonly manifests as worsening dyspnea, declining pulmonary function, and hypoxemia. While these manifestations undoubtedly impact quality of life, they have been shown to correlate with depression and anxiety (114–116). Despite these findings, few epidemiologic studies of mental health comorbidities have been performed in IPF. While 3.4% of patients with a diagnosis code for IPF also had a diagnosis code for depression in an investigation of insurance claims (62), IPF cohort studies suggest a prevalence of 12–49% (115, 116). Anxiety was shown to affect approximately 10% of IPF patients in a single center study (116).

A small IPF cohort study based in Europe suggested that increasing duration of disease correlates with standardized depression scores (117), while an ILD cohort study based in Australia showed that an increasing number of comorbid conditions also correlates with increasing depression scores (116). The impact of depression and anxiety on outcomes remains unclear in IPF. In addition, the ideal screening tool to detect depression and anxiety in this patient population has yet to be validated.

CONCLUSION

Idiopathic pulmonary fibrosis remains a devastating diagnosis for patients and their families, and its management requires a multipronged approach. Comorbidity assessment and management is a cornerstone of comprehensive management of IPF and we have reviewed the most commonly associated comorbidities that clinicians should consider (**Table 1**). Aggressive management of comorbidities is promoted by IPF centers of excellence across the country and may explain some of the improved survival associated with these centers (118). We strongly believe that proper comorbidity assessment and management can improve quality of life and has the potential to improve patient survival in IPF.

AUTHOR CONTRIBUTIONS

JO and HC contributed to the conception and writing of this review. Both authors have reviewed and approved the submitted work.

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REVIEW
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Diagnosis of Idiopathic Pulmonary Fibrosis "Pragmatic Challenges in Clinical Practice"

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The past few years have signaled a major breakthrough on the management of idiopathic pulmonary fibrosis (IPF). Finally, we have drugs in our arsenal able to slow down the inexorable disease natural course. On the other hand, the latter evidence has increased the responsibility for a timely and accurate diagnosis. Establishment of IPF diagnosis directly affects the choice of appropriate treatment. The current diagnostic guidelines represent a major step forward providing an evidence-based road map; yet, clinicians are encountering major diagnostic dilemmas that inevitably affect therapeutic decisions. This review article aims to summarize the current state of knowledge on the diagnostic procedure of IPF based on the current guidelines and discuss pragmatic difficulties and challenges encountered by clinicians with regards to their applicability in the everyday clinical practice.

Keywords: idiopathic pulmonary fibrosis, diagnosis, challenges, pragmatic, clinical practice

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) represents the most common form of idiopathic interstitial pneumonia (IIPs) and is characterized by the gravest prognosis with a median survival of 3–5 years (1), irrespective of treatment. The past 10 years, large multicenter placebo-controlled clinical trials have significantly shifted the therapeutic dial of IPF (2–4) from harmful agents to Ref. (5) to therapies able to slow down the disease progression (2–4). It is important to note that the efficacy of the antifibrotic agents, pirfenidone, and nintedanib has been tested only in the context of IPF; thus, accurate and timely diagnosis seems to be imperative. It is important to note that disease diagnosis does not represent anymore an academic exercise since it directly influences and guides therapeutic decisions.

DIAGNOSIS OF IPF

Histologically, IPF is characterized by the pattern of usual interstitial pneumonia (UIP), which is denoted by spatial and temporal heterogeneity (6). It is of utmost importance to highlight the fact that UIP pathology is not exclusive to IPF, as it may characterize other diseases including chronic hypersensitivity pneumonitis (HP), connective tissue disorders-associated ILDs, asbestosis, or drug toxicity. In other words, all IPF have UIP pathology, but not all UIP are IPF. High-resolution

computed tomography (HRCT) revolutionized the diagnosis of IPF. The presence of honeycombing in a predominantly peripheral and bibasilar distribution has a sufficient positive-predictive value (PPV) for underlying UIP pathology obviating the need for tissue confirmation (7–9). Even in that case, exclusion of other causes of UIP is mandatory to finally establish IPF diagnosis. Furthermore, IPF diagnosis represents a dynamic process and, therefore, close monitoring of the patient is mandatory. In particular, therapeutic decisions can be altered based on disease natural course and treatment responsiveness on an individual basis. In addition, disease diagnosis could be revisited in light of emerging symptoms compatible with connective tissue disorder or exposure to potentially harmful environmental agents.

The patient with IPF typically presents with progressive dyspnea on exertion of insidious onset and non-productive cough. The most characteristic clinical finding is the presence of Velcro rales that can be proven an extremely useful diagnostic tool for early disease diagnosis (10). Clubbing is almost found in 30–50% of patients; yet, its prevalence is much higher following disease progression and development of pulmonary hypertension. Multisystemic manifestations in the context of IPF are highly uncommon and should alert the physician toward alternative diagnoses. Median time from onset of symptoms to first evaluation in an ILD center quite often exceeds 1 year and the length of delay has been associated with increased mortality (11, 12).

Pulmonary function tests usually exhibit a restrictive pattern with concomitant reduction in diffusing capacity of carbon monoxide (DLco). However, the absence of restriction does not exclude the diagnosis of IPF, especially in the context of combined pulmonary fibrosis and emphysema, which is characterized by relatively preserved lung volumes with disproportionately reduced DLco (13). There are also cases discovered early and presumably with an initial FVC > 100% that do not fulfill the criteria of a restrictive pattern, nevertheless, fibrosis is evident on HRCT.

According to current diagnostic criteria (1), HRCT plays a pivotal role in the diagnostic procedure. There are three diagnostic categories based on HRCT appearance: UIP pattern, possible UIP pattern, and inconsistent with UIP. The definition of each category is based on morphological as well as distribution characteristics. UIP pattern is characterized by the presence of reticular abnormalities and honeycombing (with or without traction bronchiectasis) with a subpleural and basal predominance in the absence of inconsistent features. The above radiological features in the absence of honeycombing constitute the possible UIP pattern. Inconsistent features can be categorized as those involving distribution (upper or mid lung and peribronchovascular predominance) and those involving morphology (extensive ground glass opacities, consolidation, mosaic attenuation, nodules, discrete cysts).

The presence of honeycombing is not synonymous with IPF, as it can be seen in other diseases as chronic HP, collagen tissue-related interstitial lung diseases, asbestosis, drug-induced lung toxicity, sarcoidosis, postradiation pneumonitis, and post ARDS. Minimal honeycombing can also be encountered in cases of fibrotic NSIP, which represents a major component of the differential list.

The distribution of honeycombing can offer significant information. Typically, in IPF, it has a subpleural and basilar distribution. Chronic HP can be a great mimic of IPF. However, sometimes in chronic HP, honeycombing can be more marked in the upper/mid lung zones giving a hint to the actual diagnosis. The same upper lobe predominance of honeycombing can be seen in sarcoidosis as well. Furthermore, in sarcoidosis, the fibrotic process often follows the expected perilymphatic route, thus creating a "swath" of honeycombing extending from the hilum to the periphery of the lung. In patients who develop fibrosis post ARDS, it has a striking anterior distribution (**Table 1**).

Besides the distribution of honeycombing, there are other findings on HRCT that increase suspicion toward certain diagnoses. Silva et al. (14) studied 66 patients with biopsy proven IPF, HP, and NSIP. The presence of lobular areas with decreased attenuation, centrilobular nodules, and cysts favored the diagnosis of HP. The best predictors of NSIP were the presence of subpleural sparing and the absence of honeycombing.

In the appropriate clinical setting in patients with a UIP pattern, IPF diagnosis can be established without the need for surgical lung biopsy (SLB). In the cases of possible UIP and inconsistent with UIP, a SLB is recommended in order to reach a final diagnosis. When SLB is necessary, close cooperation with the thoracic surgeon is necessary in order to point the optimal sites for biopsy in order to increase the possibility of an accurate diagnosis. Areas of honeycombing should be avoided in lung sampling since they may reveal non-specific end-stage lung damage and absence of spatial and temporal heterogeneity suggestive of UIP. Samples should be obtained from at least two different

TABLE 1 | Differential diagnosis of radiological honeycombing.

Causes of honeycombing	Comments
Idiopathic pulmonary fibrosis	Distribution predominantly bibasilar and subpleural
Collagen tissue disease	Honeycombing mainly seen in rheumatoid arthritis-ILD
Chronic hypersensitivity pneumonitis	Honeycombing can be seen predominantly in the upper/middle zones. Clues to diagnosis: mosaic attenuation, air trapping in expiratory scans
Asbestosis	Distribution predominantly bibasilar and subpleural. Clues to diagnosis: pleural plaques (±calcification), subpleural lines
NSIP	When seen, honeycombing is minimal. Clues to diagnosis: subpleural sparing, central/peribronchovascular predominance of findings
Sarcoidosis	In rare cases, the distribution is bibasilar and subpleural. Typically, honeycombing is seen in upper/middle zones extending from the periphery toward the hilum. Clues to diagnosis: perilymphatic nodules, hilar/mediastinal lymphadenopathy (±calcification), progressive massive fibrosis
Radiation	Confined to radiation port
End-stage ARDS	Usually involves anterior lung (barotrauma)
Drug toxicity	

lobes, because of the possibility of discordant findings (UIP in one lobe and NSIP in another). In such cases, the UIP pattern drives diagnosis and prognosis as well (15, 16).

Regarding histopathology, current guidelines have classified patients into four categories: UIP, probable UIP, possible UIP, and not UIP (1). Specific combinations of HRCT and SLB pattern with the integration of clinical data are evaluated by a multidisciplinary (MDT) team in order to achieve a final diagnosis.

MDT approach is acknowledged as a major advance in IPF diagnosis. It refers to the constructive exchange of views between a respiratory physician, radiologist, and pathologist with expertise in the field of ILDs. The added value of MDT diagnosis is its association with higher levels of diagnostic confidence and better interobserver agreement (17, 18). Walsh et al. (19) were the first to evaluate the agreement between different multidisciplinary teams in diffuse lung diseases after the 2013 ATS/ERS update (20) on the classification of IIPs. Inter-MDT agreement was acceptable for cases of IPF (weighted kappa coefficient, $\kappa w = 0.71$), while it was moderate for NSIP ($\kappa w = 0.42$) and rather disappointing for HP ($\kappa w = 0.29$) reflecting the lack of diagnostic guidelines for the last two clinical entities. This indirectly impacts the accuracy of IPF diagnosis as well, given the fact that NSIP and HP are frequently major components of its differential.

A PRAGMATIC APPLICATION OF GUIDELINES IN EVERY DAY CLINICAL PRACTICE

The 2011 guidelines are a clear step forward considering that they provide clear guidance on an evidence-based approach. The most crucial caveat of these guidelines is that, in a significant percentage of "real-life" patients with IPF, lack clinical practicality regarding diagnosis, prognosis, and therapeutic decisions.

Challenge 1: Interpretation of HRCT

High-resolution computed tomography plays a pivotal role in disease diagnosis and determines the need of SLB to establish a definite diagnosis. However, accurate identification of honeycombing is not straightforward even amongst thoracic radiologists. Interobserver agreement has been proven poor, and this has been validated in recent studies (21–24). This problem is further accentuated on a community level.

Possible UIP Pattern

An increasing number of studies has shown that in patients with a high suspicion of IPF, a possible UIP pattern retains sufficient PPV for underlying UIP pathology in order to obviate the need for tissue based diagnosis. Raghu et al. (25) studied 315 patients with IPF study that had both HRCT and SLB samples. As expected, UIP pattern had a high PPV for UIP pathology (97.3%). This high PPV was retained for patients with a possible UIP pattern (94%). Though a selection bias is quite obvious given that this evidence refers to patients screened for recruitment into clinical trials and thus should not be generalized; yet, this study highlights the importance of pretest clinical probability. Chung et al. (26) studied 201 patients with pulmonary fibrosis that were subjected to lung biopsy within 1 year of chest CT. Patients with possible UIP on CT scan were more likely to have histologic probable/definite UIP comparing to patients with indeterminate UIP on CT scan (82.4 vs 54.2%, $p = 0.01$). Finally, in the INPULSIS trials, a significant proportion of patients (31.9%, $n = 338$) were enrolled based on possible UIP pattern (with traction bronchiectasis) and no confirmation by SLB. This group of patients exhibited the same progression of disease based on the annual decline of FVC compared to patients with honeycombing on HRCT and/or confirmation by SLB as well as similar treatment response to nintedanib (27). This observation adds further to the notion that in the appropriate clinical setting, possible UIP pattern carries sufficient PPV for UIP pathology.

Recently, a study by Brownell et al. offered valuable new information on this topic (28). Avoiding selection bias, they elegantly showed that the PPV of possible UIP for predicting UIP pathology directly depends on the pretest probability of IPF and the prevalence of IPF in the examined population. In the derivation cohort, possible UIP had a specificity of 91.2% and a PPV of 62.5%. By using two key clinical predictors (male sex, increasing age) and a radiographic predictor (total traction bronchiectasis score), the PPV increased above the acceptable threshold of 90%.

Inconsistent with UIP Pattern

According to guidelines, even if histology is that of a typical UIP pattern when the HRCT appearance is inconsistent with UIP, the diagnosis of IPF is deemed only to be possible. However, the term inconsistent seems to be a misnomer. IPF is actually a great mimic. UIP pathology can exhibit a wide variety of radiological expressions ranging from the typical UIP pattern with peripheral, bibasilar honeycombing, to a pattern resembling HP with areas of mosaic attenuation or to a pattern characterized by extensive ground glass opacities (29, 30). Interestingly, NSIP pathology seems to be much more consistent regarding its radiological expression (31). The key point is that an inconsistent with UIP radiological pattern does not rule out the diagnosis of IPF, but mandates histological confirmation regardless the pretest probability of the patient. In the study by Brownell et al., the maximum PPV achieved for the inconsistent UIP pattern regarding IPF diagnosis was just 38% (28). **Figure 1** summarizes a proposed algorithm for IPF diagnosis based on recent findings as described previously.

Challenge 2: Interpretation of SLB

The interpretation of histological findings is subject to the same limitations as HRCT. The pathologic distinction between UIP and fibrotic NSIP can be especially difficult and is the main reason (>50%) for interobserver variation in the evaluation of diffuse parenchymal lung disease (32). In the same study, the median prevalence for diagnoses with low confidence (<70% likelihood) was 18% while diagnoses made with 100% confidence were reached in only 39% of cases. Thus, it is not surprising that the pathological diagnosis can be reconsidered in almost 20% of cases following integration of clinical and HRCT data (17). The variability in interpretation is accentuated

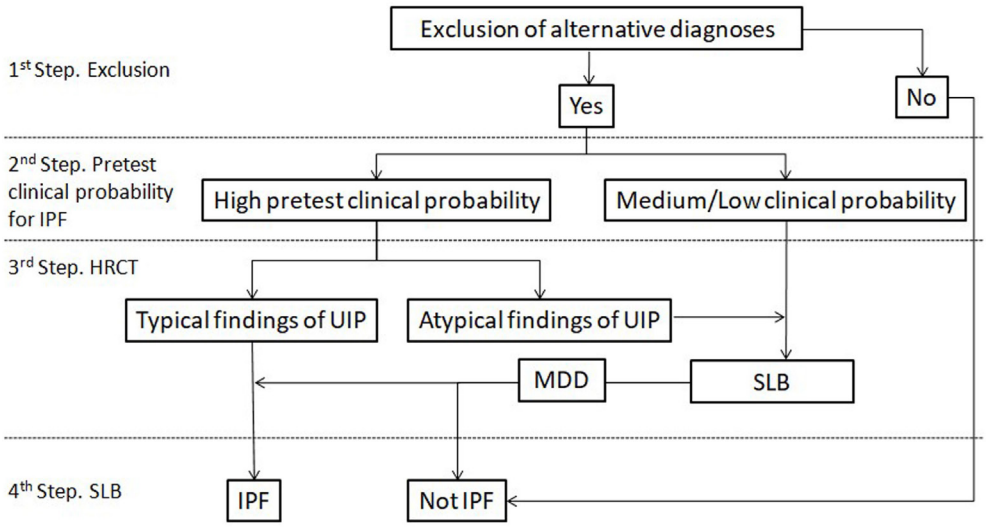

FIGURE 1 | Proposed diagnostic algorithm for idiopathic pulmonary fibrosis (IPF). Typical findings of UIP are radiological signs of lung fibrosis (honeycombing, traction bronchiolectasis, and/or irregular reticular pattern) with a predominantly peripheral/subpleural and bibasilar distribution. Atypical findings of UIP are according to the current definition of inconsistent with UIP pattern. HRCT, high-resolution computed tomography; SLB, surgical lung biopsy.

between thoracic and general pathologists with a poor level of agreement (κ: 0.21), which was shown to have direct clinical implications (33). Also, it is known that biopsies from different sites can produce discordant results, specifically, NSIP vs UIP histology (15, 16). Consequently, sampling error is a possibility and in cases with a definite UIP radiology pattern and NSIP on histology, the radiologic diagnosis actually prevails over histology (34). Finally, histology patterns in interstitial lung diseases are not exclusive to certain diseases. In fact, the same histology can correspond to different diseases and furthermore to the same disease but with strikingly different progression and natural course. SLB is not a gold standard and the risk vs benefit ratio should be carefully examined in each patient.

Challenge 3: Safety of SLB

Surgical lung biopsy in patients with ILDs as it can trigger an acute exacerbation (35, 36), regardless disease severity (37). An alarming observation is that the same parameters that increase the likelihood of IPF (increased age and male sex) (28, 38, 39) represent risk factors that increase mortality following SLB in patients with ILDs (40, 41). Actually, a provisional diagnosis of IPF was identified as a risk factor for increased mortality. Other risk factors are the presence of comorbidities, hypoxemic respiratory failure, severe physiological impairment, pulmonary hypertension, rapidly progressive disease (42).

Two large series (40, 41) reported postoperative hospital mortality rates (1.7%) similar to those reported in patients with lung cancer undergoing lobectomy. The actual postoperative mortality may vary depending on the nature of the procedure (elective vs non-elective) and the risk factors for the individual patient. Thus, the decision to proceed to SLB [*via* video-assisted thoracoscopic surgery (VATS)] should be carefully considered on an individual basis, weighing risks vs diagnostic benefit.

Challenge 4: Current Clinical Practice

By strictly adhering to current guidelines, a large number of patients (almost 50% with a suspicion of IPF) will need to be subjected to SLB. Clinical practice seems to have endorsed the facts that SLB carries a small but significant risk and that the possible UIP pattern in a patient with a high pretest probability of IPF retains sufficient PPV for UIP pathology. It is common practice that in patients with a high clinical probability of IPF (28, 38, 39) (male sex, increased age, and/or extent of fibrosis), we establish a working diagnosis of IPF (43, 44) without resorting to surgical biopsy. Biopsies are reserved for patients in whom establishing a tissue-based diagnosis is clinically meaningful and are fit enough to undergo such a procedure. This is vividly depicted in the study by Hutchinson et al. (40). In a UK study held between 1997 and 2008, only 4.5% of new cases with a provisional diagnosis J84.1 were subjected to SLB (40). Given the fact that the ICD-10 of J84.1 does not accurately describe the IPF population (45, 46), this percentage is likely to be even smaller for actual IPF cases. We eagerly wait to see how the above will be translated in the upcoming guidelines for the diagnosis of IPF.

FUTURE DIRECTIONS

Bronchoscopic Lung Cryobiopsy (BLC)

Bronchoscopic lung cryobiopsy is dynamically emerging during the past few years as an alternative diagnostic tool to SLB, claiming the same diagnostic efficacy and reduced mortality

(47). Cryobiopsies are considerably larger and have minimal crash artifacts as opposed to forceps biopsies when performed by experienced bronchoscopists in appropriate organized centers. Therefore, they allow confident recognition of histological patterns. The vital question that should be addressed is their safety and diagnostic yield against lung biopsies obtained *via* VATS. A recent meta-analysis including 16 studies with BLC (642 patients) and 14 studies with VATS (1,594 patients) (48) reported comparable diagnostic yields for BLC (83.7%) and VATS (92.7%). With regards to safety profile, BLC was associated with severe bleeding in 4.9% of cases and pneumothorax in 9.5% of cases while short-term mortality was similar between BLC (0.7%) and VATS (1.8%). Similar findings have been demonstrated by earlier studies (49, 50). In order to generalize the use of BLC beyond expert centers, it is important to standardize the procedure (e.g., size of cryoprobe, number and site of biopsies, degree of sedation), offer proper training, since it is an operator-dependent procedure (51) and prospectively evaluate safety and diagnostic profile of BLC as opposed to VATS.

Biomarkers

According to the Bayesian diagnostic approach of IPF, it would be very useful to have biomarkers that would increase the pretest probability of IPF. Ideally, these biomarkers would not only have diagnostic value but would also offer clinically relevant prognostic information regarding not only the natural course of the disease but also response to therapy. While for pulmonary embolism, d-dimers are an established diagnostic indicator in a complex disease as IPF, it is unlikely that just one "diagnosticator" will suffice. Considering that IPF diagnosis represent the least critical question for clinicians (given the major improvements of HRCT), in the real-world clinical practice, an ideal biomarker would be the one who could fulfill the unmet need for timely prediction of disease progressiveness and treatment responsiveness. In line with this, most of the studied biomarkers were mostly used as disease prognosticators rather than disease-specific diagnostic tools. Matrix metalloproteinase-(MMP)-7 represents the most extensively studied molecular biomarker that showed promising prognostic value in several independent cohorts of patients with IPF. Despite the fact that elevated MMP-7 levels clearly discriminated patients with IPF from those with HP (52); yet, they showed lack of discriminatory ability between IPF and RA-ILD (53). Further studies using highly standardized sample collection procedures and collection matrices are needed to produce reproducible and reliable diagnostic and prognostic cutoff thresholds (54). The latter observation represents an amenable need for precision medicine approaches (55, 56).

TABLE 2 | Key points for idiopathic pulmonary fibrosis diagnosis.

- Exclusion of other causes is mandatory
- Equally important is to define pretest clinical probability based on age, sex, extend of fibrosis
- Even in the absence of honeycombing, in a patient with a high pretest clinical probability, the presence of other signs of lung fibrosis (traction bronchiolectasis, irregular reticulation) in a predominantly bibasilar/subpleural distribution have sufficient positive-predictive value for usual interstitial pneumonia (UIP) pathology
- Atypical features (inconsistent with UIP) mandates the need for surgical lung biopsy (SLB), regardless pretest clinical probability
- SLB is not a diagnostic panacea, it can be potentially hazardous especially when performed non-electively and should be performed after carefully estimating the clinically meaningful benefit vs risk on an individual basis

Conclusion

The ILD community has made significant progress in understanding IPF. With the development of antifibrotic agents, accurate diagnosis is crucial. Guidance is needed to focus on practical implementation of current guidelines in a real-world clinical setting. An integral first step of the diagnostic process is the exclusion of alternative diagnoses. Equally important is the definition of the pretest probability for every patient with suspected IPF. The diagnostic significance of possible or even definite UIP pattern is completely different when facing a 45-year-old female or a 70-year-old male. Possible UIP pattern seems to carry sufficient PPV in patients with a high pretest probability of IPF. SLB should be considered in patients with inconsistent with UIP pattern after evaluating the individualized benefit risk ratio (**Table 2**). BLC seems an attractive, safer alternative to SLB; yet, standardization and prospective evaluation of the process is required in order to "escape" from expert centers and be embraced by common practice. Finally, biomarkers are sorely needed to fulfill the unmet need of current clinical practice: early prediction of disease progressiveness and treatment responsiveness that will timely guide therapeutic decisions.

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REVIEW
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Genetics in Idiopathic Pulmonary Fibrosis Pathogenesis, Prognosis, and Treatment

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Idiopathic pulmonary fibrosis (IPF), the most common form of idiopathic interstitial pneumonia (IIP), is characterized by irreversible scarring of the lung parenchyma and progressive decline in lung function leading to eventual respiratory failure. The prognosis of IPF is poor with a median survival of 3–5 years after diagnosis and no curative medical therapies. Although the pathogenesis of IPF is not well understood, there is a growing body of evidence that genetic factors contribute to disease risk. Recent studies have identified common and rare genetic variants associated with both sporadic and familial forms of pulmonary fibrosis, with at least one-third of the risk for developing fibrotic IIP explained by common genetic variants. The IPF-associated genetic loci discovered to date are implicated in diverse biological processes, including alveolar stability, host defense, cell–cell barrier function, and cell senescence. In addition, some common variants have also been associated with distinct clinical phenotypes. Better understanding of how genetic variation plays a role in disease risk and phenotype could identify potential therapeutic targets and inform clinical decision-making. In addition, clinical studies should be designed controlling for the genetic backgrounds of subjects, since clinical outcomes and therapeutic responses may differ by genotype. Further understanding of these differences will allow the development of personalized approaches to the IPF management.

Keywords: idiopathic pulmonary fibrosis, MUC5B, pulmonary fibrosis, interstitial lung disease, telomeres

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common of the idiopathic interstitial pneumonias (IIPs). IPF is characterized by progressive scarring of the lung parenchyma, which leads to dyspnea and declining pulmonary function and eventually to respiratory failure. The median survival after diagnosis of IPF is 3–5 years (1). In 2011, the American Thoracic Society/European Respiratory Society issued a new classification scheme in which they defined IPF as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown etiology, occurring mainly in older adults and associated with radiological and/or histopathological pattern of usual interstitial pneumonia (UIP) (2). The prognosis of IPF remains poor despite recently approved medical therapies (3, 4).

Numerous epidemiologic and genetic studies illustrate that genetic and environmental factors contribute to the risk of IPF (5, 6). The most convincing early evidence to support a genetic basis for IPF came from twin studies and studies focusing on familial clustering of the disease, a syndrome termed familial interstitial pneumonia (FIP) (7–9). Recent studies have identified several specific

genetic variants that confer risk for development of IPF (10, 11). Discovery of disease-associated genetic variants has improved our understanding of the ways inherited risk factors influence disease risk. However, fundamental questions persist regarding the ways in which complex genetic risk factors interact with environmental exposures to influence disease pathogenesis.

In this review, we briefly discuss the current literature regarding the role of common and rare variants in disease pathogenesis and prognosis and how this may influence clinical management in the future. Genetic variants and loci associated with IPF involve abnormalities in alveolar stability, host defense, cell–cell barrier function, and cell senescence, all of which are all thought to contribute to the pathogenesis of IPF. We conclude by discussing how treatment decisions might be affected by these findings and how better understanding of genetic variation and disease could allow for a more personalized approach to the treatment of IPF.

Rare and Common Variants Associated with IPF

Genetic variants, both rare and common, are associated with sporadic and familial forms of pulmonary fibrosis. Numerous rare variants (those with minor allele frequency of <0.1%) play a role in FIP (≥2 members of the same family with interstitial pneumonia; FIP) (**Table 1**). Familial studies have identified FIP-associated variants related to alveolar stability [*SFTPC* (12, 13), *SFTPA1* (14), *SFTPA2* (15), ATP-binding cassette-type 3 (*ABCA3*) (16), and *NAF1* (17)] as well as five genes linked to telomere biology [*TERT* (18), *TERC* (18), *DKC1* (19), *TINF2* (7, 20), *RTEL1* (21–23), and PARN] (24).

Common variants (defined as minor allele frequency of >5%) also appear to play a role in FIP risk (1). The most widely replicated risk variant (rs35705950), located in the promoter region of *MUC5B*, was initially identified in a combined linkage and association study (41) and has been strongly associated with IPF and FIP. Two large GWAS of IPF subjects (both familial and sporadic) with controls have been conducted in pulmonary fibrosis (10, 11). In addition to confirming the role of *TERT* at 5p15, *MUC5B* at 11p15, and the 3q26 region near *TERC*, the GWAS identified seven newly associated loci, including *FAM13A* (4q22), *DSP* (6q24), *OBFC1* (10q24), *ATP11A* (13q34), *DPP9* (19q13), and chromosomal regions 7q22 and 15q14-15 among others that have been nominally associated (**Table 2**).

Rare variants are thought to be highly penetrant and to have a greater effect size, but given their low frequency, they account for a smaller proportion of overall disease risk in the general population (51). Alternatively, in general, common variants have a smaller effect size but are present at higher frequency and, in aggregate, may contribute to a larger proportion of disease risk (**Figure 1**). However, the *MUC5B* promoter variant rs35705950 is a common variant with a large effect size and therefore accounts for a substantial risk in IPF. In fact, it has been estimated the *MUC5B* promoter variant accounts for 30% of the risk of developing IPF (41, 51).

TABLE 1 | Rare variants in idiopathic pulmonary fibrosis.

Gene	Gene function	Pathological consequence of mutation	Reference
SFTPC	Component of surfactant fluid	Altered trafficking and disrupted proteostasis, increased endoplasmic reticulum (ER) stress	(25–27)
SFTPA2	To modulate innate and adaptive immunity	Increase in ER stress	(15, 25, 28)
ABCA3	Transport of lipids across plasma membrane	Retention of lipids in the ER, ER stress, and apoptotic signaling	(29–31)
TERT	Enzyme in telomerase complex	Telomere shortening	(7, 18, 27, 32–36)
TERC	Template in telomerase complex	Telomere shortening	(7, 18, 27, 32–37)
DKC1	Stabilization of the template in telomerase complex	Telomere shortening	(19, 27, 38)
TINF2	Telomere maintenance	Telomere shortening	(20, 39)
RTELI	DNA helicase	Telomere shortening	(21, 22, 40)
PARN	mRNA stability	Telomere shortening	(21, 24)

Alveolar Stability

Surfactant proteins are synthesized in the endoplasmic reticulum (ER) of alveolar type II cells (AECII) and transported to and stored in the lamellar bodies until secretion into the alveolar space (25, 26). Rare variants identified in the genes encoding surfactant protein C and A (*SFTPC*, *SFTPA1*, and *SFTPA2*) have been associated with pulmonary fibrosis (53). SP-C is a small hydrophobic protein produced by AECIIs that requires the C-terminus for initial folding steps in the ER before secretion into the alveolar space (26). *SFTPC* rare variants are mutations that lie in the BRICHOS domain within the C-terminus of SP-C. The BRICHOS domain is critical for proper folding and trafficking (5, 26). Coding mutations in this region lead to accumulation of misfolded protein resulting in increased ER stress and activation of the unfolded protein response (26, 54). Mutations in the gene that encodes surfactant protein A (*SFTPA2*) have also been linked to FIP (15) and have been associated with increased ER stress as well (28, 55). Rare variants have also been identified in another gene involved with surfactant processing, *ABCA3*, in FIP families (16, 56). *ABCA3* is a transporter protein mainly expressed in AECIIs and is involved in the transport of lipids across plasma membranes (29, 57). In AECIIs, *ABCA3* mutations cause abnormal processing, trafficking, and functionality of the *ABCA* protein, leading to retention of lipids in the ER, ER stress, and apoptotic signaling (30). These mutations are expressed in a recessive manner, where as mutations in *SFTPA2* and *SFTPC* are dominantly expressed (56).

In 2011, Lawson et al. (58) demonstrated that fibrotic remodeling in response to low-dose bleomycin was more severe in mice in which ER stress was induced, either through mutant SFTPC in AECIIs or by administration of tunicamycin, a chemical known to induce ER stress. In addition to effects on apoptosis, ER stress may induce biological pathways involved in cell differentiation (59, 60) through which epithelial cells acquire phenotypic characteristics of mesenchymal cells, a process known as

TABLE 2 | Common variants in idiopathic pulmonary fibrosis (IPF).

Risk allele(s)	Gene	Gene function	Observed effect of risk variant on survival in IPF	Reference
rs408392 rs419598 rs2637988	*IL1RN*	Inhibitor of pro-inflammatory effect of IL-1alpha and IL-1beta		(27, 42)
rs4073 rs2227307	*IL8*	Pro-inflammatory cytokine	Reduced	(43, 44)
rs2609255	*FAM13A*	Signal transduction		(10)
rs3775291	*TLR3*	Pathogen recognition and activation of innate immunity	Reduced	(45)
rs2736100	*TERT*	Enzyme in telomerase complex maintaining telomere length	Reduced	(10, 27, 46, 47)
rs2395655	*HLA-DRB1*	Major histocompatibility complex—immune system		(48)
rs2076295	*DSP*	Tightly links adjacent cells		(10)
rs11191865	*OBFC1*	Stimulates the activity of DNA polymerase-alpha-primase		(10)
rs35705950	*MUC5B*	Influence on rheological properties of airway mucus, mucociliary transport, and airway defense	Improved	(10, 11, 27, 41, 49, 50)
rs7934606	*MUC2*	Mucin production		(10)
rs111521887 rs5743894 rs2743890	*TOLLIP*	Regulator of innate immune responses mediated by toll-like receptor and the transforming growth factor β signaling pathway	Reduced	(11)
rs1278769	*ATP11A*	Phospholipid translocation		(10)
rs7144383	*MDGA2*	Cell–cell interaction		(11)
rs1981997	*MAPT*	Promotes microtubule assembly and stability		(10)
rs17690703	*SPPL2C*	Protein cleavage		(11)
rs12610495	*DPP9*	Cell–cell adhesion		(10)
rs1800470	*TGFB1*	Set of peptides that controls proliferation, differentiation, and other functions in many cell types		(11)

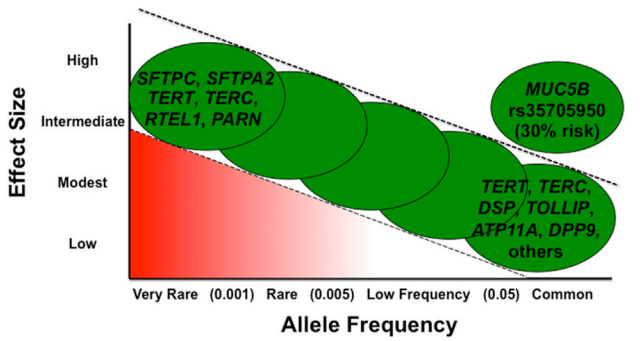

FIGURE 1 | Adapted from figure published previously in BMC Medicine with permission (52).

epithelial-to-mesenchymal transition (EMT), in IPF lungs (61). EMT is hypothesized to increase the number of cells responsible for collagen production and matrix deposition thereby leading to fibrosis (13, 59, 60). To date, published data suggest that ER stress predisposes to AECII apoptosis and subsequent lung fibrosis. Surfactant proteins have been recognized as crucial in maintaining lung alveolar structure and function. However, the precise role of alveolar stability, ER stress, and EMT in IPF pathogenesis remains an area of active investigation.

Cell Senescence

Telomeres are repetitive nucleotide sequences at the ends of chromosomes that protect them from progressive shortening during the normal cell replication process (62). Telomerases restore telomere length and consist of two major components: telomerase reverse transcriptase (encoded by *TERT*) and telomerase RNA (encoded by *TERC*) (18, 37). Mutations in telomerase components were initially identified in the setting of dyskeratosis congenita (DKC), a rare inherited syndrome of telomere shortening characterized by oral leukoplakia, abnormal skin hyperpigmentation, and dystrophic nails, with pulmonary fibrosis present in about 20% of patients; bone marrow failure can also be a complication of DKC (32). More recent studies have found an association between numerous genes in the telomerase maintenance pathway and FIP, including those related to catalytic activity (*TERT* and *TERC*) (7, 32) and telomere stabilization (*DKC1*, *PARN*, and *RTELI*) (19, 21). These pathogenic variants cause dysfunction of telomerase activity leading to accelerated telomere shortening (32, 63) in peripheral blood and the lung (32–34). Thus far, *TERT* variants are the most frequently identified rare variants associated with pulmonary fibrosis; they are found in ~15% of FIP (7, 32) and in 1–3% of sporadic cases (34). A recent whole-exome sequencing study identified *TERT*, *RTEL1*, and *PARN* variants previously associated with FIP to be associated with sporadic IPF, further supporting the role of telomere dysfunction in IPF pathogenesis and highlighting the genetic commonalities between FIP and sporadic IPF (64).

Telomere dysfunction has further been implicated in IPF as evidence has suggested that short telomeres are not exclusively related to telomerase rare variant mutations. One study found that 25% of sporadic IPF subjects and 24% of familial IPF subjects, without identified mutations for *TERT* or *TERC*, had short telomeres. In addition, all subjects within this specific study who

had a mutation in *TERT* or *TERC* and pulmonary fibrosis also had short telomeres (33).

The mechanisms by which telomere defects provoke lung disease are not fully understood. Defects in telomere maintenance have been linked to epithelial cell senescence and an impaired response to epithelial injury (65). During successive cycles of cell division, telomere shortening occurs and eventually leads to activation of the DNA-damage pathways, which result in apoptosis or senescence (32). In certain situations, cellular senescence is appropriate, but premature senescence can impair lung epithelial homeostasis and lead to stimulation of a lung remodeling response (66), resulting in fibrotic lesions (63). One study demonstrated increased epithelial cell senescence in IPF lung tissue by measuring B-galactosidase staining (a marker of senescence) and found that B-galactosidase staining was positive in all IPF cases but was not present in normal lung (67) supporting a role for senescent epithelial cells in IPF pathogenesis. Future studies are necessary to clarify the precise role of cellular senescence in lung injury response and fibrotic remodeling in IPF.

Host Defense

In 2011, genome-wide linkage analysis and targeted genetic sequencing identified a single nucleotide polymorphism (SNP) on chromosome 11 that was associated with both FIP and IPF (41). The SNP, rs35705950, was found to be a gain-of-function variant associated with increased expression of *MUC5B*. Heterozygous (GT) and homozygous (TT) individuals had an odds ratio for developing disease of 6.8 and 20.8 for FIP, and 9.0 and 21.8 for IPF, respectively, supporting the strength of the SNP's association with development of both IPF and FIP (41). *MUC5B* encodes Mucin 5B, which is a major gel-forming mucin in mucus and expressed by airway epithelial cells (68, 69). The association of the *MUC5B* promoter polymorphism with IPF has been replicated and confirmed in nine independent cohorts (10, 11, 49, 50, 70–74), including in a 2013 GWAS (OR for T minor allele = 4.51; 95% CI = 3.91–5.21; $P = 7.21 \times 10^{-95}$) (10). Additional genotyping studies have noted that the *MUC5B* variant is associated with disease in a Mexican cohort of IPF patients, but not in Asian cohorts (75). Most recently, a study of select loci in various European cohorts, including Czech and Greek IPF patients, also confirmed the association between rs35705950 and IPF (76).

MUC5B expression in IPF is localized in the distal airway, respiratory bronchiole, honeycomb cyst (77), and the bronchiolar epithelium (78). Overexpression of *MUC5B* in these areas of the lung and especially in the honeycomb cysts, which are a histopathological finding in IPF (77), further supports the notion that *MUC5B* is important in the pathogenesis of IPF. Evans and colleagues (51) hypothesize that IPF is caused by recurrent injury/repair/regeneration at the bronchoalveolar junction secondary to overexpression of MUC5B, mucociliary dysfunction, retention of particles, ER stress, and disruption of normal reparative and regenerative mechanisms in the distal lung (51).

Interestingly, the *MUC5B* promoter polymorphism may be specific to IIP, since studies have illustrated that rs35705950 is not associated with increased risk of sarcoidosis and scleroderma-related ILD, two other fibrotic lung diseases (10, 50, 70). However, recent data have shown that *MUC5B* rs357057950 variant is associated with radiographic evidence of interstitial lung abnormalities (ILA) studied in the Framingham cohort (79–81). Increasing age and number of copies of *MUC5B* promoter polymorphism were associated with ILA progression, which has been linked to increased mortality (80, 81). In addition, there are some data to suggest that rs357057950 genotype may be associated with higher likelihood of radiographic UIP pattern in the setting of fibrotic IIP (82).

The mechanism by which variants in *MUC5B* confers risk of lung fibrosis is an active area of investigation. Given that mucins play a role in innate immunity (68, 83), immune dysregulation could be a possible mechanism by which increased mucin expression contributes to the pathophysiology of IPF (84). Alternatively, IPF may be a disease of mucociliary clearance in which overexpression of *MUC5B* leads to impaired ciliary function, thereby allowing retention of particles and, subsequently, recurrent lung injury (51).

Several studies have also implicated the human leukocyte antigen (HLA) region in IPF (85–89). The HLA region is located on chromosome 6p21.31 (90), and its main function is regulation of immune response. The DRB1*15:01 allele has been shown to be more prevalent among IPF patients and associated with greater impairment of gas exchange (89). Recently, a genome-wide imputation-based association analysis identified two risk alleles, DRB1*15:01 and DQB1*06:02, found to be associated with fibrotic idiopathic interstitial pneumonias (48). Although not definitive, HLA association with IPF may suggest that autoimmunity may play a role in pulmonary fibrosis; further characterizing the pathophysiologic connection between this genetic variation and disease this remains an area of active investigation.

Epithelial Integrity

The 2013 GWAS by Fingerlin et al. (10) identified multiple susceptibility loci for fibrotic IIP, including two cell–cell adhesion molecules, *DSP* and *DPP9*. *DSP* gene expression was increased in lung tissue of individuals with IIP and varied by genotype for a variant in intron 5 (10, 91). *DSP* encodes for desmoplakin, a critical component of desmosome structure important in cell–cell adhesion. Desmosomes mechanically link cells and stabilize tissue architecture. In addition, they are involved in the regulation of cell proliferation, differentiation, migration, and apoptosis (92). The association between *DSP* variants and IPF, as well as the relationship between *DSP* variants and lung expression of this gene, was confirmed more recently by Mathai et al. (91) IPF lung has higher gene expression of *DSP*. However, IPF subjects with the rs2076295 variant were found to have lower *DSP* expression, suggesting that differential *DSP* expression may play a role in a subset or sub-phenotype of IPF (91). This association further implicates the airway epithelium in the pathogenesis of IPF, as *DSP* appears to be localized primarily to the airway epithelia and not to alveolar epithelial cells. The role of *DSP* in IPF pathogenesis remains an area of active investigation.

PROGNOSIS

Genetic variants, both rare (telomere related) and common (*MUC5B* and *TOLLIP*), may play a role in predicting disease outcomes and have prognostic implications. Short telomeres (<10th percentile adjusted for age) have been identified in a considerable portion of IPF patients, regardless of genetic mutations (33, 34). Patients with shorter telomeres have worse transplant-free survival in multiple independent cohorts (46, 93). Furthermore, a small observational study suggested that increased rates of bone marrow suppression and medication-related complications following lung transplantation are more common in IPF patients with telomerase mutations and/or short telomeres (94). Telomere length testing has been suggested as a component of pretransplant workup in IPF patients, although further prospective study is required before these observations can be utilized routinely in patient care (95).

Common polymorphisms, *MUC5B* and *TOLLIP*, have also shown promise as prognostic indicators (11, 50). A retrospective study of two separate IPF cohorts demonstrated improved survival in patients with the rs35705950 variant (49). In addition, carriers of at least 1 T allele of *MUC5B* polymorphism were found to have at least 50% improved survival and better lung function compared to those with the GG genotype (49). These findings were consistent with previous studies, which demonstrated an association between *MUC5B* variant and less severe pathological changes (96) and slower decline in FVC (50). Similarly, a *TOLLIP* variant was also associated with differential survival. The minor allele at rs5743890 (G) in TOLLIP is protective and associated with reduced susceptibility to IPF. However, those who developed IPF despite having the protective allele had increased mortality (11). At this time, there are no clinical guidelines suggesting genetic testing in the routine care and counseling of IPF patients (95), and further research is needed to identify the clinical implications of these preliminary findings.

TREATMENT

Approaches to therapy in IPF have been limited by the poorly understood pathophysiology of this progressive disease. In addition, the unpredictable clinical course of IPF, lack of validated biomarkers, and low clinical predictive value to animal models (97) have been barriers to identifying therapies. Despite these challenges, recent advances in understanding the pathophysiology of IPF have allowed for identification of novel treatment targets. Currently, two available medications, pirfenidone (4) and ninetedanib (3), have been shown to reduce the rate of lung function decline among IPF patients. However, neither approved drug is curative.

With survival-associated variants (e.g., *MUC5B* and *TOLLIP*) (74), it is possible that genotypes will define subtypes with differential responses to therapy. Identifying distinct sub-phenotypes in IPF may enable the application of targeted therapy on a pathway-specific basis. For example, it may be possible to use telomere length or *TERT* genotype to identify a group of patients who would benefit from telomere-directed therapy (95). Oldham and colleagues (98) found that some carriers with *TOLLIP* polymorphism may benefit from treatment with oral *N*-acetylcysteine (NAC). More specifically, of those that received NAC, subjects with TT genotype for rs3750920 (*TOLLIP*) had decreased risk for the trial's composite end point of death, hospitalization, or 10% decrement in forced vital capacity. In contrast, subjects with the CC genotype for rs3750920 had increased risk for the composite endpoints of the NAC intervention study (98). While NAC has not been shown to be effective in IPF in aggregate (99), it is possible that patients have differential response to this therapy (or other therapies) based on *TOLLIP* genotype (100). More prospectively designed studies are needed before genetic variation can be utilized routinely when choosing therapies for individual patients.

CONCLUSION

This review focuses on the relationship between genetic variants and IPF. In addition to sequence variation, epigenetic changes (such as DNA methylation) (101–104) and gene expression changes are associated with disease risk and phenotype (103, 105, 106). Further studies are necessary to better understand the relationships between genetic variation and epigenetic and gene expression variation in terms of disease risk and phenotype.

Given the growing body of evidence that genetic variants influence disease risk as well as disease progression and clinically meaningful patient outcomes, it will be critical to account for genetic variation in future clinical trials. Such prospective studies and analyses that focus on the relationship between genotype and therapeutic response will be crucial in personalizing and improving IPF therapy.

AUTHOR CONTRIBUTIONS

AK and SM researched and wrote first draft of manuscript; DS edited and revised document. All the authors read and approved final version of manuscript.

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REVIEW
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Acute Exacerbation in Interstitial Lung Disease

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Acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) has been defined as an acute, clinically significant deterioration that develops within less than 1 month without obvious clinical cause like fluid overload, left heart failure, or pulmonary embolism. Pathophysiologically, damage of the alveoli is the predominant feature of AE-IPF which manifests histopathologically as diffuse alveolar damage and radiologically as diffuse, bilateral ground-glass opacification on high-resolution computed tomography. A growing body of literature now focuses on acute exacerbations of interstitial lung disease (AE-ILD) other than idiopathic pulmonary fibrosis. Based on a shared pathophysiology it is generally accepted that AE-ILD can affect all patients with interstitial lung disease (ILD) but apparently occurs more frequently in patients with an underlying usual interstitial pneumonia pattern. The etiology of AE-ILD is not fully understood, but there are distinct risk factors and triggers like infection, mechanical stress, and microaspiration. In general, AE-ILD has a poor prognosis and is associated with a high mortality within 6–12 months. Although there is a lack of evidence based data, in clinical practice, AE-ILD is often treated with a high dose corticosteroid therapy and antibiotics. This article aims to provide a summary of the clinical features, diagnosis, management, and prognosis of AE-ILD as well as an update on the current developments in the field.

Keywords: acute exacerbation, interstitial lung disease, idiopathic pulmonary fibrosis, definition, diagnosis, management

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INTRODUCTION

Interstitial lung diseases (ILD) are a heterogeneous group of diseases. Despite various types of clinical presentation, disease progression, and prognosis, the common feature in most ILDs is a fibrotic destruction of the lung parenchyma. Within the clinical course of ILD, an acute exacerbation [acute exacerbations of interstitial lung disease (AE-ILD)] can occur at any time and is associated with significant morbidity and mortality (1–5). Initially, AE-ILD was described in idiopathic pulmonary

Abbreviations: AE-CTD, acute exacerbation of connective tissue disease related interstitial lung disease; AE-HP, acute exacerbation of chronic hypersensitivity pneumonitis; AE-IIP, acute exacerbation of idiopathic interstitial pneumonia; AE-ILD, acute exacerbations of interstitial lung disease; AE-IPF, acute exacerbation of idiopathic pulmonary fibrosis; AE-NSIP, acute exacerbation of non-specific interstitial pneumonia; AE-RA, acute exacerbation of rheumatoid arthritis and interstitial lung disease; ALI, acute lung injury; BAL, bronchoalveolar lavage; CCL, CC-chemokine ligand; CTD-ILD, connective tissue-related interstitial lung disease; DAD, diffuse alveolar damage; DLCO, diffusing capacity of the lung for carbon monoxide; FVC, forced vital capacity; HP, hypersensitivity pneumonitis; HRCT, high-resolution computed tomography; HSP, heat shock protein; IIP, idiopathic interstitial pneumonia; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; KL-6, Krebs von den Lungen-6; NSIP, non-specific interstitial pneumonia; RA-ILD, interstitial lung disease in patients with rheumatoid arthritis; UIP, usual interstitial pneumonia.

fibrosis (IPF), and according to the official American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Society IPF guideline, an acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) has been defined as an acute clinical worsening of dyspnea which develops within less than 1 month without an alternative etiology (6).

Pathophysiologically, AE-ILD resembles an acute lung injury (ALI), which presents histopathologically as diffuse alveolar damage (DAD) in most cases (7). However, DAD is not only found in autopsy studies of patients with IPF but also in patients with connective tissue-related ILD (CTD-ILD), idiopathic fibrotic non-specific interstitial pneumonia (NSIP), and chronic hypersensitivity pneumonitis (HP) (8, 9). Besides the histopathological DAD, AE-ILD and ALI have more clinical features in common, such as an increased oxygen requirement and new bilateral infiltrates on high-resolution computed tomography (HRCT) (e.g., ground-glass opacification/consolidation) (10–12).

While AE-IPF is increasingly recognized better and is perceived as a severe event with high mortality, there is only a limited amount of clinical data on AE-ILD in non-IPF ILD. The aim of this review is to provide a summary of the definition, clinical features, diagnosis, prognosis, and management of AE-ILD. Furthermore, this review will update the current developments in the field of AE-ILD not only in IPF but also in non-IPF ILD.

DEFINITION

Especially in IPF, great efforts have been made to establish a clear definition and diagnosis criteria for AE-IPF (6, 10, 13). In 2007, the IPF Clinical Trials Network (IPFnet) described the clinical presentation, radiological and histopathological findings of AE-IPF and developed diagnostic criteria based on the published literature (13). Just recently, an international working group revised an update on the definition of AE-IPF (**Table 1**) (10). In this document, AE-IPF is defined by a clinically significant respiratory deterioration developing within typically less than 1 month, accompanied by new radiologic abnormalities on HRCT such as diffuse, bilateral ground-glass opacification, and the absence of other obvious clinical causes like fluid overload, left heart failure, or pulmonary embolism (10). In contrast to the previous definition, the authors promote discrimination between a triggered AE-IPF (e.g., infection, post-procedural/postoperative or drug toxicity) and an idiopathic AE-IPF, where no trigger is identified (10). The revised definition aims to be broader and thus allow more inclusion possibilities. In the event of a clinical deterioration with unknown cause, where the criteria for AE-IPF are not met, the term "suspected AE-IPF" can be used (10). This might be the case if there are only unilateral ground glass abnormalities on HRCT or if HRCT data are even missing (13).

CLINCAL FEATURES AND DIAGNOSTIC EVALUATION

Unfortunately, so far, there is no existing official definition of AE-ILD in non-IPF ILD. Since an AE-ILD in non-IPF patients resembles AE-IPF (14–16), in the clinical setting it might be reasonable to apply the definition of AE-IPF to all AE-ILD. Still, it should be pointed out, that the current definition of AE-IPF refers exclusively to IPF and that the authors of the working group report decided against a definition including other ILD (10).

The clinical presentation of AE-ILD is usually a rapid worsening of respiratory symptoms with increased dyspnea within less than 1 month (10, 13). Additional findings can be cough, increased sputum production, fever, and flu-like symptoms (1, 14, 15, 17). Since many patients present with a severe hypoxemia in the arterial blood gas analysis and respiratory failure, admission to the intensive care unit and assisted ventilation is often required (13). Established criteria for a presenting abnormal gas exchange is a Pa_{O2}/Fi_{O2} ratio <225 or a decrease in Pa_{O2} of ≥10 mmHg over time (13).

TABLE 1 | Revised and previous definitions and diagnostic criteria for AE-IPF.

Diagnosis of AE-IPF	Revised diagnosis	Previous diagnosis
Definition		
	An acute, clinically significant, respiratory deterioration characterized by evidence of new widespread alveolar abnormalities	An acute, clinically significant, respiratory deteriorartion of unidentifiable cause
Diagnostic criteria		
– Previous diagnosis	Previous or concurrent diagnosis of IPF	Previous or concurrent diagnosis of IPF
– Clinical presentation	Acute worsening or development of dyspnea typically of less than 1 month	Unexplained worsening or development of dyspnea within 30 days
– Computed tomography findings	New bilateral ground-glass opacity and/or consolidation superimposed on a background pattern consistent with usual interstitial pneumonia (UIP) pattern	New bilateral ground-glass abnormality and/or consolidation superimposed on a background reticular or honeycomb pattern consistent with UIP pattern
– Exclusion of differential diagnosis	Deterioration not fully explained by cardiac failure or fluid overload	Exclusion of alternative causes, including left heart failure, pulmonary embolism and an identifiable cause of acute lung injury
– Concomitant Infection		No evidence of pulmonary infection by endotracheal aspirate or bronchoalveolar lavage

AEIPF, acute exacerbation of idiopathic pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis.

Still, establishing the diagnosis of AE-ILD often comprises a challenge. In order to get to this diagnosis, various diagnostic tests should be performed and differential diagnosis like myocardial infarction, pulmonary embolism, or fluid overload need to be excluded (**Figure 1**). An elementary part of the diagnosis is the HRCT, which should be carried out in all patients who are clinically stable. The important finding in AE-ILD is newly developed, bilateral alveolar infiltrates like ground-glass opacification with or without consolidation on HRCT (**Figure 2**) (6, 14, 16). Three suggested HRCT abnormality patterns are peripheral, multifocal and diffuse ground glass, with the latter two being associated with histologically DAD (13, 18). Several studies have shown that the extent of disease on HRCT seems to be related with the clinical outcome (1, 19–21). If there is no previous HRCT scan available,

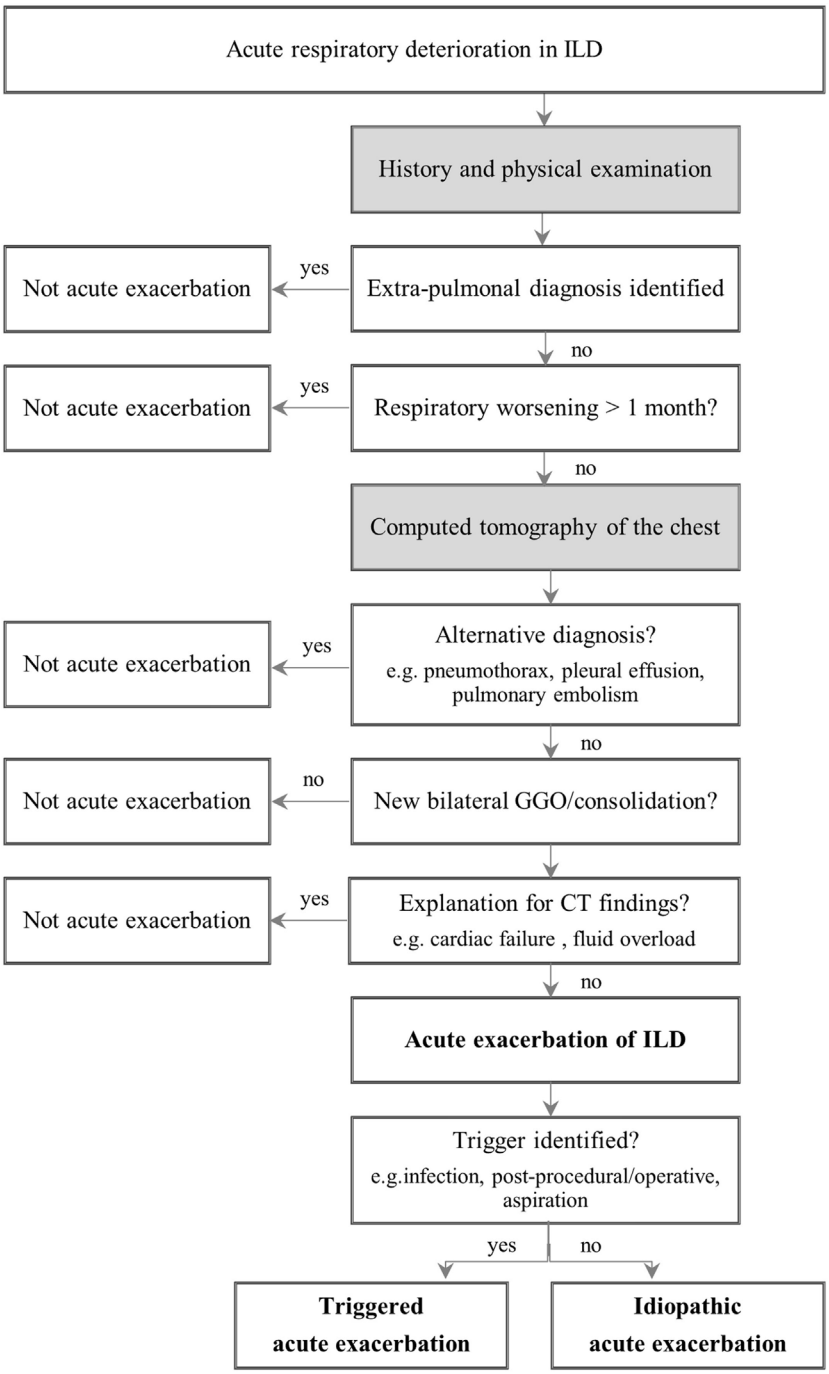

FIGURE 1 | Diagnostic approach to acute exacerbation in interstitial lung disease. Adapted from Ref. (10). Abbreviation: ILD, interstitial lung disease; GGO, ground glass opacity; CT, computed tomography.

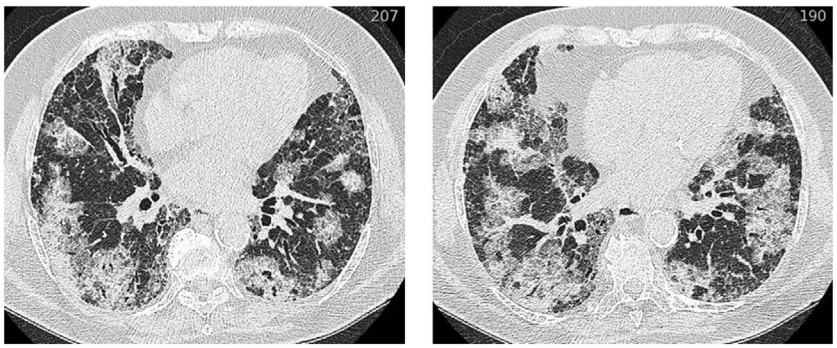

FIGURE 2 | HRCT of an acute exacerbation in IPF. Axial HRCT of a patient with IPF at the time of an acute exacerbation shows extensive bilateral ground-glass opacification. Abbreviation: HRCT, high resolution computed tomography; IPF, idiopathic pulmonary fibrosis.

bilateral ground-glass opacity and/or consolidation on a background of usual interstitial pneumonia (UIP) pattern is sufficient to confirm the radiographic diagnostic criteria of AE-IPF (10). The term "suspected AE-IPF" should be used if there are only unilateral ground glass abnormalities (13).

Histologically, most AE-ILDs are characterized by a DAD, while alternative histological appearances comprise organizing pneumonia, alveolar hemorrhage, and unspecific inflammatory changes (1, 13, 15, 22, 23). In early stages, the acute injury of the lung is characterized by an interstitial edema and hyaline membranes (10). Furthermore, type II pneumocyte hyperplasia and fibroblast foci have been reported in biopsies/autopsies as well as squamous metaplasia and honeycombing with and without hyaline membranes (24).

It has been reported, that patients with AE-ILD present with elevated inflammatory laboratory values such as increased white blood cell count, elevated values of erythrocyte sedimentation rate, and C-reactive protein and increased lactate dehydrogenase (14, 23–25). Although bronchoalveolar lavage (BAL) is not done routinely in AE-ILD, it has also been shown that AE-IPF and AE-HP are associated with an increase in neutrophils in BAL (1, 9, 17, 24–27). Rarely, lymphocytosis has been observed (23, 28), and reactive type II cells hyperplasia has been dectected on BAL (24). Furthermore, BAL is still the subject of research in terms of investigating the pathogenesis of AE and identifying possible prognostic factors.

EPIDEMIOLOGY

Acute exacerbations of interstitial lung disease can occur at any time during the disease, and in some cases it can be the presenting manifestation of an ILD (1, 13, 17). The exact frequency is unknown and the reported incidence rates of AE-ILD broadly vary, most likely due to differences in definition, ILD-entity and disease severity (10, 29). Furthermore, due to incomplete clinical information, definite AE-ILD cannot be confirmed in some cases, although AE-ILD is the suspected and the most probable diagnosis (10). The impact of this relevant difference was investigated in a *post hoc* analysis of the STEP-IPF trail, where a definite AE-IPF occurred in 40 per 1,000 patient-years but combining definite and suspected AE-IPF raised the number to 200 per 1,000 patient-years (30). In a recently published central adjudication on three randomized controlled trials, only 33.2% of the investigator-reported AE-IPF met the criteria (31). A meta-analysis of six randomized-controlled clinical trials identified a weighted average of 41 AE-IPF per 1,000 patient-years (32). In the INPULSIS I and II trial, the 1-year incidence of AE-IPF in the placebo-arm was 7.6% (33).

Compared to clinical trials, retrospective studies report even higher 1-year incidences of AE-IPF, ranging from 7 to 19.1% with highest risk in advanced IPF (1, 2, 34–36). Retrospective analyses of studies from the US and Japan identified the incidences of AE-IPF in approximately 52 per 1,000 patient-years (37, 38). In a registry-based US study, the annual rate of AE in IPF was 133 per 1,000 patient-years (39).

There is much less data on the frequency of AE-ILD in non-IPF ILD compared to AE-IPF. However, the majority of studies indicate that patients with IPF are at a higher risk for developing AE compared to non-IPF ILD (40–43). The estimated 1-year incidence of AE-NSIP is reported to be 4.2%, and the estimated 1-year incidence of AE-CTD ranges from 1.25 to 3.3% (14, 16). Within CTD-ILD, AE seems to be most common in patients with rheumatoid arthritis ILD (RA-ILD) (16). Since the frequency of a UIP pattern is higher in RA-ILD compared to other ILDs, the higher number of AE-RA may be explained by the observation that a UIP pattern *per se* is associated with a higher risk of AE-ILD. Thus, in patients with CTD-ILD and RA-ILD with UIP pattern, a 1-year incidence of 5.6 and 11.1% was found, respectively (14). Furthermore, the 2-year incidence of AE-HP was 11.5% among patients with chronic HP and UIP-like lesions on surgical biopsies (9).

Moreover, ethnicity may play a role, since AE-ILD were initially observed and reported in Japan and Korea, and the literature still is dominated by reports from Asian countries (1, 14, 19, 35). However, two randomized, controlled studies did not support this observation (33, 44, 45).

PATHOGENESIS AND ETIOLOGY

The onset and development of an AE-ILD is unpredictable and until now, it is uncertain, whether an AE-ILD is triggered by an intrinsic factor causing a progression of the underlying disease or a response to an external factor (e.g., infection, aspiration,

pulmonary emboli, mechanical stretch) or both (6, 10). Most likely, environmental and genetic factors interact individually leading to AE-IPF in only a subset of patients (13). Concerning the parallels between AE-IPF and acute respiratory distress syndrome, the IPF lung may be generally more vulnerable to intrinsic and extrinsic triggers (10). Still, further research is needed to identify the underlying causes and potential biomarkers for AE-ILD.

Epithelial Injury

During AE-IPF, alveolar injury and loss of epithelial cell integrity may be involved leading to an increased fibrin production and remodeling (13, 46). Morphologically, this leads to neutrophilia in BAL and histopathological DAD (8, 17, 24). Neutrophilic processes are potentially transmitted *via* α-defensins, as they have been shown to be upregulated in patients with AE-IPF (47, 48). α-defensins belong to a family of antimicrobial and cytotoxic peptides contained in mammalian neutrophils (49, 50). Supporting the hypothesis of epithelial injury and proliferation during AE-IPF, a gene expression study of lung tissue detected an increased expression of cyclin A2 and α-defensins together with widespread apoptosis in lungs of patients suffering from AE-IPF in comparison to stable IPF and healthy controls (47). Furthermore, α-defensins were increased in the peripheral blood of patients with AE-IPF, suggesting a potential role as biomarker (47). In a later study including patients with idiopathic interstitial pneumonia (IIP), elevated plasma levels of α-defensins in AE-IIP compared to stable IIP were also seen, but they were not useful as biomarkers due to a lack of specificity (51). Therefore, further studies are needed to clarify the role of α-defensins as biomarker.

Moreover, it could be shown that fibrocytes, which are increased in stable IPF, are even more elevated during AE-IPF (52). Fibrocytes, CD45 and collagen-1 positive cells, are mesenchymal derived progenitor cells which can migrate into injured tissue and can differentiate to fibroblast-like cells playing a role in wound repair, tissue regeneration and pulmonary fibrosis (53, 54). Patients with fibrocytes >5% of total blood leukocytes had a significantly worse survival compared to patients with fibrocytes <5% (52).

Another theory includes the involvement of alternative, so called M2, activation of macrophages in AE-IPF. M2 macrophages play an important role in tumor progression and wound healing (55), and seem to be associated with ILD (56–58). It could be shown that the pro-inflammatory chemokines CXCL1 and Interleukin 8 (produced by classically activated macrophages) and the anti-inflammatory chemokines CC-chemokine ligand (CCL)2, CCL17, CCL18, CCL22, and Interleukin 1ra (produced by alternatively/M2 activated macrophages) were elevated in BAL of patients with AE-IPF in comparison to stable IPF (26). High CCL18 levels in BAL at baseline were further highly predictive for a future AE-IPF (26).

Further markers have been studied, including Krebs von den Lungen-6 (KL-6) and surfactant protein D, which were both identified to be elevated in AE-IPF compared to stable IPF (59) and KL-6 was also increased during the event of AE-HP (60). Furthermore, an elevated serum level of KL-6 at baseline was identified as a predictor for developing AE-ILD in both, IPF and combined pulmonary fibrosis and emphysema (34, 61). KL-6 is a mucin-like glycoprotein, which is mainly expressed in type II penumocytes and bronchial epithelial cells (62). In ILD, a high expression of KL-6 has been detected in regenerating type II cells likely being the primary source of serum KL-6 (63).

Increased levels of Interleukin 6 and Interleukin 8 were also detected in patients with AE-IPF and an increase in either of them was identified to be associated with worse outcome (59, 64). Total protein C, thrombomodulin, plasminogen activator inhibitor 1 (59), and leptin (65) have also been shown to be elevated in the serum of patients with AE-IPF in comparison to stable IPF. In chronic HP, patients with elevated levels of KL-6 and surfactant protein D as well as increased neutrophils in the BAL fluid also have a higher risk for developing AE-HP.

Autoimmunity

Heat shock protein (HSP) 47 is a human collagen-specific molecular chaperone, which is involved in the early stages of biosynthesis and secretion of collagen molecules (66). In AE-IPF, it has been shown that HSP47 serum levels were significantly higher in comparison to stable IPF (67). Furthermore, immunohistochemical analysis detected more HSP47 expression in DAD than in UIP tissues (67). Interestingly, another study identified anti-HSP70 IgG autoantibodies in 25% of patients with IPF and anti-HSP70 positivity was associated with a higher mortality and risk for AE-IPF (68). Mortality among patients with positive anti-HSP70 antibodies was significantly higher compared to patients with negative antibodies (68).

Supporting autoimmune involvement in AE-IPF, one study identified annexin 1 as an autoantigen which increased antibody production and T cell response in AE-IPF with the N-terminus of annexin 1 potentially playing a role in the pathogensis of AE-IPF (69).

Infection

There is an increasing number of findings indicating that infection, both viral and bacterial, might be involved in some cases of AE-ILD. First, in a minority of patients with IIP suffering from AE-IIP, viral ribonucleic acid or a rise in specific immunoglobulins was detected by polymerase chain reaction or pan-viral microarray (70–74). Moreover, changes in the respiratory microbiome were recently identified showing an increased bacterial burden in BAL during an AE-IPF (27). Patients with AE-IPF experienced a markable change in the respiratory microbiome with an increase in *Campylobacter* sp. and *Stenotrophomonas* sp., as well as a significant decrease in *Veillonella* sp. compared to stable IPF (27). The hypothesis of an underlying infection is supported by the fact, that AE-IPF occurs more often between December and May (30, 75) and in the majority of studies an immunosuppressive therapy increases the risk for developing AE-IPF (30, 37, 76).

Microaspiration

Microaspiration might also have a connection to the development of AE-ILD (10). In a *post hoc* analysis of the placebo-treated IPF patients in three clinical trials, none of the patients developing AE-IPF was on an anti-acid therapy (77). Furthermore, patients with AE-IPF had significantly higher levels of pepsin in BAL compared to stable controls, suggesting an involvement of occult aspiration (78).

RISK FACTORS

Several clinical risk factors are discussed playing a potentially crucial role in developing an AE-IPF. First of all, a functionally and clinically advanced stage of disease appears to be an important risk factor. In this context, a low forced vital capacity (FVC) seemed to be the most stable risk factor (2, 26, 30, 34, 35). Other clinical risk factors include a recent decline in FVC (35, 38, 79), a low diffusing capacity of the lung for carbon monoxide (DLCO) (9, 26, 30), a low total lung capacity (9), a low 6-min walking distance (30), an impaired baseline oxygenation (30, 38), an increased dyspnea (30, 35), and a previous AE-IPF (37, 79). However, it should also be considered, that the apparent association between advanced IPF and risk of AE-IPF may be biased by the fact that in advanced disease an AE may have more obvious clinical consequences, while it may even be overlooked in less advanced disease.

In 2007, Selman et al. discriminated IPF patients as rapid or slow progressors based on the duration of symptoms before first presentation (80). Although this study did not analyze AE-IPF, the authors stated that the rapid progression of AE-IPF does not correspond to an AE-IPF (80). Still, some links might be present as the rapid progressors showed a higher rate of fibroblast migration than slow progressors and survival was significantly reduced (80). Until now there is no proof for the theory that rapid progressors and AE-IPF could have a connection but obviously, there is not enough data in this field. Given the association between a recent decline of FVC and an increased risk of AE-IPF (35, 38, 79), it would be further interesting to acknowledge daily variability of FVC. In this context, daily home spirometry is a promising clinical tool to follow the clinical course of IPF patients more closely and potentially detect AE-IPF earlier (81).

Although AE-ILD can occur in different histological forms of ILD, UIP-like lesions were identified to be associated with a higher risk for AE-ILD in patients with chronic HP and CTD-ILD (9, 14). Additional risk factors including male gender (15), a co-existing pulmonary hypertension (36), coronary artery disease (30), a higher body-mass-index (35), and exposure to increased ozone and nitrogen dioxide levels (37) have been reported. Some studies observed a higher risk in former smokers (9, 34), but this finding is inconsistent (2, 26, 82). Similarly, there are different findings concerning age as a potential risk factor: in IPF, younger patients seem to be at a higher risk for AE-IPF (26), whereas a study on CTD-ILD identified higher age as a risk factor for developing AE-CTD (16).

In a retrospective study on patients with ILD and lung cancer undergoing chemotherapy, 21.9% of the patients experienced AE-ILD during the time from diagnosis to the end of the chemotherapy treatment period (83). The authors suggested tegafur–gimeracil–oteracil potassium (S-1) and etoposide as relatively safe options in these patients. Moreover, there is one case-report about a patient with primary lung cancer and subclinical IPF, developing an AE-IPF after hypofractionated stereotactic radiotherapy (84).

A surgical biopsy is another important risk factor triggering AE-ILD. Whereas the incidence rates of developing an AE-ILD after a surgical biopsy for ILD-diagnosis finding are reported to be less than 2.5% (40, 41, 43, 85), AE-ILD after pulmonary resection due to lung cancer can occur in 3–32% (40, 86–88). A decreased FVC and DLCO seemed to be additional risk factors for patients with ILD developing a respiratory deterioration after lung surgery (43, 89). Pulmonary surgery itself seems to be a risk factor, but AE-ILD has also been reported in non-pulmonary surgery and throughout major surgeries the incidences was 3.3% in a study from Korea (90, 91). In IPF, there might be an association between AE-IPF and BAL, as few, individual cases of AE-IPF following BAL exist (92, 93). Furthermore, as this will become increasingly important in the future, the risk of AE-ILD after cryobiopsy needs to be investigated. However, data in this field is still limited and so far, only single cases of AE-ILD following cyrobiopsy have been reported (94, 95).

PROGNOSIS

Acute exacerbations of interstitial lung disease is a life-threatening event and the mortality rate is high. It is assumed that between 35 and 46% of deaths in IPF are caused by AE-IPF (35, 96, 97). In a large number of studies, the in-hospital mortality in AE-IPF is estimated over 50% (1–4, 17, 19, 20, 36, 70, 98) and the median survival after AE-IPF is between 1 and 4 months (2, 36, 75, 82). In IPF, the 1-month mortality ranges between 37 and 53% (37, 99), and the 3-month mortality rate ranges from 63.8 to 73.7% (5, 19, 99). The existing data suggest that patients with IPF have a worse survival compared to ILD patients other than IPF; nonetheless, AE-ILD is also fatal in non-IPF ILD (5, 74). In a study including IPF and non-IPF patients, the overall survival after admission for AE-ILD was 67% at 1 month and 40% at 3 months (4). Similarly to IPF, the highest overall mortality rate of AE-ILD is seen in AE-HP (75–100% mortality) (9, 15). Mortality of AE-ILD in other ILDs ranges from 34 to 83% (5, 16).

Some potential prognostic factors have been identified. First of all, lower baseline pulmonary function parameters (FVC and DLCO) as well as a more impaired oxygenation are associated with a worse outcome in AE-IPF (2, 35, 75). Furthermore, a higher fibrosis score or more extensive disease on HRCT seems to be of prognostic relevance (1, 19–21). A lymphocytosis >15% in the BAL might be another prognostic factor for a favorable outcome in patients with AE-IIP (28). Several markers in the blood could also be potential prognostic markers including lactate dehydrogenase (9, 19, 75), C-reactive protein (2), KL-6 (19, 21), circulating fibrocytes (52), and anti-HSP70 autoantibodies (68). Just recently, Kishaba et al. developed a staging system for AE-IPF, which includes some of these prognostic factors (19).

TREATMENT

So far, there is a lack of evidence based data on effective therapies in AE-ILD. In clinical practice, AE-ILD is often treated with high-dose systemic corticosteroid therapy and antibiotics (9, 14, 16). In AE-IPF, the current international guidelines give a weak recommendation on the treatment with corticosteroids emphasizing that this recommendation is based on anecdotal reports of benefit and the high overall mortality in AE-IPF (6). The authors further point out, that there was a consensus to promote supportive care as an important therapy strategy (6). This includes palliation of symptoms, e.g., with opioids,

and supply of oxygen in hypoxemia. Still, there are different opinions on the length of supportive care, and regarding the use of mechanical ventilation (10). Based on an estimated 90% in-hospital mortality, the international guidelines on the management of IPF make a weak recommendation against the use of mechanical ventilation in the case of respiratory failure due to the underlying lung disease (6). The authors point out that this decision has to be made case-by-case together with the physician, the patient, and the family and in accordance with the individual goals of care (6). As a bridge to lung transplantation, mechanical ventilation, or extra-corporal membrane oxygenation may be appropriate and successful in selected patients (6, 100). In a recent retrospective cohort study, the mortality rate in IPF patients undergoing mechanical ventilation significantly decreased from 58.4% in 2006 to 49.3% in 2012 (101). This reminds us to carefully analyze every single patient before a decision for or against mechanical ventilation is taken.

Several studies on different therapy regimens in AE-IPF, other than high-dose intravenous corticosteroids mono, have been published (**Table 2**). In smaller, observational studies, it could be

TABLE 2 | Medical treatment of AE-IPF other than high-dose intravenous corticosteroids mono therapy.

Treatment	Reference	Study design	Number of patients	Treatment/intervention	Clinical outcome
Tacrolimus	Horita et al. (82)	Single-center, retrospective study	15	Steroids mono versus combination steroids plus tacrolimus	Significantly better survival in tacrolimus-group
Cyclosporine	Inase et al. (102)	Single-center, retrospective study	14	Steroids mono versus steroids followed by cyclosporine	Cyclosporine seemed to prevent re-exacerbation and improve survival (no data on significance level)
	Homma et al. (103)	Retrospective study	44	Effect of treatment with steroids mono versus steroids plus cyclosporine before AE-IPF	Significantly better survival in cyclosporine-group
	Sakamoto et al. (104)	Single-center, retrospective study	22	Steroids mono versus combination of steroids plus cyclosporine	Significantly better survival in cyclosporine-group
Rituximab	Donahoe et al. (105)	Pilot- phase I/II-study; historical controls	31	Steroids mono versus combination of steroids plus rituximab/ therapeutic plasma exchanges and IVIG in severely ill IPF	Significantly better 1-year survival in rituximab group
PMX	Seo et al. (99)	Open-label pilot trial	6	Combination of steroids plus PMX	Potential beneficial effect of treatment with PMX
	Abe et al. (106)	Multi-center, retrospective study	160	Combination of steroids plus PMX	PMX improved oxygenation and may improve survival in IP patients with AE
	Abe et al. (107)	Single-center, retrospective study	45	Steroids mono versus combination of steroids plus PMX	PMX treatment significantly improved oxygenation
	Oishi et al. (108)	Single-center, retrospective study	50	Steroids mono versus combination of steroids plus PMX	Significantly better 1-year survival in PMX group
	Oishi et al. (109)	Single-center, retrospective study	26	Stable IPF and healthy controls versus combination of steroids plus PMX in AE-IPF	PMX treatment significantly improved oxygenation
Thrombomodulin i.v.	Isshiki et al. (110)	Single-center, retrospective study	41	Steroids mono versus combination of steroids plus recombinant human soluble thrombomodulin	Thrombomodulin treatment significantly improved 3-month survival
	Kataoka et al. (112)	Single-center, retrospective study	40	Combination of steroids and cyclosporine versus combination of steroids and cyclosporin plus recombinant human soluble thrombomodulin	Thrombomodulin treatment significantly improved 3-month survival
	Tsushima et al. (111)	Single-center, combined prospective and retrospective study	20	Combination of steroids plus recombinant human soluble thrombomodulin	Thrombomodulin treatment significantly improved oxygenation
	Hayakawa et al. (113)	Single arm, non-randomized prospective clinical trial; historical controls	23	Steroids mono versus combination of steroids plus recombinant human soluble thrombomodulin	Thrombomodulin plus steroid pulse therapy improved oxygenation and may improve overall survival
	Abe et al. (114)	Single-center, prospective, non-randomized study	22	Steroids mono versus combination of steroids plus recombinant human soluble thrombomodulin	Thrombomodulin treatment significantly improved 3-month survival
Procalcitonin-guided antibiotic therapy	Ding et al. (115)	Single-center, prospective, randomized study	68	Clinically guided versus procalcitonin-guided antibiotic therapy	Procalcitonin-guided antibiotic therapy had no benefits on survival

(*Continued*)

TABLE 2 | Continued

Treatment	Reference	Study design	Number of patients	Treatment/intervention	Clinical outcome
Cyclophosphamide	Akira et at (20)	Single-center, retrospective study	58	Steroids mono and combination of steroids mono plus cyclophosphamid	No data on treatment-related outcome
	Fujimoto et al. (21)	Multi-institutional, retrospective study	60	Steroids plus cyclophosphamide and steroids plus cyclosporine	No data on treatment-related outcome
	Yokoyama et al. (116)	Single-center, retrospective study	11	Steroids mono and combination of steroids mono plus cyclophosphamide and combination of steroids mono plus cyclosporine	No data on treatment-related outcome

AE-IPF, acute exacerbation of idiopathic pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis; i.v., intravenous; IVIG, intravenous immunoglobulin; PMX, polymyxin B-immobilized fiber column.

shown that the combination of a steroid-pulse therapy with oral tacrolimus (82) or cyclosporine (102–104) was superior to the corticosteroid mono therapy in terms of prognosis in IPF. Other studies identified a positive effect of a treatment with rituximab with plasma exchange and intravenous immunoglobulin (105), polymyxin B-immoblilized fiber column perfusion (99, 106–109), and intra-venous thrombomodulin (110–114). Still, the benefits seen in these studies have to be critically assessed since these were all observational studies with either a historical control or a parallel, untreated control arm, potentially excluding very ill patients from the experimental arm (10). One randomized trial investigated the benefit of a procalcitonin-guided antibiotic therapy compared to a clinical-driven antibiotic therapy but no difference in mechanical ventilation and mortality was seen (115). Several studies reported about a combination therapy of corticosteroids with other immunosuppressant drugs like cyclophosphamide, but it remains unclear whether this is beneficial (20, 21, 116).

Currently, there are indications that an anti-acid therapy could have a protective effect against AE-IPF, as in an analysis of patients from the placebo arm of three large clinical trials, an anti-acid therapy was reported to have a potentially preventive effect on the development of AE-IPF (77). The same potentially preventive effect applies for antifibrotic drugs, although, so far, there is no sufficient data on whether an antifibrotic therapy with nintedanib or pirfenidone should be paused or continued in the event of an AE-IPF. In a phase II trial, patients receiving pirfenidone had a significant reduction in AE-IPF compared to placebo (117). However, a subsequent phase II trial could not reproduce this finding (44). After AE-IPF was not included as an endpoint in the three phase III trials ASCEND and CAPACITY (118, 119), a pooled analysis recently showed that patients receiving pirfenidone had a lower risk for respiratory-related hospitalization compared to healthy controls (120). Interestingly, there is data that the perioperative use of pirfenidone might prevent postoperative AE-IPF (121). In contrast to pirfenidone, AE-IPF was consequently included in the nintedanib clinical study program as a key secondary endpoint; however, the role of nintedanib on AE-IPF needs to be fully understood. Whereas the phase II trial of nintedanib identified a delay in time to the first investigator-reported AE in the nintedanib arm (122), only one of the two INPULSIS phase III twin-trials showed a significant effect of nintedanib on AE-IPF (33). Pooled analysis of the data showed a highly significant prolongation of the time to first AE in IPF due to treatment with nintedanib, thus confirming its preventive effect (45, 123). Still, at this point, more data is needed to validate the effect of pirfenidone and nintedanib in AE-ILD.

Apart from these potentially effective therapeutic approaches, there are a number of drugs that seem to have no preventive effect on AE-IPF, including acetylcystein mono therapy (124), sildenafil (125), bosentan (126), interferon-gamma 1b (127), warfarin (128), ambrisentan (129), and imatinib (130). A combination "triple" therapy (prednisone, azathioprine and acetylcysteine), might even increase the risk for developing AE-IPF (131).

As in AE-IPF, AE-ILD in non-IPF ILD is often treated with a high dose, systemic corticosteroid therapy together with broad-spectrum antibiotics (9, 14, 16). There are some studies additionally using cyclosporine A or cyclophosphamide, but there are no reports on whether there was any benefit in these therapies (9, 16). Similar to IPF, treatment with intra-venous thrombomodulin significantly improved 3-month survival in AE-NSIP (114).

In order to address all therapeutic approaches in this context, one study should be mentioned focusing on a non-steroid approach in AE-IPF: in the event of AE-IPF, prior immunosuppression was immediately stopped and patients were only treated with best supportive care and broad-spectrum antibiotics (132). The median survival of all patients was 1.73 months. Analyzing the single event of AE-IPF, 50% of AE-IPF episodes were survived. Overall, 35.3% of the patients survived AE-IPF and the 1-year survival of the survivors was 83%. Interestingly, patients who had never been treated with immunosuppressant drugs before had a significantly better survival. The 1-year survival in the "never treated" group was 65%, whereas the patients, who had a history of immunosuppression, had a 1-year survival of 17%. Unfortunately, no comparison with a high-dose steroid therapy during AE-IPF was investigated in the study. Nevertheless, this underlines again the lack of evidence based data on therapy strategies in AE-ILD and the necessity for further studies in this field.

FUTURE

Acute exacerbations of interstitial lung diseases are severe events with a high mortality rate. Therefore, it is important to gain further

knowledge in this field. As it has been shown that in IPF, an early referral to a specialized center is crucial for survival in general (133), in AE-ILD an early diagnosis and referral might also be important for the patients' prognosis. Therefore, effort should be made to detect early signs of AE-ILD and identify patients who are at a higher risk for developing AE-ILD.

Potential treatment options should be studied in randomized, controlled trials. The currently revised definition of AE-IPF will hopefully allow a more uniform diagnosis, which will help to conduct well designed clinical trials in IPF (10). However, it should be emphasized that, even if it largely follows the framework of AE-IPF guidelines, an official, uniform definition for AE-ILD is needed in the future.

Biomarkers, which can be obtained in an easy and harmless way, are needed to identify patients at a higher risk for developing AE-ILD before symptoms and HRCT features are present. Since biomarkers in BAL might be difficult to obtain in a severely ill patient, serum markers are particularly interesting because of their easier accessibility. Furthermore, daily home spirometry might be a potential tool to understand the clinical course of AE-PF better and even possibly detecting AE-IPF in an early stage (81). There is evidence that home spirometry can potentially improve endpoint efficacy in clinical trials of IPF-therapeutics (134). Therefore, an effort should be made to design studies in that field analyzing the benefit of daily home spirometry in patients with ILD and help establishing home spirometry in clinical, daily routine.

CONCLUSION

Acute exacerbations of interstitial lung disease is a life-threatening event with a high in-hospital mortality rate. The clinical presentation of AE-ILD is similar in non-IPF and IPF, but AE-ILD in non-IPF ILD is less common and the clinical course is less fatal compared to IPF. The new working group report on AE-IPF supports that there are both, idiopathic and triggered AE (e.g., triggered by an infection) (10). So far, there is no evidence as to whether a triggered AE-ILD has a worse prognosis than an idiopathic AE-ILD. Due to the lack of evidence-based therapy options, more studies in this field are urgently needed.

AUTHOR CONTRIBUTIONS

GL and JB wrote the manuscript and have approved the final version of the manuscript for submission.

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Integrating Patient Perspectives into Personalized Medicine in Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a progressive and ultimately fatal disease which has a major impact on patients' quality of life (QOL). Except for lung transplantation, there is no curative treatment option. Fortunately, two disease-modifying drugs that slow down disease decline were recently approved. Though this is a major step forward, these drugs do not halt or reverse the disease, nor convincingly improve health-related QOL. In daily practice, disease behavior and response to therapy greatly vary among patients. It is assumed that this is related to the multiple biological pathways and complex interactions between genetic, molecular, and environmental factors that are involved in the pathogenesis of IPF. Recently, research in IPF has therefore started to focus on developing targeted therapy through identifying genetic risk factors and biomarkers. In this rapidly evolving field of personalized medicine, patient factors such as lifestyle, comorbidities, preferences, and experiences with medication should not be overlooked. This review describes recent insights and methods on how to integrate patient perspectives into personalized medicine. Furthermore, it provides an overview of the most used patient-reported outcome measures in IPF, to facilitate choices for both researchers and clinicians when incorporating the patient voice in their research and care. To enhance truly personalized treatment in IPF, biology should be combined with patient perspectives.

Keywords: idiopathic pulmonary fibrosis, health-related quality of life, personalized medicine, patient-reported outcomes, personomics, patient experiences

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INTRODUCTION

> *Give different ones [therapeutic drinks] to different patients, for the sweet ones do not benefit everyone, nor do the astringent ones, nor are all patients able to drink the same things* ***Hippocrates*** (1)

Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonia (2). IPF is characterized by progressive decline of lung function, with a median survival of only 3–5 years (3). Common symptoms as breathlessness, cough, and fatigue have a major impact on the quality of life (QOL) of patients (4). IPF occurs more often in men than women and usually affects elderly patients, aged 50 years and above (3). There are two approved anti-fibrotic drugs that slow down disease decline, but these drugs do not halt or reverse the disease, and ultimately IPF remains a fatal disease (5, 6). The heterogeneity in disease behavior and response to therapy in IPF has (further) stimulated research to identify possible distinct underlying genetic, molecular, and environmental factors associated with IPF (7, 8).

The potential to enhance personalized treatment has prompted excitement also in the IPF field (7). Until now, the focus of personalized medicine has been on physiology and the use of this biological information to predict response to treatment and to develop targeted therapy (9). In this process, patient factors should not be overlooked. For real personalized treatment patient perceptions and preferences should also be taken into account. In this article, we focus on recent insights and methods on how to integrate patient perspectives into personalized medicine.

Impact of Disease

Idiopathic pulmonary fibrosis is a heterogeneous disease, with a highly variable disease course (10, 11). Additionally, different phenotypes of IPF exist. Most patients have a slow disease progression, while some patients display relative stable periods followed by acute exacerbations and a small group of patients experiences a rapid decline in lung function (12). Uncertainty about the disease course and prognosis can cause emotional distress and anxiety, and, as a result, IPF has a major impact on most patients' health-related quality of life (HRQOL). HRQOL can be defined as a patient's perceived well-being affected by disease and treatment of the disease (13). IPF affects patients in almost every domain of life; hence, the burden of the disease is high, not just for patients but also for their partners and families. Patients often struggle with loss of independence because of functional limitations and deteriorating symptoms. Not only can breathlessness, cough, and fatigue diminish QOL, but also other symptoms such as sleep disorders, loss of appetite, and psychological problems can (14–18).

Most clinical trials in IPF that have been performed so far, have shown no convincing improvement of patient HRQOL (5, 6, 19). To date, the main focus in research has been to stabilize or improve physiological outcomes rather than HRQOL. Physiological parameters, such as lung function, do not correlate well with HRQOL measurements (20, 21). To our knowledge for parameters as imaging and biomarkers, relationships with HRQOL have not yet been established. Thus, decline in lung function does not adequately reflect the perceived impact of the disease on patients' lives.

Every person has a different lifestyle, personal circumstances, and coping strategies. These factors can play an important role in how a disease manifests itself; hence, the same disease affects each person in a different way (16, 22, 23). Medication may show promising results at group level in randomized controlled trials, but still in some individual patients, treatment may fail (22). For example, the side effects of medication may outweigh the positive effects of medication in daily practice, or the burden of treatment might be too high for patients. To improve and personalize treatment of IPF, we should also include patient perspectives and QOL.

Personomics

Personalized, stratified, or precision medicine is a broad term which can be referred to as "delivering the right treatment to the right patient at the right time" (24). Personalized medicine has gained increasing attention during the past decade (22, 25). However, the concept is not new; Hippocrates already mentioned the importance of a personalized approach to diagnosis and treatment in the fifth century BC, stating that "individuality of human beings affects predisposition to disease and response to treatment," and also noting that "not all patients are able to drink the same therapeutic drinks" (1, 26). His concepts already include the notion that experiences with treatment differ among patients. This idea is also acknowledged by Britten et al., who suggest that because individuals are more than their genetic profile, the main concept of stratified medicine is too limited at the moment (22). Personalized treatment comprises not only "biology," but should also focus on patient perspectives, needs, experiences, personality, environment, lifestyle, and other personal circumstances (**Figure 1**) (9, 22). Accordingly, the term "personomics" has been introduced to capture a patient's life circumstances that may alter disease behavior and response to treatment (23). Below we will briefly touch on the role of biology and other aspects of personalized medicine as shown in **Figure 1**, but the focus will be on patient perspectives.

Current View of Personalized Medicine in IPF

In other fields, especially oncology, personalized medicine has dramatically changed clinical practice during the last few years. Biomarkers have been used to develop targeted therapy and allocate patients to individual treatment plans (27–29).

Currently, the diagnosis of IPF is based on clinical, radiological, and pathological findings (3). The exact etiology of IPF is, however, incompletely understood. One of the proposed hypotheses is the concept of dysfunctional wound healing: repeated epithelial injury and dysfunctional regeneration possibly in combination with a dysregulated immune system normally facilitating wound healing leads to fibrogenesis and, as a consequence, excessive scarring of the lung tissue (11, 30). Epithelial injury might be caused by risk factors such as cigarette smoking, micro-aspiration of gastric content, and lead to development of IPF in susceptible individuals (11). At present, it is assumed that multiple biological pathways and complex interactions between genetic, molecular, and environmental factors are involved in the pathogenesis of IPF. Improved understanding of the pathogenesis of IPF has led to the identification of potential molecular biomarkers (7, 11, 31–33). Genome-wide association studies found genetic mutations that correlate with disease risk and possibly also disease progression (34–37); subsequently, the first examples of drug–gene interactions in IPF were found (38). To date, the value of biomarkers in IPF has not been fully clarified, and, therefore biomarkers or genetic endotyping are not yet used in clinical practice (7, 33).

Novel studies in IPF suggest that the "respiratory microbiome" is also involved in IPF pathogenesis, disease progression, and mortality (39–41). Patients with IPF have a higher bacterial burden and abundance of specific pathogens in the lung microbiome than the normal population. Furthermore, interactions have been found between specific gene expression and an altered lung microbiome in IPF, which is the first evidence for host-environmental interactions in IPF (42, 43). The lung microbiome may serve as a prognostic factor in the future, and clinical trials aimed at altering the microbiome of patients with IPF have already started (44).

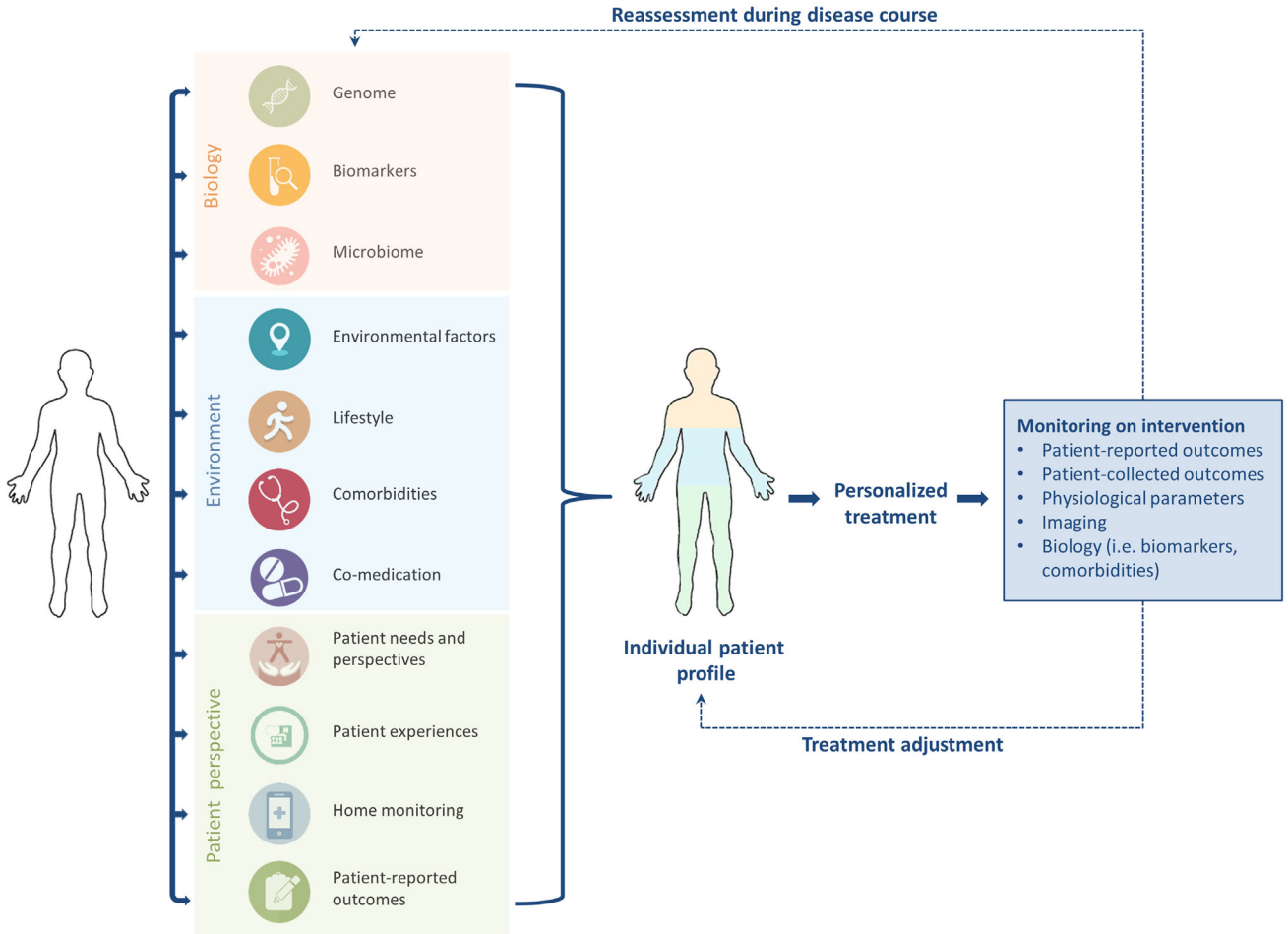

FIGURE 1 | To enhance tailored treatment in idiopathic pulmonary fibrosis, "biology" should be combined with patient factors to generate an individual patient profile. Close monitoring, timely reassessment, and treatment adjustment during the disease course are required to optimize personalized care.

A detailed description of (molecular) biology and its current role and potential in the IPF field is beyond the scope of this review.

HOW TO INTEGRATE PERSONOMICS INTO PERSONALIZED MEDICINE

Patient Needs and Perspectives in IPF Care

The importance of engaging patients in IPF care has gained increasing attention during the last several years (45). Recent qualitative studies have reported a need for better education about IPF, information about specific treatment options and palliative care, and access to specialist centers and specialist nurses. Additionally, more support for caregivers is warranted (16, 17, 46–48). These recommendations underscore the idea that not only pharmacological treatment but also non-pharmacological treatment options such as oxygen therapy, pulmonary rehabilitation, psychological support, and palliative care, are an important part of personalized management. With regard to pharmacological treatment, it is important to assess the needs and perspectives of patients before starting treatment, thereby enhancing shared decision-making. For instance, some side effects of disease-modifying drugs might have a devastating impact on one patient, but be far less bothersome to other patients (22). At the moment, over-use and under-use of medication, compliance problems, and waste of medication are not unusual in IPF (22, 49, 50). Non-adherence to medication could therefore be prevented when patients' preferences and lifestyle are taken into account (9). Since patient preferences and needs may change because of disease progression or personal circumstances, an important aspect of disease management is iterative evaluation of the situation of individual patients (16, 46, 51). Only in this way can "holistic" personalized care be given in IPF.

Comorbidities and Co-Medication

Holistic care also means looking further than the lungs. IPF is associated with a number of pulmonary and extra-pulmonary comorbidities, such as pulmonary hypertension, respiratory

infection, cardiovascular disease, emphysema, lung cancer, diabetes mellitus, venous thromboembolism, and gastroesophageal reflux (52–56). Comorbidities are more prevalent in patients with IPF than in the normal population and have a negative influence on QOL and survival (54, 56–58). Hence, early identification and treatment of comorbid conditions have the potential to improve QOL, functional outcomes, and survival for patients with IPF (53). Kreuter et al. (54) proposed the "IPF comorbidome," which visually displays prevalence of comorbidities and their strength of association with mortality in patients with IPF. This comorbidome could be used to predict prognosis for individual patients with IPF, and thus enhance personalized treatment.

Moreover, extra attention should be paid to the frail, elderly patients who have multiple comorbidities and functional impairment (55). As a consequence, these patients might have a higher risk of harmful side effects of disease-modifying medication and should be closely monitored during treatment. Besides, polypharmacy may play an important role in this group of patients. It is generally known that polypharmacy decreases medication compliance, increases risk of adverse drug events, and might lead to impaired functional status and cognitive impairment in elderly patients (59). Furthermore, co-medication can also interfere with disease-modifying medication, and subsequently increase side effects or reduce treatment efficacy (60). Accordingly, co-medication could play an important role in the choice of pharmacological treatment in IPF. Expected risk–benefit ratio, comorbidities, and co-medication should be taken into account before pharmacological treatment is started in individual patients.

MEASURING QOL AND MONITORING TREATMENT RESPONSE

It remains challenging how to measure patients' disease burden, experiences, and response to treatment in IPF. For this purpose, it is important to receive structured patient input throughout the whole disease course, starting already when the diagnosis is established. At present, digital solutions can facilitate more collaboration with patients in monitoring disease behavior, their experiences, and response to therapy (**Figure 2**).

Patient-Reported Outcome Measures (PROMs) in IPF

A PRO is defined as "any report of the status of a patient's health condition that comes directly from the patient, without interpretation of the patient's response by a clinician or anyone else" (61). Patient-reported outcome measures (PROMs) can be used to measure (HR)QOL, assess symptoms, and evaluate disease progression. There is a difference between generic and disease-specific PROMs. Disease-specific PROMs are developed to assess symptoms and (HR)QOL in a specific disease, whereas generic PROMs address more general questions and can be used in the whole population (62). One of the most commonly used generic PROMs in IPF trials are the short-form 36 and the Euroqol-5D, which is also a widely accepted instrument for economic evaluation in healthcare (63, 64). An overview of the most widely used PROMs in IPF is given in **Table 1**.

Disease-Specific PROMs

Although PROMs can play an important role to improve care for IPF, only a few well-validated, disease-specific questionnaires have been developed (19). Until a few years ago, most questionnaires used in clinical trials in IPF were originally intended for other chronic diseases (64, 65, 68). The validity of these questionnaires, such as the Saint George Respiratory Questionnaire (SGRQ) and COPD Assessment Test, has been confirmed in patients with IPF (66, 68). For the SGRQ, even an adapted version, the SGRQ-I, has been developed (67). This revised PROM consists of questions from the original SGRQ that were most relevant for patients with IPF. The reliability and validity of the SGRQ-I are comparable with the SGRQ. However, PROMs which are developed in a target population from the start, are thought to be more precise in capturing changes in HRQOL for this group of patients

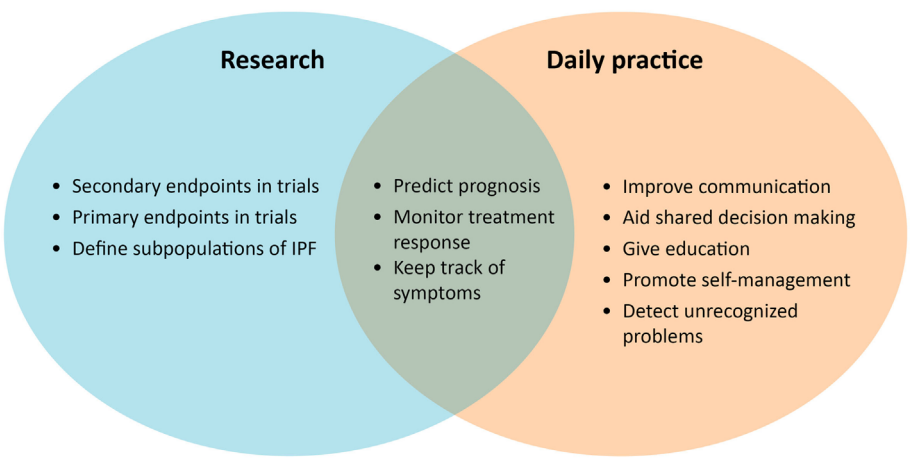

FIGURE 2 | Patient-reported and recorded outcomes can be used to enhance personalized treatment.

TABLE 1 | Overview of most used patient-reported outcomes in IPF.

Patient-reported outcome measure	Description	Validation studies and MCID	Advantages	Disadvantages
Disease-specific				
SGRQ (65)	Fifty-item questionnaire with three domains assessing HRQOL in chronic respiratory diseases	Validated in IPF; MCID in IPF: five to eight points (66)	Used in many clinical trials in IPF	Originally developed for COPD and asthma; lengthy, difficult questionnaire
SGRQ-I (67)	IPF-specific version of original SGRQ; contains 34 items	Validity comparable with SGRQ	Questions more relevant for IPF than SGRQ	Responsiveness and MCID not known yet; limited experience
CAT (68)	Composed of eight symptom items on a 0–5 response scale	Validated in IPF	Simple and quick instrument	Originally developed for COPD; limited experience in IPF
K-BILD (21)	Fifteen-item health status questionnaire in ILD with three domains	Validated in IPF MCID in IPF: five points (69)	Brief developed in ILD including IPF patients	Limited experience in clinical trials, though increasingly used
L-IPF (70) (revised version ATAQ-IPF)	Contains two modules with different domains	Currently in validation process	Adapted with feedback from patients	Not available yet
IPF-PROM (71)	Concise questionnaire to asses QOL in IPF	Study is ongoing	Developed with patients and caregivers	Not available yet
PESaM (72)	Generic and disease-specific module; evaluates patients' expectations, experiences, and satisfaction with disease-modifying drugs	Currently in validation process	Developed together with IPF patients	Not validated yet; responsiveness unknown
IPF-PREM (73)	Questionnaire to assess experiences with care delivery	Study is ongoing	Measures experiences of patients	Not available yet
Domain-specific				
UCSD (74)	Contains 24 items on a 0–5 response scale assessing dyspnea in the last week	Validated in IPF; MCID in IPF: eight points	Already used in different IPF trials; valid to assess change in dyspnea in IPF	Takes considerably more time compared with other dyspnea measures; not originally developed in IPF
mMRC (75)	Consists of one question with five grades for the level of dyspnea	Not validated in IPF	Quick, easy tool for use in daily practice; relates to disease progression	Responsiveness in IPF unclear; not originally developed in IPF
BDI-TDI (76)	BDI scores three components of dyspnea on baseline; TDI measures changes compared with baseline	Not validated in IPF; MCID in COPD: one point (76)	Measures both baseline and change over time	Only interview-administered or computerized version; not originally developed in IPF
Borg Scale (77)	Level of dyspnea scored on a scale from 0 to 10	Not validated in IPF; MCID in COPD: one point (78)	Useful during 6-min walk test in daily practice	Only measures dyspnea during exertion, does not measure dyspnea over time; not originally developed in IPF
HADS (79)	Consist of 14 items in the subscales anxiety and depression	Not validated in IPF; MCID in COPD: 1.5 points (79)	Reliable screening tool for anxiety and depression	Should not be used as diagnostic test; not originally developed in IPF
CQLQ (80)	Consists of 28 cough-specific questions in six domains	Validated in IPF; MCID in IPF: five points	Comprehensive; responsive outcome measure	Good validity for total score in IPF, but not for all domains; limited experience in IPF; not originally developed in IPF
LCQ (81)	Chronic cough quality of life questionnaire with 19 items in three domains	Not validated in IPF; MCID in chronic cough: 1.3 points (82)	High reliability; ability to detect a response to change	Limited experience in IPF; not originally developed in IPF

IPF, idiopathic pulmonary fibrosis; MCID, minimal clinically important difference; ILD, interstitial lung disease; HRQOL, health-related quality of life; SGRQ, Saint George Respiratory Questionnaire; K-BILD, Kings' Brief Interstitial Lung Disease health status questionnaire; L-IPF, living with idiopathic pulmonary fibrosis; ATAQ-IPF, a tool to assess quality of life in IPF; IPF-PROM, idiopathic pulmonary fibrosis—patient-reported outcome measure; PESaM, patient experiences and satisfaction with medication; IPF-PREM, idiopathic pulmonary fibrosis—patient-reported experience measure; UCSD, University of California San Diego shortness of breath; mMRC, modified Medical Research Council; BDI-TDI, baseline and transition dyspnea indexes; HADS, Hospital Anxiety and Depression Scale; CQLQ, Cough Quality of Life Questionnaire; LCQ, Leicester Cough Questionnaire.

(58). One of the first questionnaires specifically developed in a population of patients with interstitial lung diseases (ILDs), among whom patients with IPF, is the Kings' Brief Interstitial Lung Disease health status questionnaire (21). This is a brief, valid questionnaire that is increasingly used in IPF and other ILD clinical trials. One of the emerging PROMs in IPF is the "living with idiopathic pulmonary fibrosis" (L-IPF) questionnaire, which is a revised, electronic version of the ATAQ-IPF (a tool

to assess quality of life in IPF). The L-IPF was adapted from the ATAQ-IPF following feedback from patients, and a validation study is underway at the moment (70). Another questionnaire which is currently being developed with the help of a multidisciplinary group of patients and carers is the IPF-PROM (71).

Domain-Specific PROMs

Additionally, domain-specific PROMs, which are questionnaires related to a specific symptom or organ, can be used to capture and objectify different aspects of disease. A few measures to evaluate breathlessness, such as the University of California San Diego Shortness of Breath Questionnaire, the modified Medical Research Council scale, the baseline and transition dyspnea indexes, and the Borg scale, are commonly used in IPF, although none were originally developed for IPF (74–77). Even though cough is a major problem in IPF, no specific cough questionnaires for IPF exist. However, the Leicester Cough Questionnaire and the Cough Quality of Life Questionnaire are currently used instead (80, 83). A widely known PROM to assess anxiety and depression is the Hospital Anxiety and Depression Scale, which is increasingly used in IPF (79). No specific fatigue questionnaires for IPF exist; however, the Fatigue Assessment Scale, originally developed for sarcoidosis, is used and might be adapted for IPF in the future (84).

PROs in Research and Daily Practice

PROs could be very helpful to enhance personalized treatment in IPF (**Figure 2**). Until now, PROMs have been mainly used for research purposes, as a secondary endpoint in clinical trials. The most used primary endpoint in IPF trials is forced vital capacity, which is accepted as a surrogate measure for mortality (85). One study showed that HRQOL, assessed with the SGRQ, is also an independent prognostic factor for mortality in IPF (86). PROMs probably reflect another dimension of disease compared with traditional physiological parameters (86). In the future, PROMs could possibly be used to predict treatment success in IPF.

PROM uses in daily practice can allow healthcare providers and patients to gain more insight into the individual disease and patient behavior. In a study of Sampson et al. (46), most patients were uncertain about their own disease course and progression and had difficulties interpreting objective hospital-based parameters. PROMs could allow both patients and healthcare providers to keep track of symptoms and disease progression easily. PRO results can even be used as a simple tool to communicate with patients, educate them, promote self-management, and aid shared decision making during the course of the disease (19, 87). A systematic review in oncology has shown strong evidence that routine collecting of PROs improved patient-centered care, patient satisfaction, and detection of unrecognized problems (88).

Patient-Reported Experience Measures in IPF

Optimal treatment requires close monitoring of the balance between the effects and side effects of disease-modifying drugs. Nonetheless, to our knowledge, a reliable measure to assess patient experiences with medication in IPF is not yet available in clinical practice. For this reason, a consortium of doctors, scientists, and patient representatives has joined forces to develop the patient experiences and satisfaction with medications (PESaM) questionnaire, which has a generic module and a disease-specific part for IPF (84). The PESaM questionnaire focuses on perceived effectiveness, side effects, and ease of use of medication and its impact on patients' lives. This patient-reported experience measure (PREM) could not only be used in future clinical trials, but also in clinical practice to help with better detection of side effects and adjustment of medication. Moreover, Russell and colleagues, together with patients, are currently developing the "IPF-PREM." This is a measure to assess patient experiences with healthcare and can possibly be used to improve the quality of care for patients (69).

Home Monitoring

Ideally, for a better tailored treatment, frequent monitoring with a low burden for the patient is needed. In the last decade, the use of e-health in chronic diseases has been growing, and shows mostly promising results (89–91). E-health involves the exchange of data between a patient and a healthcare provider using information and communication technologies (92). By using e-health tools, patients may better understand their health condition and become actively involved in the management of their own disease. It allows frequent monitoring in between regular visits and collection of PROs at home (93). Recently, a study showed that daily home spirometry in a population of patients with IPF was highly feasible and informative (94). Home-based spirometry predicts disease decline and mortality better than hospital-based measurements. Routine home spirometry could be very helpful to identify patients with rapid decline in lung function and to evaluate response to treatment. The authors suggest that daily home spirometry will allow for more individualized patient care. The feasibility of home-based spirometry in IPF was confirmed by Johannson et al. (95), who additionally showed that home spirometry might reduce sample size as well as the length of future clinical trials. Another promising example of home monitoring in IPF is the longitudinal follow-up of physical activity with activity trackers worn by patients at home (96). Decline in physical activity can provide reliable, objective data on disease progression and could be integrated into a home monitoring program. A comprehensive home monitoring program, consisting of an e-health tool combined with home spirometry and online collecting of PROs, has the potential to enhance trial design, stimulate self-management, allow for early treatment adaption to minimize side effects, prevent hospital admissions, and subsequently improve personalized management and QOL for patients with IPF.

CONCLUSION

The potential to enhance personalized treatment has prompted excitement also in the IPF field. In the future, patients' genetic,

biomarker, and microbiome profiles may guide clinical trial design and treatment decisions. In this process, patient perspectives should not be overlooked. Only by integrating biological information with patient-reported and patient-collected information, will we be able to realize truly personalized treatment.

AUTHOR CONTRIBUTIONS

All authors conceptualized and designed the review, CM and MW wrote the paper, PH and MK provided critical feedback and input. All authors agree to be accountable for the content of the work and approved the manuscript.

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REVIEW
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Idiopathic Pulmonary Fibrosis: Aging, Mitochondrial Dysfunction, and Cellular Bioenergetics

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At present, the etiology of idiopathic pulmonary fibrosis (IPF) remains elusive. Over the past two decades, however, researchers have identified and described the underlying processes that result in metabolic dysregulation, metabolic reprogramming, and mitochondrial dysfunction observed in the cells of IPF lungs. Metabolic changes and mitochondrial dysfunction in IPF include decreased efficiency of electron transport chain function with increasing production of reactive oxygen species, decreased mitochondrial biogenesis, and impaired mitochondrial macroautophagy, a key pathway for the removal of dysfunctional mitochondria. Metabolic changes in IPF have potential impact on lung cell function, differentiation, and activation of fibrotic responses. These alterations result in activation of TGF-β and predispose to the development of pulmonary fibrosis. IPF is a disease of the aged, and many of these same bioenergetic changes are present to a lesser extent with normal aging, raising the possibility that these anticipated alterations in metabolic processes play important roles in creating susceptibility to the development of IPF. This review explores what is known regarding the cellular metabolic and mitochondrial changes that are found in IPF, and examines this body of literature to identify future research direction and potential points of intervention in the pathogenesis of IPF.

Keywords: aging, mitochondrial dysfunction, lung fibrosis, bioenergetics, mitophagy, senescence

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INTRODUCTION

Aging is life's natural destiny. It is biologically defined by a progressive impairment in vital functions coupled with diminished fitness to adapt to environmental stimuli and respond to stress (homeostenosis) (1). Among the predictable cellular alterations that underlie homeostenosis, metabolic dysregulation and alterations in mitochondrial function have been shown to contribute to the aging phenotype. Aging is also associated with increased susceptibility to a wide range of chronic diseases. Lung pathologies are no exception, and the prevalence of several interstitial lung diseases, most notably idiopathic pulmonary fibrosis (IPF), has been found to increase considerably with age. IPF is a progressive and irreversible lung disease, generally diagnosed in the sixth decade of life, whose etiology remains unknown and therapeutic options remain limited (2). Many of the changes in bioenergetics and mitochondrial function seen with aging are also seen in the fibrotic lung and may contribute to IPF (**Table 1**).

TABLE 1 | Mitochondrial changes in the fibrotic lung.

Feature	Change	Model	Reference
Mitochondrial reactive oxygen species	Increased	Bleomycin mouse model Asbestosis mouse model	(44)
Mitochondrial respiration	Decreased ETC complex activity, lower OCR	Human idiopathic pulmonary fibrosis (IPF) lung tissue Human lung fibroblasts Human AECII Alveolar macrophages MHV68 model of lung fibrosis	(5, 15, 42, 43, 63, 66, 69)
ATP production	Decreased	IPF lung fibroblasts IPF lung myofibroblasts IPF total lung	(24, 43, 69)
mtDNA	Increased oxidative damage, insufficient mtDNA repair	IPF total lung Murine AECII Bleomycin mouse model Asbestosis mouse model	(5, 44, 48)
Mitochondrial biogenesis	Decrease	IPF total lung Bleomycin mouse model	(15)
Mitochondrial dynamics	Imbalanced	MHV68 mouse model of fibrosis	(5)
Mitophagy alterations	Reduced levels of mediators of mitochondrial quality control in epithelial cells and fibroblasts Increased mitophagy in macrophages	IPF AECII IPF lung fibroblasts IPF pulmonary macrophages Bleomycin mouse model MHV68 mouse model of fibrosis	(5, 24, 25, 30)
Decreased expression of SIRT3	Acetylation of mitochondrial proteins	IPF total lung Bleomycin mouse model Asbestosis mouse model	(48, 52)

ETC, electron transport chain; OXPHOS, oxidative phosphorylation; AECII, alveolar type II epithelial cells; OCR, oxygen consumption rate.

MITOCHONDRIAL ALTERATIONS WITH AGING AND IPF

Over the past few decades, our understanding of the function and role of mitochondria has moved beyond the initial view of mitochondria as nothing more than a cellular energy generator producing ATP through oxidative phosphorylation (OXPHOS). Mitochondria are now understood to have their own life cycle consisting of biogenesis, fission/fusion dynamics, and recycling through macroautophagy (mitophagy), and to act as an important bidirectional signaling platform, communicating with the nucleus as well as other organelles. Multiple signaling pathways converge and interact to regulate the linked processes of mitochondrial energetics, biogenesis, production of reactive oxygen species (ROS), mitochondrial DNA (mtDNA) preservation and repair, and mitophagy. Dysregulation of many of these regulatory mechanisms that control mitochondrial function have recently been identified in the epithelial cells, fibroblasts, and macrophages in IPF lungs (3). Mitochondrial dysfunction in IPF lung cells contributes to maladaptation to cellular stress, creating vulnerability to injury and promoting the development of pulmonary fibrosis (3–6) (**Figure 1**).

MITOCHONDRIAL BIOGENESIS

Mitochondrial biogenesis, the process of producing additional mitochondria and by extension cellular energy production capacity, is under the control of master regulators PPARγ coactivator-1α (PGC-1α) and PGC-1β, which are nutritional sensors able to induce expression of nuclear respiration factors 1 and 2 (NRF). NRFs upregulate expression of the nuclear and mitochondrial mechanisms necessary for mitochondrial biogenesis (7). With aging, the capacity for mitochondrial biogenesis declines through age-related reduction in upstream activators of PGC-1α and PGC-1β such as AMP-activated protein kinase (AMPK) as well as p53-mediated, senescence-associated repression of these key regulators of mitochondrial biogenesis (8, 9). Moderate exercise, calorie restriction, and resveratrol supplementation have been shown to increase PGC-1α activity and ameliorates aging-related decline in mitochondrial biogenesis (10–12). Expression of PGC1a is reduced in lungs from IPF patients and in fibrotic mouse lungs after bleomycin treatment. Highlighting the critical role of mitochondrial biogenesis in the susceptibility to lung fibrosis, mice deficient in PGC1a are more susceptible to bleomycin-induced lung fibrosis. Thyroid hormone (T4) signaling is known to restore mitochondrial health and function through a PGC-1α-dependent pathway (13, 14). Concordantly, experimental provision of aerosolized T3 and a T4 mimetic attenuated bleomycin- and TGF-β-induced fibrosis in mouse models. T3 supplementation was also associated with reduced alveolar epithelial cell apoptosis, improvement in mitochondrial electron transport chain function, and normalization of swollen mitochondrial morphology. This improvement was

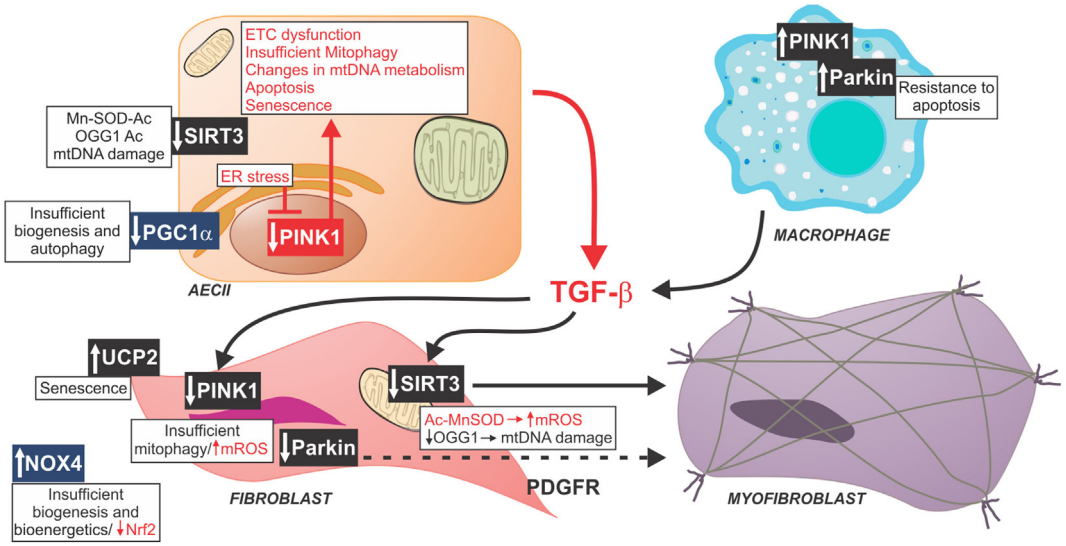

FIGURE 1 | Schematic of profibrotic pathways mediated by mitochondrial dysfunction. Reduced PGC1α in fibrotic lungs has been associated with insufficient biogenesis and diminished autophagy. Similarly, decreased PINK1, a key modulator of mitophagy, and decreased SIRT3 have been found in AECII from fibrotic lungs associated with mitochondrial dysfunction, and increased activation of TGF-β. Profibrotic macrophages exhibit increased rates of mitophagy and resistance to apoptosis resulting in greater release of TGF-β. TGF-β affects mitochondrial function in fibroblasts through decreasing PINK1, Parkin, and SIRT3. Idiopathic pulmonary fibrosis fibroblasts have increased mtDNA damage, mitochondrial dysfunction, impaired mitochondrial biogenesis, and increased rate of senescence. All of these factors contribute to in fibroblast-to-myofibroblast differentiation. Abbreviations: Ac, acetyl group; ETC, type II alveolar epithelial cell electron transport chain, Mn-SOD, manganese super oxide dismutase; mtDNA, mitochondrial DNA, mtROS, mitochondrial reactive oxygen species; NOX4, NADPH oxidase 4; Nrf2, nuclear factor (erythroid-derived 2)-like-2 factor; OGG1, 8-oxoguanine DNA glycosylase 1; PDGFR, platelet-derived growth factor receptor; PINK1, PTEN-induced putative kinase 1; UCP2, uncoupling protein 2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

not seen in PGC-1α knockout mice. These investigations were conducted in response to the finding of increased level and activity of iodothyronine deiodinase 2 in IPF lungs, which is the enzyme responsible for conversion of inactive T4 to the physiologically active T3 form. This is presumed to be an adaptive response to increase the availability of active T3 in fibrotic IPF lung (15).

Idiopathic pulmonary fibrosis and experimental models of pulmonary fibrosis are associated with telomere shortening and DNA damage, which activates the DNA damage sensor poly[ADP-ribose] polymerase 1 (PARP-1) and the checkpoint inhibitor p53. These signaling mechanisms feedback to reduce activation of PPARγ coactivators and reduce mitochondrial biogenesis (9, 16). Impaired mitochondrial biogenesis in IPF potentially creates a mismatch in cellular energy demand and production capacity with resulting mitochondrial dysfunction. Additionally, the ROS-producing enzyme NADPH oxidase-4 (Nox4), which is upregulated in IPF lung fibroblasts, represses mitochondrial biogenesis through direct effect on NRF2 and mitochondrial transcription factor A (TFAM) independent of PGC-1α (17, 18). While these pathways have been demonstrated to play a role in age and disease-related alterations in cellular bioenergetics, and the downstream consequences, such as ROS production, DNA damage, and induction of senescence, are features of lung fibrosis, additional translational investigations in IPF lungs are needed to firmly establish the role of these perturbations in the development of IPF.

MITOPHAGY

Mitophagy is a selective and adaptive response that targets mitochondria for turnover, regulates the number of mitochondria to match cellular energy needs, and removes damaged and dysfunctional mitochondria that can cause cellular stress. Mitophagy and mitochondrial turnover are important processes in maintaining cellular integrity and health. Mitochondrial damage, whether from metabolic alterations, increased ROS production, or an accumulation of mtDNA damage and mutations affecting transcription, results in a reduction of mitochondrial transmembrane potential, reduced ATP production, further increases in ROS, and leakage of mitochondrial contents into the cytosol that, if left unchecked, would eventually lead to cellular injury and apoptosis (19–23).

Selective mitophagy of damaged mitochondria occurs through PTEN-induced putative kinase 1 (PINK1)-Parkin signaling with PINK1 acting as a sensor of mitochondrial membrane depolarization and subsequently activating Parkin, which labels the dysfunctional mitochondrion for trafficking to the autophagosome (19, 24). With aging, a decrease in PINK1 has been observed coupled with a decrease in markers of autophagy and an increase in the size of mitochondria (5, 25). These findings demonstrate that capacity for mitophagy declines as we age.

Deficient mitophagy has been associated with IPF and development of pulmonary fibrosis in response to injury (5, 16, 25–27). Deficiency or dysfunction of multiple mediators of

mitophagy has been implicated in the pathobiology of IPF making maintenance or augmentation of mitophagy an attractive avenue of potential intervention. Our work has shown that type II alveolar epithelial cells from IPF lungs are deficient in PINK1 resulting in an accumulation of dysmorphic mitochondria with reduced transmembrane potential, reduced electron transport chain (ETC) activity, increased ROS production, and increased opening of the mitochondrial permeability transition pore. PINK1 knockout was also found to be sufficient to recapitulate this mitochondrial phenotype and confer vulnerability to fibrosis in response to lung injury (5). PINK1 expression has been found to diminish with age and persistent endoplasmic reticulum stress. ER stress has been shown to induce expression of ATF3, a transcriptional repressor of PINK1, in alveolar epithelial cells (28). Interestingly, deficiency of PINK1 in lung epithelial cells was also associated with upregulation of markers of senescence (p16 and p21) and increased levels of TGF-β expression, a key mediator of fibrogenic processes in IPF (5, 28).

As adaptive responses, autophagy and mitophagy are essential to maintaining normal fibroblast function and preventing apoptosis. Impairment of mitophagy, mediated by Parkin deficiency, in IPF lung fibroblasts has been associated with increased deposition of extracellular matrix under profibrotic conditions such as exposure to TGF-β. Defects in mitophagy and autophagy result in increased production of ROS and activation of platelet-derived growth factor receptor (PDGFR)/mammalian target of rapamycin (mTOR) signaling pathways that enhance fibroblast to myofibroblast transformation (24). Supporting these data, Parkin-deficient mice develop more severe lung fibrosis. Induction of autophagy and treatment with antioxidants reduced fibroblast to myofibroblast differentiation, myofibroblast proliferation, and fibrogenesis suggesting that this process is driven by increased mitochondrial ROS (24, 25). Indeed, we now recognize that a subset of surfactant protein C mutations, which causes a familial form of pulmonary fibrosis, acts through mistrafficking of surfactant protein C, accumulation within endosomes, and a late block of macroautophagy and mitophagy within type II alveolar epithelial cells. This results in proteostasis and an accumulation of dysfunctional mitochondria with increased mitochondrial mass but decreased transmembrane potential, which may increase susceptibility to pulmonary fibrosis when an additional "second hit" stressor is present (29).

TGF-β has been shown to variably affect key mediators of mitophagy and promote fibrogenesis. Treatment of alveolar epithelial cells with TGF-β results in initial stabilization of PINK1 on the surface of mitochondria and induction of mitophagy, which appears to be an adaptive response to increased ROS production due to TGF-β's effect on the ETC (30). Longer term exposure to TGF-β results in impaired mitophagy through downregulation of phosphatase and tensin homolog (PTEN), and a subsequent reduction in PINK1 expression (25). Another potential activator of autophagy is T4, which is a well-known activator of AMPK and inhibitor of mTOR (15). Supporting this role of T4 signaling in PINK1-mediated mitochondrial homeostasis and mitophagy, the therapeutic effect of inhaled T3 in mouse models of lung fibrosis required an active PINK1 signaling pathway (15).

On the contrary, pulmonary macrophages isolated from IPF lungs demonstrate increased mitophagy. IPF macrophages are a source of activated TGF-β, and macrophage resistance to apoptosis is necessary for disease progression. IPF macrophages activate TGF-β by producing mitochondrial H_2O_2 through activation of the pro-survival kinase, protein kinase B (Akt1). Increased Akt1 activation and ROS production induce mitophagy as a protective measure and prevent macrophage apoptosis, which stabilizes macrophages to release additional TGF-β and promote local fibroblast activation and proliferation. Blocking mitophagy in alveolar macrophages is protective against bleomycin-induced fibrosis (27). This finding exemplifies the role of mitophagy as an adaptive survival response that, in this case, serves to preserve and promote macrophage signaling.

MITOCHONDRIAL OXPHOS

The processes of mitochondrial respiration and OXPHOS are the highly efficient and preferable means of ATP production, but a natural byproduct of these processes is superoxide and hydrogen peroxide production, which is produced in increasing quantities by dysfunctional mitochondria (31). Mitochondrial ROS (mtROS) serve as a signaling mechanism acting at multiple points throughout the cell that serves to inactivate phosphatases, activate select kinases, and facilitate hypoxia-inducible factor 1-α (HIF-1α), p53, and NF-κB signaling pathways (32–36). Interestingly, mtROS levels have a biphasic effect. At low levels, mtROS stimulate an increase in antioxidant capacity, referred to as mitohormesis, which has a protective effect and has been associated with longevity and resistance to cellular injury. In higher concentrations or with sustained production, ROS propagates free radicals that cause oxidative damage to lipids, proteins, and DNA (37).

Control of OXPHOS and energy production occurs through cooperative signaling mechanisms at mitochondrial, cytosolic, and nuclear levels in order to balance OXPHOS, energetic demands of the cell, and nutrient supply. This complex process limits production of ROS. Additionally, mitochondria have multiple mechanisms for reducing mtROS, but effective regulation of ROS production and mitigation declines with aging and potentiates susceptibility to lung injury and fibrosis (18, 38, 39). Aging is associated with lower ATP production and increased ROS (40). Moreover, older animals have been shown to accumulate higher levels of oxidized proteins in response to lung injury suggesting waning cellular antioxidant defense systems (41).

A profibrotic environment promotes mitochondrial dysfunction in pulmonary epithelial cells. Studies in Mv1Lu cells demonstrate that treatment with TGF-β1 downregulates mitochondrial ETC function, particularly at complex IV, resulting in loss of mitochondrial transmembrane potential and increased mtROS production (42). Increased ROS serves to oxidize and activate latent TGF-β1 creating a self-reinforcing cycle with potential to recruit fibroblasts and promote fibrogenesis (30). This correlates with the findings that type II alveolar epithelial cells from IPF lungs have reduced ETC complex I and IV activity (5), and IPF fibroblasts have reduced ATP content and reduced rate of oxygen consumption indicating poor mitochondrial function (43).

While the effectiveness of antioxidant supplementation in preventing diseases of aging such as IPF has been disappointing

and, in some cases, detrimental, overexpression of an antioxidant protective mechanism in mitochondria has shown effectiveness in abrogating the fibrotic response to bleomycin-induced lung injury. Mice that overexpress mitochondria-targeted human catalase predictably show lower levels of mtROS, but also display reduced mtDNA damage and fragmentation with decreased pulmonary fibrosis in response to oxidative injury from asbestos or bleomycin (44). This raises the possibility that mitochondrial-targeted antioxidants may prevent or abrogate the development of IPF by scavenging mtROS at the source.

mtDNA DAMAGE

Mitochondrial DNA is significantly more susceptible to oxidative injury than nuclear DNA, likely due to proximity to the source of ROS, lack of protective histones, and relative paucity of mtDNA repair mechanisms (45). Indeed, oxidative DNA damage is a key mechanism of injury in the commonly used model of bleomycin-induced pulmonary fibrosis (46). In comparison with the nucleus, mitochondria contain fewer and less efficient mechanisms for correcting mutations and repairing DNA damage. Mitochondrial transcription factor A (TFAM) is a key regulator of mtDNA transcription and replication, which also functions to induce *U*-shaped bends in mtDNA. These protective nucleoids can act to sequester damaged DNA and prevent transcription until base excision repair enzymes can correct the damage (47). The base excision repair enzyme, 8-oxoguanine DNA glycosylase 1 (OGG1), plays a critical role in repair of mtDNA oxidative damage, OGG1 deficiency or inactivation predisposes to pulmonary fibrosis (26, 48). The processes of mtROS production and mtDNA damage are intricately linked, and oxidative injury and cellular stress drive apoptotic and senescence pathways that may contribute, at least in part, to both aging and to lung injury and fibrosis. Additional studies in IPF lung-derived cells or tissues are needed to establish the role of mtDNA damage in IPF.

Owing to the evolutionary origin of mitochondria as bacterial endosymbionts, mtDNA contains CpG-rich sequences, which have the potential to act as damage-associated molecular patterns and activate innate immune system mechanisms when they escape the mitochondrial milieu. This pathway may contribute to a pro-fibrotic microenvironment (49). Recently, it has been shown that IPF fibroblasts undergo glycolytic reprogramming associated with release of mtDNA into the cellular interstitium and ultimately into circulation. Furthermore, increased plasma levels of mtDNA were associated with an increase in all-cause mortality suggesting potential use of circulating mtDNA as a biomarker for progression or severity of IPF (50).

SIRTUINS ARE KEY MEDIATORS OF MITOCHONDRIAL HEALTH IMPLICATED IN IPF

Deficiency of the mitochondrial NAD-dependent deacetylase, SIRT3, has been shown to confer susceptibility to epithelial cell apoptosis and fibrosis in both bleomycin and asbestos models of lung injury (48). SIRT3 is an active enzyme in ROS detoxification as well as mtDNA protection and repair pathways, and its dependence on NAD+ as a cofactor directly links SIRT3's function to cellular bioenergetics and TCA cycle activity (51). In the mitochondrion, SIRT3 is implicated in regulation of two redox mechanisms. SIRT3 deacetylates and activates manganese superoxide dismutase (MnSOD) and isocitrate dehydrogenase 2 (IDH2) (52, 53). Low levels of SIRT3 result in increased acetylation and decreased MnSOD activity and an accumulation of mtROS and mtDNA oxidative damage (53). The matrix protein IDH2 is a key provider of the reducing agent, NADPH, necessary for proper functioning of protective antioxidant pathways (52). The mtDNA repair function of OGG1 is also regulated by SIRT3. In the absence of SIRT3, OGG1 is increasingly acetylated and deactivated resulting in unchecked mtDNA damage and lung epithelial cell apoptosis (44, 48).

SIRT3 knockout mice have also been shown to develop increased expression of TGF-β and fibrosis in multiple organs as they age. This has been shown to occur through increased acetylation and deactivation of glycogen synthase kinase-β and stabilization of profibrotic transcription factors, such as smad3 and β-catenin, independent of SIRT3's effect on MnSOD activity (54). Multiple SIRT3-dependent pathways play important roles in preventing mitochondrial damage and vulnerability to fibrosis.

Another sirtuin, SIRT1, has been established to play a role in preventing both aging-related functional decline and age-related diseases such as IPF (55). SIRT1 is a nuclear NAD-dependent deacetylase, but one of its key functions is in modulating mitochondrial biogenesis and function through nuclear-to-mitochondrial signaling pathways (56). These pathways have demonstrated how nuclear DNA damage and telomere shortening can drive mitochondrial dysfunction. Telomerase reverse transcriptase deficiency and shortened telomeres are strongly linked with the development of pulmonary fibrosis, and this chromosomal shortening activates the DNA damage sensor and checkpoint inhibitor p53. Activated p53 represses expression of a key mediator of mitochondrial biogenesis, PPARγ coactivator-1α (PGC-1α) resulting in inefficient OXPHOS and increased ROS production (57). SIRT1 deacetylates and inactivates p53 restoring normal mitochondrial function (9, 56). The nuclear DNA damage sensor, poly[ADP-ribose] polymerase 1 (PARP-1), consumes NAD+ when activated and decreases activity of SIRT1 and PGC-1α. SIRT1 and PGC-1α have also been shown to translocate to the mitochondria and may assist in stabilization of mtDNA (16). Fasting and caloric restriction, long of interest in increasing longevity, and reducing functional decline with aging, increase SIRT1 protein levels and activity (51). SIRT1 translation is inhibited by the microRNA miR-34a, which is highly expressed in models of lung injury. SIRT1 deficiency results in increased acetylation of p53, which acts to induce expression of miR-34a, creating a self-reinforcing cycle and predisposing to development of fibrosis (58). In a bleomycin model of lung injury, upregulation of SIRT1 attenuated fibroblast activation and the development of fibrosis (59). Sirtuins clearly have complex functions in modulating mitochondrial biogenesis, function, ROS production, and mtDNA protection, and this area of investigation has potential to shed new light on the pathogenesis of IPF and other diseases of aging.

METABOLIC REPROGRAMMING IN IPF

Fibrotic lung tissue in IPF has been shown to have increased metabolic activity as demonstrated by increased FDG uptake on PET scan, and increased PET activity correlates with disease progression and mortality (60). Cell-type specific metabolic changes occur in the IPF lung (**Figure 2**). Type II alveolar epithelial cells have downregulation of genes involved in lipid synthesis and metabolism identified through single cell RNA sequencing (61). Fibroblasts have been shown to undergo a metabolic shift away from the highly efficient method of ATP production, OXPHOS, to the less efficient method of glycolysis despite adequate oxygen to continue OxPhos (62–67). Additionally, alveolar macrophages in fibrotic lung tissue increase fatty acid oxidation in response to the shift to glycolysis (66). A similar metabolic shift was initially observed in cancer cells and was eponymously dubbed the Warburg effect after its discoverer (68). The Warburg-like metabolic reprogramming observed in IPF cells results in increased glucose uptake and an accumulation of TCA cycle metabolites and byproducts that act as signaling mechanisms. Kottmann and colleagues demonstrated that glycolytic flux increases lactate production and lowers the local tissue pH resulting in increased activation of TGF-β, stabilization of the transcription factor HIF-1α, and increased transcription of lactate dehydrogenase 5 (LDH5), which synergizes with TGF-β to induce differentiation of fibroblasts to myofibroblasts (62). LDH5 produces lactate, which can be exported by monocarboxylate transporter (MCT)-4 and may be taken up by adjacent cells expressing MCT-1. Shuttled lactate can be oxidized to pyruvate providing additional energy through the TCA cycle and driving OXPHOS. This phenomenon is termed the reverse Warburg effect and promotes fibroblast proliferation and increased levels of mtROS.

Primary myofibroblasts derived from IPF lungs and lung fibroblasts treated with TGF-β demonstrate increased lactate contents. Additionally, IPF myofibroblasts showed increase in expression of glycolytic enzymes including 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKB3), an enzyme that increases levels of fructose-2,6-bisphosphate and drives glycolysis (63). Glycolytic reprogramming in fibroblasts is, at least partially, driven by TGF-β. Treating normal fibroblasts with TGF-β has been shown to increase transcription of phosphofructokinase (PFK) and hexokinase 2, two key rate-limiting glycolytic enzymes (63). Increased lactate production may be due to shunting of glycolytic products toward anaerobic metabolism due to concurrent mitochondrial dysfunction.

By contrast, a recent study comparing IPF lung tissue to normal human lung using a combined metabolomic and microarray analysis of key metabolic enzymes showed a decrease in the late-stage glycolytic products, fructose 1,6-bisphosphate and phosphoenolpyruvate, and a decrease in PFK and PFKFB3, suggesting an overall reduction in glycolysis in IPF whole lung tissue (69). These findings are in concordance with recent observations in fibroblasts derived from IPF lungs. IPF fibroblasts trended toward having a lower rate of glycolysis, as measured by extracellular acidification rate, and had lesser glycolytic flux in response to TGF-β stimulation compared to normal lung fibroblasts (43). The latter finding may represent a diminished capacity to respond to TGF-β after chronic exposure to this profibrotic cytokine *in vivo*.

Despite these differences, it is known that TGF-β can mediate metabolic reprogramming. TGF-β induces expression of the facultative glucose transporter, glucose transporter 1 (GLUT1),

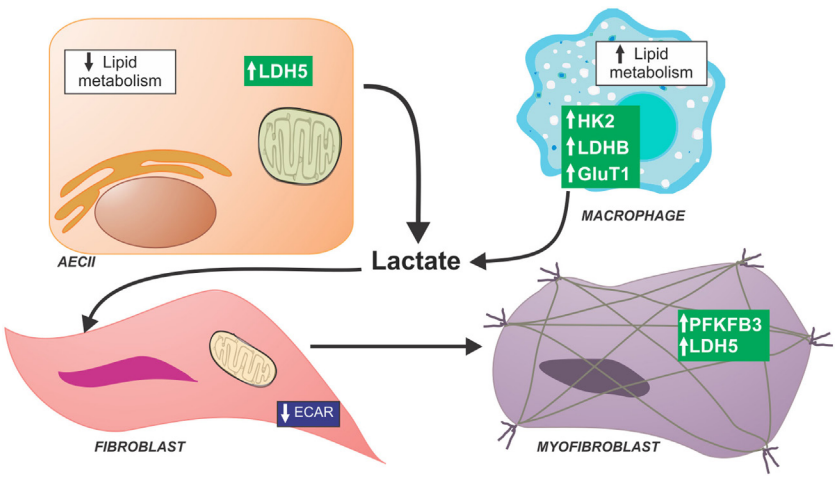

FIGURE 2 | Schematic of metabolic dysfunction in lung fibrosis. Idiopathic pulmonary fibrosis (IPF) lung have a generalized decrease in late stages of glycolysis compared with normal lungs; however, increased levels of lactate were found in IPF lungs suggesting that all products of glycolysis are shuttled toward lactate production. Several studies have been focused in metabolic alterations of specific cell compartments in the IPF lung. IPF lung fibroblasts show decreased glycolytic function determined by extracellular acid production. IPF myofibroblasts have been reported to have higher expression of glycolytic enzymes and lactate content. Single cell RNA sequence data in alveolar epithelial cells show low expression of enzymes necessary for lipid metabolism. By contrast, alveolar macrophages isolated from bleomycin-treated mice show glycolytic reprogramming and increase fatty acid oxidation. Abbreviations: AECII, type II alveolar epithelial cell; GluT1, glucose transporter 1; HK2, hexokinase 2; LDH5, lactate dehydrogenase 5; LDHB, lactate dehydrogenase B; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3.

via canonical Smad and PDGFR signaling resulting in increased cellular glucose uptake. Inhibiting GLUT1 upregulation is protective against bleomycin-induced fibrosis (64), and GLUT1 activation of AMPK is integral to fibroblast activation (67). Since metabolic reprogramming appears to be a critical step in promoting fibroblast-to-myofibroblast differentiation, preventing this shift is an intriguing target in attenuating IPF. However, further studies are needed to confirm the metabolic changes in different cell compartments in the IPF lung and determine how these alterations might modify disease progression.

MITOCHONDRIAL DYSFUNCTION AND SENESCENCE

Inducing senescence and apoptosis in fibroblasts are important mechanisms in the wound healing response after injury (70); on the contrary, the aberrant deposition of extracellular matrix seen in IPF is associated with an accumulation of apoptosis-resistant senescent cells (39, 43, 71, 72). Mitochondrial dysfunction has been shown to induce cellular senescence, a process termed mitochondrial dysfunction-associated senescence. In addition, mitophagy defects seem to contribute to a cellular senescence phenotype, which we have observed in the setting of mitochondrial dysfunction induced by PINK1 deficiency *in vivo* (28). Dysfunctional mitochondria are less efficient at oxidizing NADH to NAD+ resulting in a reduced NAD+/NADH ratio, activation of AMPK and p53 resulting in a senescent phenotype (73). Senescent IPF lung epithelial cells, fibroblasts, and myofibroblasts secrete a cell type-specific profile of pro-fibrotic and pro-inflammatory cytokines known as the senescence-associated secretory phenotype (SASP) (72, 74). Senescent fibroblasts exhibit an increased level of the ROS-generating enzyme Nox4 and low levels of the key mediator of cellular antioxidant response pathways, NFE2-related factor 2, and this redox imbalance plays a role in maintaining the senescent phenotype. Treatment with a Nox inhibitor restores the redox balance and results in a decrease in senescent cells and a reduction in fibrosis (18). *In vitro*, mouse model, and *ex vivo* studies in human IPF lung cells have all shown improvement in fibrosis with treatment with a combination of antioxidants (quercetin) and senolytic agents (dasatinib) (72, 74). This approach shows promise as a therapeutic option.

FUTURE DIRECTIONS: TARGETING MITOCHONDRIA AS AN ANTIFIBROTIC THERAPY

Research has extensively shown that both aging and IPF are associated with alterations in cellular bioenergetics and mitochondrial homeostasis. Many of these mitochondrial pathways, especially those involved in maintenance of redox balance, mtDNA protection, and reversal of senescence, have shown promise as therapeutic targets in attenuating or ameliorating fibrosis *in vitro* and in animal models of pulmonary fibrosis. These early investigations of the role of altered bioenergetics in the development of fibrotic lung disease pave the way for translational research to implicate these pathways in human IPF. Other pathways, specifically those involved in maintaining normal mitophagy and turnover of dysfunctional mitochondria and mitochondrial-targeted antioxidants, present potential avenues for altering the natural history of pulmonary fibrosis but require further investigation to identify a point of intervention. Few potential interventions appear ready for human trials to restore mitochondrial biogenesis and mitophagy in alveolar epithelial cells and use of combination senolytic and antioxidant agents to remove senescent cells from injured lung and prevent propagation of SASP signaling (75). These approaches benefit from a history of therapeutic use and safety in treatment of human disease.

There is considerably more to learn about the role of mitochondrial dysfunction in the development and maintenance of the pro-fibrotic conditions that drive IPF, regarding the interplay between mitochondrial and nuclear signaling in overall cellular dysfunction, telomere shortening, and mtDNA damage, and the varying effects of mitochondrial dysfunction in the different cell types (epithelial cells, macrophages, fibroblasts, and myofibroblasts) involved in the development of IPF. Additionally, cellular perturbations seen with normal aging such as decreased macroautophagy and cellular proteostasis have the potential to incite mitochondrial dysfunction and changes in cellular bioenergetics. Development of animal models capable of isolating defects in mitochondrial homeostasis to a particular cell type may be revelatory of the role each cell type plays and shed light on the optimal targets for intervention. Particular attention to the effects of mitochondrial dysfunction on telomere length in the resident progenitor cells of the lungs responsible for regenerating the alveolar epithelium may present novel approaches for preventing stem cell exhaustion in IPF.

While many of these pathways have shown potential as therapeutic targets in isolation, an integrated approach to the bioenergetic and homeostatic changes that are seen with aging and IPF, such as telomere shortening, reduced mitochondrial biogenesis, increased ROS production, and increased nuclear and mtDNA damage, is essential to determining the pathways and therapeutic targets that are most likely to affect the natural history of IPF.

AUTHOR CONTRIBUTIONS

DZ wrote the manuscript along with MB. The manuscript was conceptualized, supervised and modified by ALM and MR. All authors offered intellectual contribution and approved the manuscript.

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REVIEW
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The Role of Immune and Inflammatory Cells in Idiopathic Pulmonary Fibrosis

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The contribution of the immune system to idiopathic pulmonary fibrosis (IPF) remains poorly understood. While most sources agree that IPF does not result from a primary immunopathogenic mechanism, evidence gleaned from animal modeling and human studies suggests that innate and adaptive immune processes can orchestrate existing fibrotic responses. This review will synthesize the available data regarding the complex role of professional immune cells in IPF. The role of innate immune populations such as monocytes, macrophages, myeloid suppressor cells, and innate lymphoid cells will be discussed, as will the activation of these cells *via* pathogen-associated molecular patterns derived from invading or commensural microbes, and danger-associated molecular patterns derived from injured cells and tissues. The contribution of adaptive immune responses driven by T-helper cells and B cells will be reviewed as well. Each form of immune activation will be discussed in the context of its relationship to environmental and genetic factors, disease outcomes, and potential therapies. We conclude with discussion of unanswered questions and opportunities for future study in this area.

Keywords: innate immunity, adaptive immunity, macrophage, lymphocyte, fibroproliferation

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrotic disease of unknown etiology characterized by the radiographic and histopathologic pattern of usual interstitial pneumonia (UIP) (1, 2). It is known to have outcomes similar to some cancers, with mortality approaching 50% within 3–5 years after diagnosis (1). Although the origin of this disease is not known, several risk factors have been identified, including cigarette smoking (3), chronic viral infections (4), gastroesophageal reflux (5), and genetic predisposition (6), which will be discussed throughout this article as appropriate. The mechanistic relationship of these risk factors to disease development and progression has yet to be determined.

The pathogenic cascade of lung fibrosis is thought to be initiated by perpetuated microinjuries to the alveolar epithelium that engenders a dysregulated wound healing response (7). Through poorly understood processes involving the recruitment and activation of myofibroblasts, normal lung tissue is obliterated by the accumulation of extracellular matrix (ECM) components (8). The basic science and translational research conducted throughout the last few decades has allowed substantial insight into the mechanisms driving IPF (9). In addition, the tireless efforts of investigators conducting clinical trials have resulted in the development of anti-fibrotic therapies with the potential to delay the rate of lung function decline in some patients (10, 11). A central concept of these developments

has been the emerging consensus that IPF does not appear to be a direct result of immune cell dysfunction but rather that immune and inflammatory cells can permit, promote, or suppress fibroproliferation driven by native lung fibroblasts (**Figure 1**). This article reviews the evidence in support of this hypothesis.

HISTORICAL PERSPECTIVE

In order to understand the controversy surrounding the role of the immune system in IPF a brief overview of the disease state is required. IPF is defined as the presence of UIP in the absence of an identifiable underlying cause. Examination of lung tissue from patients with IPF reveals a paucity, but not absence, of inflammatory cells, when compared to pathologies, such as non-specific interstitial pneumonia, acute respiratory distress syndrome, organizing pneumonia (OP), and granulomatous processes such as hypersensitivity pneumonitis (HP) (12). Similarly, CT scan criteria specify that large areas of ground glass opacities—typically reflective of inflammatory process—are inconsistent with UIP (1). Classical signs of autoimmunity are absent on physical examination and laboratory testing, as is clinical evidence of an identifiable antigen-driven immune response (1). In addition, the long history of failed immunotherapies, such as administration of interferon gamma (13), neutralization of TNFα (14), and suppression of acute inflammatory responses with low dose Prednisone and Azathioprine (15), suggests that IPF does not result from a primary immunopathogenic process. These clinical observations have been interpreted by some sources as indicating the pathogenesis of IPF lacks an immune component (9). However, this array of findings is unlikely to suggest that the immune system is not involved. On the contrary, the worsening of clinical outcomes by classical immunosuppression suggests, if anything, that certain immune responses might be protective and others might be harmful. Thus, better understanding of all forms of immunity has the potential to advance the understanding of IPF.

INNATE VS ADAPTIVE IMMUNITY

The immune response is stratified into innate processes, which respond immediately to chemical or physical patterns of the stimulus, and adaptive immunity, which involves a highly specific antigen-driven response. Both arms of the immune system appear to be activated in IPF. The data supporting this concept are presented below.

INNATE IMMUNE CELLS

The innate immune system forms the first line of defense against pathogens. Its recognition of antigens is mainly dependent on pattern recognition by innate immune receptors. These cell populations are central to both host defense and tissue homeostasis. Macrophages and neutrophils are among the best studied innate immune cells in regard to IPF, though a contribution of monocyte-derived cells, such as fibrocytes and myeloid-derived suppressor cells (MDSCs), and of innate lymphoid cells (ILCs), has also been proposed. It should also be noted that parenchymal cells, such as epithelial cells and fibroblasts, also show abnormalities in innate

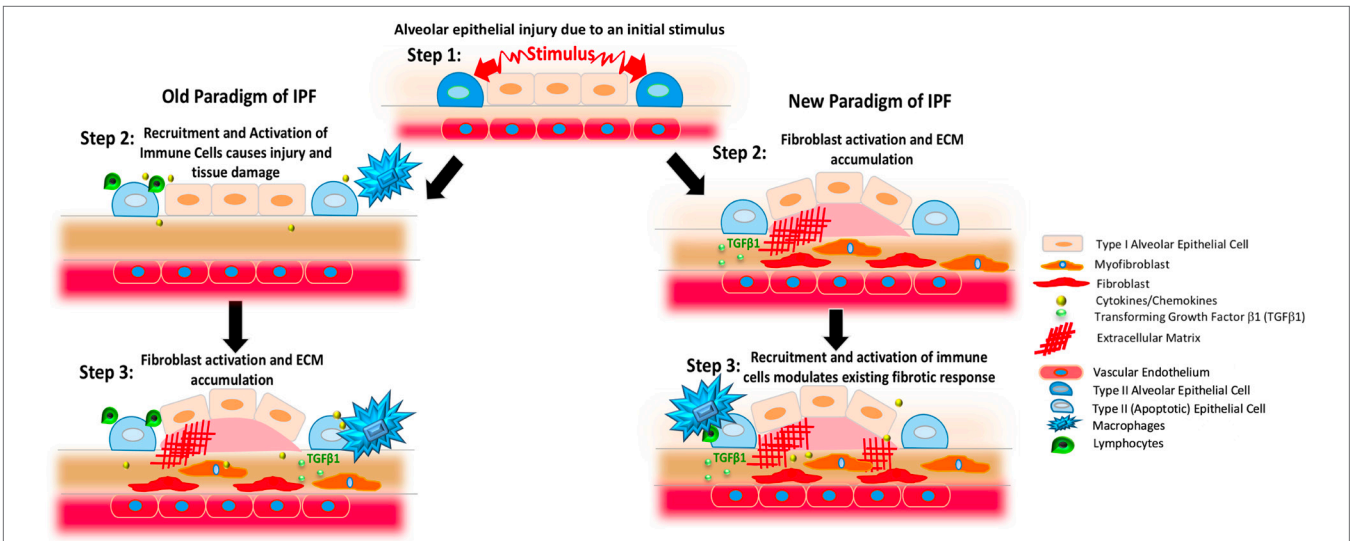

FIGURE 1 | Changing paradigms regarding the proposed pathogenesis of idiopathic pulmonary fibrosis (IPF). The left column presents previous concepts of IPF. In this setting, an initial stimulus affects the alveolar epithelium (blue and pale pink shapes). As shown in the middle panel on the left, this process results in the generation of apoptotic epithelial cells (light blue) and the recruitment and activation of various immune cells including various populations of macrophages (blue) and lymphocytes (green) that produce cytokines and chemokines (yellow). As shown in the third panel on the left, these inflammatory cells and substances induce the activation of fibroblasts (red) and myofibroblasts (orange) to result in TGFβ1 activation (small light green circles) and the deposition of excessively stiff and biochemically abnormal extracellular matrix (ECM, red crosshatched lines). Observations gleaned from clinical trials and experimental modeling have refined this paradigm, however, to result in the scheme shown on the right. In this newer model, the stimuli affecting the lung epithelium leads to fibroblast activation and ECM accumulation that can occur without a primary immunopathogenic component (second panel on left). Once the fibrotic response is established, resident and recruited immune cells, such as macrophages and lymphocytes, modulate existing responses through a variety of mechanisms.

immune activation (16). However, because these stromal populations are not considered to be classical or professional immune cells, their potential and largely speculative contribution to the immunopathogenesis of IPF will not be discussed in this review.

Macrophages

Macrophages are innate immune cells that not only act as antimicrobial phagocytes in the lungs but also play a key role in the pathogenesis of fibrotic lung disease (17). Of the immunopathogenic mechanisms discussed in this review, macrophage-driven processes are among the most extensively studied with reports of fibrosis-promoting properties dating back nearly 50 years (18). Macrophages can regulate both injury and repair in various models of fibrosis and macrophage heterogeneity has emerged as an important area of study in IPF (9). Prior classification schemes proposed the existing of two phenotypes, namely classically activated M1 macrophages that arise in response to INFγ and TNFα, and alternatively activated M2 macrophages that arise in response to stimulation with IL-4, IL-10, IL-13, and TGFβ1 (19). The central concept has been that M1 macrophages suppress, and M2 macrophages promote, fibroproliferation and uncontrolled repair (17). While recent evidence suggests that a dichotomous stratification oversimplifies the functional heterogeneity of these highly plastic cells (20), the M1/M2 distinction is useful when considering functional distinction in broad terms. In this context, a relative excess of M1 macrophages leads to epithelial cell death and failure of repair such as that seen in acute exacerbation of IPF (AE-IPF), while an excess of M2 macrophages leads to the aberrant and dysregulated repair responses that characterize progressive fibrosis (21). At least one endogenous macrophage-driven pathomechanism identified in AE-IPF is characterized by M2 macrophage activation and upregulation of M2 cytokines (22). Detailed studies performed in several mouse models of IPF demonstrate the heterogeneous and highly plastic nature of lung macrophages, with a contribution from both long-lived resident alveolar macrophages (23), as well as from interstitial macrophages that are at least partially bone marrow derived (24). While the difference in surface marker expression prevents direct translation of highly detailed studies of macrophage subtypes in the mouse, synthesis of the currently available data reveals that the accumulation of cells expressing various scavenger receptors and fibrosis-promoting markers is a common feature of many forms of lung fibrosis including IPF (21, 25, 26).

Macrophages display many functions that frame them as a central contributor to fibrotic responses. As early as the 1980s, alveolar macrophages obtained from patients with IPF were shown to stimulate fibroblast accumulation *via* a paracrine mechanism involving the production of soluble mediators typically associated with alternative activation (18). More recent work using lung-derived macrophages confirms the fibroblast-stimulating properties of macrophages (21), and also reveals that circulating monocytes in patients with IPF appear to be programmed with this property prior to actually entering the lung (21). Further studies using animal modeling reveal that removal (27, 28) or repolarizing (27) of interstitial macrophages is both preventative and therapeutic in several mouse models of IPF. This latter mechanism is the conceptual basis for administration of the large pentraxin protein serum amyloid P to patients with IPF (27, 29), which is currently under investigation for multiple forms of fibrosis including IPF. While several studies indicate that macrophages might also participate in other forms of lung fibrosis *via* the regulation of epithelial cell activation (30), this area remains largely unexplored in the context of IPF.

A large body of evidence supports the concept that macrophages produce soluble mediators that regulate fibrotic responses (17) but the mechanism(s) through which they adopt this activation state remains incompletely determined. As increasing body of evidence, however, indicates that interactions with dead or dying cells may be involved (31). In this process, called "efferocytosis," macrophages (either lung resident or recruited) participate in the engulfment of apoptotic cells causes the transcriptional activation of TGFβ (32). In fact, alveolar macrophages produce TGFβ1 in both humans (33) and mice (30) and Cre-mediated removal of TGFβ1 expression in LysM-expressing cells prevents collagen accumulation and histologic evidence of remodeling in several commonly used animal models (30). These results suggest one potential mechanism through which macrophages contribute to fibrosing lung disease. However, in addition to their role in apoptotic cell clearance and TGFβ production, macrophages produce cytokines, such as TNFα, IL-1, IL-6, IL-8, IL-10, and IL-12, and chemokines, such CXCL1, CXCL2, CXCL9, CXCL10, CXCL12, CCL5, CCL17, and CCL18 (34). Their production of lipid mediators such as eicosanoids might contribute to fibrosis (35), though this function has not been specifically studied in either IPF samples or currently used mouse models (36). Macrophages also participate in ECM remodeling through the secretion of matrix metalloproteinases (37) and by direct ingestion and recycling of collagen (38). In other clinical contexts and modeling systems, macrophages are known to direct the metabolic fate of adjacent cells (39), which might carry substantial implications for fibrosis where glycolytic reprogramming has been observed to drive fibroblast activation (40). Macrophages participate in surfactant recycling (41) which could be of critical importance given the known association between mutations in surfactant proteins and susceptibility to IPF (42). Macrophages produce angiogenic factors such as vascular endothelial growth factor (43), which can be both pro- (44) or anti-fibrotic (45), depending upon the timing of expression and the target cell. Macrophages have been shown to rescue intestinal stem cell phenotypes *via* the delivery of WNT-containing exosomes (46) and while a similar effect has yet to be seen in IPF, given the recently reported association between innate immune activation and lung progenitor cell survival (47), it is possible that similar functions may exist in IPF. The potential role of macrophages in pulmonary fibrosis is illustrated in **Figure 2**.

These data reveal a robust and important relationship between macrophages and fibroproliferation in the IPF lung and lead to the critical question of whether therapies targeting macrophage activation might stabilize or restore lung function in patients with IPF. The few clinical trials conducted in this area have yielded disappointing results. For example, as direct suppression of M1 responses with TNFα neutralization with Etanercept failed to improve clinical outcomes (14). Similar results were seen in a study that sought to inhibit macrophage recruitment *via* treatment

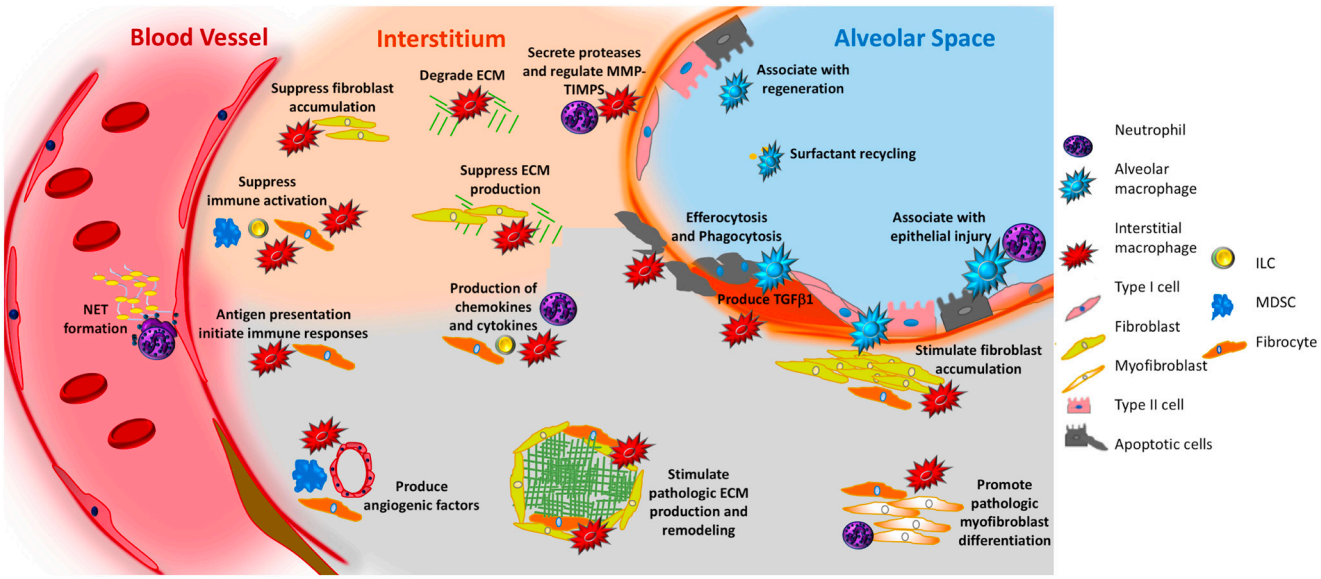

FIGURE 2 | Potential role of innate immunity in pulmonary fibrosis. In response to interactions with pathogen-associated molecular patterns or danger-associated molecular patterns, or to stimulation with various mediators, macrophages—both alveolar (aqua irregular shape) and interstitial (red irregular shape)—can adopt fibrosis modifying properties. These functions include production of TGFβ1, production of soluble mediators that cause fibroblast accumulation and activation, production of TIMPS and MMPS that participate in extracellular matrix (ECM) remodeling, production of angiogenic factors, secretion of lipid mediators, regulation of structural cell injury and stem cell renewal, and surfactant recycling. Neutrophils (purple polymorphonuclear circle) produce neutrophil elastase (NE), TIMPS, and MMPs that dictate whether ECM accumulates or is degraded. Neutrophils also participate in the formation of neutrophil extracellular traps, which may promote fibrosis *via* the production of TGFβ1 and subsequent myofibroblast activation. Circulating fibrocytes (orange spindle shaped cells) are bone marrow-derived mesenchymal cells that enter the lung *via* the vasculature. Once in the lung they adopt multiple functions that would be expected to modulate fibrogenesis including the ability to differentiate into fibroblasts and myofibroblasts, production of ECM, contraction of wounds, participate in antigen presentation, production of chemokines and cytokines, and regulation of angiogenesis *via* production of soluble mediators. Myeloid-derived suppressor cells (MDSC, blue) are immunosuppressive cells that show an association with ECM remodeling and pulmonary hypertension. Innate lymphoid cells (ILCs) produce cytokines that may regulate fibroblast accumulation and ECM production. In the above figure, the functions of each cell are depicted in font matching the cell's color. Note that cells are not drawn to scale.

with Carlumab, a monoclonal antibody targeting chemokine C-C chemokine ligand 2 (48). While these data could be viewed as negative, they are in fact incredibly helpful as they reveal that targeting the alternative activation state or specific function of macrophages, rather than the M1 phenotype or broadly active aspect such as recruitment, is more likely to be of benefit in a complex disease such as IPF. This concept is the conceptual basis for the ongoing Phase II trial of Pentraxin 5, an acute phase reactant that interferes with innate immune activation by binding to debris and inhibiting Fcγ receptor driven inflammatory process in phagocytic cells (49). The mechanism(s) though which the extrinsic features of the lung microenvironment might result in sterile inflammation and fibrosis are presented below.

ACTIVATION OF PROFESSIONAL IMMUNE CELLS IN IPF: PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs) VS DANGER-ASSOCIATED MOLECULAR PATTERNS (DAMPs)

Pathogen-Associated Molecular Patterns

A dominant mechanism through which innate immune cells adopt fibrosis-promoting properties likely involves the recognition of innate immune agonists by pattern recognition receptors (PRRs). Ligands for PRRs fall into two classes. Those derived from invading microorganisms are called "PAMPs" and those derived from injured cells and tissues are called "DAMPs" (50). While both inflammatory and parenchymal cells contain PRRs, we will, for the sake of clarity, restrict this particular review to the professional immune cells that have been classically accepted as the first line of host defense (51). Because IPF is not believed to result from a primary infectious process, until recently, the concept of PAMPs has been little studied (31). However, over the last two decades, data gleaned from human and animal studies have linked certain viruses and bacteria with IPF.

Viruses

Several viruses are proposed as playing a role in the development of IPF. For example, Epstein–Barr virus (EBV, a member of the Herpes family) is enriched in bronchoalveolar lavage (BAL) fluid and lung biopsy tissue of IPF patients when compared with healthy controls (52, 53), and is thought to act *via* upregulating TGFβ1 expression and inducing mesenchymal properties in lung epithelial cells (54). In addition, Cytomegalovirus is thought to accelerate existing fibrosis in bleomycin-treated mice by enhancing TGFβ1 activation and increasing detection of both phospho-SMAD2 and Vimentin (55). A strong association with

Herpesvirus saimiri was seen in a human study, wherein the infected epithelial cells demonstrated evidence of IL-17 expression of viral origin (56). Considering the potential contributions of viruses, the use of adjuvant antiviral therapy in IPF has shown potential benefit both in animal models (57) and a small human study (58) although data in this regard are limited.

Bacteria

The potential contribution of bacteria to IPF pathogenesis is also an area of active investigation. Specifically, a relationship between total bacterial load and poor prognosis was observed in a study in which enriched detection of organisms, such as Haemophilus, Streptococcus, Neisseria and Veillonella, was found in BAL fluid of IPF patients (59). In addition, BAL samples from patients with IPF contain augmented concentrations of certain strains of Staphylococcus and Streptococcus (60), the latter of which was shown in profiling studies to associate with increased NOD receptor signaling and poor outcomes (61). The source(s) of these bacteria is not clear though given the association of IPF with GERD (62), one possibility is that ongoing microaspiration leads to repeated inoculation with oral and gastric organisms. Thus, the concept of the microbiome is gaining traction in IPF and forms the basis for studies examining antibiotics as a novel treatment approach (63).

Danger-Associated Molecular Patterns

Immune responses are also initiated in the absence of pathogen recognition. Here, damage to previously intact cells and tissue results in the accumulation of substances with the potential to function as "DAMPs." In uninjured tissues, DAMP-mediated inflammatory processes contribute to homeostasis by allowing the regulated removal of debris, thereby facilitating the resolution of injury and the achievement of repair (31). Abnormal responses to DAMP recognition has been described as one form of immunosenescence (64) and it is intriguing to consider this concept in relationship to the telomerase mutations that are associated with the IPF disease state. Excessive accumulation of DAMPs, however, activates PRRs to engender a microenvironment rich in sterile inflammation (65). These responses may differ from PAMP-driven inflammatory responses (66). Another form of innate immune ligands derived from homeostatic mechanism (HAMPs) has recently been described (67), but because these moieties have not been studied in the context of IPF, they will not be discussed further in our review.

A number of substances are classified as DAMPs. The easiest to conceptualize may be intracellular components such as nucleic acids and organelles that are passively released from necrotic cells. DAMPs might also be actively released by cells *via* the exocytosis of membrane bound vesicles or endosomes. Still another class of DAMPs is generated by the transformation of inert proteins such as collagens into signaling molecules such as collagen fragments. These entities are recognized by innate immune receptors that for the most part have the ability to be activated *via* pathogens as well (31). The activation of these receptors can be protective or harmful depending on the nature of the ligand and the specific receptor. For example, animal modeling reveals that mice lacking toll-like receptor 3 (TLR3) suffer increased fibrosis in the bleomycin model, and the Leu412 Phe polymorphism in the gene encoding TLR3 (which recognizes dsRNA as well as free RNA) has been implicated in a rapidly progressive form of IPF (68). Mice lacking toll-like receptor 4 (TLR4) manifest increased fibrosis in the bleomycin model, and treatment with TLR4 agonists ameliorates fibrosis and remodeling in this setting *via* a mechanism involving lung progenitor cell renewal (47) and augmentation of TGFβ1 and IL-17 production (69). However, because TLR4 inhibition is protective in other settings (70, 71), the role of this PRR is not fully known. A connection between TLR4 and IPF may exist, however, as enrichment of several endogenous ligands for TLR4, such as high mobility group box 1 (72, 73), tenascin-C (74–76), S100 protein (73), and hyaluronan fragments (77), has been reported in the BAL or lung tissue of patients with IPF (78). Furthermore, the finding that a mutation in toll interacting protein, an adaptor protein for toll-like receptor 2 (TLR2) and TLR4, increases susceptibility to IPF (79), suggests a potential protective role for this pathway.

In terms of pathogenic responses, much more information is available. For example, NACHT, LLR, and PYD domains-containing protein 3 (NALP3) inflammasome activation leads to IL-1β production and fibrosis in bleomycin treated animals (80, 81). While the relevance of these results to the human disease state is not directly established, lung tissue from patients with IPF show increased concentrations of uric acid (82), and BAL from these patients contained an increase in free ATP (83), both of which are known inflammasome activators (81, 84). Inflammasome activation in IPF may also occur *via* toll-like receptor 9 (TLR9) as detection of this PRR (85) and its endogenous ligand mitochondrial DNA are both increased in IPF (86). In fact, direct exposure of previously normal fibroblasts to either endogenous TLR9 agonists such as unmethylated CpG-rich DNA derived from mitochondria (mtDNA) (86) or synthetic TLR9 agonists enacts a transition to an αSMA expressing, myofibroblastic phenotype (87). However, because mice with ubiquitous deletion of TLR9 develop worsened fibrosis in several experimental settings (88), likely due to inflammatory nature of these models, the role of TLR9 in IPF has been difficult to understand. Thus, this is an area that would benefit from additional studies and improved models that more accurately represent the microenvironment of the diseased human lung.

Neutrophils

Neutrophils are innate immune cells that possess several functions through which they might participate in fibrosis. Neutrophilia in BAL fluid has been associated with early mortality in IPF (89) and concentrations of the neutrophil chemoattractant, CXCL8, are increased in IPF (90). Furthermore, levels of alveolar epithelial marker, cytokeratin 19, in BAL fluid correlated to neutrophil concentrations, suggesting an association between neutrophils and epithelial injury in this context (91).

Neutrophils might also contribute to fibrosis *via* their regulation of ECM turnover. Neutrophil elastase (NE), the main proteolytic product of alveolar neutrophils, is increased in BAL fluid of IPF patients (92). NE generates DAMPs by degrading various ECM components, such as collagens I, II, III, IV, fibronectin, laminin, and elastin (93, 94), and *ex vivo* work demonstrates that NE can induce fibroblast proliferation and myofibroblast

differentiation (95). NE deficient mice are protected from the fibrosis seen in both the bleomycin and asbestos models (93, 95), and the NE inhibitor, Sivelestat, is protective in the bleomycin model (96). Neutrophils also control ECM homeostasis through their regulation of the net balance between MMPs and TIMPs (97, 98), particularly the pro-fibrotic MMP-2, MMP-8, and MMP-9 (99). Batimastat, a synthetic inhibitor of MMP, when used in bleomycin-induced mice, resulted in reduced MMP-2, MMP-9, and TIMP-1 level in BAL fluid and was, therefore, useful in preventing pulmonary fibrosis (100), though the relevance of these findings to human IPF remains unclear.

One newly described fibrosis-promoting function of neutrophils is the generation of extracellular neutrophil traps. These pro-inflammatory collections of chromatin and neutrophils regulate both immune cell function (101) and fibroblast activation (102). While enhanced detection of intrapulmonary neutrophil extracellular traps (NETs) has been reported in both the bleomycin model and in some forms of fibrotic ILD (102), a specific association with IPF has yet to be fully described. Further studies are warranted to understand whether NETS play a role in IPF pathogenesis.

To summarize, neutrophils are innate immune cells that are associated with the production of cytokines and chemokines, presence of injury, regulation of ECM turnover, and generation of NETs. All of these functions would be expected to result in fibroblast activation and ECM accumulation (**Figure 2**). However, because the pathology of UIP is not characterized by neutrophil accumulation—and, in fact, the presence of neutrophils would lead to a pathologic diagnosis other than UIP—the role of neutrophils remains unclear.

Fibrocytes

Fibrocytes are circulating, bone marrow-derived mesenchymal progenitor cells that can migrate into lung tissue and further differentiate into fibroblasts and myofibroblasts (103). These cells are believed to derive from monocyte-based progenitors. They comprise only a small fraction of circulating leukocytes in normal humans, but are found abundantly in pathologic conditions characterized by macrophage-driven inflammation and persistent fibroblast activation such as IPF (104). These cells express hematopoietic and progenitor cell markers, CD45 and CD34, and also produce ECM proteins such as collagens I and III, vimentin and fibronectin (105). Similarly, they can be induced to express αSMA (106) and participate in the contraction of artificial wounds (107, 108). However, despite their documented ability to both produce ECM and display functions of myofibroblasts in a variety of modeling systems, consistent evidence of these properties in human lung tissue remains scarce. Thus, in recent years, attention has focused on alternate functions of these highly plastic cells. Fibrocytes not only express receptors for chemokines, such as CCR3, CCR5, CCR7, and CXCR4, but also produce inflammatory cytokines, TNF-α, IL-6, IL-8, and IL-10, and chemokines, MIP-1α, MIP-1β, MCP-1, and GRO-α (109, 110). They participate in antigen presentation (111, 112) and angiogenesis (113), and in some settings are able to control the activation of adjacent fibroblasts *via* paracrine means (114). Thus, fibrocytes display an array of functions that would be expected to influence the development and progression of lung fibrosis (**Figure 2**), though their specific contribution to IPF is currently not defined and requires further investigation.

Myeloid-Derived Suppressor Cells (MDSCs)

Myeloid-derived suppressor cells are a heterogeneous group of immature myeloid immune cells, which appear to be related to poor prognosis in certain forms of cancers (115). MDSCs play a role in the immune system through their suppressive action on T cells (116). They promote regulatory T cell (Treg) expansion and restrain T-cell activation (117) and are associated with a number of diseases characterized by fibrosis and pathologic remodeling. Thus, it is perhaps not surprising that one recent study found that elevated concentrations of peripheral blood MDSCs, defined by the surface markers HLA-DR, CD33, CD11b, CD14, and CD66b, reflect poor lung function in patients with IPF (118). Another experiment with bleomycin-induced mice showed that MDSCs triggered vascular remodeling and pulmonary hypertension, and that preventing their recruitment *via* neutralization of CXCR2 normalized the pulmonary pressure (119). While the relationship between vascular abnormalities and parenchymal fibrosis remains poorly understood, accumulating evidence suggests that these events might significantly impact parenchymal homeostasis during injury and repair (45). Thus, therapeutic strategies targeting the activity of MDSCs or restricting their accumulation and expansion in peripheral blood may be a novel approach to disease modification, though more work is needed to understand their relevance to human disease (118).

Innate Lymphoid Cells

Innate lymphoid cells are newly identified lymphoid cell populations that do not express the recombination activating gene and are classified into three subgroups: ILC1, which include natural killer cells that produce IFN-γ as well as CD127lo and CD127hi ILCs (120); ILC2, which produce type 2 cytokines, such as IL-5 and IL-13; and lastly, ILC3 that produce IL-17 and IL-22 (121). ILCs in the lung interact with epithelial cells, natural killer T cells, and myeloid cells to form an immune system network (122). ILC2 are activated quickly by environmental antigens and pathogens to release large quantities of IL-13, thereby suggesting a potential role in pulmonary fibrosis (123). ILCs have been identified in the IPF lung (124) though studies of their role in this disease remain in the nascent stages. Thus, this is an area that would benefit from additional study.

ADAPTIVE IMMUNE RESPONSES: T CELLS AND B CELLS

As noted above, the role of lymphocytes in fibrosis is poorly understood and controversial. The failure of IPF to improve in response to lymphocyte-modulating therapies, and the observation that lymphocytes are not required for the development of experimentally induced fibrosis in mice (125), has contributed to this situation. However, substantial and outcome predictive abnormalities in lymphocyte subsets and activation have been

described in the lungs and blood of IPF patients, and animal modeling shows that certain lymphocyte populations are sufficient to induce or modify mammalian lung fibrosis. Thus, lymphocytes might participate in fibrosis *via* as yet undefined mechanisms. The evidence supporting this concept is reviewed below.

T Lymphocytes

Relative to samples obtained from normal individuals, lung tissue and BAL fluid from patients with IPF are enriched for several population of T lymphocytes (126). In the tissue, these lymphoid aggregates contain CD3+ T lymphocytes (127) and evaluation of the peripheral blood supports these findings to some extent. Specifically, transcriptional profiling of PBMCs found that a signature characterized by reduced expression of T cell regulatory genes related to the immune checkpoint CTLA-4 was associated with reduced event free survival (128) and these findings were recently recapitulated in a landmark study involving six independent IPF cohorts from centers across the US and Europe (129). These findings recapitulate an earlier study in which reduced expression of the costimulatory molecule CD28 on circulating T cells was seen to be a predictor of poor outcomes (130). Because treatment with the lymphocyte-modulating agent Azathioprine results in impaired function of molecules that function as immune checkpoints (131), it is intriguing to speculate that the worsened outcomes seen in the Azathioprine-treated subjects in the PANTHER trial (15) relates to aberrant activation of CD4+ T lymphocytes. In this light, it is also interesting that the checkpoint inhibitors used as cancer immunotherapy can cause inflammatory ILD (132) though to date a specific relationship with IPF has not been shown. CD4+ T lymphocytes are divided into several subpopulations, among which the best studied in IPF are T-helper cells, as shown in **Figure 3**. T-helper cells comprise several classically defined subpopulations based on their pattern of cytokine expression. In the below paragraphs, the data regarding specific T-helper populations in the context of IPF will be presented.

Th1/Th2 Cells

Perhaps the most studied concept in T-helper biology as it relates to pulmonary fibrosis is the contributions of Th1 and Th2 cells. Animal modeling, and some human data, suggests that the relative proportions of these populations might enact the balance between injury and repair. Here, Th1 cells and their secretory products are thought of as being anti-fibrotic and Th2 cells and their mediators are considered pro-fibrotic (133). For example, Th1 cells release IL-12, which is a potent inducer of IFNγ, and several studies have reported a relative reduction in IFNγ levels in the BAL or circulation of patients with IPF (134). In addition, a bleomycin study in mice found that IL-12-attenuated pulmonary fibrosis *via* modulation of IFNγ production, thus presenting a protective role of Th1 associated mediators in fibrosis (135). These findings are supported by other work showing that attenuation of Th1 differentiation *via* targeting of the transcription factor T-bet increased bleomycin-induced lung injury (136). Conversely, studies that focused on the Th2 cytokines IL-4 and IL-13 showed them to stimulate fibroblast proliferation, collagen production, and fibroblast to myofibroblast differentiation—thereby rendering Th2 cells fibrogenic (137). In addition, detection of Th2 cells and their secretory mediators appears to be enhanced in the lungs and blood of patients with IPF (137–140). However, systemic administration of recombinant

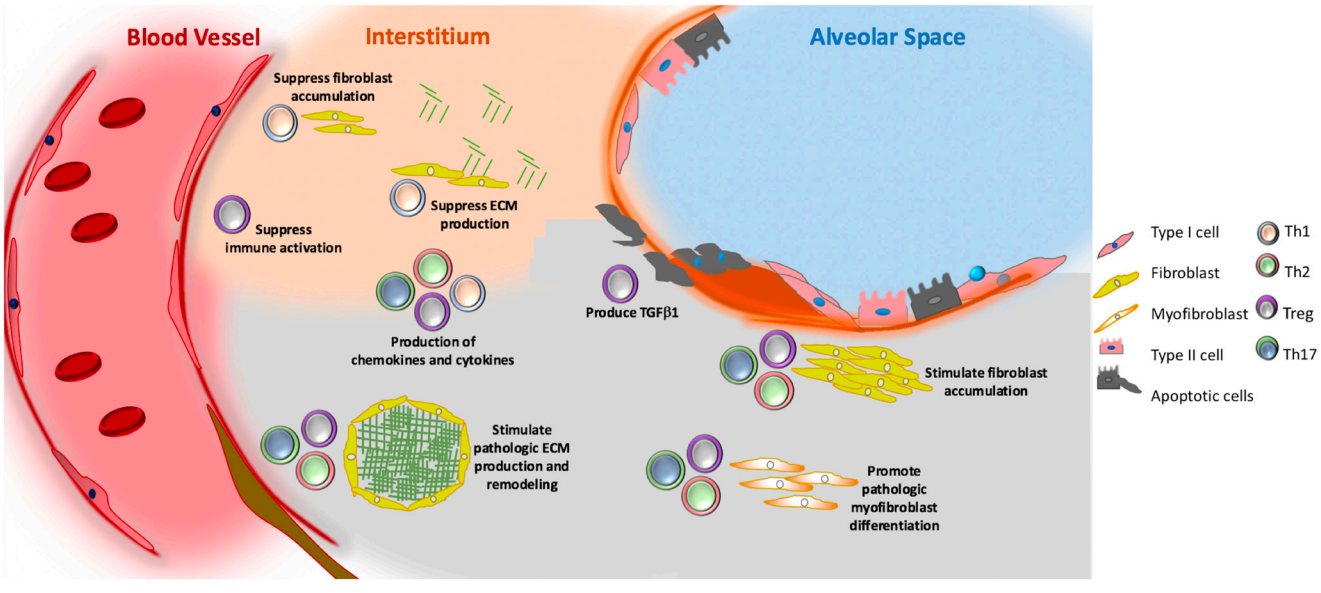

FIGURE 3 | Putative role of adaptive immunity in idiopathic pulmonary fibrosis. Th1 (grey) cells may suppress fibroblast responses through the secretion of pro-inflammatory cytokines, such as interferon gamma and TNFα. Th2 (pink) and Th17 (green) cells stimulate fibroblast proliferation, activation, and extracellular matrix (ECM) production through their secretion of IL-4 and IL-13 (Th2) and IL-17 (Th17). Tregs (purple) may either promote fibrosis through production of PDGFβ and TGFβ1, or suppress fibrosis *via* poorly understood effects on fibrocyte accumulation. Note that image is not drawn to scale.

IFNγ (which would simulate the presence of Th1 biology) and monoclonal antibody-mediated neutralization of IL-13 (which would specifically target Th2 responses) failed to demonstrate efficacy in randomized, placebo-controlled trials of patients with IPF (141, 142). Therefore, the concept of the Th1/Th2 balance as a central mediator of IPF may require re-evaluation and the development of strategies to better and more efficaciously target their secretory products. The potential role of Th1 and Th2 cells is shown in **Figure 3**.

Th17 Cells

The role of Th17 cells in IPF is also incompletely defined. Th17 cells produce cytokines, such as IL-17 and IL-22, which are host-defensive cytokine in many infectious conditions but also promote inflammatory pathology in various diseases such as autoimmune conditions (143). As shown in **Figure 3,** the functions of IL-17 include stimulation of ECM production, collagen disposition, and mediation of TGF-β signaling (144). Increased detection of IL-17 in the lung tissue, BAL, and serum of IPF patients suggests a potential relationship with disease. These human findings are supported by murine studies in which administration of IL-17A is sufficient to induce collagen accumulation and fibrotic lesions (144, 145), and that neutralization of IL-17 can reduce fibrosis in several animal models (146, 147). Furthermore, in silica-induced lung fibrosis, neutralization of IL-17A delayed T-cell-driven immune responses and consequently slowed the progression of lung inflammation and fibrosis (148). Interestingly, recent work has expanded the concept of IL-17 in fibrosis beyond lymphocytes, as one recent studying in an experimental model of HP found neutrophils and monocytes/macrophages to be a dominant source of IL-17A (149). Similar findings have not been described in IPF. While IL-22, another product of Th17 cells, appears to be protective in the bleomycin model (150) BAL concentrations do not differ between IPF and control (151). Because anti-IL-17 treatment has not been tested in IPF, the efficacy of neutralizing Th17 cells and their secretory products as a therapeutic approach in IPF is currently not known.

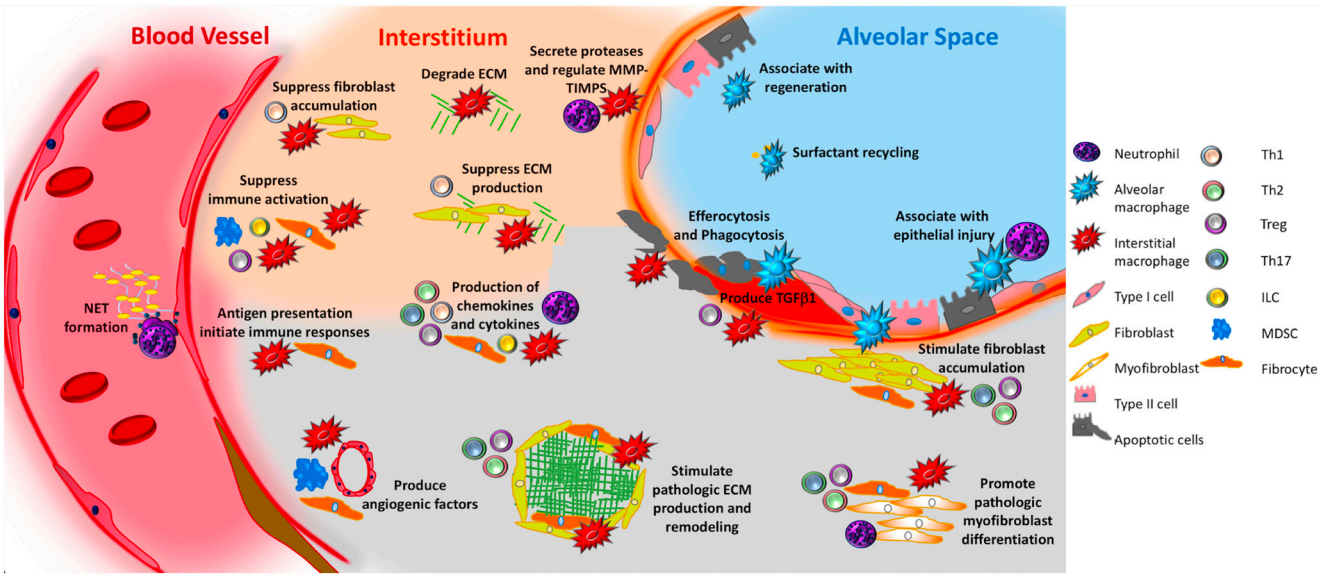

FIGURE 4 | Unifying schematic of immunopathogenic mechanisms of idiopathic pulmonary fibrosis (IPF) reveals that many important fibrosis-promoting processes may be regulated by input from both the innate and adaptive immune systems. Currently available data suggest that innate mechanisms may dominate. For example, both alveolar and interstitial macrophages respond to innate immune ligands present in pathogen-associated molecular patterns or danger-associated molecular patterns to assume adopt fibrosis modifying properties, including production of TGFβ1, paracrine regulation of fibroblast accumulation and activation, production of TIMPS and MMPS that participate in extracellular matrix (ECM) remodeling, production of angiogenic factors, secretion of lipid mediators, regulation of structural cell injury and stem cell renewal, and surfactant recycling. Macrophages might also suppress fibrosis by stimulating a regenerative program in epithelial stem cells, by regulating MMPs and TIMPS, and by directly degrading collagen and ECM. Neutrophils may promote fibrosis *via* the formation of neutrophil extracellular traps (NETs), which may promote fibrosis *via* the production of TGFβ1 and subsequent myofibroblast activation. However, neutrophils may also suppress fibrosis by regulating the MMP:TIMP balance and producing neutrophil elastase (NE) which degrades ECM. Circulating fibrocytes possess several fibromodulatory, including the ability to differentiate into fibroblasts and myofibroblasts, production of ECM, contraction of wounds, participate in antigen presentation, production of chemokines and cytokines, and regulation of angiogenesis *via* production of soluble mediators. Myeloid-derived suppressor cells (blue) display show an association with ECM remodeling and pulmonary hypertension. Innate lymphoid cells (ILCs) produce cytokines that may regulate fibroblast accumulation and ECM production. In terms of the adaptive immune response, Th1 cells may suppress fibroblast responses through the secretion of pro-inflammatory cytokines while Th2 and Th17 cells stimulate fibroblast proliferation, activation, and ECM production. Tregs either promote fibrosis through production of PDGFβ and TGFβ1, or suppress fibrosis *via* poorly understood effects on fibrocyte accumulation. B cells are not shown in this figure given the largely speculative nature of their role in IPF. The redundancy and opposing effects of these functions likely accounts for the failure of IPF to respond to classical forms of immunosuppression. Given the pronounced contribution of the innate immune system, interventions targeting the recognition of, or response to, innate immune ligands might be of benefit.

Regulatory T Cells

The role of Tregs in pulmonary fibrosis has been gaining acceptance in the recent years. Due to their ability to produce both IL-10 and TGFβ1, Tregs have the potential to both promote or suppress fibrosis depending on the context. For example, a now seminal 2009 study reported marked suppression of functional CD4+, CD25high, FoxP3+ cells in the BAL and peripheral blood of IPF patients (152), thereby showing, for the first time, a relationship between impaired Tregs and IPF. However, more recent studies in this area have actually shown an increase in Tregs, and an imbalance of the Treg/Th17 axis in IPF patients (153). In addition, a population of aberrantly activated Tregs identified by expression of the neuroimmune molecule Semaphorin 7a+ was sufficient to engender TGF-β1 induced fibrosis in the adult mouse lung (154) *via* undefined mechanisms. A synthesis of this information suggests that recruited or lung resident Tregs might be fibrosis-suppressive or fibrosis-stimulatory depending on their interaction with the local milieu (155). This concept is supported by experimental data from a bleomycin model showing that Tregs may stimulate TGFβ1 production and collagen accumulation when present during the injury phase, but might reduce these endpoints when present during the later stages of the model (155). These studies are complemented by data from an LPS model of lung injury showing that Tregs suppress fibrocyte recruitment and fibrosis *via* interruption of the chemokine C-X-C motif ligand 4/stromal cell-derived factor 1 (CXCL4/SDF1) axis (156), but promote fibroblast activation *via* production of PDGFβ in a model of silicosis (157). In summary, Tregs play a controversial role in pulmonary fibrosis and depending on the stage of fibrosis, they can be both harmful as well as protective. The putative contribution of Tregs to mammalian lung fibrosis is depicted in **Figure 3**.

B Cells

B cells represent another arm of the adaptive immune system. Functioning primarily as producers of antibodies, increased detection of CD20+ B cells has been reported for IPF lungs (158). A variety of novel autoantibodies targeting neoepitopes have been reported in IPF (159–163), with many of the targets being structural cell proteins, such as desmoplakin (164) and periplakin (159). In addition, while the presence of clinically relevant positive serology effectively rules out IPF as a diagnosis, recent work demonstrates that patients with low level titers of autoantibodies might have worsened clinical outcomes than those patients lacking these findings (160). Presence of autoantibodies can be linked to poor survival as seen in a recent study wherein high levels of anti-vimentin were associated with worsened pulmonary function and prognosis (165). Similar findings were observed with the presence of anti-HSP70 autoantibodies in patients with IPF (166). Further evidence of a potential role for humoral responses in the pathogenesis of IPF is provided by the detection of antibodies targeting BPI fold containing family B, member 1 (167) though to date the mechanistic impact of these observations remain elusive. While B cell subtypes and function have not been specifically phenotyped in large-scale clinical studies, evidence does exist supporting a role for B cells in some forms of this disease. For example, detection of B lymphocyte stimulator, which is also known as B-cell-activating factor (BAFF), is enriched in the lungs and blood of patients with IPF (168). The potential of B cells to serve a mechanistic role in this disease is shown by a retrospective study in which stable outpatients with end stage ILD who received CD20 targeted therapy (which removes B cells) showed a trend toward improved lung function (169). A beneficial role of B-cell-targeted therapies is further supported by the finding that patients with respiratory failure due to an AE-IPF showed clinical improvement after undergoing combined plasmapheresis (which would be presumed to remove offending antibodies) and anti-CD20 therapy (170). These human observations are complemented by animal modeling in which neutralization or genetic ablation of BAFF attenuates pulmonary fibrosis (168). However, because data gleaned in other models show that B cells may actually suppress fibrotic response (171), precise understanding of the relationship between B cells and IPF remains elusive and the potential contribution these cells to IPF pathogenesis remains a query in need of further study. Due to the largely speculative nature of their relationship with fibrosis, B cells are not included in **Figure 3**.

BOX 1 | Unanswered questions regarding the immune and inflammatory cells in idiopathic pulmonary fibrosis (IPF).

- To what extent do data obtained from mouse models reflect the situation in the fibrotic human lung? Can mimetics be developed that more accurately simulate the IPF disease state?
- Do events in the peripheral blood truly reflect events occurring in the diseased lung?
- Do the innate immune abnormalities seen in IPF represent a unique form of immunosenescence?
- Can therapies targeting macrophage activation stabilize or restore lung function in patients with IPF?
- Does the altered microbiome cause pathogen-associated molecular pattern-driven innate immune activation in IPF and are antimicrobial therapies efficacious in IPF?
- Does perpetuated microinjury cause danger-associated molecular pattern (DAMP)-driven innate immune activation in IPF and are therapies targeting DAMPs and their receptors efficacious in IPF?
- Are neutrophil extracellular traps an important part of IPF pathogenesis?
- What is the role of fibrocytes and myeloid-derived suppressor cells in IPF?
- Do innate lymphoid cells participate in IPF?
- How does the relative balance of T-helper cells participate in IPF and can this contribution be targeted in a safe and efficacious manner?
- Are B cells involved in the development of IPF?
- Can immune events detected in the circulation be used to guide personalized therapies in IPF?

CONCLUSION

As can be seen, the cells of the immune system show a rich and multifaceted contribution to IPF. When viewed in contrast with the epithelial cells that are believed to be the primary site of injury, and with fibroblasts, which are viewed as the driver of matrix deposition and remodeling, the more heterogeneous contribution of the innate and adaptive immune systems shown in **Figure 4** is nuanced and unlikely to respond to a single intervention. This aspect, combined with the relative ease of isolating immune cells and substances from the BAL and circulation renders

the immune system an attractive area for the development of immunopathogenesis-based personalized therapies based on easily accessible biomarkers. Areas of particular interest and important questions in this context, which are shown in **Box 1**, would benefit from concerted efforts performed in large-scale multicenter recruitment efforts, leveraging of existing datasets and registries, and the generation of improved modeling systems that more faithfully recapitulate the complex microenvironment of the fibrotic human lung and improve the understanding and treatment of IPF on a global scale.

AUTHOR CONTRIBUTIONS

OD wrote manuscript, JW made figures and wrote manuscript, MM wrote manuscript, and EH wrote manuscript. All authors approved final version.

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REVIEW
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Impact of Transcriptomics on Our Understanding of Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a lethal fibrotic lung disease characterized by aberrant remodeling of the lung parenchyma with extensive changes to the phenotypes of all lung resident cells. The introduction of transcriptomics, genome scale profiling of thousands of RNA transcripts, caused a significant inversion in IPF research. Instead of generating hypotheses based on animal models of disease, or biological plausibility, with limited validation in humans, investigators were able to generate hypotheses based on unbiased molecular analysis of human samples and then use animal models of disease to test their hypotheses. In this review, we describe the insights made from transcriptomic analysis of human IPF samples. We describe how transcriptomic studies led to identification of novel genes and pathways involved in the human IPF lung such as: matrix metalloproteinases, WNT pathway, epithelial genes, role of microRNAs among others, as well as conceptual insights such as the involvement of developmental pathways and deep shifts in epithelial and fibroblast phenotypes. The impact of lung and transcriptomic studies on disease classification, endotype discovery, and reproducible biomarkers is also described in detail. Despite these impressive achievements, the impact of transcriptomic studies has been limited because they analyzed bulk tissue and did not address the cellular and spatial heterogeneity of the IPF lung. We discuss new emerging technologies and applications, such as single-cell RNAseq and microenvironment analysis that may address cellular and spatial heterogeneity. We end by making the point that most current tissue collections and resources are not amenable to analysis using the novel technologies. To take advantage of the new opportunities, we need new efforts of sample collections, this time focused on access to all the microenvironments and cells in the IPF lung.

Keywords: interstitial lung diseases, idiopathic pulmonary fibrosis, transcriptomics, biomarkers, microenvironment, microarray, RNAseq

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INTRODUCTION

Our understanding of idiopathic pulmonary fibrosis (IPF), a chronically progressive scarring lung disease, with a significant genetic component, has dramatically changed in the last two decades. This has happened because after years of formulating hypotheses based on animal models, or analogies from other diseases, pulmonary researchers shifted their focus to analyzing the human lung. The increased availability of well-characterized human tissues and the emergence of high throughput transcriptomic profiling technologies facilitated a new era in IPF research, one in which novel hypotheses are based on observations from human lungs. The sheer size of the data, and its unbiased nature, reintroduced serendipity in pulmonary fibrosis research, and thus led to numerous,

previously unexpected observations, novel hypotheses and paradigm shifts. In this perspective, we provide an overview of the impact of transcriptomics on our understanding of IPF. We highlight the timeline of major discoveries (**Figure 1**) with a focus on mechanisms and pathways, novel biomarkers and disease classification, non-coding RNAs, and disease microenvironments.

BRIEF HISTORY

The history of transcriptomics in pulmonary fibrosis, is a story of ever increased technological throughput, enhanced sophistication of data analysis and availability of human samples. Gene expression microarrays, which allowed the parallel analysis of hundreds and later thousands of genes, emerged in the second half of the last decade of the twentieth century (1, 2). When the first publication of the application of microarray analysis to pulmonary fibrosis in mice was published in 2000 (3), microarrays could profile ~6,000 transcripts, the statistical approaches were not widely accepted, and human tissues were not available. Two years later, the first analysis of human lungs in 2002 included only eight samples, used a classification algorithm and did not mention a *p*-value (4). These papers were exciting and novel but very limited in numbers of samples and sophistication of analytical approaches.

Even several years later, studies that aimed at classifying disease included relatively low numbers of samples (5–10). These studies were more sophisticated in data normalization, visualization, and the wide adaptation of statistical approaches to address multiple testing (11, 12). Tissue availability has only increased when NIH-NHLBI established the Lung Tissue Research Consortium, a multicenter publicly available lung tissue repository (13). The expanded availability of tissues allowed application of microarray platforms to hundreds of samples (14, 15) as well the public availability of data through the Lung Genomics Research Consortium (16). Development of RNAseq for deeper sequencing than with microarray platforms resulted in routine profiling of the whole transcriptome including coding and non-coding RNAs, detection of larger dynamic ranges of transcripts, and identification of novel transcripts and variants (17, 18). This further allowed analysis of low-input and degraded RNA samples that enabled research on lung microenvironments and archived tissues (19, 20). Currently, when approaching a transcriptomic study, investigators do not have to be limited by sample or technological feasibility. Instead, they can follow a rational approach to design (**Figure 2**). The key insights below largely follow aspects of this outline.

MECHANISMS AND PATHWAYS

Transcriptomics studies revealed numerous novel molecules and pathways highly relevant for IPF pathogenesis. Here, we describe the most prominent findings, while a more complete list is available in **Table 1**.

Matrix Metalloproteinases

Development of IPF was initially explained as fibroblast proliferation, higher expression of tissue inhibitor proteinases (TIMPs), and reduced activity of matrix metalloproteases (MMP) (55). The

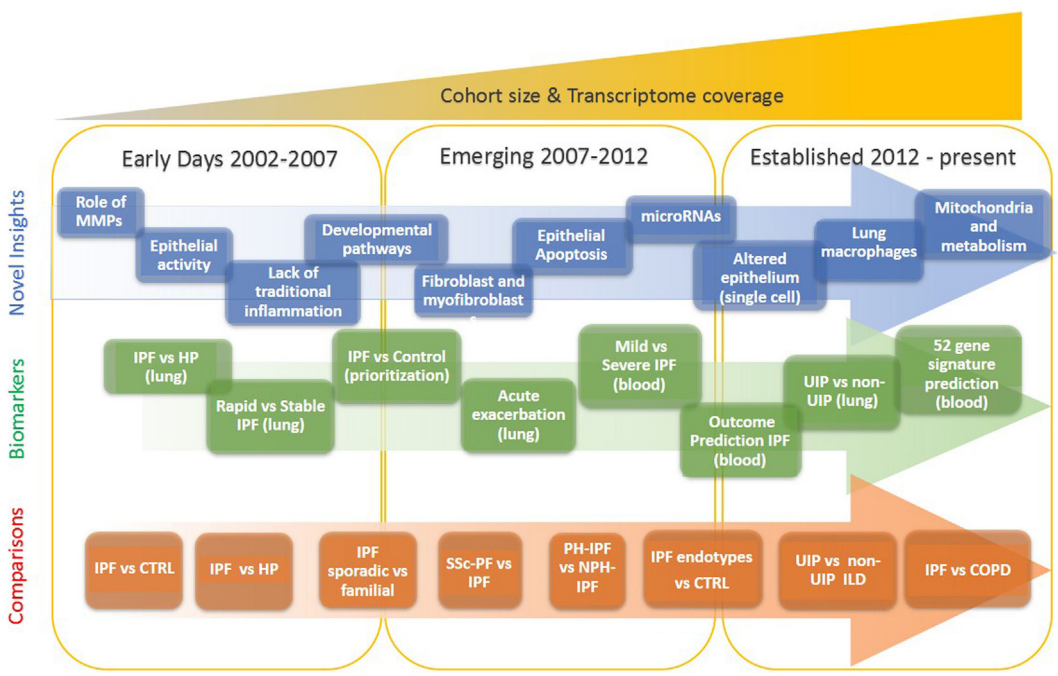

FIGURE 1 | Evolution of idiopathic pulmonary fibrosis (IPF) transcriptome analysis. The progression of IPF transcriptomic research is that of increased complexity, more genes studied, more sample studied, and more detailed phenotypes. In the early days, a few thousand genes were analyzed on a small number of samples and limited analytical approaches. During the emerging period investigators studied tens of samples, mostly on microarrays that profiled of all protein coding mRNAs. In the established period, the numbers of samples are in hundreds, all transcribed RNA is measured, and analytical methods are sophisticated.

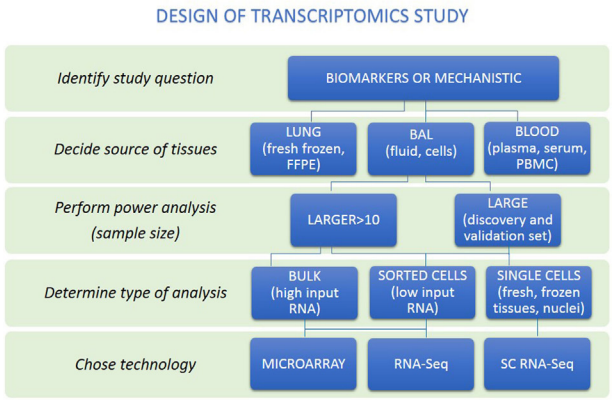

FIGURE 2 | A rational approach to design of transcriptomics study. An overview of steps to help researchers make appropriate study design is presented. First, distinguish whether study aims to identify biomarkers or mechanisms. Then, the source of tissues together with power analysis to calculate sample size to be able to answer research question should be performed. Decision about the type of analysis should be made (bulk, sorted cells, or single cells). Last, the technology to perform transcriptome analysis should be chosen.

first study that analyzed human lungs contradicted this paradigm (**Figure 1**; **Table 1**). Instead of the expected downregulation, authors found that MMPs were among the most increased genes in IPF lungs including MMP1, MMP2, MMP7, and MMP9 (4). MMP7 was localized predominantly in alveolar epithelium, and MMP7 knockout mice were relatively resistant to fibrosis (4). In addition to MMP7, MMP1 (4), MMP3 (28), MMP19 (29), and MMP28 (56) have been found to be increased in lung epithelial cells of patients with IPF, with diverse and sometimes opposing roles (57, 58).

While their exact roles have not been fully elucidated, the initial unexpected observation that MMPs are increased in the IPF lung, has been validated numerous times. It is now well accepted that MMPs affect numerous signaling pathways that together contribute to the profibrotic environment in the IPF lung and may also serve as effective biomarkers (see below).

Genes Expressed in Lung Epithelium

Transcriptomic analysis of bulk tissue depends on follow-up analyses to decipher the cellular origin of differentially expressed genes. One of the most surprising findings in IPF transcriptomics was that cellular origin of large number of genes that distinguish the IPF lung from controls ended up being the alveolar epithelium (59, 60) (**Figure 1**; **Table 1**). Among the first examples were MMP7, and later SPP1, a protein known to be expressed in inflammatory and bone cells, that in IPF is increased in the epithelial cells adjacent to myofibroblasts foci (22).

Other genes increased in IPF and unexpectedly localized to the alveolar epithelium adjacent to fibrotic regions include N-cadherin (5), HIF-1-alpha (31), IGFBP-4 (9), CCNA2 (10), TAGLN (33), CRLF1 (34), EGFR (35), and DIO2 (54). Among decreased genes, reduced expression of CAV1 (6) and AGER (52) in IPF compared with control lungs was thought to reflect changes in epithelial function or loss of type I alveolar epithelial cells (**Table 1**).

Of particular interest in this context, is a study that demonstrated that IPF patients with increased expression of cilia genes exhibited also increased MMP7 and MUC5B, as well as microscopic honeycombing but not myofibroblast foci on histological examination, suggesting that they represented a distinct IPF endophenotype (61) (**Table 1** and see below).

Fibroblasts and Fibroblast Foci Related Gene Expression

Genes associated with myofibroblasts, a hallmark of lung histology in IPF, have been described as early as 2002 in bulk tissue analysis (4). Analysis of lung fibroblasts treated with TGFB1 revealed responses to TGFB1 and smooth muscle like myofibroblast phenotype switching (62) that was similar to what was observed in the IPF lung. Fibroblasts isolated from IPF lungs exhibited increased expression of IGFBP3 and IGFBP5 (43), TWIST1 (48), WNT5A (45), COMP (63), and FOXF1 (38). Increased Vascular cell adhesion molecule 1 gene expression in IPF lungs negatively correlated with lung function (39). Another TGFB1 induced gene, FKBP10, a collagen chaperone, was also increased in IPF and IPF lung fibroblasts and contributed to Collagen synthesis (40). Recently, TAZ, a transcriptional coactivator important in development, was shown to be increased in the fibroblastic foci and to contribute to fibrotic response through TAZ-mediated regulation of CTGF (42) (**Figure 1**; **Table 1**).

Of particular interest are genes downregulated in IPF lungs and IPF fibroblasts, as they may represent key features lost during disease. RXFP1, a relaxin/insulin-like family peptide receptor is significantly decreased in IPF tissues and fibroblasts and correlates with disease severity. A relaxin-like peptide, CGEN25009 was effective at decreasing bleomycin-induced, fibrosis *in vivo* (41). Similarly, PTPN11, a ubiquitously expressed SH2 domain-containing tyrosine phosphatase, was decreased in IPF lungs and IPF fibroblasts. Overexpression of constitutively active PTPN11 reduced the responsiveness of fibroblasts to profibrotic stimuli, and viral delivery of PTPN11 to wild-type mice blunted bleomycin-induced pulmonary fibrosis (50) (**Figure 1**; **Table 1**).

The WNT Pathway in IPF

Perhaps, one of the most intriguing finding in IPF lungs gene expression was the aberrant activation of developmental pathways and especially the WNT/β-catenin pathway in IPF (**Figure 1**; **Table 1**) (64, 65). In 2003, the first observation of β-catenin expression in fibroblastic foci, as well as its expression and colocalization with WNT downstream target genes, CCND1 and MMP7 in adjacent proliferative bronchiolar lesions was reported (64). Subsequently, increased WNT1, WNT7b, WNT10b, FZD2 and FZD3, β-catenin, and LEF1 were found in IPF lungs (23). WNT1, WNT3a, β-catenin, and GSK3B were mainly localized to alveolar and bronchial epithelium with increased expression of WNT targets CCND1 and MMP7. Increased expression of WISP1, a WNT inducible signaling protein, was found in IPF lungs. WISP1 had profibrotic effects *in vitro*, and WISP1 neutralizing antibodies blunted fibrosis *in vivo* (47). Inhibition of WNT/β-catenin

TABLE 1 | Summary of relevant idiopathic pulmonary fibrosis (IPF) genes identified by transcriptome profiling.

Gene ID[a]	Gene name	Direction of expression	Tissue localization	Relevant pathway	Reference
Expressed in lung epithelium in IPF					
MMP7	Matrix metallopeptidase 7	Increased	Lung (alveolar epithelial cells and fibroblasts), peripheral blood and BAL	Extracellular matrix degradation, defensins, SPP1, and WNT/β-catenin pathway	(4, 5, 21–27)
MMP3	Matrix metallopeptidase 3	Increased	Lung, epithelial cells	Extracellular matrix degradation, β-catenin pathway	(28)
MMP19	Matrix metallopeptidase 19	Increased	Lung, epithelial cells	Extracellular matrix degradation, PTGS2 pathway	(29)
MMP1	Matrix metallopeptidase 1	Increased	Lung, epithelial cells	Extracellular matrix degradation, mitochondrial function/HIF-1-alpha pathway	(30)
SPP1	Osteopontin	Increased	Lung (epithelial cells)	Extracellular matrix degradation	(9, 22)
IGFBP-4	Insulin-like growth factor binding protein 4	Increased	Lung (alveolar and basal cells)	IGF1 pathway	(5, 24)
CCNA2	Cyclin A2	Increased	Lung (alveolar epithelial cells)	Cell cycle regulation	(10)
HIF1A	Hypoxia-inducible factor-1 alpha	Increased	Lung (alveolar epithelial cells)	Hypoxia, p53/VEGF pathways	(31)
CAV1	Caveolin-1	Decreased	Lung	Cell cycle regulation, TGF-b/JNK pathway	(6)
SYN-2	Syndecan-2	Increased	Lung, alveolar macrophages	TGF-b pathway	(32)
TAGLN	Transgelin	Increased	Lung, ATII cells	TGF-b pathway	(33)
CRLF 1	Cytokine receptor-like factor 1	Increased	Lung, ATII	Th1 cells inflammatory response	(34)
EGFR	Epidermal growth factor receptor	Increased	Lung, epithelial cells	Reepithelization	(35)
LYCAT	Lysocardiolipin acyltransferase	Decreased	Lung (epithelial cells), peripheral blood mononuclear cell (PBMC)	Mitochondrial membrane potential	(36)
SERPINF1 (PEDF)	Pigment epithelium-derived factor	Increased	Lung	Angiogenesis	(37)
Fibroblasts related gene expression in IPF					
FOXF1	Forkhead box F1	Increased	Lung	COL1/ARPC1 pathway	(38)
VCAM-1	Vascular cell adhesion molecule 1	Increased	Lung, fibroblast foci and blood vessels	TGF-b/ERK/Cyclin D pathway	(39)
FKBP10	FK506-binding protein 10	Increased	Lung, fibroblasts, and CD68 (+) macrophages	TGF-b/Col I synthesis	(40)
RXFP1	Relaxin/insulin-like family peptide receptor 1	Decreased	Lung	TGF-b	(41)
TAZ	Transcriptional coactivator with PDZ-binding motif	Increased	Lung	CTGF and Col1 pathways	(42)
IGFBP3, IGFBP5	Insulin-like growth factor binding proteins 3 and 5	Increased	Lung	IGF pathway	(43)
WNT pathway in IPF					
WNT1, 3a, 5a, 7b, 10b, Fzd2 and 3, β-catenin, Lef1, Gsk-3β	Wingless and others	Increased	Lung, fibroblasts, alveolar and bronchial epithelium	Wnt signaling	(23, 44, 45)
LRP5	Wnt co-receptor	Increased	Lung, PBMC	Wnt and TGF-b pathway	(46)
WISP1	Wnt1-inducible signaling protein-1	Increased	Lung	Wnt signaling	(47)
Apoptotic response in IPF					
TWIST1	Twist basic helix–loop–helix transcription factor 1	Increased	Lung—fibroblastic foci	Apoptosis/PDGF pathway	(48)
CXCL12	Chemokine ligand 12	Increased	Lung	Inflammation	(8)
TNSF10, BAX, CASP6	Apoptotic regulators	Altered expression	Lung	Apoptosis	(49)
SHP2 (PTPN11)	SH2 domain-containing tyrosine phosphatase-2	Decreased	Lung	Apoptosis/Tyr and Ser/Thr kinase pathways	(50)
Host defense implicated in IPF					
DEFA3–4	Defensin alpha 3 and 4	Increased	Lung and peripheral blood	Host defense	(10, 51)
AGER (RAGE)	Advanced glycosylation end product-specific receptor	Decreased	Lung and peripheral blood	Inflammation	(24, 52)
Mitochondria-related genes in IPF					
PINK1	PTEN-induced putative kinase 1	Decreased	Lung	Dysfunction of mitochondria	(53)
DIO2	Iodothyronine deiodinase 2	Increased	Lung	TH pathway/mitochondrial biogenesis	(54)

pathway attenuated lung fibrosis in mice, suggesting an essential role of WNT/β-catenin pathway in IPF development (46, 66).

While many of these observations were focused on epithelial cells, WNT5A, a member of the non-canonical signaling pathway was increased in IPF lung fibroblasts, with multiple observations suggesting its role in determining fibroblast phenotype in IPF (45, 67, 68).

Aging, Metabolism, and Mitochondria-Related Molecules

Mitochondrial dysfunction is emerging as one of the key features of IPF. Gene expression data revealed decreased PINK1, a key regulator of mitophagy, and analysis of IPF lungs revealed accumulation of dysfunctional mitochondria in alveolar epithelial cells. Findings from PINK1 knockout confirmed these results, and established a role for impaired mitophagy in IPF (53) potentially through TGFB1 effects (69).

High expression of DIO2, an enzyme that activates thyroid hormone in IPF lungs, and a predisposition to fibrosis among DIO2 knockout mice, led investigators to treat bleomycin treated mice with thyroid hormone or a small molecule agonist (54). Thyroid hormone reversed bleomycin-induced mitochondrial injury both *in vivo* and *in vitro* and augmented resolution of fibrosis in mouse models of pulmonary fibrosis. This effect was dependent on intact PPARGC1A and PINK1 pathways suggesting that the antifibrotic effect of thyroid hormone was mediated through restoration of mitochondrial homeostasis (54).

Changes in expression of genes encoding numerous metabolic enzymes from IPF lungs associated with glucose, fatty acid and citric acid metabolism suggesting on large alterations in mitochondria function (70). Similar findings were found in fibroblasts and alveolar macrophages (71, 72). More detailed review of age-related perturbations in genome and epigenome associating with plausible roles of mitochondria in pathogenesis were published elsewhere (73, 74).

GENE EXPRESSION PATTERNS AS TOOLS FOR DISEASE DIAGNOSIS, CLASSIFICATION, AND OUTCOME PREDICTORS

Transcriptomics studies have also been used to identify disease class related gene expression patterns in the lung, as well as to prioritize protein biomarkers found in the blood stream, or to identify peripheral blood mononuclear cells (PBMCs), gene expression patterns that correlate with disease clinical attributes. The studies are summarized in **Table 2**.

Disease Classification

An early suggestion that lung gene expression can be used to classify disease emerged from comparison of lungs of patients with IPF from those with fibrotic hypersensitivity pneumonitis (HP) using transcriptome analysis (5). The enrichment pathway analysis of the HP signature revealed T-cell activation, inflammation,

TABLE 2 | Summary of gene signatures that classify interstitial lung diseases.

# Genes	Tissue origin	Disease comparison	Sample size	Year	Reference
407	Lung	Idiopathic pulmonary fibrosis (IPF) vs HP	15 (IPF) 12 (HP)	2006	(5)
332/6	Lung	Sporadic IPF vs familial, IPF vs non-specific interstitial pneumonitis (NSIP)	16 sporadic IPF (2 NSIP) 10 familial (4 NSIP)	2007	(8)
242/335	Lung, fibroblasts	CTRL vs (SScPF; SScPAH; iPAH; IPF)	33 (15 severe PF, 6 moderate/severe PF and PAH, 4 moderate PF with PAH, 7 PAH), 10 IPF	2011	(75)
<50	Lung	SSc/IFP; IPF vs NSIP	≤10	2007, 2011	(8, 75)
22	Lung	IUP vs (non-IUP, sarc, HP)	77 training set (39 IUP, 38 non-IUP), validation set 48 (22 IUP, 26 non-IUP)	2015	(19)
4,734	Lung	PH-IPF and PAH vs CTRL	18 (PAH), 8 (PH-IPF)	2010	(76)
74	Lung	Chronic lung disease	13 data sets	2015	(77)
>1,500/32[a]	LCM lung	PH-IPF vs CTRL, PH-chronic obstructive pulmonary disease (COPD) vs CTRL, PH-IPF vs PH-COPD	LCM pulmonary arterioles (n = 8)	2014	(78)
255	LCM lung	PH-IPF vs NPH-IPF	8 PH-IPF, 8 NPH-IPF	2013	(79)
2,490[b] 337[c] 214[d]	Lung	IPF vs COPD vs CTRL	19 IPF, 49 COPD	2016	(18)
3 Gene clusters	Lung	IPF vs COPD vs CTRL	319 (3 data sets)[e]	2015	(15)

[a]*32 small DEGs overlap between PH-IPF and PH-COPD.*
[b]*2,490 DEGs between IPF and CTRL.*
[c]*DEGs between COPD and CTRL.*
[d]*DEGs overlap between IPF and COPD.*
[e]*4,259 mRNA and 438 microRNA and also includes 669 clinical variables.*

and humoral immune response pathways, whereas the IPF gene signature showed enrichment for cell adhesion, extracellular matrix, and lung development pathways (80).

Analysis of lung samples obtained from patients with sporadic IPF, familial pulmonary fibrosis with a usual interstitial pneumonia (UIP) pattern, and non-specific interstitial pneumonitis (NSIP) revealed similarities on gene expression patterns and pathways and a minimal difference between IPF and NSIP (**Table 2**) (5, 8). Similar findings were found when systemic sclerosis (SSc) associated pulmonary fibrosis and IPF were compared (75).

A recent study used supervised machine learning algorithms to distinguish lung biopsy samples with UIP from non-UIP (NSIP, sarcoidosis, and HP) identified a 22 gene signature (specificity 92%, sensitivity 64–82%). This approach was solely based on transcriptional data concordant with UIP pathological findings without integration of clinical information, or comparison to patient-level diagnoses by multidisciplinary teams, the current diagnostic gold standard (19). The same group continued improving genomic classifiers to differentiate UIP from non-UIP and demonstrate high robustness toward lung tissue collection using transbronchial biopsy (81, 82) (**Figure 1**; **Table 2**).

Lung Gene Expression Profiles Associated With Disease Activity and Severity

Idiopathic pulmonary fibrosis has different patterns of progression, from stable disease lasting for long periods of time to rapid progression, and acute exacerbations that are highly lethal. Despite a very small number of samples, differentially expressed genes were found in end-stage lungs obtained from patients with rapid and slow progression defined by length of symptoms (**Figures 1** and **2**; **Table 3**) (7). Similar findings were also found in a study aimed to identify genes that defined progression by rate of deterioration in pulmonary function tests (9). SFTPA1, SPP1, and HSPA1A were among top increased genes and correlated with worst survival in IPF in agreement with previous reports (83, 84).

The study of acute exacerbations of IPF has been limited, because of lack of tissue availability. Using a unique resource of rapid lung autopsies (88) investigators compared lung gene expression profiles of acute exacerbations, stable end-stage IPF, and controls (10). They did not find any significant evidence for infection or overt inflammation in acute exacerbation lungs, but they did find increased expression of CCNA2, and DEFA3 and DEFA4, antimicrobial proteins of the alpha-defensin family known to be cleaved by MMP7 (25) and evidence for widespread epithelial apoptosis.

A more sophisticated effort to identify disease endotypes based on tissue gene expression, incorporated clinical and histological information in the analysis (61). This determined that patients with increased expression of cilia-related genes, such as DNAH6, DNAH7, DNAI1, and RPGRIP1L, exhibited also increased expression of SPP1, MMP1, MMP7, PLUNC, MUC5B, as well as more microscopic honeycombing on histology but no myofibroblastic foci (61) (**Table 3**). Interestingly, MMP7 has previously been shown to attenuate ciliated cell differentiation during wound repair (27). Another effort to identify disease activity genes studied gene expression commonalities between IPF disease progression in humans and bleomycin-induced lung fibrosis in rats (14). They identified the largest overlap in differentially expressed genes between lung transcriptome of bleomycin-induced fibrosis and IPF human lungs and identified 12 genes (C6, CTHRC1, CTSE, FHL2, GAL, GREM1, LCN2, MMP7, NELL1, PCSK1, PLA2G2A, and SLC2A5) as translational markers of disease activity. Of those markers, four classified IPF patients based on disease severity (14).

Cross Disease Endotypes

The availability of large datasets such as the LGRC, allowed also analysis of multiple chronic lung disease in parallel. Recently, applying a novel computational approach named integrative phenotyping framework, investigators discovered novel endotypes of chronic obstructive pulmonary disease (COPD) and IPF (15). They integrated clinical phenotype data with mRNA and microRNA data and identified novel patient clusters. The genes that characterized the patients in the intermediate clusters were enriched with inflammatory and immune pathways, suggesting that patients from those clusters could have a mechanistically distinct autoimmune endotypes (15). Similarly, the same group integrated mRNA, microRNA, and splicing gene variants to identify convergent transcriptional regulatory networks in IPF and COPD (18). The p53/hypoxia pathway emerged as a convergent pathway in COPD and IPF. A recent study performed meta-analysis of 13 published data

TABLE 3 | Summary of gene signatures that predict idiopathic pulmonary fibrosis (IPF) progression [rapid vs slow (stable)].

# Genes	Tissue origin	Sample size (IPF)	Year	Reference
437	Lung	26 (rapid progressors), 88 (slow progressors)	2007	(7)
579	Lung	23 (stable), 8 (acute exacerbation)	2009	(10)
134	Lung	6 (stable), 6 (progressive)	2009	(9)
472	Lung	119 (training), 111 (validation)	2013	(61)
468/12[b]	Bleomycin rat/IPF human	100 (human), 73 (rats)	2015	(14)
1,428/2,790/13[a]	Peripheral blood mononuclear cell (PBMC)	130 (mild vs ctrl; severe vs ctrl; mild vs severe)	2012	(51)
118	PBMC	45 (training), 21 and 75 (validation)	2015	(85)
52	PBMC	45 (discovery), 75 (validation), and 425 (validation)	2013, 2017	(86, 87)

[a] *13 DEGs between mild and severe IPF.*

[b] *12 is set of translational markers.*

sets including cystic fibrosis, COPD, IPF and asthma, environmental conditions (smoking, epithelial injury), and control to identify general markers of chronic lung disease (77). Increased inflammatory, wounding, defense response and regulation of cell proliferation pathways, and decreased immune response pathways were observed (77). While intriguing, all of these studies were limited by lack of resolution with regard to cellular admixture and depth of clinical phenotyping (**Figure 1**; **Table 2**).

Prioritization of Protein Biomarkers

Genome scale transcriptome studies facilitated the development of protein-based biomarkers for IPF diagnosis (**Figures 1** and **2**; **Table 4**). A comparison of proteins in the blood flow of patients with IPF to control using a targeted proteomic approach identified a signature of MMP1, MMP7, MMP8, IGFBP1, and TNFRSA1F (24) that was able to distinguish IPF from controls with high sensitivity and specificity. MMP 1 and MMP 7 were also increased in the lungs of IPF patients and able to differentiate IPF patients from other chronic lung disease including hypersensitivity pneumonitis and sarcoidosis.

Indeed, MMP7, which emerged out of the first microarray analysis of human IPF lungs, was replicated as predictive of increased mortality in multiple cohorts of IPF patients (14, 26, 90–92, 95). Similar experimental strategy, following a lung gene expression finding with assessment of a protein in the peripheral blood, has been applied to many molecules including SPP1 (22), COMP (63), CXCL13 (93), CCL8 (94), and others (**Table 4**).

Peripheral Blood Gene Expression Patterns

The transcriptome of the peripheral blood is highly appealing because of information about disease presence and outcome. It represents a safe and accessible alternative to availability of samples from the lung. Microarray gene expression profiles of whole blood RNA (51) distinguished IPF patients from controls, and among IPF patients, 13 genes were changed with increased disease severity as assessed by DLCO but not FVC (**Figures 1** and **2**; **Table 3**) (51). Interestingly, alpha-defensins identified in acute exacerbations in the lung (10) were also associated with disease severity in the peripheral blood.

A subsequent study aimed to identify PBMC gene expression profiles predictive of increased mortality in patients with IPF (86). The authors performed microarray analysis on RNA isolated from PBMCs in discovery and replication cohorts of IPF patients. They identified a 52-gene outcome-predictive signature that distinguished two patient groups with significant differences in transplant free survival in both cohorts. Interestingly, increased mortality was associated with decreases in the T-cell co-stimulatory molecules CD28, ICOS, LCK, and ITK, potentially highlighting the role of potential T-cell aberrations and maybe the role of immunosenescence in IPF. Remarkably, the outcome-predictive accuracy of a score calculated based on the 52-gene signature was recently validated in a six cohorts study containing 425 IPF patients (87). Adding the 52-gene risk score to the Gender, Age, and Physiology index significantly improved its mortality predictive accuracy, suggesting that the genomic signature improved on the performance of validated clinical markers. Analysis of longitudinal changes in the signature revealed that while the 52-gene risk score tracked changes in FVC, patients never shifted their risk profile. However, in a subset of treated patients, a shift in the risk score was also accompanied by functional improvement, suggesting that the 52-gene signature may be indicative of response to the therapy. These datasets were also used in manuscripts that applied Weighted Gene Co-expression Network Analysis to identify gene expression modules that correlate with outcome (85) or microbiome changes (96) (**Table 3**). The impressive accuracy and replication should drive experiments that test the value of these biomarkers prospectively and assess in detail shift in circulating inflammatory cells in IPF using unbiased methods such as single-cell RNAseq.

ROLE OF NON-CODING RNAs IN IPF

Until recently considered the dark matter of the genome, the significant role of non-coding RNAs in human health and disease is increasingly appreciated (97). We will focus here on microRNAs, as their role has been extensively studied in pulmonary fibrosis.

MicroRNA Changes Reveal Loss of Differentiation

MicroRNAs are small non-coding RNAs that regulate gene expression by either initiating RNA degradation or inhibiting translation through binding to the 3′ UTR of their target gene. Acting as rheostats, many microRNAs regulate the general responsiveness of a cell to a certain stimulus by affecting numerous genes and frequently serving as gate keepers of feed forward

TABLE 4 | Summary of single genes—biomarkers of idiopathic pulmonary fibrosis (IPF) progression.

Gene ID	Gene name	Tissue origin	Sample size (IPF)	Year	Reference
MMP7	Matrix metallopeptidase 7	Lung, serum, plasma, BAL	13 (lung), 74 (plasma, lung, BAL)	2002, 2008	(4, 24)
			20 (BAL)	2009	(89)
			214 (plasma, 140 derivation and 101 validation)	2012	(26)
			65 (serum), 1,227 (serum), 97 (plasma)	2016, 2017	(90, 91, 92)
SPP1	Osteopontin	Lung, BAL	18	2005	(22)
COMP	Cartilage oligomeric matrix protein	Lung	115	2013	(63)
CXCL13	C–X–C motif chemokine 13	Lung, plasma	92, 94	2014	(93)
CCL8	Chemokine (C–C motif) ligand 8	Lung, BAL, plasma	8 (lung), 86 (BAL, plasma)	2017	(94)

loops. The expression of approximately 10% of the microRNAs is different in IPF compared with control lungs (98, 99). The microRNA expression patterns observed in IPF are similar to those observed in the developing lung. Comparison of fetal, IPF and control lungs revealed that miR-487b, miR-409-3p, miR-154, miR-154*, miR-134, miR-299–5p, miR-410, miR-382, miR-377, and miR-296 were increased in IPF or fetal lungs compared with controls (99). A time course systems biology analysis of microRNAs changed during postnatal lung development suggested that close to 40% were also changed in IPF (100). In the same vein, comparison of microRNA signatures in IPF and non-small cell lung cancer revealed significant similarities and numerous microRNAs that changed in the same direction. Notably, over 20 microRNAs including members of the miR-30, let-7, miR-29 families were decreased in IPF and lung cancer, commonly increased microRNAs included miR-155, miR-21, miR-205, and miR-31 (101). While the cellular origin and exact effects of all of these common microRNA changes are unclear, together with the observations about lung development, microRNA changes in the IPF lung suggest a loss of the differentiated organ regulatory networks potentially as a result of desynchronized aging (102, 103).

IPF MicroRNAs and TGFB1

One of the most recurrent themes in microRNAs in IPF, is that they are both regulated by and regulators of TGFB1 signaling. Thus, in many cases, a change in the expression of a microRNA disrupts the careful balance of self-limited activation of TGFB1. Let-7d, a microRNA known to regulate epithelial cell differentiation, is a good example. It is decreased in IPF lungs, it is inhibited by TGFB1 through direct effect of SMAD3, and when it is inhibited, it ceases to inhibit HMGA2, allowing amplification of TGFB1 signaling and early fibrotic changes *in vivo* and *in vitro* (98). Similarly, miR-21, a microRNA increased in IPF lungs, is induced by TGFB1 and is an inhibitor of SMAD7, a regulatory SMA that inhibits TGFB1 signaling pathways (104). A larger number of TGFB1 inducible microRNAs, localized to chromosome 14q32, were also increased in IPF lungs (99). Other microRNAs regulating or regulated by TGFB1 were found to be changed in IPF lungs include miR-30, miR-199, miR-29, miR-26, miR-155, miR-326, and others (105). While, it can be safely said that microRNA changes in IPF seem to result in lowering the cell profibrotic threshold, it has to be mentioned that they were obtained in isolation, for one microRNA at a time, but in the IPF lung, at least when analyzed in bulk, they happen simultaneously. To understand better the effects of microRNA perturbations, careful dissection of the cellular, spatial, and temporal changes, as well as their integrated effects is required.

miR-29, the Ultimate Antifibromir

Of microRNAs differentially expressed in IPF, the miR-29 family is probably the most extensively studied both mechanistically and as a therapeutic target, because of its known inhibitory effects on extracellular matrix proteins, and growth factors such CTGF and IGF1 (106). miR-29 family members are decreased in cardiac, renal and liver fibrosis, keloid, fibrotic Crohn's disease, and other fibrotic conditions (107–113). miR-29 family microRNAs are decreased in IPF lungs (114), they regulate numerous genes related to fibrosis (115) and seem to regulate profibrotic signals from the extracellular matrix to fibroblasts (116). Both gene delivery of miR-29 *via* a transposon method (117) or using a miR-29b mimic (118) augmented resolution of bleomycin-induced pulmonary fibrosis. While most of these studies focused on the role of miR-29 in fibroblasts, two recent studies suggested that miR-29 could be important in prevention of pulmonary fibrosis (119) or bronchopulmonary dysplasia (120) through beneficial effects on alveolar repair. Regardless of the cell specificity of the effect, miR-29 supplementation seems a viable option as an antifibrotic therapy.

TABLE 5 | Data and tissue repositories.

Name	Website	Reference
Lung Tissue Research Consortium	http://www.ltrcpublic.com/	(126)
Lung Genomics Research Consortium	http://www.lung-genomics.org/	(126)
Lung development map	https://www.lungmap.net/	(127, 128)
Cell differentiation analysis (scRNAseq)	http://www.cs.cmu.edu/~jund/scdiff/index.html	(129)

IPF MICROENVIRONMENTS

The IPF lung is characterized histologically by its regional, temporal and cellular heterogeneity, meaning that normal looking regions are interspersed with diseased regions, different regions may appear at different stages of disease (121, 122), and both the cellular content and the phenotype of known cells are dramatically altered in the IPF lung. Transcriptomic profiles of bulk tissue homogenates do not capture this complexity. They also do not allow understanding how cells influence each other in the remodeled IPF microenvironment. Improving the cellular and spatial resolution of transcriptomics using single cells and tissue microenvironments is critically important to decipher what happens in the IPF lung.

Tissue and Cellular Heterogeneity Are Starting to Emerge

Transcriptome analyses performed on bulk lung tissue detected strong gene expression signals, leading to discovery of IPF relevant signaling pathways (**Figures 1** and **2**). However, it is unclear whether alteration in transcriptome signals represented core features of disease or was dominated by changes in cellular admixture. Increased gene expression changes observed in the IPF lung were frequently assigned to cell types, based on prior knowledge or follow-up studies, as in the case of MMP7, SPP1, WISP1, COMP, TWIST1, PINK1, and the others mentioned earlier. In most cases, such analysis was done after the fact, using low throughput technologies such as immunohistochemistry, and was dependent on prior knowledge and availability of reagents. Only few studies analyzed transcriptomic gene expression

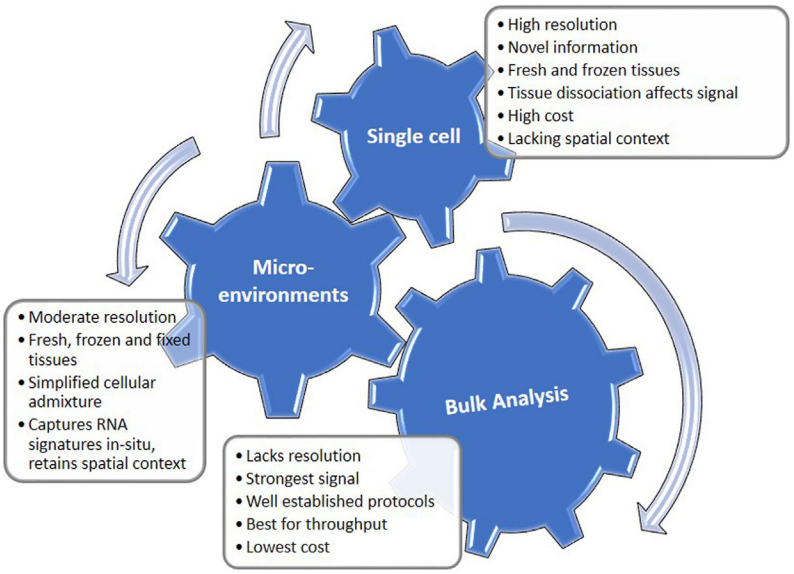

FIGURE 3 | Triangulation of transcriptomic data to understand disease. Single cell, microenvironment, and bulk tissue transcriptomic analysis have their advantages and disadvantages. When applied together, they can help in understanding regulatory networks in the tissue.

in well-defined IPF microenvironments. Comparison of the transcriptome of hyperplastic vs conserved epithelial cells and dense fibrotic lung regions, using laser capture microdissections identified previously unrecognized MMP19, as a molecule increased in hyperplastic epithelial cells, with an antifibrotic role (29). Two studies reported solely gene expression profiles of pulmonary vasculature and showed differential gene expression for IPF patients with and without coexistent PH (79) and for PH-IPF and COPD (78) (**Table 2**). Two clusters of co-regulated genes related to bronchiolar epithelium or lymphoid aggregates were identified when whole lung transcriptome was correlated with tissues histology and clinical variables (123). The first study to apply single-cell RNAseq of sorted epithelial cells from IPF patients or controls revealed distinct epithelial cell types in IPF lung and complete lack of some "normal" epithelial cells (124). Using transcriptomic profiling of flow-sorted cells, monocytes shown to differentiate into alveolar macrophages and continuously express profibrotic genes over the course of fibrosis. Thus, selective targeting of alveolar macrophage differentiation within the lung may decrease fibrosis and avoid global monocyte or tissue-resident alveolar macrophage depletion (125). Besides transcriptomics profiling of sorted and single cells isolated from fresh lung, the RNAseq of archival formalin-fixed paraffin-embedded lung biopsy from IPF patients is possible (20). This allows analysis of specific areas of lungs and their interaction observed microscopically (epithelium and fibroblastic foci), usage of clinical variables (survival) and overcoming the availability of fresh lung tissues.

While lung microenvironment studies are still rare, the rapid emergence of methods for high throughput sequencing of single cells, the improved ability to perform sequencing from IPF microenvironments, the improved analytical methods, and the success of old fashioned analyses of bulk tissue should encourage investigators to perform larger studies focusing on understanding temporo-spatial multicellular networks in IPF.

CONCLUSION AND FUTURE DIRECTIONS

The progress of transcriptomics in IPF is characterized by increased sophistication and complexity (**Figure 1**). Transcriptomics studies facilitated multiple shifts with regard to the role of MMPs, developmental pathways, microRNAs, and the importance of alveolar epithelial and myofibroblast regulatory networks in IPF. They have also had significant impact on the discovery and prioritization of validated biomarkers (**Figure 1**). However, most of these studies used low sample number and lack validation cohorts. NIH NHLBI funded efforts led to generation of publicly available datasets of multi-omics data generated from carefully characterized human and mouse samples (**Table 5**). They contain, mainly bulk tissue, but also limited amounts of sorted cells and single-cell transcriptomic profiles. With the advent of novel technologies for single cell and microenvironment transcriptomic profiling, we have a unique opportunity to triangulate IPF regulatory and transcriptional networks by analyzing the lung from a verity of perspectives, use available bulk data, as well as profiles of disease microenvironments and single cells (**Figure 3**). This will allow integration of information and resolution of the cellular, temporal, and spatial complexities of the IPF lungs and thus better therapeutics and diagnostics. In 2014 following a series of meetings sponsored by NIH-NHLBI,

the Pulmonary Fibrosis Foundation and the American Thoracic Society Assembly of Respiratory, Cell and Molecular Biology convened a series of meetings that recommended among other things, an open access biorepository for IPF research (126). While various registries have been formed, new centralized efforts to obtain IPF lung tissues have not been renewed. This is a problem, because most current tissue collections are not amenable to analysis using the novel technologies. To take advantage of the new opportunities, to continue the momentum of transcriptomic success we need new efforts of sample collections, this time focused on access to all the microenvironments and cells in the IPF lung.

AUTHOR CONTRIBUTIONS

MV and NK substantially contributed to review design; data analysis and interpretation. Both the authors participated in writing and revising the review, approved the final work, and agreed to be accountable for all aspects of the review.

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REVIEW
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Mesenchymal Stem Cells for the Treatment of Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF) is an inexorably progressive lung disease of unknown origin. Prognosis is poor, with limited treatment options available, and the median survival remains just 3–5 years. Despite the use of pirfenidone and nintedanib for the treatment of IPF, curative therapies remain elusive and mortality remains high. Regenerative medicine and the use of cell-based therapies has recently emerged as a potential option for various diseases. Promising results of preclinical studies using mesenchymal stem cells (MSCs) suggest that they may represent a potential therapeutic option for the treatment of chronic lung diseases including IPF. Encouraging results of Phase 1 studies of MSCs various have reduced safety concerns. Nonetheless, there is still a pressing need for exploratory biomarkers and interval end-points in the context of MSCs investigation. This review intends to summarize the current state of knowledge for stem cells in the experimental and clinical setting of IPF, present important safety and efficacy issues, highlight future challenges and address the need for large, multicenter clinical trials coupled with realistic end-points, including biomarkers, to assess treatment efficacy.

Keywords: idiopathic pulmonary fibrosis, mesenchymal stem cells, treatment, safety, efficacy

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INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is a progressive debilitating lung disease of unknown etiology (1–4). The disease is characterized by a combination of histological changes including extracellular matrix (ECM) deposition, phenotypic changes of fibroblasts and alveolar epithelial cells, formation of fibroblastic foci, and scattered areas of aberrant wound healing interspersed with normal lung parenchyma (1, 5–14).

Current evidence suggests that the areas of fibrosis seen in lungs of patients with IPF share many features with normal aging lung, such as genomic instability, telomere attrition, mitochondrial dysfunction, cellular senescence, and immune dysregulation (10, 15, 16). Due to the inefficacy of immunomodulatory and immunosuppressive agents in the past, the role of the immune system in the pathogenesis of IPF remains poorly understood (17–22). However, highly activated and proliferative CD4+ cells and functional impairment of T regulatory cells (Tregs) in patients with IPF, suggest a link between immunity and pulmonary fibrosis (10, 23, 24).

There are two approved compounds for the treatment of IPF: pirfenidone and nintedanib**.** Pirfenidone is an antifibrotic compound with an unclear mechanism of action targeting several molecules including transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin 6 (25). Nintedanib is a tyrosine-kinase inhibitor, targeting vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and platelet derived growth factor receptor (PDGFR) (22).While the use of pirfenidone and nintedanib have been shown to slow the progression of IPF (26–28), both compounds have significant side effects and neither is curative (28–30). Morbidity and mortality from IPF remains high and thus there is a pressing need for alternative therapeutic options for this complex disease (7, 31–33). The US National Institutes of Health database lists 493 complete or ongoing clinical trials of MSCs (34). Toward this end, regeneration and cell therapies such as the use of mesenchymal stem cells (MSCs) have emerged as a potential option.

MSCs are multipotent cells able to differentiate into a number of different cell lines and exert immunomodulatory, anti-proliferative, and anti-inflammatory effects. Their multipotency, migratory ability, and immunoprivileged state has led to extensive research efforts for therapeutic applications in several diseases including cardiac ischemia (35–39), ischemic acute renal failure (37), sepsis (40), autoimmune disorders (41), severe graft-vs. -host disease (42), pancreatic islet and renal glomerular repair in diabetes (43), fulminant hepatic failure (44), chronic lung diseases (45–48), and acute lung injury (49–53) (**Table 2**).

MSCs are easily harvested from many tissues (peripheral blood, adipose tissue, bone marrow, and umbilical cord) and may be expanded *in vitro* with minimal modifications. MSCs represent the most extensively studied stem cell population (54). Research supports the immunomodulatory, anti-inflammatory, and potentially anti-fibrotic properties of MSCs (49, 55, 56). Importantly, MSCs are "immune privileged," lacking expression of class II major histocompatibility complex (MHC-II). Therefore, allogeneic use of MSCs is possible (57).

PRECLINICAL STUDIES

Recent studies on the pathophysiology of IPF suggest that early alveolar injury activates abnormal alveolar epithelial cells and stimulates the release of mediators including matrix metalloproteinases and TGF beta-1 (2, 58–61). These mediators activate cytokines and chemokines including IL-1 and IL-13 leading to the phenotype of abnormal wound healing (62–65). Therefore, IPF is considered a complex and multifactorial disease characterized by alveolar epithelial injury and alveolar collapse, fewer alveolar epithelial type II cells, alveolar stem cell exhaustion, and myofibroblast deregulation due to living on a fibrotic matrix (66, 67).

Several experimental studies have been conducted in order to investigate the effect of MSCs from various organs, mainly from bone marrow with a dosage ranging between 0.1×10^6 and 4×10^6 cells, in pathways associated with lung injury and pulmonary fibrosis and several end-points had been set (68, 69) (**Table 1**). The majority of studies recorded substantial improvement in histopathology (56, 64, 70–82), decrease to Ashcroft score (70–72, 77, 79, 80, 83) and lung collagen content (56, 70–81), reduced pulmonary transforming growth factor-b (TGFb) levels (56, 70–81, 84) and decreased BAL neutrophil count (76, 77, 80, 81, 85) following to MSCs administration. Importantly, both bone marrow and amnion-derived MSCs reduced TGFb levels (84). However, to this end, data are still conflicting regarding levels of tumor necrosis factor-a (TNF-a) (56, 71, 83–85), interleukins IL-1, IL-6 (71, 77, 81, 84, 86) and metalloproteinases MMP-2, MMP-9, MMP-13 (56, 71, 83, 84) following administration of MSCs (4, 87–89).

The majority of studies investigating the effect of donor MSCs on BLM-induced pulmonary fibrosis have used young male mouse models (90, 91). Young mice, however, undergo spontaneous resolution of BLM-induced pulmonary fibrosis in some studies (69, 90, 91). Although IPF is primarily a disease of individuals over the age of 50, most studies have also utilized young female mice to evaluate the molecular patterns and potential therapeutic targets for patients with IPF (92–94). Interestingly, in one study of bleomycin induced fibrosis in mice, MSCs were found to improve survival when compared with pirfenidone (64). This study also reported downregulation of IL-2, IL-1b, TNF-α, and TGFβ leading to a reduction in inflammation (64). In addition, downregulation of MMPs was noted with a reduction in collagen deposition and fibrosis (64).

Collectively, MSCs seem to exert pleiotropic effects in the site of lung injury including anti-inflammatory, immunomodulatory, antifibrotic effects (65), engagement in paracrine signaling (95), activation of resident stem cells, and differentiation into local cell types (56, 65, 74, 77, 79, 83, 85, 96–98). Preclinical studies have shown MSCs to be efficacious in the treatment and prevention of lung fibrosis (65, 69). Nonetheless, concerns remain regarding the activity of MSCs within a pro-fibrotic microenvironment (99–102). While some preclinical studies suggest that MSCs might promote fibrosis, to date, no human studies have found a similar pro-fibrotic effect (37, 42, 91, 100, 101, 103–112).

TABLE 1 | Main results of preclinical studies of mesenchymal stem cell therapy in experimental pulmonary fibrosis based on end-points set.

End-point	Outcome	Studies
Histopathology	Significant improvement	(56, 64, 70–82)
Ashcroft score	Decrease	(70–72, 77, 79, 80, 83)
Lung collagen content	Decrease	(56, 70–81)
TGF-b	Decrease	(56, 70–81, 84)
BAL neutrophil count	Decrease	(76, 77, 80, 81, 85)
TNF-a	Conflicting	(56, 71, 83–85)
IL-1, IL-6	Conflicting	(71, 77, 81, 84, 86)
MMP-2, MMP-9, MMP-13	Conflicting	(56, 71, 83, 84)
Survival compared with pirfenidone	Improved	(64)

BAL, bronchoalveolar lavage, IL, interleukin, MMP, metalloproteinases, TGF-b, transforming growth factor-beta, TNF-a, tumor necrosis factor-a.

TABLE 2 | Results of clinical human studies of mesenchymal stem cell therapy.

Study	Disease model	Cell type	Delivery and dose	Safety results	Efficacy results
(42)	Acute graft versus host disease	Allogeneic BM-MSCs; HLA matched and mismatched	IV, 1.4 × 10^6 cells/kg	No adverse effects reported	Complete response in 30 of 55 patients. Partial response in 9 of 55 patients
(37)	Myocardial infarction	Allogeneic BM-MSCs; Non-HLA matched	IV, 0.5, 1.6, or 5.0 × 10^6 cells/kg	No difference in adverse events compared with placebo	Decreased arrhythmic events. Decreased PVCs. Improved post-event ejection fraction. Improved overall clinical status. Improved FEV_1 percent predicted
(41)	Refractory lupus	Allogeneic BM-MSCs; Non-HLA matched family members	IV, 1 × 10^6 cells/kg	No adverse events reported	Improved SLE disease activity index score. Improved 24-h proteinuria
(112)	Ischemic cardiomyopathy	Allogeneic vs. autologous BM-MSCs; Non-HLA matched (allogeneic)	Endocardial, 20, 100, or 200 × 10^6 cells	One patient in each arm hospitalized for heart failure. No statistically significant difference in adverse events between arms	Improvement in 6MWT and QOL index with autologous MSCs. CT evidence of reverse LV remodeling in both arms. Improved LV and diastolic volumes with allogeneic MSCs
(110)	COPD	Allogeneic BM-MSCs; Non-HLA matched	IV, 100 × 10^6 cells/ infusion Four monthly infusions	No difference in adverse events compared with placebo	No effect seen on frequency of COPD exacerbation or PFTs. Decreased circulating C-reactive protein in patients with high baseline levels
(103)	IPF	ADSCs-SVF	Endobronchial, 0.5 × 10^6 cells/kg of body weight in 10cc; 3 dosages over 3 months	No difference in adverse events compared with placebo. No ectopic tissue formation	Cell-treated patients did not deteriorate in both functional parameters and indicators of quality of life
(109)	IPF	Allogeneic placental MSCs	IV, 1 & 2 × 10^6 cells/kg; one dose	Minor and transient acute adverse events	Stable lung function. No evidence of worsening fibrosis
(53)	ARDS	Allogeneic BM-MSC	IV, 1, 5, or 10 × 10^6 cells/kg; 3 patients per dosage arm	No adverse events Serious adverse events after infusion (3 patients), non-MSC related	None
(105)	IPF	Allogeneic BM-MSC	IV, one dose: 20 × 106 ($n = 3$) 100 × 106 ($n = 3$) and 200 × 106 cells ($n = 3$)	No treatment-emergent serious adverse events. Two non-treatment related deaths due to progression of IPF	(Exploratory results): 3.0% mean decline in % predicted FVC and 5.4% mean decline in % predicted DLCO
(113)	IPF	ADSCs-SVF	Endobronchial, 0.5 × 10^6 cells/kg of body weight in 10cc; 3 dosages over 3 months	No difference in adverse events compared with placebo. No ectopic tissue formation	Median overall progression-free survival 26 months. Median overall survival 32 months. All patients alive for at least 2 years after first administration

ADSCs-SVF, autologous adipose derived stromal cells-stromal vascular fraction; BM-hMSCs, human bone marrow-derived mesenchymal stem cells; IPF, Idiopathic pulmonary fibrosis; PD-MSCs, placenta-derived mesenchymal stem cells.

CLINICAL TRIALS

Early clinical studies of MSCs in patients with IPF have shown promising safety profiles (30, 103, 114). Phase 1 clinical trials have been conducted for safety of MSC therapy. A phase Ib study of endobronchially administered autologous adipose-derived MSCs showed not only acceptable safety outcomes, but also improvements in quality of life parameters (103). The recently published longitudinal outcomes of this study also demonstrated an acceptable safety profile, 100% survival rate 2 years after first administration and a median overall progression-free survival of 26 months (113). Furthermore, studies of intravenously administered placental derived MSCs (105, 109) found that administration of up to 2 × 10^6 cells per kilogram was safe in subjects with moderately severe IPF (109). Importantly, the authors reported only minor and transient alterations in peri-infusion hemodynamics and gas exchange, reducing the concerns for embolization of stem cells to an already compromised pulmonary vasculature. Subjects were followed for six months with no observed decline in forced vital capacity (FVC), diffusing lung capacity for carbon monoxide (DLCO), six-minute walk test (6MWT), or CT fibrosis score (90). The AETHER trial also showed favorable safety outcomes for the intravenous delivery of a single dose of allogeneic MSCs in patients with IPF up to 2 × 10^8 cells (105). Although this was an underpowered study for the detection of significant changes in functional indices, the mean decline in % predicted FVC and DLCO were below the thresholds for disease progression (1, 115). ReCell, an FDA approved phase 1b multidose, randomized, double-blind trial of 10 × 10^6 cells delivered intravenously to patients with IPF, has not yet begun enrollment.

OUTSTANDING CHALLENGES

While it now appears that it is safe to use MSCs in patients with IPF, many questions and challenges remain. In the preclinical realm, there is a need for animal models more representative of chronic IPF (91, 116) for the continued study of how MSCs exert their effects. Bleomycin induced pulmonary fibrosis is still considered the best available animal model for preclinical testing (91, 117). However, there is increasing criticism that potential therapies usually administered the first 7 days following bleomycin exposure may act mainly through prevention of the inflammatory cascade rather than reversal of fibrosis, thus limiting their applicability to human IPF (69). First, improved animal models will enable the identification of biomarkers that may be useful as measures of disease activity and/or treatment effect. Second, the timing of treatment for best effect needs to be better elucidated. Furthermore, the most efficacious source of MSCs and the role of age need to be more fully explored. One report suggesting that adipose-derived MSCs from young, but not old, mice prevent bleomycin induced lung fibrosis in an aged mouse model (118) highlights the need for further research in this area.

Several challenges in the clinical setting also remain to be addressed. The optimal source of MSCs, the best route of administration, the number and timing of administrations, and the appropriate dosing interval. Thus, allogeneic human bone marrow-derived and autologous adipose derived MSCs have been the most studied in the context of IPF. There are limited studies on endogenous stem cells from the lungs of patients with IPF and concerns remain surrounding the risk of biopsy and the potential for intervention-induced IPF exacerbation and the possibility of detrimental effects on lung function from biopsy. Lung tissue obtained at the time of lung transplant remains the best tool for study.

It is also critical to characterize appropriate endpoints to assess treatment effectiveness in these patients (100, 119–129). Molecular biomarkers would be the optimal choice for the assessment of cell based therapies. Finally, well-designed and meticulously conducted multicenter randomized clinical trials of MSCs for the treatment of IPF are needed to assess efficacy.

MOVING FORWARD

While preclinical trials suggest that MSCs may be effective in the treatment of IPF, and early clinical trials suggest that they are likely to be safe in the population, insufficient data exists at this time to definitely state that the use of MSCs for the treatment of IPF is either safe or efficacious. Despite this lack of evidence, cell based therapies are being aggressively marketed to this vulnerable patient population. A recent study found that as of August of 2016, there were at least 351 stem cell related businesses registered in the United States. These sites offer unproven, experimental treatments for a wide variety of conditions (130, 131). In the case of IPF, desperate patients and their physicians continue to succumb to an onslaught of marketing and branding of as yet unproven "stem cell" treatments. Unfortunately, these businesses are also almost wholly unregulated (132). Publication of case reports of harm arising from the misuse of unproven treatments support increased government oversight in the interest of patient safety.

CONCLUSION

Idiopathic Pulmonary Fibrosis (IPF) is a debilitating lung disease characterized by a progressive decline in lung function ultimately resulting in death. The lack of curative treatments for this disease has created an urgency for other potential therapeutic options. Preclinical studies suggest that because MSCs have immunomodulatory, anti-inflammatory, and potentially anti-fibrotic properties, they may be efficacious in the treatment of IPF. Early clinical trials have shown that MSCs may be safely administered to patients with IPF, but large multicenter randomized trials still need to be performed.

AUTHOR CONTRIBUTIONS

AT, RT and TK wrote the initial manuscript. The manuscript was supervised and significantly modified by MG, AT, DB. KM, IN, VA offered significant intellectual contribution. All authors approved the final form of the manuscript.

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REVIEW
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Autotaxin in Pathophysiology and Pulmonary Fibrosis

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Lysophospholipid signaling is emerging as a druggable regulator of pathophysiological responses, and especially fibrosis, exemplified by the relative ongoing clinical trials in idiopathic pulmonary fibrosis (IPF) patients. In this review, we focus on ectonucleotide pyrophosphatase-phosphodiesterase 2 (ENPP2), or as more widely known Autotaxin (ATX), a secreted lysophospholipase D (lysoPLD) largely responsible for extracellular lysophosphatidic acid (LPA) production. In turn, LPA is a bioactive phospholipid autacoid, forming locally upon increased ATX levels and acting also locally through its receptors, likely guided by ATX's structural conformation and cell surface associations. Increased ATX activity levels have been detected in many inflammatory and fibroproliferative conditions, while genetic and pharmacologic studies have confirmed a pleiotropic participation of ATX/LPA in different processes and disorders. In pulmonary fibrosis, ATX levels rise in the broncheoalveolar fluid (BALF) and stimulate LPA production. LPA engagement of its receptors activate multiple G-protein mediated signal transduction pathways leading to different responses from pulmonary cells including the production of pro-inflammatory signals from stressed epithelial cells, the modulation of endothelial physiology, the activation of TGF signaling and the stimulation of fibroblast accumulation. Genetic or pharmacologic targeting of the ATX/LPA axis attenuated disease development in animal models, thus providing the proof of principle for therapeutic interventions.

Keywords: autotaxin (ATX), lysophosphatidic acid (LPA), lysophosphatidic acid receptor (LPAR), g-proteins, pulmonary fibrosis

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INTRODUCTION

ATX was first identified as an autocrine motility-stimulating factor, isolated from the supernatant of highly metastatic melanoma cells (1). Its cDNA cloning revealed that ATX was homologous to ectonucleotide pyrophosphatase-phosphodiesterase 1 (ENPP1), possessing phosphodiesterase activity *in vitro* (2); ATX was thus classified as ENPP2 in the ENPP (1–7) protein family, being the only secreted and not transmembrane member (3). In addition, several years later it was discovered that ATX is identical to the long elusive plasma lysoPLD (4, 5), and is now considered responsible for the synthesis of the majority of extracellular LPA (**Figure 1**).

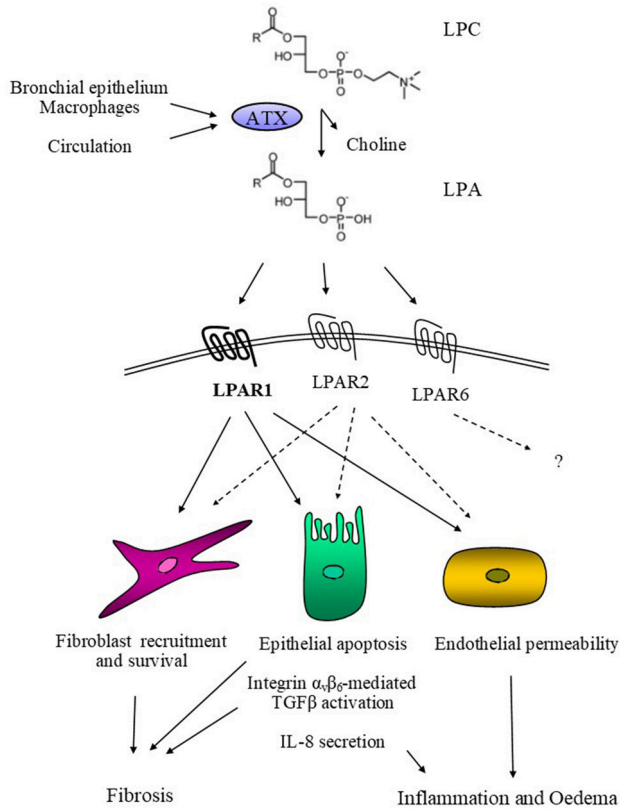

FIGURE 1 | Schematic representation of ATX's mode of action in pulmonary fibrosis. ATX, derived from the bronchial epithelium and alveolar macrophages or extravasated from the circulation, catalyses the hydrolysis of LPC and the local production of LPA. In turn, LPA activates its cognate receptors LPAR1, possibly LPAR2, and hypothetically LPAR6, activating the corresponding G-protein-mediated signal transduction cascades. As a result, LPA induces epithelial apoptosis, the initiating pathogenetic event in modeled pulmonary fibrosis and possibly IPF. LPA also induces IL-8 secretion from epithelial cells, promoting inflammation, while it also stimulates endothelial permeability, thus promoting pulmonary oedema. Moreover, LPA stimulate the $\alpha_v\beta_6$-mediated TGFβ activation leading to the activation and trans-differentiation of pulmonary fibroblasts, for which LPA is additionally a pro-survival and chemotactic factor.

THE *ENPP2*/*Enpp2* GENE; EXPRESSION AND REGULATION

ENPP2 consists of 27 exons and resides in the human chromosomal region 8q24 (6, 7), a region with frequent somatic copy number alterations in cancer patients, containing potential susceptibility loci for various types of cancers (8, 9). The 8q24 locus has been suggested to regulate the expression of the proto-oncogene *MYC,* also residing in the region (10). *In silico* analysis of publicly available genomic data at The Cancer Genome Atlas (11) indicated genetic alterations, mostly amplifications, of *ENPP2* in cancer patients, with the highest rates observed in ovarian (33%), breast (20%), liver (20%), and lung (11%) carcinomas (12). Moreover, a number of single nucleotide polymorphisms (SNPs) that associate with cancer susceptibility have been detected in or around *ENPP2* (9). Promoter regions of *ENPP2* were found hyper-methylated in primary invasive breast carcinomas (13), while inhibition of histone deacetylases 3 and 7 with trichostatin A also attenuated *ENPP2* expression in colon cancer cells (14), suggesting that *ENPP2* expression can be also amenable to epigenetic regulation. In mice, the highly (93%) homologous *Enpp2* gene is located in chromosome 15 and has a similar structure (15, 16).

A variety of cell types and/or tissues have been reported to express *ENPP2*/*Enpp2;* the highest mRNA levels in healthy conditions have been observed in adipose tissue, brain, and spinal cord, testis and ovary, followed by lung, kidney, and pancreas (15, 17–19), suggesting that ATX/LPA may participate in the homeostasis of these tissues. In disease states, increased mRNA expression has been reported in a large variety of cancer types and cell lines, as well as in different cell types in chronic inflammatory disorders (20).

Several transcription factors have been suggested to control *ENPP2*/*Enpp2* transcription in different cell types and pathophysiological states: Hoxa13 and Hoxd13 in mouse embryonic fibroblasts (21), v-jun in chick embryo fibroblasts (22), c-jun in soft tissue sarcomas (23), Stat3 in breast cancer cells (24), AP-1 in keratinocytes and neuroblastoma cells (25, 26), NFAT1 in melanoma and carcinoma cells (27, 28), as well as NF-kB in keratinocytes and hepatocytes (26, 29, 30). *Enpp2* mRNA stability has been reported to be controlled by the RNA-binding Proteins HuR and AUF1 (31), adding an extra level of regulation.

Several extracellular, mainly pro-inflammatory, factors have been suggested to stimulate *ENPP2*/*Enpp2* expression, many through the transcription factors indicated above: TNF in synovial fibroblasts, hepatocytes, hepatoma cell lines, and thyroid cancer cells (32–35), IL-1β in thyroid cancer cells (34), IL-6 in dermal fibroblasts (36), as well as galectin 3 in melanoma cells (27). Different TLR ligands, including LPS, CpG oligonucleotides and poly(I:C), were shown to stimulate *ENPP2* expression in THP-1 monocytic cells, likely involving an IFN autocrine-paracrine loop (37, 38). Lysophatidylcholine (LPC), a major component of cell membranes and oxidized lipoproteins as well as the enzymatic substrate of ATX, is a potent inducer of *Enpp2* expression in hepatocytes (32). On the other hand, the enzymatic product of ATX, LPA, as well as sphingosine 1 phosphate (S1P), have been suggested to create a negative feedback loop on *Enpp2* expression or activity, under certain conditions (34, 39).

ATX ISOFORMS, STRUCTURE, AND ENZYMATIC ACTIVITY

Alternative splicing of *ENPP2*/*Enpp2* exons 12 and 21 leads to five, all catalytically active, protein isoforms, named α to ε (15, 40). Isoform β is the most abundant one, likely accounting for the majority of ATX/LPA reported pathophysiological effects. Isoform δ is also abundant, lacking an exon 19 encoded tetrapeptide of unknown function, also missing in isoform ε. Isoform γ is brain specific, and contains an exon 21 encoded 25 aa insert of unknown function (20). Isoforms α and ε are much less abundant, while they contain a 52 aa polybasic insert, encoded by exon 12, that has been shown to bind to heparin and heparin

sulfate proteoglycans (41). Proteolytic cleavage of a N-terminal hydrophobic sequence that functions as a signal peptide (42, 43) and glycosylation at asparagine residues (42–45), are necessary for secretion and optimal enzymatic activity.

ATX can be found catalytically active in most biological fluids, such as serum/plasma, bronchoalveolar lavage fluid (BALF), blister fluid, cerebrospinal fluid, synovial fluid, peritoneal fluid, and urine (20). The major source of serum ATX is likely the adipose tissue, as conditional genetic deletion of *Enpp2* in adipocytes resulted in a 38% decrease of plasma LPA (17), whereas ubiquitous heterozygous deletion results in a 50% reduction (46–48). Moreover, ATX has been shown to be secreted, in healthy conditions, from bronchial epithelial cells (49) and high endothelial venules (19), as well as choroid plexus epithelium cells (43), activated astrocytes and oligodendrocytes in the brain (50). Intriguingly, ATX has been also detected in exosomes (51), cell derived vesicles that have been suggested to mediate intercellular or cross-tissue signaling.

ATX consists of two N-terminal somatomedin B-like (SMB) domains, a central phosphodiesterase (PDE) domain and a nuclease-like domain (NUC) in its C-terminus (16, 52, 53). The SMB domains, stabilized by four pairs of disulphide bonds, likely mediate ATX binding to integrins, thus localizing LPA production to the cell surface (19, 52, 54–56). The PDE domain, which interacts with both SMB and NUC domains, contains the active catalytic site consisted of a threonine residue (Thr209/210, for mouse and human, respectively) and two zinc ions coordinated by conserved aspartate and histidine residues. It contains a hydrophobic lipid-binding pocket that can accommodate various LPC and LPA species and an open tunnel that could serve as an exit to LPA (53).

LPC, the enzymatic substrate of ATX, is highly abundant in the circulation, predominantly associated with albumin and lipoproteins (57). LPC is synthesized through the hydrolysis of phosphatidylcholine (PC) by phospholipases (PLA2, PLA1) and lecithin cholesterol acyltransferase (LCAT) enzymes (58). ATX has a preference for shorter and unsaturated fatty acid chains, depending on divalent cations such as Co^{2+} or Mn^{2+} (20, 53). Although ATX can also hydrolyze sphingosylphosphorylcholine (SPC, the precursor of S1P) and nucleotides *in vitro*, genetic and pharmacologic studies in mice established that the main enzymatic activity of ATX *in vivo* is LPC hydrolysis and the production of extracellular LPA (20, 53).

LPA, RECEPTORS, AND SIGNALING

LPA consists of a glycerol backbone, a single fatty acyl chain of varying length and saturation, and a free phosphate group as a polar head. It can be found in most biological fluids, mostly following the expression profile of ATX (57, 59). LPA levels in serum are much higher than those in plasma, due to the release of LPC and other phospholipids from activated platelets during coagulation and their hydrolysis by ATX (60, 61). Moreover, the LPA concentration in plasma (∼0.7 μM) is significantly lower than LPC's (∼200 μM), while the predominant LPA species (18:2 > 20:4 > 18:1) are not analogous to the corresponding LPC ones (16:0 > 18:1/18:0 > 20:4); similar observations were made in BALFs (62). This can be likely explained by the slow release of LPA from ATX, due to its high affinity for LPA (39, 63), as well by the rapid turnover of LPA, as shown after the pharmacological inhibition of ATX *in vivo* (64, 65). Although there are other biosynthetic routes for LPA production, any increases in the extracellular LPA content of biological fluids and local sites can be attributed to the lysoPLD activity of ATX (58). On the other hand, a group of membrane-associated lipid-phosphate phosphatases (LPPs) have been suggested as negative regulators of LPA levels, adding an extra layer of regulation of its effects (66, 67).

LPA signals through at least six type I rhodopsin-like receptors (LPARs) that exhibit widespread, but differential, tissue distribution, as well as overlapping specificities (68). The orphan GPR87 and P2Y10 receptors (69, 70), as well as the receptor for advanced glycation end products (RAGE) (71) and the intracellular peroxisome proliferator-activated receptor γ (PPARγ) (72), have also been suggested to mediate LPA signaling. Little is known on LPARs functional conformation and possible associations; LPAR1 has been detected in lipid rafts (73, 74) and suggested to heterodimerize with CD14 (74) and CD97 (75).

LPARs couple with G-proteins, crucial molecular switches activating numerous signal transduction pathways (76). G-protein coupled receptors (GPCRs) is the largest family of cell-surface molecules involved in signal transduction, and their aberrant function has been linked with various human diseases, thus representing almost 50% of current therapeutic targets (77). Many *in vitro* studies, extensively reviewed elsewhere (20, 57, 61), have shown that LPA: stimulates the mitogenic Ras-Raf-MEK-ERK pathway and the PI3K pathway promoting cell survival through $G_{\alpha i}$; induces RhoA-mediated cytoskeletal remodeling, as well as cell migration and invasion through $G_{\alpha 12/13}$ in cooperation with the $G_{\alpha i}$-mediated Rac activation pathway; activates phospholipase C, through $G_{\alpha q}$, with consequent production of second messengers. Of note, most *in vitro* effects of LPA were reported at concentrations much higher than the physiological concentrations, as found in healthy biological fluids, suggesting that they likely concern pathophysiological situations with increased levels of LPA. Overall, any LPA effect in each cell type will depend on its local concentration, regulated by ATX and LPPs, the levels of possible agonists and antagonists and the relative abundance of the different receptor subtypes.

ATX/LPA IN PATHOPHYSIOLOGY

Ubiquitous genetic deletion of ATX and abrogation of LPA production resulted to embryonic lethality in mice due to malformations of the vascular and neural systems (46–48, 78), indicating a major role for ATX in **development**; reviewed in Moolenaar et al. (79). Of note, the embryonic phenotype of ATX knock out mice did not resemble the phenotype of any of the individual LPA receptors knock out mice (68), suggesting that coordinated LPA signaling through various receptors is necessary for the observed ATX effects in embryonic development; non-catalytic effects of ATX in development are also possible especially in the neural system (50, 80). Accordingly, elevated

ATX levels have been detected in human pregnancy, further modulated in pregnancy-related pathophysiological conditions (81–86).

Notwithstanding the necessity for ATX in embryonic life, induced genetic deletion or long-term pharmaceutical targeting of ATX in adult mice was shown to be well tolerated (18), indicating that the majority of ATX/LPA (>80%) is dispensable in **adult healthy life**. The remaining ATX-mediated LPA levels, together possibly with LPA produced via other routes (58) are likely adequate to maintain a healthy tissue homeostasis. Given the importance of ATX in embryonic development but not in adult life, the overexpression of ATX in a pathophysiological condition suggests ATX/LPA as a developmental pathway aberrantly re-expressed in pathophysiological situations.

One of the main features of the embryonic lethal phenotype of ATX knock out mice was the aberrant vascular system, as also seen upon ATX knockdown in zebrafish (87) and in line with the suggested role of LPA in **vascular homeostasis** (88, 89). A similar phenotype was also seen in the embryos of transgenic mice overexpressing ATX (90) and LPP3 knock out mice (91, 92) that sustain much higher levels of LPA than wt mice, suggesting that LPA levels should be tightly regulated during development. Of note, $G_{a13}{}^{-/-}$ embryos display similar impairments in the vasculature as the $Enpp2^{-/-}$ embryos (46, 93), suggesting G_{a13} as the predominant G-protein mediating ATX/LPA effects in the vasculature. In adult life, LPA has been suggested to modulate endothelial cell physiology, through the stimulation of the expression of angiogenesis related genes and the modulation of their permeability (88, 89, 91). Beyond endothelial cells, LPA has a plethora of effects on other cells of the vessel wall, as well as on blood cells including platelets. Moreover, LPA is generated during mild oxidation of LDL, while its levels accumulate in atherosclerotic plaques, suggesting a role for ATX/LPA in **atherosclerosis** (94, 95).

The possible involvement of ATX/LPA in atherosclerosis is further underscored by the fact that the adipose tissue is a major source of systemic ATX, while its effects, through LPA, can classify ATX as an adipokine. Although the effects of ATX/LPA in adiposity are not clear (17, 96, 97), the ATX-LPA pathway has been suggested to participate in **obesity** related **insulin resistance** and the regulation of **glucose homeostasis** (98), with many implications for the pathogenesis of different metabolic disorders. However, the autocrine and/or paracrine effects of ATX/LPA in metabolism and the consequent effects in disease pathogenesis have not yet been fully explored.

ATX was first isolated due to its ability to promote the motility of melanoma cells (1). Accordingly, many xenograft studies have shown that ATX knock down in melanoma cells, as well as pharmacological inhibition of ATX and LPAR antagonism, attenuate the metastasis of melanoma cells in the lungs of mice, well establishing a role for ATX/LPA in **metastasis**; reviewed in Leblanc and Peyruchaud (99). Beyond melanomas, interaction of ATX with integrin $\alpha_v\beta_3$ on tumor cells, has been reported to control the metastasis of breast cancer to the bone [reviewed in (56, 100)].

Transgenic over-expression of *Enpp2*, as well as *Lpar 1*, *2,* or *3*, in the mammary gland resulted in spontaneous breast cancer development (101), indicating a role for the ATX/LPA axis in **breast cancer**. However, spontaneous carcinogenesis was only observed in aged mice, suggesting that ATX/LPA act synergistically with oncogenic age-related signals. Notwithstanding the conflicting reports on ATX levels in breast cancer, the source of ATX in breast cancer was suggested to be the adjacent mammary fat pads, rather than the cancer cells themselves (102), suggesting that ATX can have paracrine effects in cancer development. In the liver, genetic deletion of *Enpp2* from hepatocytes attenuated **hepatocellular carcinoma** (HCC) development, revealing ATX/LPA autocrine effects in hepatocyte metabolism (32, 103). Increased ATX expression has been reported in many other types of cancer, including thyroid and ovarian (20, 104).

Increased ATX levels have been also reported in neuroblastomas and glioblastomas (50) and given the abundant expression of the brain specific isoform ATXγ as well the neuronal defects of the $Enpp2^{-/-}$ mice, a role for ATX/LPA in brain cancer seems likely, but it remains yet unexplored. However, another major role for ATX/LPA was revealed in the brain, as it was shown that PLA2/ATX-dependent LPA/LPAR1 signaling is crucial for the initiation of neuropathic pain (105, 106). Moreover, ATX was shown to modulate oligodendrocyte physiology and differentiation via catalytic and non-catalytic functions (50, 107). In this context, increased ATX and LPA levels have been reported in the sera and cerebrospinal fluid (CSF) of **multiple sclerosis** patients (108–110), while pharmacologic inhibition of ATX attenuated the development of experimental autoimmune encephalomyelitis (111).

Besides multiple sclerosis, ATX/LPA were shown to have a role in the pathogenesis of other chronic inflammatory diseases. Conditional genetic deletion of ATX from synovial fibroblasts or pharmacologic inhibition attenuated the development of inflammatory **arthritis** in animal models (33, 112), suggesting a major role for ATX/LPA in rheumatoid arthritis (113, 114). TNF-induced ATX secretion from synovial fibroblasts was shown to result in increased production of LPA which in turn stimulated, in an autocrine mode, cytoskeletal re-organization, proliferation, and migration of synovial fibroblasts (33), the main effector cells in disease pathogenesis. Moreover, increased ATX staining was noted in lymphoid aggregates, in line with the suggestion that ATX can be an adhesive substrate for homing lymphocytes, facilitating their transmigration across endothelial layers in different modes (19, 115–118). Further to the possible regulation of immune responses by ATX/LPA, LPA was recently shown to convert monocytes to macrophages (119).

Chronic inflammation of the liver, due to cytotoxic, viral or metabolic stimuli, was shown to stimulate ATX secretion from hepatocytes, while LPA was shown to activate hepatic stellate cells and to amplify pro-fibrotic signals (32). Conditional genetic deletion of *Enpp2* from hepatocytes or pharmacological inhibition of ATX, attenuated the development of fibrosis in a cytotoxic model (32). Increased ATX expression has been reported in patients with chronic liver diseases of different etiologies, suggesting ATX as a diagnostic marker of different forms of **liver fibrosis** (32, 120). ATX/LPA have been also

implicated in the fibrosis of other tissues, such as renal fibrosis (121) and skin fibrosis (36, 122).

ATX/LPA IN PULMONARY FIBROSIS

Enpp2 has been suggested, using genome-wide linkage analysis coupled with expression profiling, as a candidate gene controlling lung function, development and remodeling (123). Accordingly, *Enpp2*$^{-/-}$ mice were found to be embryonically lethal (46–48, 78), while *Lpar1*$^{-/-}$ mice were shown to have reduced alveolar septal formation during development (124). In adult life, ATX is constitutively expressed by bronchial epithelial cells, in both humans, and mice, and can be detected in BALFs (49, 125). However, a 50% reduction of systemic ATX levels in the heterozygous *Enpp2*$^{+/-}$ mice or genetic abrogation of bronchial *Enpp2* expression had no major phenotypic effect in the lungs of mice, suggesting that tissue homeostasis in health does not require large amounts of LPA (49, 126). On the other hand, transgenic overexpression of *Enpp2* from the bronchial epithelium or from the liver resulting to 200% increases of ATX systemic levels, had no gross phenotypic effect in the lung either, suggesting that ATX/LPA are not sufficient to induce lung damage *per se* (126).

Subsegmental allergic challenge of asthma patients induced ATX/LPA levels in their BALFs (127, 128), while pharmacologic inhibition of ATX resulted in a marked attenuation of Th2 cytokines and allergic lung inflammation in a triple-allergen mouse asthma model (128); conflicting reports have suggested both pro-inflammatory and anti-inflammatory roles for LPAR2 (128–130). Therefore, a role for ATX/LPA in asthma seems likely and consistent with early reports on LPA effects in the proliferation and contraction of airway smooth muscle cells (131, 132).

Increased ATX staining has been detected in lung tissue samples from IPF and fibrotic non-specific interstitial pneumonia (fNSIP) patients, compared to other interstitial diseases and especially control samples (49). ATX localized mainly within the hyperplastic bronchiolar epithelium, but it was also detected weakly on alveolar epithelium around fibroblastic foci, interstitial macrophages, and fibroblast like cells. On the contrary, ATX was minimally localized within both the inflammatory components of cellular NSIP lung samples and in areas of loose connective tissue, called Masson bodies, representing the pathogenic hallmark of cryptogenic organizing pneumonia. These two latter forms of idiopathic interstitial pneumonias have a propitious prognosis and an excellent treatment response to corticosteroids, indicating that ATX up-regulation is closely associated with more progressive and irreversible forms of pulmonary fibrosis, such as IPF/UIP and fNSIP (49). Of note, as ATX has been suggested to bind to integrins at the surface of platelets and cancer cells (52, 54, 56), it cannot be excluded that ATX can bind to the surface of lung cells via integrins, thus avoiding clearance while exerting locally-produced LPA effects. In turn, the levels of specific LPA species have been found moderately increased in BALFs and exhaled breath condensates collected from IPF patients (133, 134); however, larger studies are needed.

A similar ATX staining profile was observed in the lungs of mice treated with bleomycin (BLM) (49), the most widely used animal model of pulmonary inflammation and fibrosis (135, 136), while increased ATX levels were detected in the corresponding BALFs (49, 62). Conditional genetic deletion of *Enpp2* from bronchial epithelial cells (CC10^{+}) and macrophages (LysM^{+}), the main pulmonary cells expressing ATX, reduced BALF ATX levels and disease severity thus confirming the pulmonary ATX sources as well as establishing a pathogenic role for ATX. However, BALF ATX levels remained relatively high, while the modeled disease was not completely attenuated, suggesting additional, extrapulmonary sources of ATX. ATX levels in BALF correlated with total protein and albumin measurements, pointing to a possible extravasation of ATX from the circulation; paradoxically, no major effects in BLM-induced fibrosis development were noted in genetically modified mice with increased or decreased serum and systemic levels of ATX (49). Nevertheless, systemic pharmacologic inhibition of ATX, both with small molecules and DNA aptamers, decreased LPA levels, and attenuated pulmonary fibrosis (49, 137, 138). It should be noted that ATX inhibition with PAT-048 (Bristol Myers Squibb; WO2012024620) was reported to have no effects in BLM-induced pulmonary fibrosis (62), most likely due to experimental settings and compound characteristics. However, the therapeutic potential of targeting the ATX/LPA axis was recently re-evaluated, where yet another ATX inhibitor was shown to prevent BLM-induced pulmonary fibrosis (139). Many more small molecule ATX inhibitors have been reported (140, 141), however they are still not tested in animal models of pulmonary fibrosis. Intriguingly, the bile salt tauroursodeoxycholate (TUDCA) was recently reported to be a partial non-competitive inhibitor of ATX (142), suggesting that the previously reported therapeutic effects of TUDCA in BLM-induced fibrosis (143), could be due to ATX inhibition.

Moreover, an autocrine pathway linking ATX, LPA signaling and b-catenin was recently reported to contribute to fibrosis progression in lung allografts, one of the primary causes of long-term graft failure after organ transplantation (144). Pharmacologic ATX inhibition or LPAR1 antagonism decreased allograft fibrosis (144), further extending the therapeutic potential of targeting the ATX/LPA axis in lung fibroproliferative disorders.

In agreement with a pathogenic role of ATX/LPA in pulmonary fibrosis, ubiquitous genetic deletion of either *Lpar1* or *Lpar2* also abrogated BLM-induced disease development (133, 145). Pharmacologic antagonism of LPAR1 was shown to be beneficial for the treatment of BLM-treated mice (146), thus stimulating the respective on-going clinical trial (NCT 02068053). Moreover, simultaneous ATX inhibition and LPAR1 antagonism has been reported to have some additive effect in melanoma metastasis (147), warranting further investigation and/or optimization. Beyond LPAR1&2, LPAR6 is the highest expressing LPAR in the lung (not published data), but its possible role in pulmonary pathophysiology and fibrosis has not been explored yet (**Figure 1**).

Reduced numbers of TUNEL$^+$ cells were noted in the alveolar and bronchial epithelium of BLM-treated *Lpar1*$^{-/-}$ and *Lpar2*$^{-/-}$ mice, suggesting that LPA, through LPAR1 and/or 2, promotes epithelial apoptosis (145, 148), the initiating pathogenetic event in this model (135) and, likely, in human patients (149). Interestingly, apoptosing epithelial cells post BLM were shown to express TNF that has a major contribution in the pathogenesis of the modeled disease (150), while TNF has been reported to stimulate ATX expression in other cell types (33, 35). Many other LPA possible effects in pulmonary epithelial cells *in vitro* have been reported and are detailed elsewhere (151), including the induction of IL-8 secretion resulting to neutrophil influx (152, 153).

LPA stimulation of normal human bronchial epithelial cells has been shown to increase stress fiber formation, and to reorganize integrin $\alpha_v\beta_6$ at their ends leading to TGF-β activation (154). Integrin $\alpha_v\beta_6$ has been shown previously to bind and activate TGF-β, a mechanism suggested to regulate pulmonary inflammation and fibrosis (155). TGF-β is the prototype pro-fibrotic factor with a well-documented involvement in the pathogenesis of both the human and the modeled disease, with effects on alveolar epithelial cell injury, myofibroblast differentiation, epithelial-to-mesenchymal transition, and ECM deposition and remodeling (156). TGF-β is produced by different cell types, including alveolar macrophages, while LPA was shown to induce TGF-β expression in pulmonary fibroblasts *in vitro* (145). Therefore, TGF-β activation and possibly expression is another important mechanism through which LPA promotes pulmonary fibrosis.

BALF isolated from BLM-treated mice stimulates the chemotaxis of pulmonary fibroblasts, which was found attenuated by more than 50% in the absence of *Lpar1* expression, indicating that LPA is a major fibroblast chemoattractant (133). The structural organization of LPAR2 has been suggested to govern gradient sensing and the directional migration of fibroblasts in response to LPA (157), while LPA-induced mTORC2-mediated PKC-δ phosphorylation was shown to be critically important for fibroblast migration and pulmonary fibrosis development (158). LPA has been reported to promote, through GPCR-mediated pathways, the cytoskeletal reorganization and proliferation of lung fibroblasts (151), mediated likely from LPAR2 (145) but not from LPAR1 (133). Moreover, LPA signaling, specifically through LPAR1, has been found to suppress, under certain conditions, the apoptosis of primary mouse lung fibroblasts induced by serum deprivation (148). Similar anti-apoptotic effects of LPA have been reported in many cell lines (151), further supporting a role for ATX/LPA in mediating pathologic fibroblast accumulation, the main pathogenetic event in IPF.

Calcium second messenger signals are essential for many critical cellular functions (159). In fibroblasts, calcium homeostasis and ionic mechanisms have been proposed to orchestrate many of their functions, including proliferation, secretion of extracellular matrix components, as well as TGF-β production and differentiation to myofibroblasts (160). In this context, transient receptor potential vanilloid 4 (TRPV4) Ca^{2+} channels have been shown to get activated in response to matrix stiffness, as found in fibrotic lungs (161), and to mediate fibroblast activation and differentiation (162). Interestingly, LPA is well known to stimulate Ca^{2+} influx and/or mobilization in many cells (163), while it was recently shown to directly activate a TRPV1 ion channel (164). Although, the activation was intracellular (164), transbilayer LPA movement has been suggested before in the activation of the nuclear PPARγ receptor (72). Therefore, LPA-induced alterations in calcium homeostasis can have dominant effects in the physiology of fibroblasts, as well as many other cell types.

One of the hallmarks of the observed protection from BLM-induced fibrosis in *Lpar1*$^{-/-}$ mice was the attenuation of BLM-induced vascular leak, indicating a major role of LPA in promoting endothelial permeability upon damage (133). Accordingly, transgenic overexpression of ATX from the liver resulting to elevated circulating LPA levels induced a bleeding diathesis (55). However, the effects of LPA on endothelial permeability remain controversial, while different LPA receptors have been proposed to mediate different effects on endothelial physiology (23, 151, 164). Endothelial dysfunction mainly characterizes the development of atherosclerosis and cardiovascular diseases, however, interstitial lung diseases have all been reported to have a lung vascular disease component (165).

Therefore, ATX-mediated LPA production promotes pleiotropic effects in pulmonary cells stimulating the development of pulmonary fibrosis (**Figure 1**). Accordingly, ATX inhibition was shown to attenuate BLM-induced pulmonary fibrosis (49, 137, 138), thus providing the proof of principle for therapeutic interventions and stimulating the on-going clinical trial. In a phase 1 study, GLPG1690, a potent and orally bioavailable ATX inhibitor exhibiting a good PK/PD profile (137), was shown to be safe and well tolerated (166), as previously shown with another compound and genetic interventions in mice (18). An exploratory phase 2a study in IPF patients (FLORA; NCT 02738801) was just completed with promising results (expected to be published soon), leading to phase IIb, currently recruiting.

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IN and CM drafted the paper. VA edited it.

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REVIEW
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The Management of Patients With Idiopathic Pulmonary Fibrosis

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Idiopathic pulmonary fibrosis (IPF), the most common form of fibrosing idiopathic interstitial pneumonia, is an inexorably progressive disease with a 5-year survival of ∼20%. In the last decade, our understanding of disease pathobiology has increased significantly and this has inevitably impacted on the approach to treatment. Indeed, the paradigm shift from a chronic inflammatory disorder to a primarily fibrotic one coupled with a more precise disease definition and redefined diagnostic criteria have resulted in a massive increase in the number of clinical trials evaluating novel candidate drugs. Most of these trials, however, have been negative, probably because of the multitude and redundancy of cell types, growth factors and profibrotic pathways involved in disease pathogenesis. As a consequence, until recently IPF has lacked effective therapies. Finally, in 2014, two large phase 3 clinical trials have provided robust evidence that pirfenidone, a compound with anti-fibrotic, anti-oxidant and anti-inflammatory properties, and nintedanib, a tyrosine kinase inhibitor with selectivity for vascular endothelial growth factor, platelet-derived growth factor and fibroblast growth factor receptors are able to slow down functional decline and disease progression with an acceptable safety profile. While this is a major achievement, neither pirfenidone nor nintedanib cures IPF and most patients continue to experience disease progression and/or exacerbation despite treatment. Therefore, in recent years increasingly more attention has been paid to preservation of quality of life and, in the advanced phase of the disease, palliation of symptoms. Lung transplantation, the only curative treatment, remains a viable option for only a minority of highly selected patients. The unmet medical need in IPF remains high, and more efficacious and better tolerated drugs are urgently needed. However, a truly effective therapeutic approach should also address quality of life and highly prevalent concomitant conditions and complications of IPF.

Keywords: idiopathic pulmonary fibrosis, pharmacologic treatment, pirfenidone, nintedanib, non-pharmacological treatment, therapy

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INTRODUCTION

The approach to treatment of idiopathic pulmonary fibrosis (IPF) has changed dramatically in the last decade. A number of factors have contributed to this, including improved, though still incomplete, knowledge of disease pathobiology, refined disease definition and diagnostic criteria, and advances in clinical trial design and conductance (1–3). Historically, corticosteroids and immunosuppressive agents have represented the standard of care for patients with IPF

based on the prevailing hypothesis that chronic inflammation may precede and progresses to pulmonary fibrosis. However, the IPFnet-sponsored PANTHER-IPF (Evaluating the Effectiveness of Prednisone, Azathioprine, and N-acetylcysteine in Patients With IPF) trial was terminated prematurely following an interim safety analysis revealing that combination (triple) therapy of prednisone, azathioprine and N-acetylcysteine was associated with increased rates of all-cause mortality, hospitalization and serious adverse events compared to placebo (4). Accordingly, this therapy no longer represents a therapeutic option in patients with IPF (5).

Current paradigm of disease pathogenesis involves recurrent alveolar epithelial cell injury followed by an aberrant wound healing response characterized by uncontrolled migration and proliferation of lung fibroblasts and differentiation of fibroblasts to myofibroblasts resulting in excessive collagen deposition, scarring of the lung parenchyma and irreversible loss of function (6, 7). Accordingly, recent clinical trials have evaluated the efficacy of compounds targeting the wound healing cascade and fibrogenesis, but, overall, with disappointing results, probably because of the multitude of mediators, growth factors and signaling pathways involved in the fibrotic process (8). More recently, two compounds pleiotropic in their mechanisms of action—pirfenidone and nintedanib—have been approved for the treatment of IPF based on their ability to slow down the pace of functional decline and disease progression in phase 3 clinical trials (9, 10). Management of physical debility and palliation of symptoms are similarly important, while lung transplantation represents a realistic therapeutic option only in a small fraction of highly selected patients.

In this article, we summarize and discuss the most recent literature on pharmacological and non-pharmacological treatment of this dreadful disease.

THE ATS/ERS/JRS/ALAT GUIDELINE DOCUMENT ON TREATMENT OF IPF

Originally published in 2011 (11), these evidence-based guidelines have been updated in 2015 to incorporate the most relevant data reported since publication of the previous document (5). For each treatment regimen, a multidisciplinary expert committee graded the certainty (e.g., the *confidence*) in effect estimate as *high*, *moderate*, *low*, or *very low* according to the GRADE (Grading of Recommendations Assessment, Development and Evaluation) methodology (12) and made a recommendation either "strong" or "conditional" *for* or *against* a given intervention. The recommendations were based, among others, on the strength of evidence, outcomes studies, and associated importance to patients, desirable and undesirable consequences of treatment, costs, feasibility of treatment, and acceptability of treatment to stakeholders. Current recommendations for treatment of IPF are summarized in **Table 1**.

Three therapeutic interventions received a *conditional* (e.g., weak) recommendation for use (e.g., pirfenidone, nintedanib, and antacid medication), and they are discussed below.

TABLE 1 | Key recommendations on pharmacological treatment of IPF according to current guideline.

	2015 Guideline	2011 Guideline
THERAPEUTIC AGENT		
Pirfenidone	Conditional recommendation for use*	Weak recommendation against use
Nintedanib	Conditional recommendation for use	Not addressed
Antiacid therapy	Conditional recommendation for use	Weak recommendation for use
Phosphodiesterase-5 inhibitor (sildenafil)	Conditional recommendation against use	Not addressed
Dual endothelin receptor antagonists (bosentan, macitentan)	Conditional recommendation against use	Strong recommendation against use
N-acetylcysteine (NAC)	Conditional recommendation against use	Weak recommendation against use
Azathioprine + corticosteroids + NAC	Strong recommendation against use	Weak recommendation against use
Warfarin	Strong recommendation against use	Weak recommendation against use
Imatinib	Strong recommendation against use	Not addressed
Selective endothelin receptor antagonist (ambrisentan)	Strong recommendation against use	Not addressed

**Conditional recommendations are synonymous with weak recommendations*

PIRFENIDONE

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) is an orally available, synthetic compound that exerts anti-fibrotic, anti-inflammatory, and anti-oxidant activities (13). While its exact mechanism of action remains to be elucidated, pirfenidone's biological effects are believed to occur mainly through suppression of tumor necrosis factor (TNF)-α, an early mediator of inflammation (14), and mediators in the transforming growth factor (TGF)-β pathway, such as the cytoplasmic Smad proteins (15), resulting in inhibition of fibroblast proliferation and differentiation to myofibroblasts, and decreased collagen production (16).

Four phase 3 randomized controlled trials have assessed the efficacy of pirfenidone in patients with IPF. In a Japanese study led by Taniguchi [Shionogi Phase 3 (SP3)], 275 patients were randomized in a 2:1:2 ratio to high-dose pirfenidone (1,800 mg/day), low-dose pirfenidone (1,200 mg/day), or placebo (17). As compared to placebo, both high-dose and low-dose pirfenidone reduced significantly the rate of decline in vital capacity (VC) (−0.16 vs. −0.09 L and −0.08 L; $p = 0.042$ and $p = 0.039$, respectively). Additional significant differences in favor of pirfenidone were observed in progression-free survival (PFS) (defined as decline in VC of >10% from baseline or death) and change in total lung capacity (TLC). Limitations of the study, however, included the change of the primary endpoint before unblinding and the handling of missing data (e.g., last

observation carried forward, which may inflate the type 1 error rate). At the time of this trial, pirfenidone had already been approved for treatment of IPF in Japan based on a secondary endpoint analysis of a previous study [Shionogi Phase 2 (SP2)] showing a significantly reduced rate of acute exacerbations (AE) of IPF (AE-IPF) in patients randomized to pirfenidone (18).

The CAPACITY (Clinical Studies Assessing Pirfenidone in IPF: Research on Efficacy and Safety Outcomes) program consisted of two nearly identical trials (PIPF-004 and PIPF-006) that evaluated the efficacy of pirfenidone in IPF patients with mild to moderate functional impairment [predicted forced vital capacity (FVC) ≥50%, predicted carbon monoxide diffusing capacity (DL_{CO}) ≥35%, either predicted FVC or predicted DL_{CO} ≤90%, and 6-minute walk test (6MWT) distance ≥150 m] (19). Study 004 enrolled 435 patients who were randomized in a 2:1:2 dosing ratio to pirfenidone 2,403 mg/day ($n = 174$), pirfenidone 1,197 mg/day ($n = 87$), or placebo ($n = 174$), whereas study 006 had only two arms (e.g., pirfenidone 2,403 mg/day, $n = 173$ and placebo, $n = 171$). The change in percentage predicted FVC from baseline to week 72 was the primary outcome in both trials. In study PIPF-004, mean FVC change at week 72 was −8.0% in the pirfenidone 2,403 mg/day arm and −12.4% in the placebo arm ($p = 0.001$). In addition, 35/174 (20%) patients in the pirfenidone 2,403 mg/day group vs. 60/174 (35%) in the placebo group had a decline in FVC of at least 10% ($p = 0.001$). In the pirfenidone low-dose group, change in FVC was intermediate to that of the pirfenidone 2,403 mg/day and placebo groups. Conversely, in study PIPF-006, the FVC change at week 72 did not differ significantly between the two groups (−9.0% in the pirfenidone group vs. −9.6% in the placebo group; $p = 0.51$). Based on these data, pirfenidone was approved by the European Medicines Agency (EMA), whereas the U.S. Food and Drug Administration (FDA) requested an additional phase 3 study to confirm drug efficacy before pirfenidone could be approved. The ASCEND (Assessment of Pirfenidone to Confirm Efficacy and Safety in IPF) trial enrolled 555 patients with IPF who were randomly assigned to either pirfenidone 2,403 mg/day ($n = 278$) or placebo ($n = 277$) (9). The primary outcome was the change in percentage of predicted FVC or death from baseline to week 52. Notably, in order to enrol patients at higher risk for disease progression, thus maximizing the likelihood of detecting a treatment effect, patients suspected to have airflow limitation [ratio of the forced expiratory volume in one second (FEV1) to FVC <0.80] were excluded while the minimum DL_{CO} for enrolment was reduced from 35 to 30% of the predicted value. Pirfenidone treatment, as compared with placebo, was associated with a relative reduction of 47.9% in the proportion of patients who had an absolute decline of ≥10% in percentage predicted FVC or who died (46/278 [16.5%] vs. 88/277 [31.8%]; $p < 0.001$), and with a relative increase of 132.5% in the proportion of patients whose FVC remained stable (63/278 [22.7%] vs. 27/277 [9.7%]; $p < 0.001$). A series of sensitivity analyses corroborated the robustness of these findings and the magnitude of pirfenidone effect (20). Pirfenidone treatment was also associated with a reduced decline in the 6-minute walk distance (6MWD) ($p = 0.04$) and improved progression-free survival (defined as the time to a decrease of ≥10% in the percentage of the predicted FVC, a decrease of ≥50 m in the 6MWD, or death, whichever occurred first; $p < 0.001$). Conversely, pirfenidone was not superior to placebo with regard to dyspnea scores ($p = 0.16$), or all-cause (4.0 vs. 7.2%; $p = 0.10$) or IPF-related mortality (1.1 vs. 2.5%; $p = 0.23$). However, a pre-specified pooled analysis of the ASCEND and CAPACITY trials revealed that pirfenidone was associated with a significant reduction of both all-cause [3.5 vs. 6.7%; hazard ratio (HR): 0.52; $p = 0.01$] and IPF-related mortality (1.1 vs. 3.5%; HR: 0.32; $p = 0.006$) at week 52 compared with placebo (9). Pooled analyses of ASCEND and CAPACITY trials and meta-analyses, which included also data from the SP2 and SP3 Japanese trials, confirmed that pirfenidone treatment reduced significant the risk of mortality compared with placebo over 120 weeks (21). In addition, pooled analysis of the phase 3 clinical trials ASCEND and CAPACITY showed that pirfenidone beneficial effect extends to non-elective respiratory-related hospitalization, which is reduced by ∼50% compared to placebo (7 vs. 12%, HR 0.52, p-value = 0.001) (22), and is consistent across a broad range of patient subsets (e.g., U.S. vs. non-U.S. patients, gender, age, race, various measures and degrees of lung function impairment, use of supplemental oxygen, smoking status, or time since diagnosis) (23, 24). Several recent publications, including "real world" experiences, have confirmed the long-term efficacy and safety profiles of pirfenidone in patients with IPF (25–27). These studies exemplified the difference between efficacy and effectiveness of pirfenidone use in patient with IPF, as pirfenidone was the first drug for IPF to show longitudinal effectiveness within the real-life clinical setting and not only within the "controlled" environment of a clinical trial (28).

Data on safety and efficacy of pirfenidone in patients with severe functional impairment (i.e., FVC% predicted <50% and/or DL_{CO} <35%) are limited. In a recent retrospective study of such patients ($n = 43$), pirfenidone was associated with a trend toward a reduced functional decline compared to the 6-month period preceding treatment initiation, but did not show any benefit after 1 year of treatment (29). At present, there are insufficient data to justify the use of pirfenidone in patients with severe functional impairment.

Common side effects of the drug include gastrointestinal intolerance (e.g., nausea, dyspepsia, vomiting, abdominal discomfort, diarrhea) and skin reactions (photosensitivity, rash), which in most cases are mild to moderate in severity, reversible, and without clinically significant sequelae. Gastrointestinal side effects can be prevented/mitigated by taking pirfenidone during a meal, following a gradual initial dosing titration, and taking prokinetic agents and/or proton-pump inhibitors, whereas avoiding direct sun exposure, applying a broad-spectrum sunscreen with high ultraviolet (UV) A and UVB protection, and wearing protective clothing generally reduces the risk of photosensitivity skin reactions (30). Rare cases of eosinophilic pneumonia have also been described (www.pneumotox.com). Pirfenidone has been granted approval for treatment of IPF by the FDA in October 2014.

NINTEDANIB

Nintedanib, previously known by its development code BIBF 1120, is an intracellular inhibitor of the tyrosine kinases vascular endothelial growth factor receptor (VEGFR) 1-3, fibroblast growth factor receptor (FGFR) 1-3, and platelet-derived growth factor receptor (PDGFR) a and b (31). By inhibiting VEGFR, FGFR, and PDGFR, nintedanib interferes with a number of processes that have been implicated in the pathogenesis of IPF, namely proliferation and migration of primary human lung fibroblasts, fibroblast to myofibroblast transformation, and TGF-β-stimulated secretion and deposition of collagen by primary human lung fibroblasts, resulting in an inhibitory effect on extracellular matrix secretion and deposition (32). In patients with IPF, the safety and efficacy of four different doses of BIBF 1120 (e.g., 50 mg once daily [$n = 86$], and 50 mg [$n = 86$], 100 mg [$n = 86$] and 150 mg [$n = 85$] all twice daily) compared with placebo ($n = 85$) were initially evaluated in the TOMORROW (To Improve Pulmonary Fibrosis With BIBF 1120), a phase 2, proof-of-concept study, in which the primary endpoint was the annual rate of decline in FVC (33). In the BIBF 1120 150 mg twice daily group, FVC declined by 0.06 L per year compared with 0.19 L per year in the placebo group, corresponding to a 68.4% reduction in the rate of decline. In addition, BIBF 1120 150 mg twice daily was associated with a lower incidence of AE-IPF (15.7 vs. 2.4 vs. per 100-patient-years; risk ratio: 0.16; $p = 0.02$) and improved quality of life as assessed by the St. George's Respiratory Questionnaire (SGRQ) compared with placebo. The INPULSIS program consisted of two parallel 52-week, phase 3 trials (INPULSIS-1 and INPULSIS-2) designed to confirm the efficacy and safety of nintedanib 150 mg twice daily in patients with IPF (10). One thousand sixty-six patients were randomly assigned in a 3:2 ratio to either nintedanib 150 mg twice daily ($n = 309$ in INPULSIS-1 and $n = 329$ in INPULSIS-2) or placebo ($n = 204$ in INPULSIS-1 and $n = 219$ in INPULSIS-2). Similar to the TOMORROW trial, the primary outcome was the annual rate of decline in FVC. Both studies met the primary endpoint. Specifically, the adjusted annual rate of change in FVC was −114.7 ml in the nintedanib group and −239.9 ml in the placebo group in INPULSIS-1 (between group difference: 125.3 ml; $p < 0.001$) and −113.6 and −207.3 ml in INPULSIS-2 (between group difference: 93.7 ml; $p < 0.001$), respectively. In both trials, the robustness of the results of the primary analysis and the magnitude of treatment effect were confirmed by a series of prespecified sensitivity analyses. In addition, nintedanib treatment, compared with placebo, was associated with a reduced risk of disease progression—defined as absolute decline in percent predicted FVC of ≥10% or death—by 47% in INPULSIS-1 (24.3 vs. 40.7%; HR: 0.53; $p = 0.0001$) and by 33% in INPULSIS-2 (29.8 vs. 42.0%; HR: 0.67; $p = 0.0054$) (34). Furthermore, in both trials, patients receiving nintedanib were more likely to be stable (e.g., to have a decline in the percentage of predicted FVC of ≤5%) at week 52 compared with patients randomized to placebo (52.8 vs. 38.2% in INPULSIS-1, $p = 0.001$; and 53.2 vs. 39.3% in INPULSIS-2, $p = 0.001$) (32). A pooled analysis and a meta-analysis of data from the TOMORROW and INPULSIS trials confirmed the beneficial effect of nintedanib in slowing down disease progression (35).

Time to first investigator-reported AE, one of the two key secondary end points (the other being SGRQ), was significantly delayed with nintedanib vs. placebo in INPULSIS-2 (HR: 0.38, $p = 0.005$) but not in INPULSIS-1 (HR: 1.15, $p = 0.67$). However, a pre-specified sensitivity analysis of pooled data from the INPULSIS trials showed that nintedanib compared to placebo delayed significantly the first adjudicated AE-IPF (either confirmed or suspected) (HR: 0.32, $p = 0.001$) (10). More extensive analysis of the INPULSIS data showed that nintedanib treatment reduces by ∼40% mortality following AE, although this result did not reach statistical significance (36). Nintedanib treatment was associated with a significantly smaller increase in the total SGRQ score (consistent with more preserved quality of life) in INPULSIS-2 (2.80 points vs. 5.48 points in the placebo group; $p = 0.02$) but not in INPULSIS-1 (4.34 points vs. 4.39 points, respectively; $p = 0.97$). In addition, in a pre-specified analysis of pooled data from the INPULSIS trials, the adjusted mean change in the SGRQ total score from baseline to week 52 was similar in the nintedanib and placebo groups. Finally, in a pre-specified pooled analysis of the INPULSIS data, the nintedanib and placebo arms did not differ significantly in terms of death from any cause (5.5 vs. 7.8%, respectively; HR: 0.70; $p = 0.14$) or death from a respiratory cause (3.8 vs. 5.0%, respectively; HR: 0.74; $p = 0.34$).

IPF is a highly heterogeneous disease and patients with varying clinical phenotypes may respond differently to antifibrotic therapies. A number of subgroup analyses however have confirmed the broad therapeutic efficacy of nintedanib in patients with IPF. Using pooled data from the INPULSIS trials, Costabel and colleagues showed that treatment effects, examined against sex, age (<65, ≥65 years), race (White, Asian), smoking status (never, ex/current), baseline FVC % predicted (≤70%, >70%), baseline SGRQ total score (≤40, >40), corticosteroid use (yes, no) and bronchodilator use (yes, no) did not differ significantly for the primary (annual rate of decline in FVC) or key secondary (time to first AE and change from baseline in the SGRQ) end points (37). In a *post-hoc* subgroup analysis of pooled data from the INPULSIS trials ($n = 1{,}061$), Raghu and colleagues demonstrated that the rate of decline in FVC in patients with possible UIP on high-resolution CT (HRCT) [i.e., reticular abnormality and traction bronchiectasis in the absence of features inconsistent with usual interstitial pneumonia (UIP)] and no confirmatory surgical lung biopsy is similar as in patients with a diagnosis of IPF according to current guidelines (i.e., honeycombing on HRCT and/or UIP on surgical lung biopsy) (38). A further *post-hoc* subgroup analysis of pooled data from the INPULSIS trials revealed that patients with IPF and preserved lung volumes (FVC >90% predicted) experience the same rate of functional decline and receive the same benefit from nintedanib as patients with more impaired lung function (FVC <90%), thus supporting the concept of offering early treatment to patients with IPF (39).

The most frequent adverse event associated with nintedanib treatment was diarrhea (∼60% within the first 3 months of treatment), which in most cases was of mild or moderate intensity

and led to premature study discontinuation in 4.5% of patients (vs. none in the placebo group) in INPULSIS-1 and 4.3% of patients (vs. 0.5% in the placebo group) in INPULSIS-2 (10). However, in both trials, the same proportion of patients in the nintedanib and placebo groups experienced serious adverse events. Nintedanib has been approved by the FDA in October 2014 and in Europe in early 2015.

ANTACID THERAPY

Gastroesophageal reflux (GER), both symptomatic and asymptomatic, occurs in a high proportion of patients with IPF, and chronic microaspiration secondary to GER is believed to play a role in the pathogenesis and progression of the disease (40, 41). Accordingly, a number of studies have explored the possibility that antacid therapy (AAT) may be beneficial in terms of slowing disease progression and even improving survival in patients with IPF. In an uncontrolled retrospective study of 204 IPF patients from two major academic medical centers in the U.S., GER medications [either proton pump inhibitors (PPI) or H2 blockers] were associated with reduced radiological fibrosis and improved survival (42). Furthermore, a *post-hoc* analysis of data from patients randomized to placebo in three IPFnet-sponsored clinical trials ($n = 242$, 124 of whom [51%] were taking either PPI or H2 blockers at the time of enrolment) showed that the use of AAT was associated with a smaller decrease in FVC (estimated change over 30-weeks of −0.06 vs. −0.12 L in patients not taking AAT; $p = 0.05$) and fewer AEs (no events vs. 9 events in patients not taking AAT; $p < 0.01$) (43). The 2015 guidelines conditionally recommend the use of GER medications in patients with IPF based on the potential benefit and favorable side effect profile of these drugs (5). However, a more recent *post-hoc* analysis of patients assigned to placebo in three clinical trials of pirfenidone (CAPACITY 004, CAPACITY 006, and ASCEND) ($n = 624$, 291 of whom [47%] received AAT) questioned the efficacy of GER medications in IPF (44). Indeed, in this study AAT did not improve progression-free survival (defined as FVC decrease ≥10%, 6MWD decrease ≥50 m, or death), FVC decline, hospitalization and all-cause and IPF-related mortality. Moreover, use of GER medications was associated with a significantly higher rate of overall infections ($p = 0.02$) and pulmonary infections ($p = 0.02$) in patients with advanced IPF (e.g., FVC <70%). The role of AAT in the treatment of patients with IPF remains highly controversial and needs to be addressed in prospective randomized trials (45). One such study, which is currently ongoing, will test the hypothesis that treatment with laparoscopic antireflux surgery in patients with IPF and abnormal GER (WRAP-IPF; NCT01982968) may slow the decline in FVC over the 48-week study duration by abolishing acid and non-acid reflux, both believed to be pathogenic in IPF (41).

MANAGEMENT OF ACUTE EXACERBATIONS

The term "acute exacerbations" (AE) refers to episodes of acute respiratory deterioration accompanied by the development of new radiologic abnormalities (i.e., ground glass opacities and/or consolidation on a background of reticulation and traction bronchiectasis with or without honeycomb changes) on chest X-ray or HRCT (46). The annual incidence of AE ranges between 4 and 20% and is significantly higher in patients with more severe disease (46). The prognosis following an AE is poor with a median survival of ∼3 months (47). At present, there are no therapies of proven efficacy for AE-IPF so that the treating physician is left with supportive care (i.e., palliation of symptoms and relief of hypoxemia with supplemental oxygen) and unproven interventions. Therefore, searching for (and ruling out) known causes of clinical deterioration, including drug toxicity, is warranted.

Corticosteroids

The 2011 guidelines make a weak recommendation *for* the use of corticosteroids in patients with AE-IPF, although randomized controlled clinical trials are lacking (11). The appropriate dose and duration of therapy remain unclear, but in most series the dose has ranged between prednisone 1 mg/kg per day orally and methylprednisolone 1 g per day intravenously for 3 days followed by a gradual taper, based on clinical response. The role of high-dose corticosteroids in AE-IPF remains highly controversial (48–50).

Antibiotics

The rationale behind the use of broad-spectrum antibiotics to treat AE-IPF is that many patients present with flu-like symptoms and have elevated neutrophil count in bronchoalveolar lavage fluid (51). More recently, in a retrospective single-center study of IPF patients hospitalized for AE ($n = 85$), Kawamura and co-workers showed that early administration of azithromycin 500 mg/day for 5 days is associated with a significantly lower mortality compared with a fluoroquinolone-based regimen (26 vs. 70%; $p < 0.001$) (52). This study however has a number of limitations, including its small sample size, retrospective nature and the choice of fluoroquinolone-treated patients as control group; accordingly, these findings need to be confirmed in prospective studies. Ding and colleagues evaluated the use of procalcitonin (PCT)-guided antibiotic treatment vs. standard clinician-determined antibiotic treatment in patients with AE-IPF (53). PCT guidance reduced significantly the duration of antibiotic use, but the duration of mechanical ventilation and overall mortality were similar in both groups.

Mechanical Ventilation

The role of invasive mechanical ventilation (IMV) and non-invasive ventilation (NIV) in the management of AE-IPF has not been formally studied and remains unclear. The 2011 guideline document makes a weak recommendation *against* the use of MV to treat AE-IPF, thus suggesting this may be a reasonable intervention only in a minority of selected patients (11); however, a recent U.S. nationwide retrospective cohort analysis suggested that mortality rates of IPF patients who received IMV or NIV for acute respiratory failure (51.6 and 30.9%, respectively, in this study) may be lower than previously reported (54). Prospective studies are needed to identify IPF patients more likely to benefit from MV and NIV.

Novel Approaches

Recombinant Human Soluble Thrombomodulin (rhTM)

It is a regulator of intravascular coagulation expressed on the endothelial cell surface (55). A number of studies have consistently shown that rhTM improves 3-month survival in patients with AE-IPF (56–58). However, these results need to be confirmed in the setting of randomized controlled trials.

Hemoperfusion With Polymyxin B Immobilized Fiber

Originally developed to remove gram-negative bacterial endotoxins, polymyxin B direct hemoperfusion (PMX-DHP) may also remove cytokines involved in lung injury (59). A number of retrospective studies, mostly from Japan, have shown that PMX-DHP improves oxygenation and survival in patients with AE-IPF, although most patients received also high-dose systemic corticosteroids (60–62). PMX-DHP is a promising therapeutic approach in patients with AE-IPF, but its safety and efficacy need to be validated in larger prospective clinical trials.

Autoantibody-Targeted Treatment

Recent evidence suggests that immune dysregulation may contribute to IPF progression and that treatments that reduce autoantibodies may be beneficial in a significant minority of patients (63). Donahoe and colleagues treated 11 patients with AE-IPF with plasma exchange and rituximab ± intravenous immunoglobulin (64). Compared to historical controls treated with high-dose corticosteroids, trial subjects had significantly better 1-year survival (1/20, 5% vs. 9/11, 82%). These data suggest considering a trial of autoantibody-targeted therapies in patients with AE-IPF.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the potential of transdifferentiation, clonality, and self-renewal. MSC properties include also immunomodulation, epithelial repair, and secretion of growth factors (65). MSCs have been shown to ameliorate inflammation and mitigate parenchymal remodeling in bleomycin-induced pulmonary fibrosis (66), but the bleomycin model recapitulates only partially the complex pathobiology of IPF (67). Therefore, the application of MSCs in patients with IPF is controversial and under study (68). In a small cohort of patients with IPF ($n = 14$), Tzouvelekis and colleagues have shown that endobronchial infusion of autologous adipose derived stem cells was not associated with serious adverse events (69). Yet, results should be interpreted cautiously before rigid conclusions can be drawn. Significant limitations severely hampering the widespread implementation of stem cell use in IPF relate mainly to our limited knowledge of the fate of these cells within the pro-fibrotic microenvironment given their mesenchymal origin and their potential to differentiate into myofibroblasts, thus causing disease progression. In addition, there are many unanswered questions including the time (early or advanced disease) and optimal route of administration (intravenous or endobronchial), source of mesenchymal stem cells (MSCs) (eg, adipose tissue, bone marrow, or umbilical cord), frequency of infusions as well as the choice of the appropriate primary end-points to show benefit (70). An FDA approved RCT investigating safety and efficacy of a single intravenous administration of allogeneic bone-marrow derived MSCs is currently recruiting patients (NCT02611167) and results are eagerly awaited.

NON-PHARMACOLOGICAL MANAGEMENT

Besides pharmacological treatment and lung transplantation, there is increasing evidence that supportive measures such as pulmonary rehabilitation, adequate nutrition, prevention of infections and timely initiation of palliative care can improve and maintain health status and quality of life of patients with IPF (**Figure 1**). The most recent advances in lung transplantation, rehabilitation, and palliative care are discussed below.

Lung Transplantation

In the last 5 years, IPF has become the most common indication for lung transplantation (71). In the 2011 guideline document, lung transplantation was strongly recommended in IPF, though in highly selected patients (11). According to the Organ Procurement and Transplantation Network (OPTN) report, the proportion of patients transplanted with IPF has increased constantly in recent years and reached 49.6% in 2015. At present, the worldwide frequency of the procedure is ∼4,000 per year with 5-year survival rates ranging from 50 to 60%, whereas 10 years survival is around 30% (72). Recipients aged 65 years or older and those with a *lung allocation score* (LAS) of 60 or higher show the lowest survival (72). Recent analyses performed after implementation of the LAS suggest that lung transplantation in patients older than 70 years of age may

FIGURE 1 | Multidisciplinary approach to the management of patients with IPF.

have outcomes comparable to those of younger patients (73). The trend in transplanting older patients may increase in the future due to greater experience of transplant centers and the raising awareness of IPF patient advocacy groups, which in the IPF Charter on patients' rights raised the point that a*ge restrictions for lung transplantations exclude many healthy, viable patients* (74).

Worldwide, bilateral lung transplants are preferentially performed (about 70% of all procedures) (72), while most patients with interstitial lung disease (ILD) undergo single lung transplant (75). Bilateral lung transplants in patients with IPF appear to be associated with longer survival, although the long-term survival advantage is counterbalanced by longer time on the waiting list and higher risk of mortality (76). There are several factors that influence the selection procedure, such as age, comorbidities, anatomical features, predicted pre-transplant survival, organ availability, and center experience, but further long-term data are needed to draw firm conclusions on this highly debated topic (77). Early referral of IPF patients to transplant is highly recommended due to their poor prognosis, high mortality on the waiting list and the unpredictable disease course. After the LAS implementation and the revision of the selection criteria (78), change over time in lung function (FVC and DL_{CO}) has become the driving factor for early referral to lung transplant and has led to a reduction of the waiting list mortality of 10–20% (72).

Overall, the median survival of patients with ILD after lung transplant is 4.7 years, significantly less than that of patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (5.5 and 8.3 years, respectively) (71, 72). Older age at the time of the transplant, prevalence of age-related comorbidities, and higher prevalence of bronchiolitis obliterans syndrome (BOS) represent the main factors that influence outcome (77). Although the incidence of pulmonary and extra-pulmonary post-transplant complications does not appear to be higher in patients with IPF than in those with other ILDs or lung diseases, there is an increasing risk for IPF patients to develop thromboembolism before and after lung transplantation (79). In addition, GER, which is highly prevalent in patients with IPF (80) and often complicates lung transplantation, is strongly associated with the development of BOS (81) and its presence should be carefully evaluated both pre- and post-transplantation (77). Fundoplication after lung transplantation and antireflux surgery pre- and post-transplantation have shown to preserve lung function and prevent reflux-associated BOS (82). On the other hand, there are no data to suggest that IPF patients are at higher risk to develop restrictive chronic lung allograft dysfunction (CLAD), a condition characterized by concomitant decrease in FVC and FEV_1, and usually accompanied by parenchymal infiltrates (77).

Pulmonary Rehabilitation

The natural history of IPF is characterized by a progressive impairment of exercise capacity and mobility, as a consequence of shortness of breath and exercise-induced hypoxemia, which induce patients to reduce and eventually avoid physical activity (83). In addition, sarcopenia, the age-related loss of muscle mass quality and strength, contributes to inactivity in these patients, which are typically 60–80 years old. In the general population and in chronic respiratory diseases, inactivity is associated with poorer health-related outcomes, including higher mortality risk (84). Exercise training in healthy subjects has been shown to positively affect the physiology of cardiovascular, respiratory, and musculoskeletal systems (85). In ILDs, pulmonary rehabilitation has been shown to alleviate respiratory and psychological symptoms, particularly dyspnea and anxiety, and improve exercise tolerance, 6MWD and quality of life scores (86–88). Possible mechanisms underlying these beneficial effects include chest expansion during deep-breathing exercises and stretching of the thoracic muscles resulting in a more efficient breathing pattern, improved respiratory muscle strength, enhanced pleural elasticity and pulmonary compliance (89).

The majority of studies on pulmonary rehabilitation in IPF combined aerobic activity (walking and/or cycling) with resistance and flexibility exercises for peripheral skeletal muscles (90). In a recent meta-analysis, 9 out of 10 of the exercise training studies examined showed a benefit in 6MWD (range 35–81 meters), peak aerobic capacity, and improvement of dyspnea and quality of life (91). Overall, supervised exercise training programs appear to provide the best results in terms of compliance and maintenance of physical activity, while home-based programs seem to be associated with a lower level of improvement (92). Pulmonary rehabilitation should be considered at any stage of IPF and in patients awaiting lung transplantation, since its timely administration correlates with a clinically significant improvement in physical activity and health-related quality of life (93). While the short-term effects of pulmonary rehabilitation on IPF outcomes are supported by several retrospective and prospective studies, its long-term effects have not been extensively studied and systematic investigation in this regard is needed.

Oxygen Treatment

Prescription of long-term oxygen treatment (LTOT) is a challenging step in the management of IPF not only for the patients but also for their relatives and treating physicians. Patients tend not to accept LTOT as the disease becomes more "visible." In addition, their training with the devices is often inadequate and problematic. Although supplemental oxygen therapy is likely to improve symptoms and overall quality of life in IPF patients, especially those with resting or nocturnal hypoxemia (11), a recent systematic review showed no effects of oxygen therapy on dyspnoea during exercise in ILD, although exercise capacity was increased (94). The *ambulatory oxygen in fibrotic lung disease* (AmbOx) trial is the first randomized control trial investigating the effects of ambulatory oxygen during daily life on health status and breathlessness in patients with ILD (95). In this study, patients with fibrotic lung disease with oxygen saturation (SaO_2) $\geq$94% at rest but $\leq$88% during a 6MWT were treated with ambulatory oxygen for a 2-week period compared to 2 weeks off. Preliminary data show that ambulatory oxygen is associated with significantly improved health status in patients with ILD (96).

TABLE 2 | Most developed drug candidates for idiopathic pulmonary fibrosis.

Compound	Mechanism of action	Study design	Primary outcome/study duration	Developmental phase/status	Clinical trial identifier
Inhaled TD139	Galectin-3 inhibitor	Phase 1: randomized, placebo-controlled, single ascending dose. Phase 2: randomized, placebo-controlled, multiple dose expansion cohort	Safety and tolerability (number of participant with adverse events over 2 weeks)	Phase I/II; completed, awaiting results	NCT02257177
PRM-151	Recombinant human Pentraxin-2 (serum amyloid P). Antifibrotic immunomodulator.	Randomized, placebo-controlled	Change in FVC % predicted from baseline through week 28	Phase II; active, not recruiting	NCT02550873
KD025	ROCK2 inhibitor	Randomized, open-label, active comparator	Change in FVC from baseline through week 24	Phase II; recruiting	NCT02688647
Tipelukast	LT receptor antagonist, PDE 3 and 4 inhibitor, 5-LO inhibitor	Randomized, placebo-controlled	Change in FVC from baseline through week 26	Phase II; recruiting	NCT02503657
PBI-4050	CTGF, α-SMA and collagen I expression inhibitor	Open-label, single-arm	Safety and tolerability (number of participant with abnormal laboratory values and/or adverse events over 9 months)	Phase II; completed, awaiting results	NCT02538536
GLPG1690	Autotaxin inhibitor	Randomized, placebo-controlled	Safety and tolerability over 12 weeks; pharmacokinetics; concentration of lysophosphatidic acid in blood/bronchoalveolar lavage	Phase II; Completed, awaiting results	NCT02738801
CC-90001	JNK inhibitor	Randomized, placebo-controlled	Change in FVC % predicted from baseline through week 24	Phase II; recruiting	NCT03142191
BMS-986020	LPA receptor inhibitor	Randomized, placebo-controlled	Rate of change in FVC at week 26	Phase II; completed, awaiting results	NCT01766817
BG00011 (formerly STX-100)	$\alpha_v\beta_6$ inhibitor	Randomized, placebo-controlled, dose escalation	Safety and tolerability (number of participant experiencing adverse events over 16 weeks)	Phase II; completed, awaiting results	NCT01371305
Pamrevlumab/FG-3019	CTGF inhibitor	Randomized, placebo-controlled	Change in FVC from baseline through week 48	Phase II; active, not recruiting	NCT01890265
Rituximab	CD20 inhibitor	Randomized, placebo-controlled	Change in titers of Autoantibodies to HEp-2 Cells over 9 months	Phase II; completed, awaiting results	NCT01969409
Lebrikizumab	IL-13 inhibitor	Randomized, placebo-controlled and active drug (i.e., pirfenidone) controlled	Rate of decline in FVC % predicted from baseline through week 52	Phase II; completed, awaiting results	NCT01872689
SAR156597	IL-4 and IL-13 inhibitor	Randomized, placebo-controlled	Absolute change in FVC from baseline through week 52	Phase II; completed, awaiting results	NCT02345070

CTGF, connective tissue growth factor; FVC, forced vital capacity; HEp-2, Human epithelial type 2; IL, interleukin; JNK, c-Jun N-Terminal Kinase; 5-LO, 5-lipoxygenase; LPA, lysophosphatidic acid; LT, leukotriene; PDE, phosphodiesterase; ROCK2, Rho associated kinase 2; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β.

Palliation of Symptoms

The aim of palliative care in IPF is to reduce the impact of symptoms on quality of life and minimize stress and psychological consequences, mainly depression and anxiety, which are related to the inexorably progressive nature of the disease. Dyspnea and cough appear early in the disease course, while fatigue, reduced appetite, and weight loss are typically seen in advanced stages of the disease. Dyspnea is strongly associated with reduced quality of life (97, 98) and has been shown to correlate with a worse prognosis (99). The mechanisms behind dyspnea are not fully understood but neuroimaging studies have shown how dyspnea and pain activate common areas in the brain and share a cerebral network (100). In a recent systematic review on the use of opioids for dyspnea in patients with IPF, Kohberg and colleagues observed that only systemic morphine administration improved significantly the

dyspnea score on a visual analog scale without severe side effects (101). The majority of these studies used an individually titrated dose between 10 and 30 mg, which appears to be associated with a beneficial effect on dyspnea. Conversely, nebulized morphine did not show any effect on dyspnea, although this was probably due to the sub-therapeutic dosage (102). The concern of morphine-induced respiratory depression was addressed by almost all studies, but only minor side effects, such as nausea and constipation, were reported (103). However, strict dosage is necessary, and the risk of tolerance should be considered. Only randomized placebo-controlled trials will clarify whether morphine is effective and safe in the treatment of dyspnea in patients with IPF.

Chronic cough is another major issue in the management of IPF, since patients are often refractory to conventional anti-tussive therapy (104). A small non-randomized study with oral corticosteroids showed a reduction of cough reflex in IPF patients (103), somehow supporting the beneficial effect of low-dose steroids observed in clinical practice (102). Thalidomide has also been investigated as a potential treatment of cough in a single-center study, but despite the positive effect on quality of life, only 20% of the subjects completed the study due to adverse events (104). Recently, Birring and colleagues assessed the safety and efficacy of PA101, a novel formulation of sodium cromoglicate delivered via a high-efficiency eFlow nebuliser, in patients with IPF and chronic cough (105). IPF patients and patients with chronic idiopathic cough (CIC) were randomized 1:1 to receive PA101 (40 mg) or placebo three times daily for 2 weeks, followed by a 2-week washout, and then crossed over to the other arm. Compared to placebo, PA101 reduced daytime cough frequency by 31% at day 14 in patients with IPF but not in those with CIC, suggesting that the mechanism of cough in IPF may be disease specific. More recently, pirfenidone has been shown to significantly reduce objective 24-h cough counts and to improve subjective measures of cough, although the study had a short follow-up period and was not placebo-controlled (106).

Access to palliative care is one of the most relevant unmet needs in the management of IPF (74). It is, therefore, strongly recommended to treat respiratory symptoms irrespective of disease severity (11). Early referral of patients to individual counseling, patient support groups and comprehensive rehabilitation programs, which should also include psychological support, can have a positive impact on perception of dyspnea and quality of life, and prepare patients to face the final stages of the disease (107, 108).

Outlook

With improved clinical and basic understanding of IPF, an evidence-based approach to treatment is evolving. However, access to approved therapies remains suboptimal and problematic. Maher and colleagues have recently reported that ∼40% of patients with confirmed IPF across Europe do not receive antifibrotic treatment (109). In particular, there appears to be a tendency to adopt a "wait and watch" approach in patients with mild functional impairment or stable disease. This observation underscores the need for increasing physician awareness of the progressive nature of IPF and the benefit associated with early treatment. In addition, compared to treated patients, a lower proportion of untreated patients had a multidisciplinary team evaluation at diagnosis, highlighting the importance of encouraging and facilitating patient referral to expert centers.

The need for safer and more efficacious treatment options has led to an exponential increase in the number of high-quality clinical trials of pharmacological interventions (**Table 2**). Yet, drug development in IPF poses major challenges, ranging from the lack of animal models that mimic all pathologic changes of IPF to the choice of the appropriate primary outcome on which to judge drug efficacy and the clinical meaningfulness of the observed effects. FVC is acknowledged as the preferred endpoint in IPF clinical trials (110), but how often the FVC should be measured remains unknown. Indeed, while the preferred time interval has been every 3 months, this may result in "missing events" in the case of patients who progress and die within this timeframe without documentation of a significant decline of their FVC (111). Recently, Russell and colleagues have shown that unsupervised daily home spirometry is a feasible and clinically informative tool for monitoring disease behavior in patients with IPF (112). From a clinical trial perspective, daily home spirometry may significantly reduce both the required sample size and duration of the trial by increasing the number of recorded measurements. Daily home spirometry may benefit particularly early phase clinical trials.

CONCLUSIONS

The management of patients with IPF is highly complex due to the progressive nature of the disease, the debilitating symptoms that severely impair quality of life and the highly prevalent comorbidities and complications. Currently, there is significant lack of knowledge regarding management of comorbid conditions including pulmonary hypertension and lung cancer as well as disease acute exacerbations that severely limit patients' survival. In the terminal phase of the disease palliative care becomes critically important. Developing a real cure for patients suffering from this terrible disease requires a close collaborative interplay between the scientific, professional, and patient community and the pharmaceutical industry. Only a comprehensive approach to disease management will eventually prove truly efficacious. Clinical trials focusing on disease biology and mechanisms by applying personalized medicine approaches seems to be the way forward (113, 114).

AUTHOR CONTRIBUTIONS

PS: conception and drafting of the article, final approval of the manuscript; AT and FB: drafting and critical revision of the article, final approval of the manuscript.

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BRIEF RESEARCH REPORT
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Bleomycin Revisited: A Direct Comparison of the Intratracheal Micro-Spraying and the Oropharyngeal Aspiration Routes of Bleomycin Administration in Mice

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Idiopathic Pulmonary Fibrosis (IPF) is a fatal disease characterized by exuberant deposition of extracellular matrix components, deterioration of lung architecture and impairment of lung functions. Its etiopathogenesis remains incompletely understood, as reflected in the lack of an appropriate therapy. Modeling the human disease in mice via the administration of bleomycin (BLM), despite the inherent limitations, has provided valuable insights into the underlying pathogenetic mechanisms, and has been instrumental for the development and validation of new pharmacologic interventions. Here we have directly compared the, most widely used, intratracheal (IT) route of administration with oropharyngeal aspiration (OA). Our results suggest that the OA route of BLM-administration can be used as a safe and effective alternative, minimizing peri-operative and experimental mortality, while preserving a solid fibrotic profile, as assessed with a plethora of standardized readout assays.

Keywords: pulmonary fibrosis, animal model, bleomycin (BLM), intratracheal (IT), oropharyngeal (OA)

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INTRODUCTION

Modelinghuman diseases in mice, despite the inherent limitations, has provided valuable insights into the underlying pathogenetic mechanisms, and has been instrumental for the development and validation of new pharmacologic interventions. In the context of Idiopathic Pulmonary Fibrosis (IPF), a fatal disease characterized by exuberant deposition of extracellular matrix components, deterioration of lung architecture and impairment of lung functions (1), the most widely used experimental model is induced by the administration of bleomycin in C57Bl6/J mice (2–4).

Bleomycin (BLM), a mixture of glycopeptides isolated from *Streptomyces verticillus*, is an anti-neoplastic/antibiotic drug for the treatment of some forms of cancer (5). It acts through DNA fragmentation, an activity modulated by many factors in different cell types including chromatin structure and DNA repair machinery, as well as antioxidant and metabolic enzymes (5).The lack of BLM hydrolase in pulmonary epithelial cells is thought to be the main reason for the observed toxicity of BLM in the lung, resulting in the development of pulmonary fibrosis as a side effect in treated cancer patients. The observed toxicity in human patients was soon translated to an animal model (6), serving the scientific community ever since. The model is characterized by alveolar epithelial cell death and the secretion of pro-inflammatory and pro-fibrotic factors, leading to fibroblast activation and collagen deposition, reproducing some, but not all, of the key features of the human disease (3, 4). The ensuing inflammation, the lack of alveolar epithelial hyperplasia

and the quick resolution, the main differences with the human disease and the major drawbacks of the BLM model, can be circumvented, in part, though the repetitive administration of BLM (7).

In order to mimic the human exposure, BLM was initially administered systemically, via intravenous or intraperitoneal injections, resulting, as in humans, to the subpleural development of fibrotic lesions (4). However, intratracheal (IT) administration of a single BLM dose, resulting in bronchiolocentric fibrotic patches, has become the method of choice (3). Furthermore, the IT delivery of BLM through a microspray aerosolizer (8) has been shown to yield more reproducible results and more homogenous distribution of fibrotic lesions than IT instillation/injection (9). Following the example of other drugs and agents (10–13), an alternative way of administering BLM in the trachea, namely oropharyngeal aspiration (OA), has been recently introduced (14, 15). However, the IT and OA routes of BLM administration have not been directly compared (3). As shown here, OA of 0.8 U/Kg BLM results in similar fibrotic responses as with 3.2 U/Kg when administered IT. OA administration reduced both the peri-operative mortality, due to the ease and speed of procedures, while the lower BLM dose employed also reduced experimental mortality.

MATERIALS AND METHODS

Mice

Mice were bred under SPF conditions at the local animal facility at "20–22°C, 55 ± 5% humidity, and a 12-h light-dark cycle; water and food were given *ad libitum*" (17). All experimentation in mice was in line with the ARRIVE guidelines and has been approved by the Veterinary service and Fishery Department of the local governmental prefecture, following the approval by the Institutional Animal Ethical Committee (IAEC; #985) of BSRC Alexander Fleming.

Pulmonary fibrosis was induced through the administration of BLM (Nippon Kayaku) to anesthetized (IP ketamine/xylazine/atropine, 100/10/0.05 mg/kg, respectively) mice. The intratracheal (IT) route was applied essentially as previously published (17), and as described in the online **Supplementary Materials and Methods**. Briefly, a MicroSprayer aerosolizer attached to a high-pressure syringe was inserted from the mouth to the carina (trachea's bifurcation) and BLM (0.08 U/mouse), or saline, was sprayed directly into the lungs of mice). The oropharyngeal (OA) route was applied as follows: the tongue of the mice was carefully pulled out using blunt forceps while the mouse's neck and thorax were stabilized on a plastic wall through a rubber band in order for the neck to be minimally stretched. The latter permitted the visualization of the trachea through a laryngoscope and a fiber- optic device. BLM (in a final volume of 50 μl) was then delivered as liquid in the oropharyngeal cavity, with a blunt ended conventional pipette tip. At the same time, the nares were blocked by a tong to prevent obligate nasal breathing and force BLM inhalation. Once BLM was administered (IT or OA), mice were placed on an electrical heating blanket to ensure speedy recovery from anesthesia and to avoid hypothermia.

Bronchoalveolar Lavage Fluid (BALF) collection and analysis, lung histopathological analysis and Quantitative RT-PCR analysis were performed with standardized protocols, as previously published (16) and as described in the on-line **Supplementary Materials and Methods**.

Respiratory mechanics were analyzed with the FlexiVent ventilator system (SCIREQ) following manufacturer instructions, as previously published (17) and as described in the on-line **Supplementary Materials and Methods**.

Statistical Analysis

Statistical significance was assessed with unpaired Student's *t*-test in comparison with control values (GraphPad Prism 6). Data are presented as means (±SEM); $p < 0.05$ (*) was considered significant.

RESULTS AND DISCUSSION

Beyond the route of administration, the severity of BLM effects highly depends on the precise genetic background of mice (i.e., C57Bl6 J vs. N, further differing between vendors), the local genetic drift of the colony and the health status of the corresponding animal house. As a result, a wide range of BLM concentrations have been employed to induce pulmonary fibrosis in mice (2, 4). As there are only a few published protocols on the OA route of BLM administration, we first tested four different BLM doses administered by OA. The starting concentration was 3.2 U/Kg, the concentration used locally for the IT route, which has been chosen after extensive testing over the years to establish a reproducible phenotype with minimal lethality. Administering 3.2 U/Kg BLM via OA, as well as to a lesser extend 1.6 U/Kg, resulted in significant mortality rates (**Figure S1A**), so these concentrations were discontinued. On the contrary, doses of 0.4 and 0.8 U/Kg were well tolerable, while the dose of 0.8 U/Kg produced statistically significant increases in all established diseases indices (**Figures S1B–H**) with minimal mortality and was thus selected for the direct comparison of IT and OA routes.

Pulmonary fibrosis was induced by IT or OA administration of BLM (at 3.2 and 0.8 U/Kg, respectively) in both male and female, 8–12 weeks old, C57Bl6/J mice. No sex effect was observed in any readout assays, so all following experimental results concern cumulative data, of randomly assigned, sex and age matched groups of littermate mice. No statistically significant difference on overall mouse survival was found between IT and OA BLM administrations (**Figures 1A,B**); however, at these doses, no mice died upon OA administration, most likely due to the lower BLM dose employed. Similarly, both routes of BLM administration, as compared to saline-treated animals, resulted in significant weight loss (**Figure 1C**), one of the traditional indicators of BLM-induced injury. However, IT administration (of BLM or saline) always resulted in peri-operative mortality (data not shown), a feature not usually reported (or even recorded), as it concerns almost exclusively handling, skill and chance. Nevertheless, OA administration is deemed

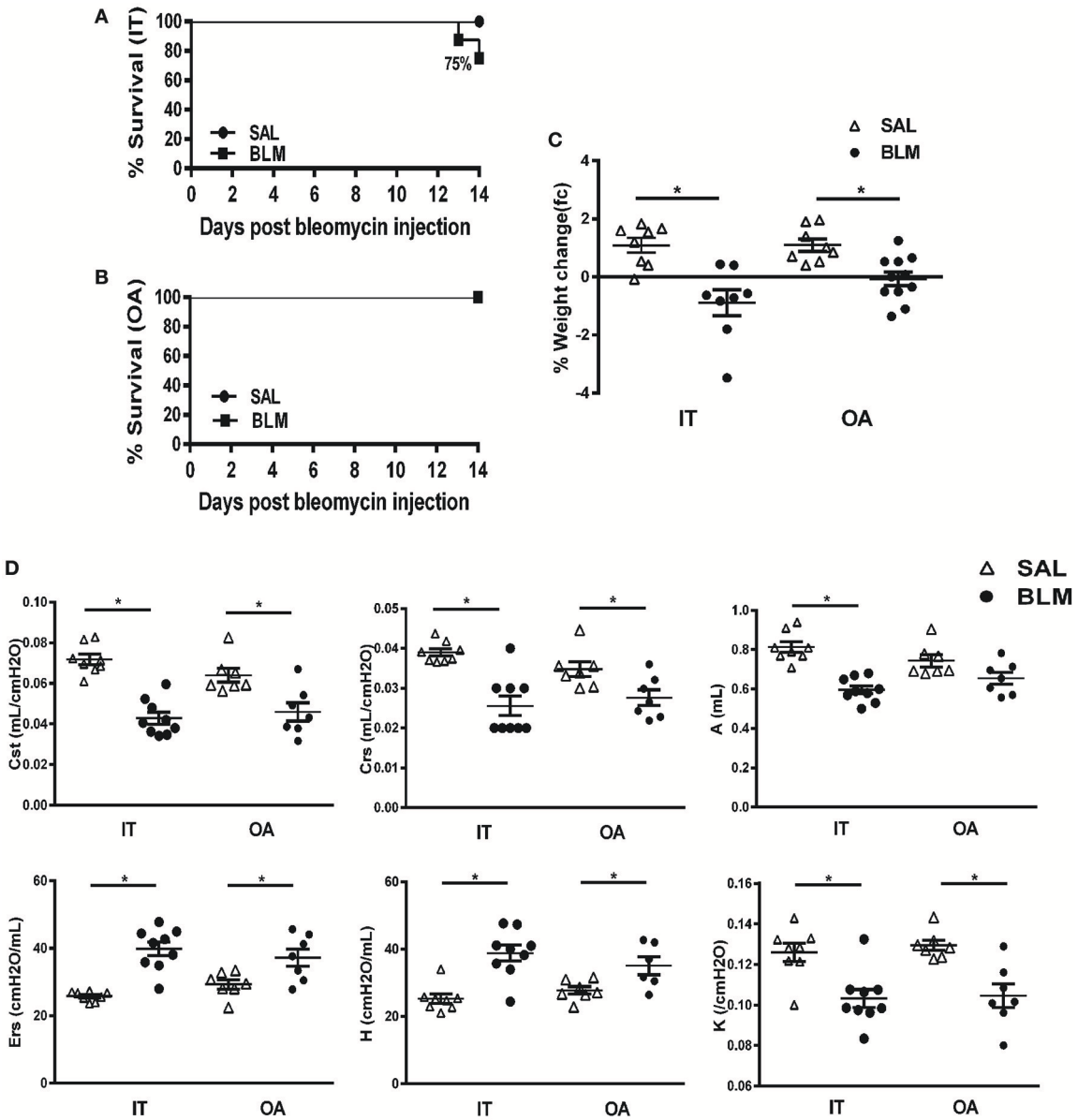

FIGURE 1 | Effects of the intratracheal microspraying (IT) or oropharyngeal aspiration (OA) routes of bleomycin (BLM) administration on mortality, weight loss and functional respiratory mechanics. 8–12 weeks-old C57BL6/J mice were challenged with BLM delivered via the IT or OA routes (at doses of 3.2 and 0.8 U/kg, respectively) and were sacrificed 14 days later. Data from two independent experiments are presented as scatter plots with horizontal bars representing mean levels (±SEM). Statistical significance was assessed with unpaired Student's *t*-test in comparison with the relative control values; $^*p < 0.05$ was considered statistically significant. **(A,B)** Kaplan-Meier plot using 14-days survival data from mice treated with BLM delivered either through IT or OA route, respectively. **(C)** Both OT and IA-treated mice demonstrated marked weight loss compared to saline-treated animals 14 days following BLM-challenge. **(D)** *In-vivo* respiratory mechanics following challenge with BLM. OA administration exerted similar to IT administration significant functional impairment on respiratory mechanics compared to saline-treated controls, as assessed by: mean static lung compliance (*Cst*), mean respirator system compliance (*Crs*), mean total lung capacity (*A*), mean respiratory system elastance (*Ers*), mean tissue elastance (*H*) and the curvature of the upper portion of the deflation limp of the pressure volume (PV) curve (*K*).

advantageous on overall experimental mice survival, with both practical and ethical benefits. Moreover, the experimental OA procedure is much easier and faster, as described in detail in **Supplementary Materials and Methods**, maximizing productivity and reproducibility, while it requires much less training.

Fourteen days post BLM (or saline) administration mice were sedated, tracheotomized and connected to a mechanical ventilator to evaluate forced-oscillation lung mechanics (**Figure 1D**). OA administration exerted similar to IT administration functional impairment on respiratory mechanics compared to saline-treated controls, as assessed by significant

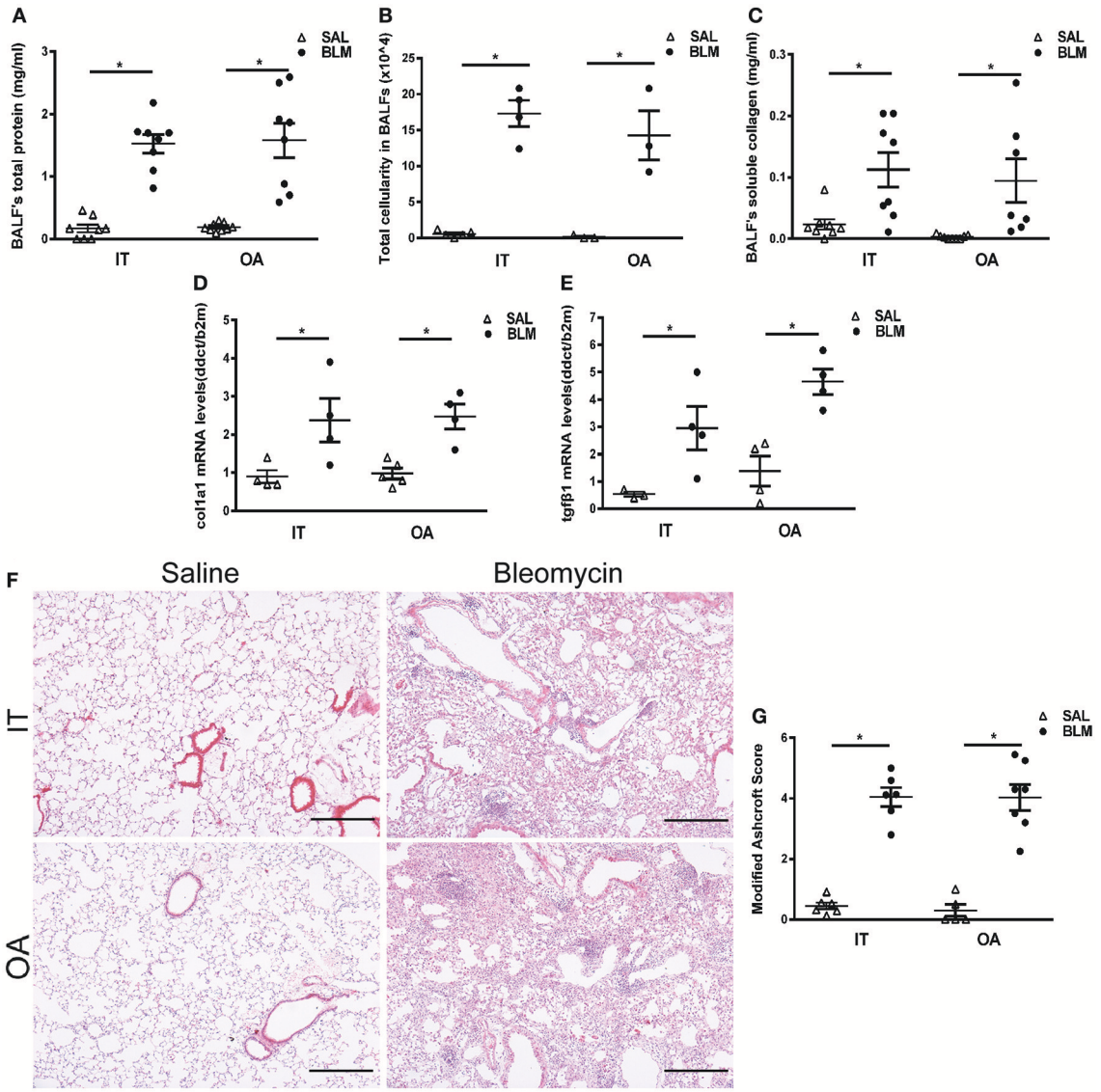

FIGURE 2 | Biochemical and histological analysis of injured lungs following intratracheal microspraying (IT) or oropharyngeal aspiration (OA) bleomycin (BLM) administration. 8–12 weeks-old C57BL6/J mice were challenged with BLM delivered via the IT or OA routes (at doses of 3.2 and 0.8 U/kg, respectively) and were sacrificed 14 days later. Data from two independent experiments are presented as scatter plots with horizontal bars representing mean levels (±SEM). Statistical significance was assessed with unpaired Student's *t*-test in comparison with the relative control values; $^*p < 0.05$ was considered statistically significant. **(A)** Increased total protein levels in BALF were observed with both routes of BLM delivery compared to saline-treated controls. **(B)** Both routes of delivery (IT and OA) produced significantly increases in bronchoalveolar lavage fluid (BALF) total cellularity compared to saline-treated animals. **(C)** Lung collagen was assessed by measuring BALF soluble collagen content with sirius red. Both routes of BLM administration were associated with substantial increases in BALF soluble collagen content compared to saline-treated animals. **(D,E)** Quantitative RT-PCR analysis of the *Col1a1* and *Tgfb* mRNA levels in whole mouse lungs challenged with BLM either through IT or OA route of delivery and saline-treated animals. Values were normalized to the expression values of *b2m*. **(F)** Representative H&E-stained lung sections. Scale bars 100 μm. **(G)** Quantitative analysis of histological changes and extent of fibrosis was performed by the modified Ashcroft score. Data represent mean scores obtained from two independent blind reviewers.

reductions in: (1) static lung compliance (*Cst*), (2) respiratory system compliance (*Crs*), and (3) total lung capacity (*A*), as well as increases in: (4) respiratory system elastance (*Ers*), (5) tissue elastance (*H*), (6) curvature of the upper portion of the deflation limb of the PV curve (*K*), (**Figure 1D**). Both static and dynamic lung compliance as well as elastance and total (inspiratory) lung capacity were found to be reliable indices of fibrotic lung injury, as recently suggested (18), well correlating with the Ashcroft score (**Table S1**). Therefore, and as the method is the most relevant to clinical measurements in human patients, not requiring additional mouse numbers, it is thus proposed as a valuable surrogate analysis of BLM-induced pulmonary fibrosis.

The route of BLM administration did not have an effect in BLM-induced vascular leak, as indicated by the total protein levels in the corresponding bronchoalveolar fluids (BALFs)

(**Figure 2A**). Similarly, no differences were detected in the total number of inflammatory cells in BALFs, upon measuring trypan blue stained cells in a hematocytometer (**Figure 2B**). Moreover, no qualitative differences in inflammation was detected either at this final endpoint with FACS analysis (data not shown). BALFs were also analyzed for soluble collagen content with sirius red, as an indirect indicator of tissue fibrosis. Again, no difference was noted upon the differential administration route of BLM (**Figure 2C**). It should be noted that measuring the hydroxyproline content of lung tissue is the most accurate method of determining lung collagen content and, as such, has been recommended as the optimal primary endpoint for fibrosis assessment (3). However, this technique requires at least half the lung, thus limiting the number of parallel analyses that can be performed (either in the mRNA or protein or enzymatic activity level accordingly) or requiring additional mouse numbers, that would still not allow for direct comparisons or correlations of collagen content with other disease indices. The estimation of collagen levels was complemented with Real Time RT-PCR assessment of Collagen 1a1 mRNA levels, again not revealing any differences between the two routes of BLM administration (**Figure 2D**). Similarly, no differences in TGF mRNA levels, the major profibrotic factor driving collagen expression and disease development in both mice and human, were noted (**Figure 2E**).

In line with the BALF assays, and as shown in representative images of H&E stained lung sections, BLM-challenge promoted extensive fibrotic changes and architectural distortion compared to saline-treated animals, irrespectively of the delivery method (**Figure 2F**); no major differences in the distribution and homogeneity of fibrotic lesions were observed. Moreover, collagen visualization with Sirius red and Mason trichrome staining of lung sections did not reveal any gross differences between the two methods either (**Figures S2A,B**); a recently reported automated histological image analysis of fibrotic lungs will further allow objective quantification of lung tissue density as a result of the deposited collagen (18). As expected, quantification of fibrosis by two blind reviewers using the Ashcroft score did not reveal any differences upon differential BLM delivery (**Figure 2G**).

Overall, as prompted by the recent American Thoracic Society workshop report on the use of animal models (3), we have directly compared for the first time the IT and OA routes of BLM-delivery in mice, using a plethora of readout assays including *in vivo* lung function measurements which are highly clinically relevant. Our results suggest that the OA route can be used as a safe and effective alternative route of BLM administration, allowing researchers to easily produce reproducible and robust kinetics of fibrotic lung injury combined with a beneficial safety profile sparing invasive surgical procedures of the IT administration, as well as the systemic effects of higher BLM doses. Moreover, the low BLM concentrations employed will allow drug testing in animals of a much better shape, without compromising a solid fibrotic profile.

AUTHOR CONTRIBUTIONS

VA designed the study; IB and IN performed all reported experiments; AT co-analyzed the data and wrote the paper, which was edited by VA and was critically commented by all authors.

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SUPPLEMENTARY MATERIAL

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