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IDIOPATHIC PULMONARY FIBROSIS: CLINICAL IMPACT OF DIFFERENT PHENOTYPES

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ABSTRACT

Background. Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, irreversible disease and carries a bad prognosis with median survival ranging from 2.5 to 3.5 years. No proven effective treatment is available other than lung transplantation. IPF occurs in middle-aged and elderly adults and is associated with the histopathologic and/or radiologic pattern of usual interstitial pneumonia (UIP). Acute exacerbation, lung cancer and pulmonary hypertension (PH) are the main complications which adversely affect the survival of IPF patients. Different from acute exacerbation which can occur at any stage of the disease, lung cancer and pulmonary hypertension (PH) are more likely in advanced disease. IPF is reported to be associated with an increased risk of lung cancer (8–14-fold compared to the general population) which represents the cause of death in up to 10% of patients with IPF.

Focal areas of neoplastic transformation are difficult to recognise by computed tomography (CT) and are often an unexpected finding in autoptic and explanted lungs. Type I alveolar epithelial cells are highly vulnerable to injury and several works have shown extensive loss of these cells in IPF, mainly by apoptotic cell death. Abnormal epithelial renewal results in the presence of different transitional reactive phenotypes. Serpin B3 and B4 [squamous cell carcinoma antigen (SCCA) 1/2] displays inhibitory activities on serine proteinases. Both isoforms protect injured cells from apoptosis induced by several kinds of stimuli, and in vivo experiments demonstrate that these Serpins can promote tumour growth.

On the other side Pulmonary hypertension (PH) represents an important complication of idiopathic pulmonary fibrosis (IPF) with a negative impact on patient survival. A frequency of 20-90% has been reported and it carries severe consequences including

decreased exercise capacity and increased mortality before and after lung transplantation. The complex biological processes that underlie pulmonary fibrosis might directly contribute to the pathogenesis of vascular remodelling which is equally patchy and heterogeneous. Endothelial injury, usually occurring through apoptotic cell death is another crucial aspect in the pathogenesis of IPF-associated PH as reported in recent studies. Several studies have implicated viral infection as a cause of epithelial injury and therefore an important factor in its pathogenesis. Among viral agents herpes viruses, in particular Epstein Barr virus (EBV), have been suggested as principal cofactors (as initiating or exacerbating agents) of fibrotic lung disease. The influence of viruses on PH associated with IPF is unknown.

In this study we aimed to investigate two main complications/comorbidities that influence so far the natural history of IPF patients and have a negative prognostic impact on survival. We decided to study IPF patients associated to lung cancer (first study) and IPF patients associated to pulmonary hypertension (second study) in order to explore their patho-biological features and the clinical impact.

First study

Aim. Given that Serpin B3/B4 related to epithelial proliferation in IPF patients remains to be investigated. The aim of the work was to study the expression of Serpin B3/B4 tissue in relation to different clinicopathological data of IPF patients.

Materials and methods. Explanted lungs from 48 IPF patients (including cases with cancer or high-grade epithelial dysplasia) were studied and compared with other diffuse parenchymal diseases and normal lungs. Immunohistochemistry for Serpin B3/B4 and Ki-67 was quantified in all cases, distinguishing stained metaplastic cells. In IPF patients correlations between Serpin expression and several clinicopathological data, including fibrotic remodelling were performed.

Results. Serpin B3/B4 and Ki-67 were significantly overexpressed in many metaplastic cells in IPF patients compared to control cases. Higher Serpin B3/B4 was found in older patients and cases with more impaired respiratory function. Serpin B3/B4 expression was related to both TGF-b and Ki-67 and was higher in patients with cancer/high-grade dysplasia. Serpin B3 was expressed in all cases, whereas Serpin B4 was expressed only in IPF.

Conclusions. Serpin B3/B4, particularly Serpin B4, appears to play an important role in aberrant epithelial proliferation. Evaluation of Serpin B3/B4 could have prognostic value in predicting disease progression, especially in patients with increased susceptibility to lung cancer.

Second study

Aim. The influence of viruses on PH associated with IPF is unknown. We aimed to investigate the influence of viruses in IPF patients focusing on aspects related to PH. A laboratory mouse model of gamma-herpesvirus (MHV-68) induced pulmonary fibrosis was also assessed.

Methods. 55 IPF explanted lung 41 controls were studied by molecular analysis to detect several viral genomes. Viral molecular data were correlated with mean pulmonary arterial pressure (mPAP) and arterial remodelling. Different clinical and morphological variables were studied by univariate and multivariate analyses at time of transplant. The same lung tissue analyses were performed in MHV-68 infected mice.

Results. A higher frequency of virus positive cases was found in IPF patients than in controls ($p=0.0003$) and only herpes virus genomes were detected. Viral cases showed higher mPAP ($p=0.01$) and poorer performance in the six minute walking test (6MWT; $p=0.002$). Increased arterial thickening, particularly of the intimal layer ($p=0.002$ and $p=0.004$) and higher TGF-β expression ($p=0.002$) were demonstrated in viral cases. The remodelled vessels showed increased vessel cell proliferation (Ki-67 positive cells) in the proximity to metaplastic epithelial cells and macrophages. Viral infection was associated with higher mPAP ($p=0.003$) and poorer performance in the 6MWT ($p=0.001$) after adjusting for other covariates/intermediate factors. In MHV-68 infected mice, morphological features were similar to those of patients.

Conclusion. Herpesviral infections may contribute to the development of PH in IPF patients.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), the most common form of the idiopathic interstitial pneumonias, is a chronic, progressive, irreversible disease and carries the worst prognosis with median survival ranging from 2.5 to 3.5 years (1). Despite major accomplishments in our understanding of the pathogenesis of lung fibrosis, the diagnosis and management of patients with IPF continues to pose significant challenges (1).

DEFINITION

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, occurring primarily in older adults, limited to the lungs, and associated with the histopathologic and/or radiologic pattern of UIP defined below (1-3). The definition of IPF requires the exclusion of other forms of interstitial pneumonia including other idiopathic interstitial pneumonias and ILD associated with environmental exposure, medication, or systemic disease (1-2).

CLINICAL PRESENTATION

IPF should be considered in all adult patients with unexplained chronic exertional dyspnea, and commonly presents with cough, bibasilar inspiratory crackles, and finger clubbing (4). The incidence of the disease increases with older age, with presentation typically occurring in the sixth and seventh decades (5). Patients with IPF aged less than 50 years are rare; such patients may subsequently manifest overt features of an underlying connective tissue disease that was subclinical at the time IPF was diagnosed (6). More men have been reported with IPF than women, and the majority of patients have a history of cigarette smoking (5; 7).

INCIDENCE AND PREVALENCE

There are no large-scale studies of the incidence or prevalence of IPF on which to base formal estimates. The incidence of IPF was estimated at 10.7 cases per 100,000 per year for men and 7.4 cases per 100,000 per year for women in a population-based

study from the county of Bernalillo, New Mexico (8). A study from the United Kingdom reported an overall incidence rate of only 4.6 per 100,000 person-years, but estimated that the incidence of IPF increased by 11% annually between 1991 and 2003 (9). This increase was not felt to be attributable to the aging of the population or increased ascertainment of milder cases. A third study from the United States estimated the incidence of IPF to be between 6.8 and 16.3 per 100,000 persons using a large database of healthcare claims in a health plan (6). Prevalence estimates for IPF have varied from 2 to 29 cases per 100,000 in the general population (6, 10). The wide range in these numbers is likely explained by the previous lack of uniform definition used in identifying cases of IPF, as well as by differences in study designs and populations. A recent analysis based on healthcare claims data of a large health plan in the United States yielded a prevalence estimate of between 14.0 and 42.7 per 100,000 persons depending on the case definition used (6). It is unknown if the incidence and prevalence of IPF are influenced by geographic, ethnic, cultural, or racial factors.

PATHOGENETIC HYPOTHESIS

The pathogenesis of IPF remains enigmatic and despite considerable recent investigation of UIP, neither the initial cause nor the reasons for progression are known. The precise factors that initiate and maintain the inflammatory and fibrotic responses observed in IPF are unknown. During the past decade there has been a shift away from the pathogenesis theory of generalized inflammation progressing to widespread parenchymal fibrosis toward a paradigm of disordered fibroproliferation and alveolar epithelial cell function. Indeed, the current hypothesis regarding its development conceptualizes ongoing multiple, small, focal, and isolated episodes of epithelial injury followed by a pathologic fibrotic-repair mechanism (11). It is probable that the disease results from a series of nonspecific insults to both the epithelial barrier and the pulmonary parenchyma (11). More substantial support comes from the histopathologic features, in particular the myxoid-appearing matrix with aggregates of actively proliferating and collagen-producing myofibroblasts termed '*fibroblast foci*' (12-13). These foci are often identified in a transition zone between the more normal uninvolved lung and the abnormal fibrotic lung. They have been interpreted to represent an organizing phase of focal acute lung injury (13) and believed to recapitulate the events that typify the healing skin wound. It is increasingly apparent

that mesenchymal cells in fibroblastic foci in IPF exhibit a variety of abnormalities compared to normal lung fibroblasts or fibroblasts from other lung diseases. The origin of pathological fibroblast foci within the IPF lesion remains puzzling. Possibilities include differentiation of resident fibroblasts, recruitment of circulating fibroblast precursors, and transdifferentiation of epithelial cells into pathological fibroblast phenotypes (14-16) (Fig. 1).
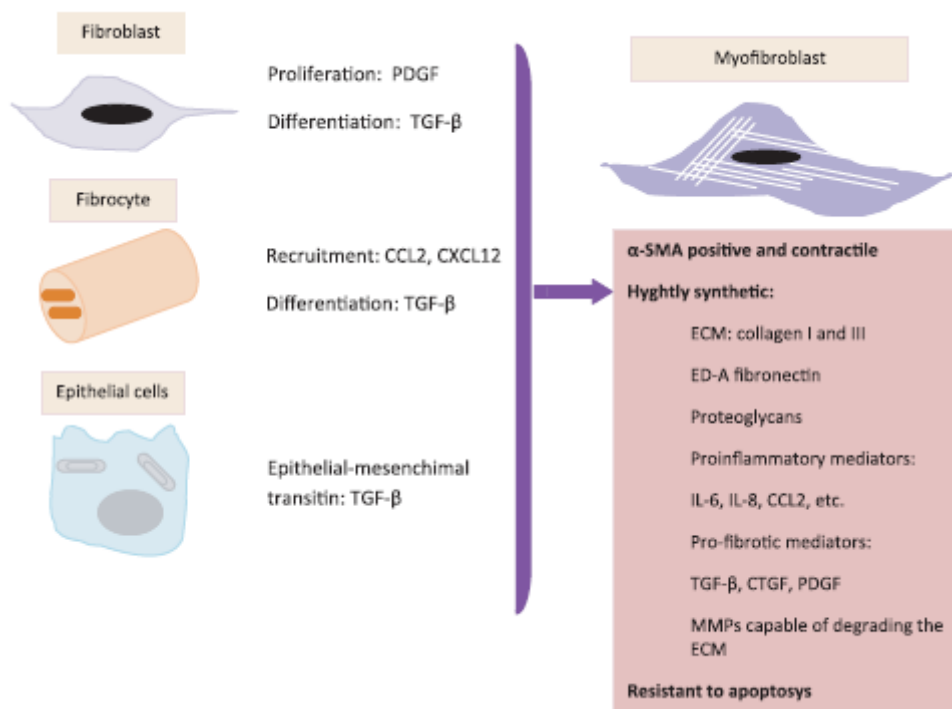

Figure 1: The primary effector cell in idiopathic pulmonary fibrosis is the myofibroblast; a cell that is highly synthetic, exhibits a contractile phenotype, and is characterized by the presence of a-smooth muscle actin stress fibres (adapted from reference 16).

A marked disruption in the integrity of the alveolar epithelium with presence of several altered phenotypes is a distinctive feature in IPF lung. Morphologic phenotypes may include: (i) hyperplastic type 2 pneumocytes, (ii) reactive large and elongated epithelial cells (putative transitional cells among type 2 and type 1 pneumocytes), (iii) flattened and attenuated epithelial cells usually overlying the fibroblastic foci, (iv) bronchiolar-type epithelium lining the enlarged airspaces of honeycomb lesions, and (v) squamous metaplasia. (Fig. 2). The reasons for all these epithelial changes are unknown, but some of them seem to be related to the initial insult, whereas others appear to represent a reactive response to lung remodelling (17). Re-establishing an intact

epithelium following injury is a key component of normal wound healing. This may require orchestrated epithelial cell responses that include proliferation and expansion of progenitor/stem cells, migration, and differentiation. The homeostatic function of an intact epithelium may be necessary to suppress mesenchymal cell activation. Idiopathic pulmonary fibrosis more likely results from an aberrant activation of alveolar epithelial cells (AEC)s after injury that provoke the migration, proliferation, and activation of mesenchymal cells with the formation of fibroblastic/myofibroblastic foci, leading to the exaggerated accumulation of extracellular matrix (ECM) with the irreversible destruction of the lung parenchyma (18)
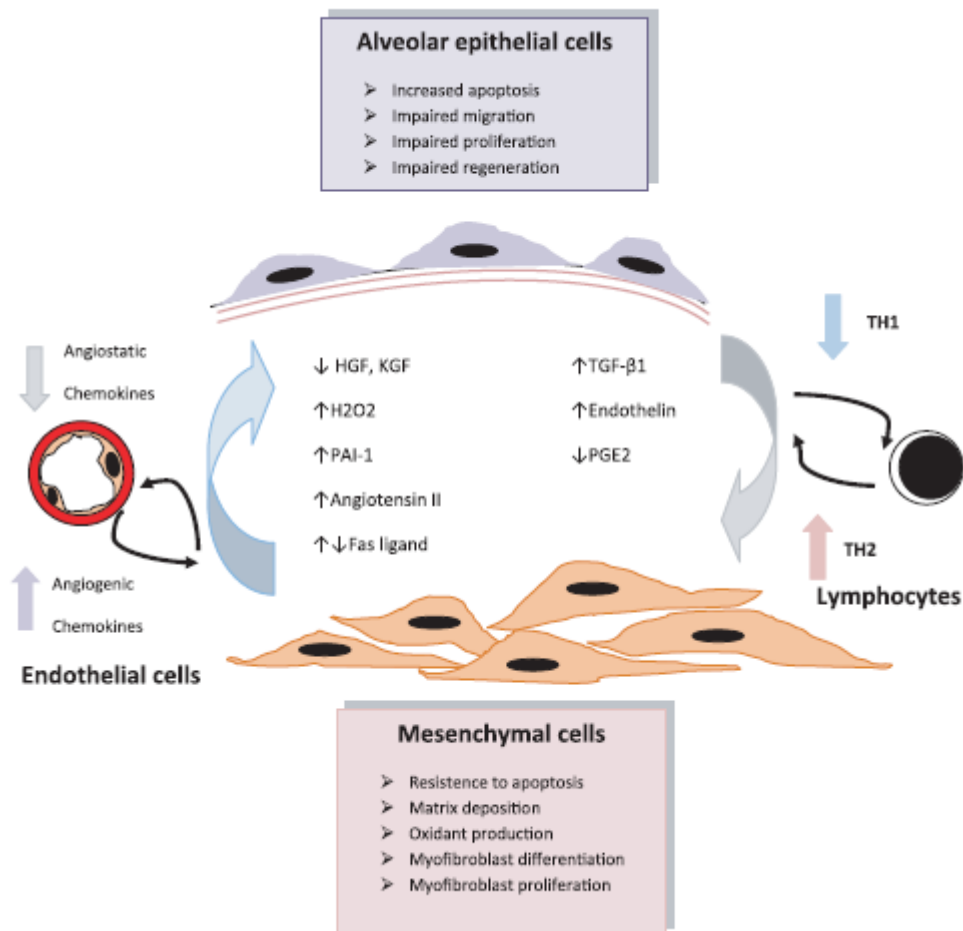

Figure 2: The pathogenesis of idiopathic pulmonary fibrosis is complex, and three major hypothesis or paradigms for the pathogenesis of idiopathic pulmonary fibrosis have emerged: dysregulated epithelial–mesenchymal interactions, aberrant angiogenesis, and the Th1/Th2 cytokine imbalance (adapted from reference 19).

The precise process by which AECs become 'activated' and communicate with fibroblasts leading to unremitting fibrosis in IPF remains poorly understood. Nonetheless, various theories have been proposed to explain this process, including the production of fibroblasts chemotactic factors by activated AECs and the release of growth factors involved in re-epithelization. These growth factors include transforming grow factor-b (TGF-b), platelet-derived growth factor (PDGF), insulin-like growth factor (ILGF)-1, and endothelin (ET)-1, among others. Thus, when the distal epithelium in the lung becomes injured and the basement membrane loses its integrity, it endeavours to re-epithelialize the surface and, as a result, growth factor is produced that can potentially recruit fibroblasts or myofibroblasts. Thus, instead of healing by repair, myofibroblasts proliferation and ECM deposition continues unabated (20). In IPF, there is loss of epithelial type I cells and a marked proliferation of epithelial type II cells; however, these cells generally do not appear to re-epithelialize the alveolar space because the basement membranes of patients with IPF remain abnormal, demonstrating microscopic alterations including duplication and fragmentation (21). These basement membrane abnormalities also permit the migration of mesenchymal cells from interstitium to the alveolar regions of the injured lung. These changes suggest that a chronic injury to the alveolar epithelium may result in perpetual nature of intra-alveolar exudates, fibrosis, and remodeling. Dysregulated interactions between epithelial and mesenchymal cells within the context of a damaged basement membrane and the surrounding ECM are increasingly recognized as a key control point in the pathogenesis of fibrotic disorders (22). Several studies have described phenotypic alterations in type II AECs including proliferation (23), bronchiolarization (24), apoptosis, and regenerative hyperplasia (25). Underlying areas of atypical and apoptotic epithelial cells are fibroblastic foci containing activated contractile myofibroblasts that secrete abundant ECM proteins (26). These myofibroblasts are closely associated with areas of epithelial cell death and regeneration. Microarray analysis identified an IPF-specific gene expression signature characterized by the up-regulation of genes indicative of an active tissue remodelling program, including ECM and a large number of myofibroblast/ smooth muscle cell-associated and epithelial cellrelated genes (18). The up-regulated development-relevant genes included several members of transcription factor families. While transcription factors active in morphogenesis and differentiation of the embryonic lung may be transiently expressed during adult lung repair, they are only rarely expressed in the normal lung. In fact, their

sustained expression is often thought of as a marker for malignant transformation. In IPF, there is increasing evidence supporting the idea that AECs are the primary source of cytokines and growth factors involved in fibroblast migration and proliferation, and myofibroblast differentiation. Thus, various studies performed by in situ hybridization and immunohistochemistry have demonstrated that in this disease, AECs are the main site of synthesis of PDGF, TGF-b, and tumor necrosis factor-a (TNF-a), all of them central pieces for the development of pulmonary fibrosis (27-29). Likewise, ET-1, a multifunctional peptide able to induce mesenchymal cell mitosis is strongly up-regulated in type 2 pneumocytes primarily in those located close to fibroblastic foci (30). Connective tissue growth factor (CTGF), a chemotactic and mitogenic factor for fibroblasts, is also up-regulated in type 2 AECs and fibroblasts in IPF lungs (31). Epithelial–mesenchymal transition is a key process in embryogenesis. An EMT-like process has been reported in cancer progression and metastasis and in fibrotic disorders (32-35). Recently, EMT was also observed in lung fibrosis and is another possible explanation for AEC activation: numerous cells co-expressing epithelial and mesenchymal markers within the expanded interstitium in IPF lungs (35). The occurrence of EMT in the lung represents a dramatic shift in cellular phenotype and requires reversal of early embryonic programs. In the lung, the formation of the various cell types lining the proximal and distal airways occurs through the differentiation of the epithelial precursor cells. Thus, EMT in IPF probably represents a dramatic reprogramming of epithelial cells (36). There is also a growing circumstantial evidence to suggest that in IPF the alveolar epithelium is prone to undergoing programmed cell death, although the mechanism for inducing epithelial apoptosis is, as yet, unknown and several different mechanisms, including TGF-b1 (which typically functions as a tumor suppressor through growth-inhibition and induction of apoptosis), oxidative stress, Fas activation, and angiotensin II have been proposed. Moreover, Telomeres are noncoding DNA sequences at the end of eukaryotic chromosomes that maintain chromosomal integrity and prevent replication of defective genes (37). When normal cells reach a critical telomere length, they exit the cell cycle and undergo senescence. The putative relationship between telomerase and human disease is demonstrated in two opposite situations. On one side of the spectrum, most human cancers are characterized by the expression of telomerase, which helps to maintain telomere length and enhance indefinite cell proliferation (38). On the other side of the spectrum, in pulmonary fibrosis, telomerase activity seems to be diminished, with consequent

premature telomere shortening. Two recent reports demonstrated that mutations in the genes encoding telomerase components are detected in patients with familial IPF and occasionally in patients with sporadic IPF (39). According to these studies, pulmonary fibrosis may be at least partially related to a telomerase deficiency and short dysfunctional telomeres, which after DNA damage leads to cell death or cell cycle arrest. Interestingly, cigarette smoking, a strong environmental risk factor for IPF, causes telomere shortening in a dose-dependent manner (40). As AECs play a critical role in the pathogenesis of IPF, and telomerase expression is generally restricted to cells with the capacity to proliferate, it has been speculated that fibrotic response in patients with short telomeres is provoked by a loss of alveolar cells (39), which may be enhanced by exposure to cigarette smoke.
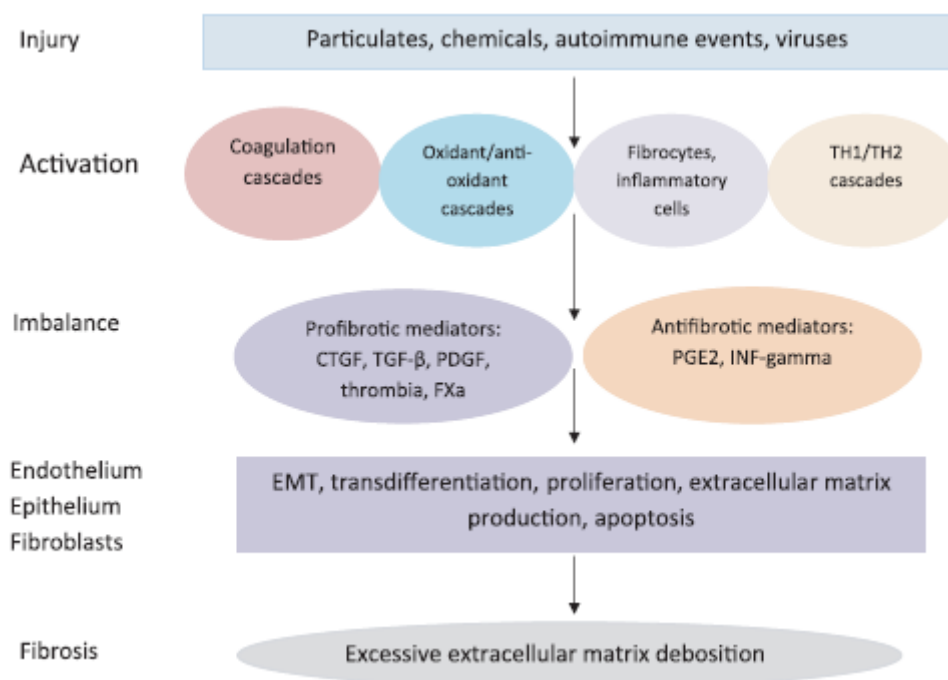

Figure 3: injury activates multiple inflammatory, cell signaling, and repair pathways which causes an imbalance in pro- and antifibrotic mediators. In turn, these mediators activate multiple cell types, causing changes in cellular functioning and cell–cell interactions that ultimately result in progressive fibrosis (adapted from reference 44).

In summary following injury, mesenchymal cells infiltrate wounds, differentiate into myofibroblasts, contractile and highly activated, secretory cells with characteristics intermediate between fibroblasts and smooth muscle cells, and secrete ECM proteins

that help to constitute provisional matrix, which serves as the 'scaffold' for normal tissue repair (41). Contraction of the provisional matrix approximates epithelial cells margins to facilitate re-epithelialization while collagen secretion stabilizes the contracted matrix. For normal healing to occur, wound myofibroblasts must undergo apoptosis (42); failure of apoptosis (cumulating evidence supports the emergence of an 'apoptosis paradox' in IPF – apoptosis susceptibility in epithelial cells and apoptosis resistance in myofibroblasts) leads to myofibroblasts accumulation, exuberant ECM production, persistent tissue contraction, and pathologic scar formation (43-45) (fig. 3).

POTENTIAL RISK FACTORS and COMORBIDITIES

Although idiopathic pulmonary fibrosis is, by definition, a disease of unknown etiology, a number of potential risk factors have been described.

Cigarette smoking. Smoking is strongly associated with IPF, particularly for individuals with a smoking history of more than 20 pack-years (46–50).

Environmental exposures. Increased risk for IPF has been found to be associated with a variety of environmental exposures (46,50-51). A significantly increased risk has been observed after exposure to metal dusts (brass, lead, and steel) and wood dust (pine) (51) Farming, raising birds, hair dressing, stone cutting/polishing, and exposure to livestock and to vegetable dust/animal dust have also been associated with IPF (47). Supporting an environmental etiology, increased numbers of inorganic particles have been detected in lymphnodes of patients with pulmonary fibrosis in autopsy studies (35). These observations must be interpreted with great caution, since epidemiologic studies of environmental risk factors are subject to a variety of biases and limitations.

Microbial agents. Several studies have investigated the possible role of chronic viral infection in the etiology of IPF. Most research has been focused on Epstein-Barr virus (EBV) and hepatitis C. Both the protein and the DNA of EBV have been identified in lung tissue of patients with IPF, usually in the alveolar epithelial cells (52,53). Tang and coworkers tested for the presence of eight herpesviruses, including EBV, in lung specimens from 33 patients with IPF, and found that one or more herpesviruses were detected in almost all IPF lungs compared with one-third of the control lungs (54). The positive viruses include EBV, cytomegalovirus, human herpesvirus (HHV)-7, and HHV-8. However, negative association studies have also been reported (55-56). Variable results have emerged from studies of hepatitis C. Elevation of serum antibodies to

cytomegalovirus has been reported (57), while associations with other viruses, including BK and JC polyomaviruses, have not been found (58). The evaluation of putative associations of virus, and other microbes, with IPF is hindered by confounding factors: patients were likely receiving immunosuppressive therapy, making infection a potential complication of therapy (55); the prevalence of EBV in general population is high: in one study, EBV DNA was detected in 96% of patients with IPF, but also in 100% of fibrotic lungs secondary to systemic sclerosis and in 71% of control lungs (59). Despite the large number of studies to date, definitive conclusions about the role of infection in IPF cannot be made.

Gastroesophageal reflux. The term "silent" microaspiration is used when patients have asymptomatic aspiration of small volumes of oropharyngeal secretions or gastric fluid into their lungs. Recently, a strong association between gastroesophageal reflux and idiopathic pulmonary fibrosis has been reported (60) but the causal relationship is unclear. Moreover GER could be a trigger to worsening fibrosis. The latter theory argues that idiopathic pulmonary fibrosis leads to progressive architectural distortion of the mediastinal structures, traction on the esophagus and the diaphragm, and weakening of the lower esophageal sphincter. Weakening of the lower esophageal sphincter would predispose patients to gastroesophageal reflux and microaspiration. Evidence contrary to this theory includes two studies that demonstrated no association between lung function and acid exposure times, and a third showing an inverse relationship between lung function and the presence of gastroesophageal reflux (61). Regardless, a weakened lower esophageal sphincter could allow for chronic microaspiration, causing repetitive injury to the already diseased lung and leading to the accelerated decline and/or acute respiratory decompensation seen in some patients with idiopathic pulmonary fibrosis (62). Nevertheless, the putative role of GER in IPF warrants further study.

Pulmonary Hypertension. A frequency of 20-90% has been reported and it carries severe consequences including decreased exercise capacity and increased mortality before and after lung transplantation (63-66). Recent studies have shown that PH measured with right-heart catheterization or transthoracic echocardiography in IPF patients is associated with low DLCO, shorter walk distances, and desaturation during exercise (64). Similar relationships have been identified when brain natriuretic peptide was used as a surrogate marker for PH in IPF patients (67).

However to date no clinical or biohumoral parameters are available as predictive markers of PH in IPF patients. Several authors have shown that PH in IPF relates poorly to the degree of pulmonary function test and is not always confined to advanced disease (65). Indeed even in end stage IPF cohorts, no association between the presence of PH and severity of the disease (both in terms of functional parameters and fibrosis extension) has been observed (65,68).

Lung cancer. An association between IPF and lung cancer was based upon the simultaneous finding of IPF and lung cancer in autopsy studies dating back several decades. As previously described in the introduction, a marked disruption in the integrity of the alveolar epithelium with presence of several altered phenotypes is a distinctive feature in IPF lung. Morphologic phenotypes may include hyperplastic type 2 pneumocytes, reactive large and elongated epithelial cells (putative transitional cells among type 2 and type 1 pneumocytes), flattened and attenuated epithelial cells usually overlying the fibroblastic foci, bronchiolar-type epithelium lining the enlarged airspaces of honeycomb lesions, and squamous metaplasia. Importantly, lung carcinomas develop in a high percentage of patients with IPF, and it may be difficult to differentiate prominent reactive epithelial proliferations from well-differentiated adenocarcinomas (69). The reasons for all these epithelial changes are unknown, but some of them seem to be related to the initial insult, whereas others appear to represent a reactive response to lung remodeling. A small number of epidemiologic reports helped advance the notion that IPF is an independent risk factor for lung cancer and recently a seven-fold increase in lung cancer was observed in IPF patients. Unsettled issues regarding the association between fibrosis and lung cancer include questions regarding the underlying mechanism of this association (70). Some studies have reported smoking history, as the common independent risk factors for the development of lung cancer or either lung fibrosis. Recently, Vancheri and Raghu have shown some similarities between the pathobiology of lung cancer and fibrosis (71). In particular they demonstrated that genetic alterations, response to growth and inhibitory signals, resistance to apoptosis, myofibroblast origin and behaviour, altered cellular signaling pathways are all fundamental pathogenetic hallmarks of both IPF and cancer.

Diabetes mellitus recent studies provided further evidence of an association between IPF and diabetes mellitus as patients treated with insuline (72)

GENETIC FACTORS

Familial pulmonary fibrosis: Although accounting for less than 5% of total patients with IPF, familial forms of IPF (i.e., those affecting two or more members of the same primary biological family) have been reported (73-76). The criteria used to define IPF in familial and sporadic cases are the same; familial IPF and sporadic IPF are clinically and histologically indistinguishable (75), although familial forms may develop at an earlier age (75) and seem to have different patterns of gene transcription (76). The evidence of a ''founder effect'' (i.e., a significant geographical clustering of cases) of familial pulmonary fibrosis in the Finnish population supports the relevance of genetic factors in the development of pulmonary fibrosis (75). There are many studies of apparent ''familial IPF'' which show that since at least half of the pedigrees demonstrate the presence of more than one type of idiopathic interstitial pneumonia (IIP).The most likely mode of genetic transmission of pulmonary fibrosis in familial cases is autosomal-dominant with variable penetrance (75,76). A linkage with chromosome 14 has been suggested (77). More strong associations with familial idiopathic interstitial pneumonia have been found with mutations in the surfactant protein C gene (78), but this association has not been found in patients with the sporadic form of the disease (79–81). Rare mutations in the gene encoding another surfactant protein, A2 (SFTPA2), have been associated with familial pulmonary fibrosis and lung cancer (82); the locus was discovered by genetic linkage in a large pedigree and two rare mutations were discovered by sequencing candidate genes within the linked interval. Recent reports by several investigators have documented that genetic variants within the human telomerase reverse transcriptase (hTERT) or human telomerase RNA (hTR) components of the telomerase gene are associated with familial pulmonary fibrosis and are present in some patients with sporadic IPF.

Sporadic pulmonary fibrosis: polymorphisms of genes encoding for cytokines (interleukin [IL]-1 a, tumor necrosis factor-a, lymphotoxin a, IL-4, IL-6, IL-8, IL-10, and IL-12, enzymes (a1-antitrypsin and angiotensinconverting enzym), profibrotic molecules (transforming growth factor-b1), coagulation pathway genes (plasminogen activator inhibitors-1 and -2), genes for surfactant protein-A and -B, immunomodulatory genes (complement receptor 1, NOD2/CARD15), and matrix metalloproteinase (MMP)-1) have been reported to have increased frequencies in patients with sporadic IPF. Many of these have also been related to disease progression. These association

studies need to be investigated in larger cohorts; at present there are no genetic factors that are consistently associated with sporadic IPF. Microarray analyses of gene expression will contribute to our understanding of pathogenesis, refinement of classification, and the targeting of candidates for therapy, but these are currently in an early phase of development (83).

HISTOPATHOLOGY

The histopathologic hallmark of IPF is a heterogeneous variegated appearance with alternating areas of normal lung, interstitial inflammation, fibrosis and honeycomb change (3) (Figure 4). These changes often affect the subpleural and paraseptal parenchyma most severely. Inflammation consists of a patchy interstitial infiltrate of lymphocytes and plasma cells associated with hyperplasia of type 2 pneumocytes and bronchiolar epithelium. The fibrotic zones are composed mainly of dense collagen with fibroblast foci. Areas of honeycomb change are composed of cystic fibrotic airspaces that are frequently lined by bronchiolar epithelium and filled with mucus and inflammatory cells. Smooth muscle metaplasia in the interstitium is commonly seen in areas of fibrosis and honeycomb change.
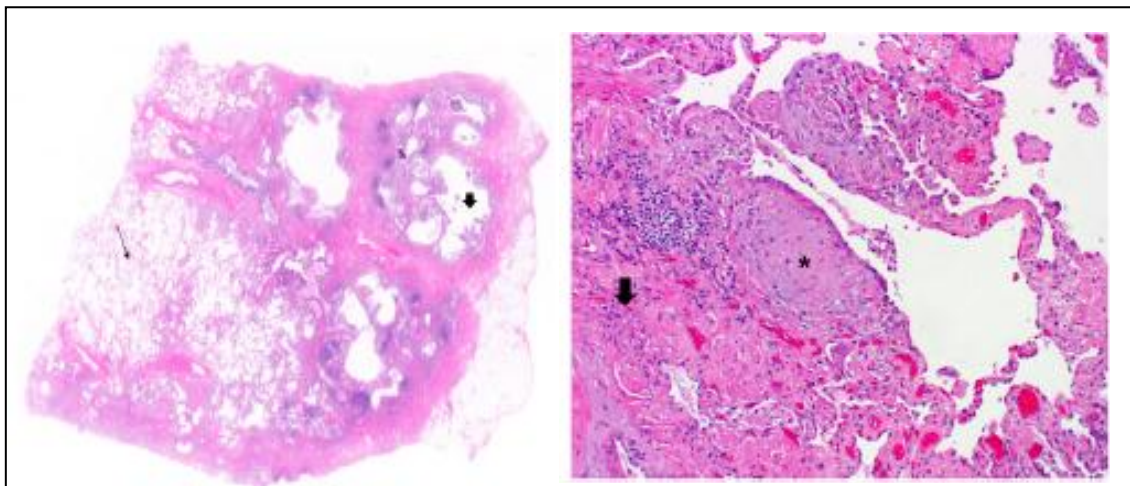

Figure 4: Surgical lung biopsy demonstrating UIP pattern: a patchy process with honeycomb spaces (thick arrow), some preserved lung tissue regions (thin arrow), and fibrosis extending into the lung from the subpleural regions. Adjacent to the regions of more chronic fibrosis (thick arrow) is a fibroblast focus (asterisk), recognized by its convex shape and composition of edematous fibroblastic tissue, suggestive of recent lung injury. (adapted from reference 84)

Some biopsies may reveal a pattern of fibrosis that does not meet the above criteria for UIP pattern (3). These biopsies may be termed "nonclassifiable fibrosis." In the

absence of histologic features diagnostic of an alternative condition, such biopsies may be consistent with the diagnosis of IPF (Tables 1 and 3) in the appropriate clinical and radiologic setting and after careful multidisciplinary discussion.

TABLE 1: HISTOPATHOLOGICAL CRITERIA FOR UIP PATTERN

UIP Pattern (All Four Criteria)	Probable UIP Pattern	Possible UIP Pattern (All Three Criteria)	Not UIP Pattern (Any of the Six Criteria)
• Evidence of marked fibrosis/ architectural distortion, ± honeycombing in a predominantly subpleural/ paraseptal distribution • Presence of patchy involvement of lung parenchyma by fibrosis • Presence of fibroblast foci • Absence of features against a diagnosis of UIP suggesting an alternate diagnosis (see fourth column)	• Evidence of marked fibrosis / architectural distortion, ± honeycombing • Absence of either patchy involvement or fibroblastic foci, but not both • Absence of features against a diagnosis of UIP suggesting an alternate diagnosis (see fourth column) OR • Honeycomb changes only‡	• Patchy or diffuse involvement of lung parenchyma by fibrosis, with or without interstitial inflammation • Absence of other criteria for UIP (see UIP PATTERN column) • Absence of features against a diagnosis of UIP suggesting an alternate diagnosis (see fourth column)	• Hyaline membranes* • Organizing pneumonia*† • Granulomas† • Marked interstitial inflammatory cell infiltrate away from honeycombing • Predominant airway centered changes • Other features suggestive of an alternate diagnosis

* Can be associated with acute exacerbation of idiopathic pumonary fibrosis.
† An isolated or occasional granuloma and/or a mild component of organizing pneumonia pattern may rarely be coexisting in lung biopsies with an otherwise UIP pattern.
‡ This scenario usually represents end-stage fibrotic lung disease where honeycombed segments have been sampled but where a UIP pattern might be present in other areas. Such areas are usually represented by overt honeycombing on HRCT and can be avoided by pre-operative targeting of biopsy sites away from these areas using HRCT. (adapted from reference 84)

HRCT FEATURES

HRCT is an essential component of the diagnostic pathway in IPF (Figure 5 and Table 2). UIP is characterized on HRCT by the presence of reticular opacities, often associated with traction bronchiectasis (85). Honeycombing is common, and is critical for making a definite diagnosis. Honeycombing is manifested on HRCT as clustered cystic airspaces, typically of comparable diameters on the order of 3–10 mm but occasionally as large as 2.5 cm. It is usually subpleural and is characterized by well-defined walls (86). Ground glass opacities are common, but usually less extensive than the reticulation. The distribution of UIP on HRCT is characteristically basal and peripheral, though often patchy. The presence of coexistent pleural abnormalities (e.g., pleural plaques, calcifications, significant pleural effusion) suggests an alternative etiology for UIP pattern. Micronodules, air trapping, nonhoneycomb cysts, extensive ground glass opacities, consolidation, or a peribronchovascular-predominant distribution should lead to consideration of an alternative diagnosis. Mild mediastinal lymph node enlargement (usually , 1.5 cm in short axis) can be seen (86). The chest radiograph is less useful than HRCT in evaluating patients with suspected IPF (87).
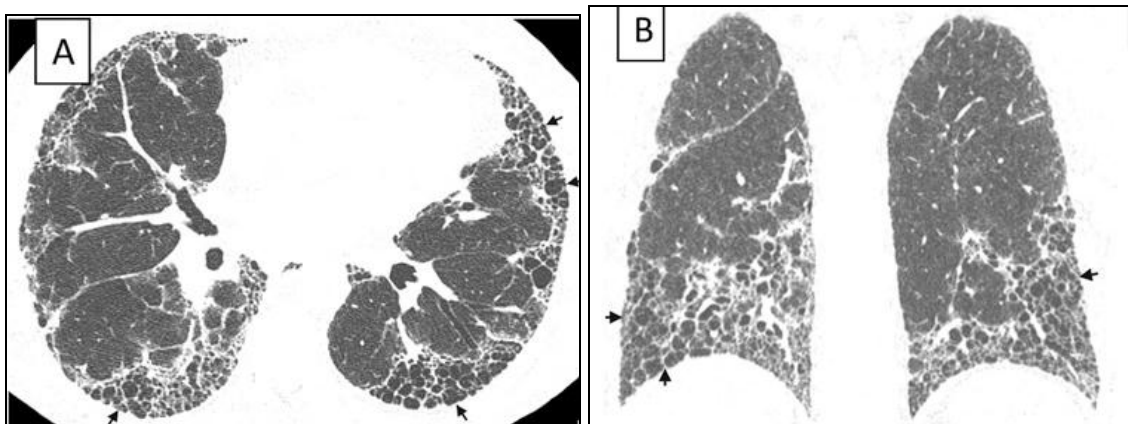
Figure 5: (A and B): UIP pattern, with extensive honeycombing: axial and coronal HRCT images show basal predominant, peripheral predominant reticular abnormality with multiple layers of honeycombing (arrows).(adapted from reference 84)

Several studies have documented that the positive predictive value of a HRCT diagnosis of UIP is 90 to 100% (87) but they are affected by selection bias (they only included patients with biopsy-proven diagnoses) Nonetheless, a UIP pattern on HRCT is highly accurate for the presence of UIP pattern on surgical lung biopsy. If honeycombing is absent, but the imaging features otherwise meet criteria for UIP, the imaging features are regarded as representing possible UIP, and surgical lung biopsy is necessary to make a definitive diagnosis. In patients whose HRCT does not demonstrate a UIP pattern, the surgical lung biopsy may still demonstrate UIP pattern on histopathology (table 3).

TABLE 2: HIGH-RESOLUTION COMPUTED TOMOGRAPHY CRITERIA FOR UIP PATTERN

UIP Pattern (All Four Features)	Possible UIP Pattern (All Three Features)	Inconsistent with UIP Pattern (Any of the Seven Features)
• Subpleural, basal predominance	• Subpleural, basal predominance	• Upper or mid-lung predominance
• Reticular abnormality	• Reticular abnormality	• Peribronchovascular predominance
• Honeycombing with or without traction bronchiectasis	• Absence of features listed as inconsistent with UIP pattern (*see* third column)	• Extensive ground glass abnormality (extent > reticular abnormality)
• Absence of features listed as inconsistent with UIP pattern (*see* third column)		• Profuse micronodules (bilateral, predominantly upper lobes)
		• Discrete cysts (multiple, bilateral, away from areas of honeycombing)
		• Diffuse mosaic attenuation/air-trapping (bilateral, in three or more lobes)
		• Consolidation in bronchopulmonary segment(s)/lobe(s)

(adapted from reference 84)

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DIAGNOSIS

Diagnostic criteria for adult patients with ILD and suspected IPF are presented in Table 3 and Figure 6. Careful exclusion of alternative etiologies through multidisciplinary discussion between pulmonologists, radiologists, and pathologists experienced in the diagnosis of ILD is of the utmost importance to an accurate diagnosis. Given the high-quality evidence regarding HRCT specificity for the recognition of histopathologic UIP pattern, surgical lung biopsy is not essential (88,89).

TABLE 3 COMBINATION OF HIGH-RESOLUTION COMPUTED TOMOGRAPHY AND SURGICAL LUNG BIOPSY FOR THE DIAGNOSIS OF IPF

HRCT Pattern*	Surgical Lung Biopsy Pattern* (When Performed)	Diagnosis of IPF?[†]
UIP	UIP	YES
	Probable UIP	
	Possible UIP	
	Nonclassifiable fibrosis[‡]	
	Not UIP	No
Possible UIP	UIP	YES
	Probable UIP	
	Possible UIP	Probable[§]
	Nonclassifiable fibrosis	
	Not UIP	No
Inconsistent with UIP	UIP	Possible[ǁ]
	Probable UIP	No
	Possible UIP	
	Nonclassifiable fibrosis	
	Not UIP	

Bold type indicates combinations of HRCT and surgical lung biopsy patterns that correspond with a diagnosis of IPF (a YES in the far right column). The combination of UIP HRCT and probable UIP or possible UIP or Nonclassifiable fibrosis (surgical lung biopsy patterns) (for example) equals a diagnosis of IPF; the combination of UIP HRCT and Not UIP (surgical lung biopsy pattern) does not make the diagnosis of IPF.
* Patterns as described in Tables 1 and 2.
‡ Nonclassifiable fibrosis: Some biopsies may reveal a pattern of fibrosis that does not meet the above criteria for UIP pattern and the other idiopathic interstitial pneumonias (1) (see text). These biopsies may be termed ''nonclassifiable fibrosis.''
† The accuracy of the diagnosis of IPF increases with multidisciplinary discussion (MDD). This is particularly relevant in cases in which the radiologic and histopathologic patterns are discordant. Timely referral to interstitial lung disease experts is encouraged. (adapted from reference 84)

In the appropriate clinical setting (this includes a thorough medical, occupational/environmental and family history, physical examination, physiological testing, and laboratory evaluation), the presence of a UIP pattern on HRCT is sufficient for the diagnosis of IPF.
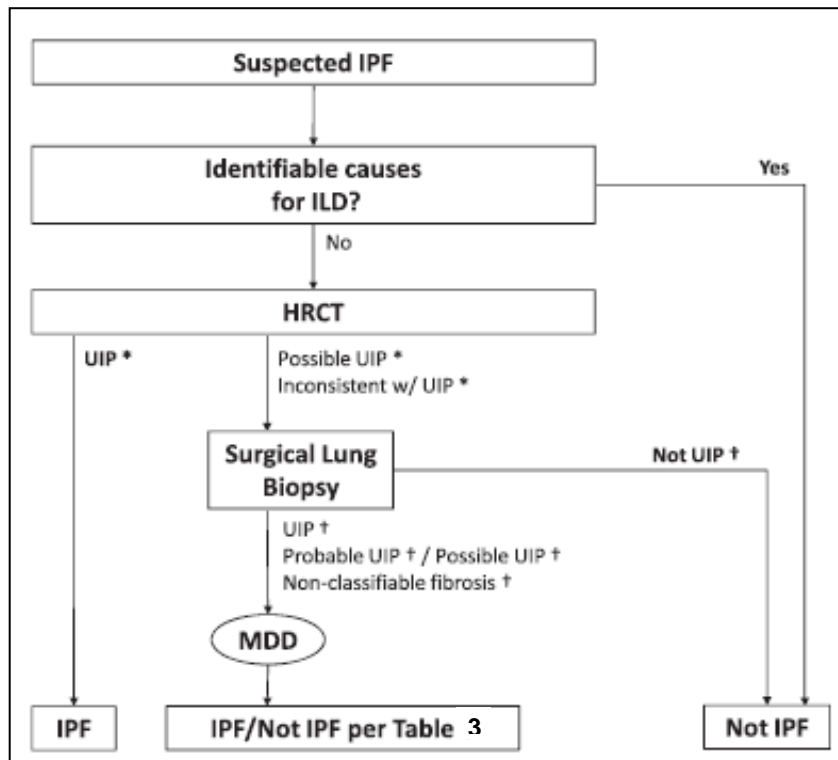
Figure 6. Diagnostic algorithm for idiopathic pulmonary fibrosis (IPF). Patients with suspected IPF (i.e., patients with unexplained dyspnea on exertion and/or cough with evidence of interstitial lung disease [ILD]) should be carefully evaluated for identifiable causes of ILD. In the absence of an identifiable cause for ILD, an HRCT demonstrating UIP pattern is diagnostic of IPF. In the absence of UIP pattern on HRCT, IPF can be diagnosed by the combination of specific HRCT and histopathological patterns. The accuracy of the diagnosis of IPF increases with multidisciplinary discussion (MDD) among ILD experts. *Refer to Table 2 for definitions. †Refer to Table 1 for definitions. (adapted from reference 84)

Diagnostic Criteria

The diagnosis of IPF requires the following:

1. Exclusion of other known causes of ILD (e.g., domestic and occupational environmental exposures, connective tissue disease, and drug toxicity)
2. The presence of a UIP pattern on HRCT in patients not subjected to surgical lung biopsy
3. Specific combinations of HRCT and surgical lung biopsy pattern in patients subjected to surgical lung biopsy

An HRCT or pathologic UIP pattern is not 100% specific to IPF (1, 3). Discordant histologic patterns on surgical lung biopsy specimens obtained from different segments have been described. This supports the obtainment of surgical lung biopsies from multiple lobes in patients with suspected IPF. In patients with severe physiologic impairment or substantial comorbidity, the risks of surgical lung biopsy may outweigh

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the benefits of establishing a secure diagnosis of IPF. The final decision regarding whether or not to pursue a surgical lung biopsy must be tailored to the clinical situation of the individual patient.

Exclusion of Other Known Causes

A careful history and physical examination focusing on comorbidities, medication use, environmental exposures, and family history is essential. Patients who meet established criteria for connective tissue disease do not have IPF. Younger patients, especially women, without clinical or serologic features at presentation may subsequently manifest clinical features of connective tissue disease. Therefore, the index of suspicion for connective tissue disease in younger patients (under the age of 50 yr) should be high. Cellular analyses of BAL can be useful in the diagnosis of certain forms of ILD. In the evaluation of patients with suspected IPF, the most important application of BAL is in the exclusion of chronic hypersensitivity pneumonitis. Transbronchial lung biopsy is useful in the evaluation of selected conditions (e.g., granulomatous disorders such as sarcoidosis). Connective tissue disease can present with a UIP pattern, and ILD has been described as the sole clinical manifestation of these conditions and can precede the overt manifestation of a specific connective tissue disease (90). Serologic testing for connective tissue disease should be performed in the evaluation of IPF and should include rheumatoid factor, anti-cyclic citrullinated peptide, and anti-nuclear antibody titer and pattern. The routine use of other serological tests such as antisynthetase antibodies (e.g., Jo-1), creatine kinase and aldolase, Sjogren's antibodies (SS-A, SS-B), and scleroderma antibodies (scl-70, PM-1) is of unclear benefit, but may be helpful in selected cases. Patients with IPF may have a mildly positive antinuclear antibody titer and/or rheumatoid factor level without any other clinical features of connective tissue. Such patients should be carefully screened for signs and symptoms of connective tissues disease (e.g., arthritis, Raynaud's phenomenon, skin changes, abnormal esophageal motility). In the absence of additional serologic or clinical evidence to support a connective tissues diagnosis, the diagnosis of IPF is appropriate.

NATURAL HISTORY OF IPF

The natural history of IPF has been described as a progressive decline in subjective and objective pulmonary function until eventual death from respiratory failure or complicating comorbidity (91). Available longitudinal studies do not allow a clear assessment of median survival in IPF. Several retrospective longitudinal studies suggest a median survival time from 2 to 3 years from the time of diagnosis (92). However, recent data from clinical trials of patient with preserved pulmonary function suggest this may be an underestimate (93). Death rates increase with increasing age, are consistently higher in men than women, and experience seasonal variation, with the highest death rates occurring in the winter, even when infections are excluded (94). Ischemic heart disease, heart failure, bronchogenic carcinoma, infection, and pulmonary embolism are also important causes of mortality in IPF (95). In general, factors that are associated with shortened survival time include: older age, smoking history, lower body mass index (BMI), more severe physiologic impairment, greater radiologic extent of disease, and the development of other complications or conditions, in particular, pulmonary hypertension, emphysema, and bronchogenic cancer (96). On CT findings, the overall extent of fibrosis has been consistently shown to correlate with disease severity parameters on pulmonary function tests and prognosis (97).

There appear to be several possible natural histories for patients with IPF and for a given patient, the natural history is unpredictable at the time of the diagnosis.

It is unknown if these different natural histories represent distinct phenotypes of IPF or if the natural history is influenced by geographic, ethnic, cultural, racial, or other factors (98).

Subclinical IPF

It is well recognized that symptoms precede diagnosis by a median of 1 to 2 years and radiographic evidence of disease may even precede symptoms, suggesting "subclinical" periods of disease that are not well characterized. Progression of asymptomatic to symptomatic IPF may occur over years to decades (89) (figure 7).
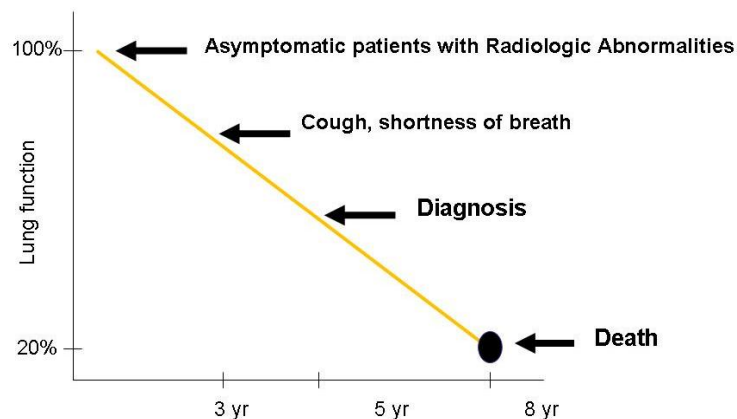
Figure 7: subclinical IPF

Slowly Progressive IPF

The classic clinical phenotype of IPF is one of slowly progressive decline in lung function and worsening dyspnea leading to death within several years of diagnosis. The mean annual rate of decline in progressive disease, as measured by the FVC, ranges from 0.13 L to 0.21 L. It appears that this slowly progressive clinical course may actually be less common than historically described (99).

Rapidly Progressive IPF

Selman and coworkers identified a subgroup of patients with IPF who displayed a rapidly progressive disease (less than 6 months of symptoms before first presentation) and showed shortened survival compared with patients following the slowly progressive clinical course. These were primarily men who were heavy cigarette smokers (100). Interestingly, the patients with an accelerated clinical course displayed a gene expression profile that differed from those with slower progression and longer survival despite having similar lung function, chest imaging, and histology findings at the time of diagnosis. Global gene expression analysis performed in a subset of patients identified a number of genes that were differentially expressed with upregulation of several functional pathways in the lungs from rapid progressor patients. These pathways seem to reflect diverse molecular mechanisms mostly operating in alveolar epithelial and mesenchymal cells and include genes involved in cell motility, myofibroblast differentiation, oxidative stress, coagulation and development. Although they can not ruled-out some effect of smoking, the differentially expressed genes among "rapid" and "slow" progressors found in this work differ from those described as associated to smoking (101,102). Of the genes increased in rapid progressors Selman and

Coworkers localize two of them. One was the adenosine A2B receptor gene, which is involved in the differentiation of human lung fibroblasts to myofibroblasts-a key process in fibrotic remodelling (103). The immunoreactive A2B receptor was mainly observed in alveolar epithelial cells and fibroblasts in ''rapid'' progressor lungs. Taken together, these results suggest a potential regulatory role for adenosine or its receptor in IPF. The second gene increased in the ''rapid'' progressor lungs was prominin-1/CD133, which is found in hematopoietic stem cells and embryonic epithelium among others. The immunoreactive protein was also expressed by reactive alveolar epithelial cells of ''rapid'' progressor lungs, supporting the notion that these cells may undergo a phenotypic shift during the pathogenesis of IPF. Decreased genes in ''rapid'' progressors included Smad6, an inhibitor of TGF-beta Smad-mediated signal transduction, and ''Rapid'' progressors also showed higher levels of active MMP-9 in the BAL fluids. In conclusion, the study by Selman represents the first identification of a distinct clinical phenotype of IPF, one that differs in clinical course and transcriptional profile, despite having similar lung function, chest imaging, and histology findings. Those findings suggest that during the development of this complex disease, genetic modifiers (and environmental factors, i.e. smoking) may play an important role in determining the eventual clinical phenotype, and that some modifiers genes are inducing a more aggressive IPF phenotype. Boon and colleagues also showed that lung molecular signatures at the time of diagnosis may identify patients with stable IPF compared with those with rapidly progressive disease (104). That molecular signatures from lung parenchyma at the time of diagnosis appear to be helpful in predicting disease progression and may prove valuable in predicting the activity of IPF (104)

Acute Exacerbation of IPF
Definition and diagnostic criteria: There is no established consensus approach to the diagnosis of acute exacerbation of IPF. Although specific criteria vary considerably among the published case series, most studies require a combination of the following: worsening of dyspnea within days to weeks (generally < 30 d); evidence of abnormal gas exchange as defined by a low partial pressure of arterial oxygen (PaO2)/percentage of inspired oxygen (FIO2) ratio or a decrease in PaO2; new radiographic opacities; and an absence of an alternative explanation, such as infection, left heart failure, or pulmonary embolism (106) (figure 8 and table 4). There is variation

in the rigor with which this last criterion, particularly with regard to the evaluation for possible infection, has been approached.
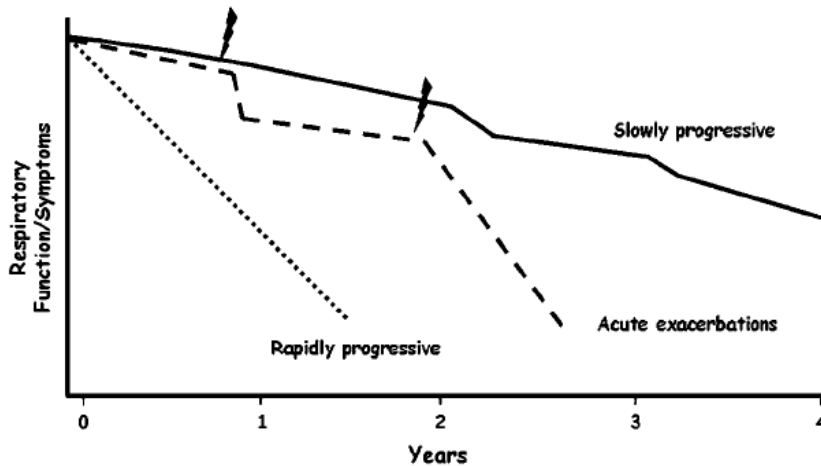

Figure 8: Natural history of IPF. There appear to be several possible natural histories for patients with IPF. The majority of patients experience a slow but steady worsening of their disease ("Slow progression"). Some patients remain stable ("Stable"), while others have an accelerated decline ("Rapid progression"). A minority of patients may experience unpredictable acute worsening of their disease (lightning bolt), either from a secondary complication such as pneumonia, or for unrecognized reasons. This event may be fatal or may leave patients with substantially worsened disease. The relative frequency of each of these natural histories is unknown. (adapted from references 105)

TABLE 4: DIAGNOSTIC CRITERIA OF ACUTE EXACERBATION

Previous or concurrent diagnosis of idiopathic pulmonary fibrosis[*]
Unexplained worsening or development of dyspnea within 30 days
High-resolution computed tomography with new bilateral ground-glass abnormality and/or consolidation superimposed on a background reticular or honeycomb pattern consistent with usual interstitial pneumonia pattern[†]
No evidence of pulmonary infection by endotracheal aspirate or bronchoalveolar lavage[‡]
Exclusion of alternative causes, including the following:
  · Left heart failure
  · Pulmonary embolism
  · Identifiable cause of acute lung injury[§]

Patients with idiopathic clinical worsening who fail to meet all five criteria due to missing data should be termed "suspected acute exacerbations."
[*] If the diagnosis of idiopathic pulmonary fibrosis is not previously established according to American Thoracic Society/European Respiratory Society consensus criteria (1), this criterion can be met by the presence of radiologic and/or histopathologic changes consistent with usual interstitial pneumonia pattern on the current evaluation.
[†] If no previous high-resolution computed tomography is available, the qualifier "new" can be dropped.
[‡] Evaluation of samples should include studies for routine bacterial organisms, opportunistic pathogens, and common viral pathogens.
[§] Causes of acute lung injury include sepsis, aspiration, trauma, reperfusion pulmonary edema, pulmonary contusion, fat embolization, inhalational injury, cardiopulmonary bypass, drug toxicity, acute pancreatitis, transfusion of blood products, and stem cell transplantation (adapted from reference 108).

Incidence: Four recent studies provide information regarding the incidence and mortality of acute exacerbations of IPF. A randomized, controlled trial of anticoagulant

therapy in IPF included rehospitalization (which included acute exacerbation) as a secondary endpoint (107). Of 56 patients followed for approximately 3 years, 32 (57%) were rehospitalized for acute exacerbation and 53% of those patients died. Importantly, subjects in this study were initially enrolled during an admission to the hospital, suggesting the population recruited was enriched for previous acute exacerbation or progressive disease. A retrospective review of an observational cohort of 147 patients with IPF identified 11 who met criteria for acute exacerbation (109). The 2-year incidence of acute exacerbation was reported at 9.6%, and mortality at 78%. The time to development of acute exacerbation from the subjects' initial visits was between 3 and 60 months. Identifying acute exacerbations in this study was hindered by the retrospective analysis, as illustrated by 14 additional cases of acute worsening that could not be included due to lack of data. In the third one, acute exacerbation was included as a secondary endpoint in a randomized, double-blinded, placebo-controlled trial of pirfenidone for the treatment of IPF (110). Of 107 patients followed for 6 months, 5 developed acute exacerbation, 1 of whom died. All cases were in the placebo group, suggesting that the incidence in untreated IPF might be higher (14%). Finally, a recent study by Kim et Coworkers find that 1- and 3-yr incidences of acute exacerbation were 14.2 and 20.7%, respectively (111).

Risk Factors: Acute exacerbations of IPF appear to occur at any time during the course of disease, and for some patients, may be the presenting manifestation of their disease. Importantly, the risk of an exacerbation does not appear to be linked to the level of pulmonary function derangement (109), although in one prospective series, patients with lower forced vital capacity had more total and respiratory hospitalizations during subsequent follow-up (95) and recently low FVC ha s found to be a risk factor (111). There is no clear association with age or smoking history, but acute exacerbations seem to be more common in men and in non-smoker (111). Several reports suggest that surgical lung biopsy may be a risk factor, although distinguishing these cases from post-surgical acute respiratory distress syndrome is difficult (112).

Clinical Features: Acute to subacute worsening of dyspnea is invariable in patients with acute exacerbation of IPF. Development of new or worsening dyspnea generally occurs within 30 days, although certain series have reported longer time courses. Cough, fever, and flulike symptoms are additional findings (113). Many patients present with severe hypoxemia, and respiratory failure requiring mechanical ventilation is common.

Laboratory Findings: Significant gas exchange abnormalities have been almost universally described (113). The most common criteria for abnormal gas exchange have been a PaO2/FIO2 ratio of less than 225 and a decrease over time in PaO2 of 10 mm Hg or greater. Systematic studies of serum and bronchoalveolar lavage fluid (BALF) from patients with acute exacerbations of IPF are rare. Exaggerated ST2 protein, IL-8, and α-defensin levels have been reported in some patients with acute exacerbations, suggesting the importance of activated T cells and neutrophils (114,115). These levels correlate negatively with lung function parameters. An increase in BALF neutrophils has also been reported (113). Serum KL-6, neutrophil elastase, and lactate hydrogenase (LDH) levels have been suggested as markers of acute exacerbation of IPF (116). Collard et al recently studied the plasma biomarker profile of acute exacerbation of IPF compared with those of stable IPF and acute lung injury. Plasma was collected from patients with stable IPF, acute exacerbation of IPF, and acute lung injury for measurement of biomarkers of cellular activity/injury (receptor for advanced glycation endproducts, surfactant protein D, KL-6, von Willebrand factor), systemic inflammation (IL-6), and coagulation/fibrinolysis (protein C, thrombomodulin, plasminogen activator inhibitor-1). Plasma from patients with acute exacerbation of IPF showed significant elevations in markers of type II alveolar epithelial cell injury and/or proliferation, endothelial cell injury, and coagulation. This profile differed from the biomarker profile in patients with acute lung injury. These findings support the hypothesis that type II alveolar epithelial cells are centrally involved in the pathobiology of acute exacerbation of IPF. Furthermore, they suggest that acute exacerbation of IPF has a distinct plasma biomarker profile from that of acute lung injury (117).

Radiology: Early reports of acute exacerbation of IPF describe diffuse ground-glass opacities on plain chest radiograph. High-resolution computed tomography (HRCT) generally demonstrates bilateral ground-glass abnormality with or without areas of consolidation, superimposed on the bibasilar subpleural reticular abnormality, traction bronchiectasis, and honeycomb change typical of usual interstitial pneumonia (UIP) pattern (Figure 9) (113). Three HRCT patterns of abnormality have been suggested: peripheral, multifocal, and diffuse ground glass (118). The multifocal and diffuse patterns have been associated with diffuse alveolar damage superimposed on UIP on surgical lung biopsy. Survival may be related to the degree of CT involvement (118).
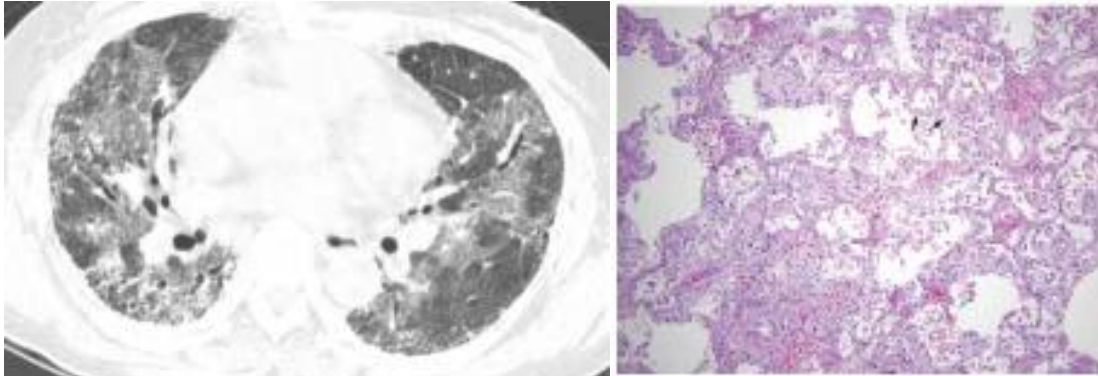
Figure 9: left: the groundglass opacities of multifocal patchy distribution have developed in both lungs. The patchy ground-glass opacities are distributed randomly and the fine reticular opacities can also be seen within the ground-glass opacity. *Right:* Higher magnification photomicrograph in an area of organizing DAD showing characteristic expansion and distortion of the alveolar septa by proliferating fibroblasts and myofibroblasts with scattered hyaline membranes (arrows) [hematoxylin-eosin, original _ 100] (adapted from references 109 and 95)

Histopathology: Diffuse alveolar damage superimposed on underlying UIP is the most commonly described finding when surgical lung biopsy is performed (Figure 9) (113, 119). Diffuse alveolar damage has been described in nearly three-quarters of the lung specimens reported in the literature, and, in all but one case, the findings were associated with histologic evidence of underlying UIP. Organizing pneumonia without other evidence of organizing diffuse alveolar damage and extensive fibroblastic foci have also been described in a few cases (119).

*Etiology: t*he etiology of acute exacerbations of IPF is unknown; we propose several hypotheses that should be tested in future research studies. First, perhaps acute exacerbations of IPF represent a distinct, pathobiological manifestation of the primary disease process, characterized by idiopathic lung injury. Second, acute exacerbations of IPF may represent clinically occult but biologically distinct conditions that go undiagnosed (e.g., viral infection, aspiration). Viral infections has been recently investigated by Collard and coworkers (120): acute exacerbation of IPF is often accompanied by fever, increased cough, and myalgia, suggesting an infectious etiology. Respiratory viruses have been considered a particularly likely cause, based on the similarities in clinical and radiological presentation between acute exacerbation of IPF and viral pneumonitis and the poor sensitivity of standard methods of viral detection. They found that the majority of cases of acute exacerbation of IPF had no evidence of an underlying viral infection. This suggests that viral infection is not a common cause of acute exacerbation of IPF. Gastroesophageal reflux is common in patients with IPF and may lead to aspiration of gastric contents, a known cause of

diffuse alveolar damage, and acute worsening (121). Third, acute exacerbations of IPF may be the sequelae of an acute direct stress to the lung, with a subsequent acceleration of the already abnormal fibroproliferative process intrinsic to IPF (122). No consensus opinion was reached regarding the relative likelihood of these competing hypotheses.

Biology: There is little known regarding the pathobiology of acute exacerbations of IPF. Disordered epithelial cell integrity, cellular inflammation, cytokines, matrix metalloproteinases (MMPs), and coagulation components are all likely involved in the pathogenesis of IPF, and rapid alteration in these processes may contribute to acute exacerbations. Loss of alveolar epithelial cell integrity and injury may play an important role in acute exacerbations, leading to the extrusion of fibrin onto the alveolar surface and remodeling. Acute exacerbation is morphologically characterized by BALF neutrophilia and histopathology showing diffuse alveolar damage. Both of these observations suggest loss of alveolar epithelial cell integrity and injury may play an important role (113). Environmental factors likely interact with a genetic variability in epithelial cell function, which may explain why only a subset of patients with IPF appears to develop acute exacerbations (123). Fibrocytes are circulating bone marrow–derived precursors that migrate to the lung in both human disease (124) and animal models of fibrosis (124). Fibrocytes can be recruited in response to chemokines generated by infection or injury and may potentiate fibrogenesis via extracellular matrix production and/or secretion of profibrotic factors (126-127). Recent evidence suggests that the percentage of circulating fibrocytes is increased in IPF compared with normal control subjects (6–10 vs. 0.5–2.4%, respectively) (128). In patients experiencing acute exacerbation, the level is further elevated. In one patient, the level of fibrocytes measured during acute exacerbation was 23.1% and this decreased to 3.7% upon recovery 6 weeks later (126). Whether fibrocyte recruitment and/or function is abnormal in acute exacerbation is unknown. MMPs regulate extracellular matrix turnover (127). Patients with rapidly progressive IPF show increases in active MMP-9 in BALF (128). Excessive MMP-9 may severely disrupt the structural and functional integrity of the alveolar–capillary basement membrane (128) and may activate latent transforming growth factor (TGF)-β, a profibrotic cytokine (129). TGF-β may be further activated by stretching of the lung through a mechanism involving integrins (130). For example, single-lung ventilation may lead to stretch-dependent TGF-β activation and acute exacerbation, a hypothesis supported by the observation that acute exacerbations after

video-assisted thorascopic surgery (VATS) were more prominent in the intraoperatively ventilated lung (113). Thus, increases in MMP-9 and TGF-β activation may promote the development of acute exacerbation in IPF. Disordered coagulation and fibrinolysis may be important components of acute exacerbations of IPF (107). Studies of patients with stable IPF have demonstrated a procoagulant and antifibrinolytic alveolar environment (131), and a similar environment has been described in the acute respiratory distress syndrome (ARDS) (132). The presence of disordered coagulation and fibrinolysis in stable IPF and the clinical and pathologic similarities of acute exacerbations with ARDS support a role for these mechanisms. A polymorphism in erythrocyte complement receptor 1 and mutations in surfactant protein genes are present in selected patients with IPF (133). Recently, the presence of heterozygous mutations in the telomerase reverse transcriptase (hTERT) and/ or RNA component (hTR) genes encoding telomerase components have been reported in several families with IPF and in one patient with sporadic IPF (134). These mutations lead to shortened telomeres that may limit the regenerative capacity of alveolar epithelial cells and contribute to the pathobiology of acute exacerbation.

Treatment: has generally consisted of high-dose corticosteroids, but there are no data from controlled trials to prove their efficacy. Cyclosporin A has been studied, but no convincing evidence of benefit has been demonstrated (135). Data from randomized clinical trials involving patients with IPF suggest possible roles for anticoagulant and antifibrotic therapies.

TREATMENT OF IPF

The recent guidelines for IPF did not find sufficient evidence to support the use of any specific pharmacologic therapy for patients with IPF. However, clinical trials of some agents have suggested a possible benefit (84). In particular:

- ❖ The recommendation against the use of the following agents for the treatment of IPF is strong:
  - ➢ Corticosteroid monotherapy
  - ➢ Colchicine
  - ➢ Cyclosporine A
  - ➢ Combined corticosteroid and immune-modulator therapy

- ➢ Interferon g 1b: A recent study by King and Collegues cannot recommend treatment with interferon gamma-1b since the drug did not improve survival for patients with idiopathic pulmonary fibrosis, which refutes previous findings from subgroup analyses of survival in studies of patients with mild-to-moderate physiological impairment of pulmonary function (136)
- ➢ Bosentan: Bosentan treatment in patients with IPF did not show superiority over placebo on 6MWD. A trend in delayed time to death or disease progression, and improvement in QOL,was observed with bosentan (137).
- ➢ Etanercept
- ❖ The recommendation against the use of the following agents for the treatment of IPF is weak; that is, these therapies should not be used in the majority of patients with IPF, but may be a reasonable choice in a minority:
  - ➢ Combined acetylcysteine and azathioprine and prednisone
  - ➢ Acetylcysteine monotherapy
  - ➢ Anticoagulation: Kubo and Colleagues found a significant improvement in survival at 3 yr in IPF patient treated with anticoagulant therapy (35% survival in the nonanticoagulant group compared with 63% in the anticoagulant group). Although the incidence of acute exacerbation was not different between the groups, the mortality associated with acute exacerbation was lower in the anticoagulant group (15 deaths in 21 acute exacerbations vs. two deaths in 11 acute exacerbations). Several methodologic issues raise concern for the study's applicability to a typical IPF population. The incidence of acute exacerbation was higher than is typical (64% in the placebo group), and the median survival of the placebo group was lower than is seen in contemporary IPF cohorts. There may have been selection bias toward more advanced and rapidly progressive disease, perhaps because these patients were recruited on initial hospitalization (107, 138).
  - ➢ Pirfenidone: in a recent study Pirfenidone was relatively well tolerated in patients with IPF. Treatment with pirfenidone may decrease the rate of decline in VC and may increase the progression-free survival time over 52 weeks. Additional studies are needed to confirm these findings (139). The collective data provide evidence that pirfenidone reduces decline in lung function in patients with idiopathic pulmonary fibrosis. In conclusion, pirfenidone has a

favourable benefit-risk profile and represents a suitable treatment option for patients with idiopathic pulmonary fibrosis (140).

➢ The recommendation for long-term oxygen therapy in patients with IPF and clinically significant resting hypoxemia is strong. Supplemental oxygen in a patient demonstrating clinically significant resting hypoxemia (commonly defined by a resting SpO2 of 88%), and extrapolation from data in COPD. The committee is not able to specify a PaO2 cut off for use of supplemental oxygen; for now this must be determined at the discretion of the treating physician. It is unknown if supplemental long-term oxygen therapy in patients who demonstrate only exertional hypoxemia improves survival.

❖ The recommendation for lung transplantation in appropriate patients with IPF is strong. Five-year survival rates after lung transplantation in IPF are estimated at 50 to 56% (141). It is unclear if the survival benefit is different in single- versus double-lung transplant recipients (142).

❖ The recommendation against mechanical ventilation in patients with respiratory failure due to IPF is weak; that is, mechanical ventilation should not be used in the majority of patients with IPF, but may be a reasonable choice in a minority.

❖ The recommendation for pulmonary rehabilitation in patients with IPF is weak; that is, pulmonary rehabilitation should be used in the majority of patients with IPF, but not using pulmonary rehabilitation may be a reasonable choice in a minority.

❖ The recommendation for corticosteroids in patients with acute exacerbation of IPF is weak; that is, corticosteroids should be used in the majority of patients with acute exacerbation of IPF, but not using corticosteroids may be a reasonable choice in a minority.

❖ The recommendation against the treatment of pulmonary hypertension associated with IPF is weak; that is, pulmonary hypertension should not be treated in the majority of patients with IPF, but treatment may be a reasonable choice in a minority

❖ The recommendation for the treatment of asymptomatic gastroesophageal reflux in patients with IPF is weak; that is, asymptomatic gastroesophageal reflux should be treated in the majority of patients with IPF, but not treating asymptomatic gastroesophageal reflux may be a reasonable choice in a minority .

Based on the evidence published to date, there is no proven pharmacological therapy for IPF. While a few studies have suggested potential benefits from some

pharmacologic agents, the recommendations made by the committee for these agents were ''weak no.'' For the well-informed patient who strongly desires pharmacologic treatment, it is suggested that the choice of agent may be made from therapies that received a weak recommendation against their use (''weak no''). Continued, concerted efforts should be made by physicians, patients, and sponsors to pursue well-designed clinical trials aimed at improving outcomes, including quality of life, in patients with IPF.

MONITORING THE CLINICAL COURSE

Monitoring of patients with IPF is necessary to proactively identify patients with progressive disease, to appreciate worsening of symptoms and oxygenation, and to detect the development of disease or treatment complications. In addition, careful assessment of the clinical course is useful in helping patients understand their disease course and in initiating timely, appropriate therapeutic interventions, including consideration of lung transplantation (figure 10)

Disease progression may be manifested by increasing respiratory symptoms, worsening pulmonary function test results, progressive fibrosis on HRCT, or acute respiratory decline. In the absence of another identifiable cause, the presence of any of the following changes is consistent with progressive disease:

- o Progressive dyspnea (objectively assessed)
- o Progressive, sustained decrease from baseline in absolute
- o FVC
- o Progressive, sustained decrease from baseline in absolute
- o DLCO (corrected for hemoglobin)
- o Progression of fibrosis from baseline on HRCT
- o Acute exacerbation
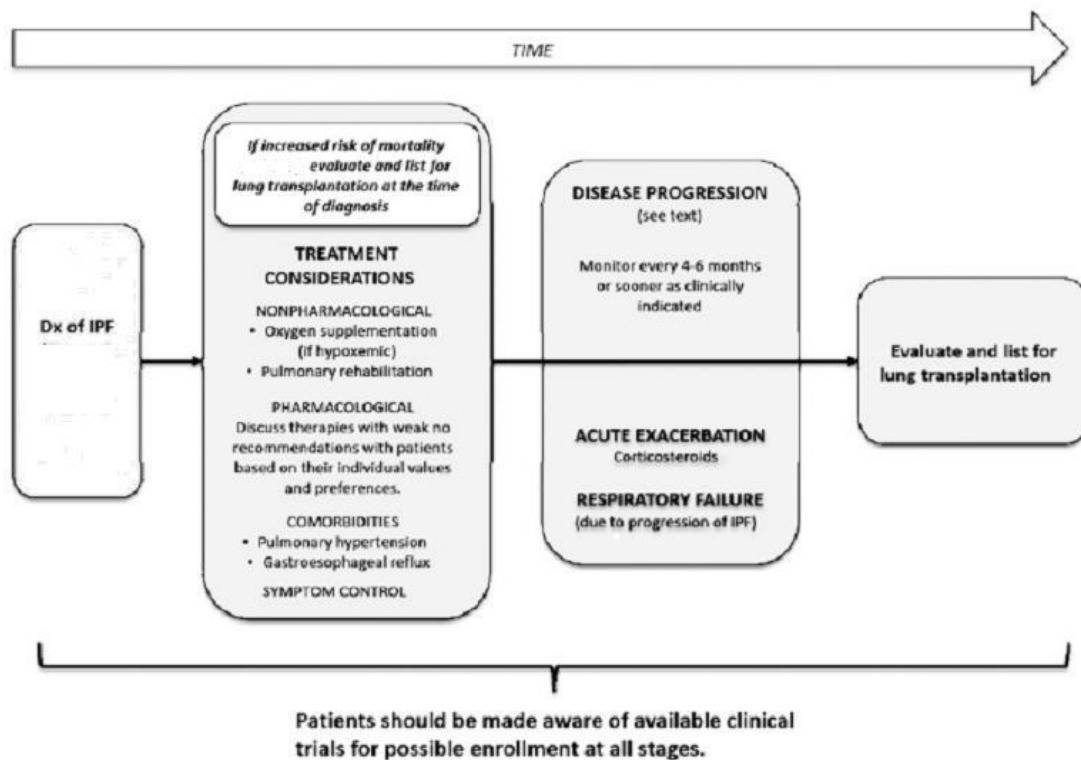- o Death from respiratory failure

Figure 10. Schematic pathway for clinical management of patients with IPF. Clinicians are required to spend adequate time with patients to discuss patients' values, preferences, and prognosis. All patients should be made aware of available clinical trials for possible enrollment. Patient at increased risk of mortality should be considered for lung transplantation. Pharmacologic treatment should be limited to a carefully selected minority of patients who are willing to accept possible adverse consequences even if expected benefits are small. See text for specific recommendations of pharmacological therapies. Oxygen supplementation (if hypoxemic) and pulmonary rehabilitation are recommended treatments (strong yes and weak yes, respectively). All patients should be monitored for disease progression and identification of complications at 4 to 6 months or sooner as clinically indicated. Corticosteroids are an appropriate treatment option for acute exacerbation (weak yes). Mechanical ventilation is not recommended for the majority of patients with respiratory failure due to progression of their disease (weak no). Symptom control (palliative care) focuses on reducing symptoms (e.g., cough and dyspnea) and providing comfort to patients, rather than treating patients' disease. Advanced directives must be discussed in the ambulatory setting (adapted from reference 84)

Evidence from several clinical cohorts to date confirms that a change in absolute FVC of 10%(with or without a concomitant change in DLCO) or a change in absolute DLCO of 15% (with or without a concomitant change in FVC) is a surrogate marker of mortality and is evidence of, in the absence of an alternative explanation, disease progression (143). Smaller (5–10%) but progressive, sustained changes in FVC may also represent progression of disease (144). On average, progression of disease is monitored over periods of 3 to 6 months, but sustained changes in symptoms, physiology, and radiology over shorter periods of time may also identify disease progression (figure 10). Of the above parameters, pulmonary function testing provides the most standardized approach to objective monitoring and quantification of disease

progression. Evidence suggests that progressive fibrosis leads to gradual decline in pulmonary function and worsening symptoms (145). The placebo arms of several large, randomized controlled treatment trials in IPF have suggested an average annual decline in FVC of approximately 0.2 liters in the overall population of patients with IPF with mild to moderate pulmonary function abnormalities at the time of enrollment (146). The rate of decline in individual patients is widely variable. A decline in absolute DLCO in the absence of an alternative explanation is consistent with progressive disease, although such a decline may also reflect changes in the pulmonary vasculature and coexistent pulmonary hypertension. Using our current techniques, longitudinal measurement of other clinical and physiological variables (e.g., TLC, P(A-a)O2) and 6-MWT variables have significant limitations and are not recommended for routine use in monitoring for disease progression at this time. Monitoring for desaturation during 6MWT is useful, however, in patients with significant exercise intolerance to assess the need for supplemental oxygen. The physiological effect of comorbidities such as coexisting emphysema on the predictive values of serial changes in pulmonary function is unclear, but is likely to be a confounding factor (147). While it is appropriate to routinely monitor disease course with FVC and DLCO measurements at 3- to 6-month intervals, a subset of patients with rapid progression or acute worsening may not have demonstrated progression during the preceding interval (95). The optimal time interval for repetition of FVC and DLCO has not been formally investigated. A flexible approach to monitoring for disease progression is required with a lower threshold for earlier repetition of FVC and DLCO in the presence of progressive dyspnea or other features of a more rapidly progressive course.

AIM OF THE FIRST STUDY

The aim of the work was to study Serpin B3/B4 tissue expression in relation to different clinicopathological data of advanced IPF patients (including cases with high grade dysplasia or cancer), mainly focusing on epithelial proliferation. To better understand the value of Serpin B3/B4 in relation to the proliferative aspect, 13 patients with non-IPF diffuse parenchymal lung diseases (DPLDs) and 10 normal subjects were included in the study. We also investigated which type of Serpin B3 and B4 isoforms play a key role in the disease.

MATHERIALS AND METHODS

Study population

The study was performed on native lungs of 48 patients consecutively transplanted for IPF from September 1995 to February 2007 at our Centre. The main clinical characteristics of subjects are summarised in Table 1. The diagnosis of IPF was based on the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus Classification (84).

Characteristics	IPF patients	Range
Sex (males:females)	33: 15	
Age at transplantation (years)	55.1 ± 7.2	39–65
Type of transplant (RtSLT: LtSLT: BLSLT)	12: 25: 11	
Dust exposure (%)	16	
Smokers (%)	83	
Smoking (pack-years)	23.7 ± 23.6	2.5–120
Functional parameters before transplantation (less than 6 months)		
DLCO (% predicted)	22.0 ± 9.5	7–43
FEV1 (% predicted)	45.2 ± 14.3	22–73
FVC (% predicted)	42.4 ± 14.2	21–66
VC (% predicted)	40.7 ± 12.7	21–66
TLC (% predicted)	47.7 ± 10.8	31–73
RV (% predicted)	62.5 ± 23.9	31–121
PaO$_2$ (% predicted)	54.2 ± 10.2	35–79
PaCO$_2$ (% predicted)	42.9 ± 9.6	27–87

*For normally distributed quantitative variables, values are expressed as mean and standard deviation, range; for categorical variables, number or percentage distributions are shown. BLSLT, bilateral single lung transplantation; DLCO, diffusing lung capacity for carbon monoxide; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; IPF, idiopathic pulmonary fibrosis; LtSLT, left single lung transplantation; PaCO2, partial pressure of carbon dioxide; PaO2, partial pressure of oxygen; RtSLT, right single lung transplantation; RV, residual volume; TLC, total lung capacity; VC, vital capacity.

In all cases, cancer and high grade dysplasia/carcinoma in situ (CIS) of different metaplastic cell types (cuboidal, bronchiolar and squamous) were also reported. For high grade dysplasia/CIS of squamous type the World Health Organization (WHO) classification was followed (148). Cuboidal cell dysplasia usually appears as a flat lesion with type 2 pneumocytes showing enlarged nuclei and evident nucleoli. Bronchial dysplasia was identified on the basis of nuclear atypia and irregular cell arrangement with frequent pseudo/multilayering, dark or coarsely uneven nuclear chromatin and one or more prominent nucleoli (149). Control group included subjects with normal lungs (non-implanted lungs due to surgical technical problems, 10 cases) and patients affected by non-IPF end-stage diffuse parenchymal lung diseases (DPLDs, 13 cases): two sarcoidosis, five lymphangioleiomyomatosis, one desquamative interstitial pneumonia, one extrinsic allergic alveolitis and four Langerhans cell histiocytosis. The other non-IPF DPLDs patients included seven females and six males, with a mean age of 46.5_14 years; the majority of them were heavy smokers. The donors were the same included in a previous study of our group:9 five males and five females, mean age 30_17 years, all non-smokers, who died of cerebral trauma and stayed less than 2 days in intensive care without evidence of lung infections or other complications.

Methods

Serpin B3/B4 and Ki-67 immunostaining

For morphological and immunohistochemical analysis at least two samples taken from different lobes were selected from specimens that showed features of excellent tissue preservation and adequate lung inflation. Four mm thick sections were cut and processed for immunohistochemical analysis of Serpin B3/B4 and Ki-67. Primary polyclonal antibody anti Serpin B3/B4 and with the primary monoclonal antibody antiKi-67 were used. Both Serpin B3/B4 and Ki-67 were evaluated using an assisted computerised morphometric analysis, counting at least 150 strongly stained metaplastic cells (range 150–350) in the two lung samples taken from two different lobes of each lung. The quantification was restricted to strongly stained metaplastic epithelial cells distinguishing bronchial, cuboidal and squamous cells, to identify the major unstable cell type. Data were expressed as number of positive cells/total cell count (%) and Ki-67 quantification represented the Ki-67 labelling index. The same

paraffin-embedded sections were processed by confocal microscopy to confirm the co-expression of Serpin B3/B4 and Ki-67.

Molecular analyses for serpin B3/B4 detection

Molecular investigations were performed in 24 IPF patients and six normal lungs, whose frozen lung tissue samples were available. Total RNA was extracted from frozen lung tissues of the IPF and control group (lung donors) using a modified RNAzol method, as previously described (150). RNA was reverse-transcribed using the Reverse Transcription System (Promega Corporation, USA) according to the manufacturer's instructions. Real-time PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Italy). The real time PCR was carried out in a 96-well microtitre plate format (Applied Biosystems). All assays were performed in triplicate to ensure their reproducibility, and a negative control was included in each run.

Statistical analysis

Statistical analyses were performed with the SAS statistical software release 9.1 (SAS Institute, USA). In all analyses, the threshold for significance was set at two-tailed 0.05. To describe quantitative variables, mean values, standard deviation, median and range were used. Normality of distributions was verified by means of the Shapiro-Wilk test. The significance of differences between mean values of Serpin B3/B4 in IPFs and the control group was verified by means of the unpaired Student's t-test. To compare the Serpin B3/B4 expression between low-grade dysplasia IPFs, high-grade dysplasia/cancer IPFs, DPLDs and normal lungs, one-way unbalanced analysis of variance was applied (GLM procedure).

Multiple comparisons were performed by means of Scheffe's test (95%CI for mean values were computed). In IPF patients, linearity of the relationships between Serpin B3/B4 value and other quantitative variables was tested by means of Pearson's correlation coefficient. As IPF patients showed significant impaired DLCO value they were categorised in two groups on the basis of median DLCO (lower and more than 20% predicted). Serpin B3/B4 expression and Ki-67 labelling index were evaluated by means of separate multiple regression mixed models using SASMIXED procedure with the restricted maximum likelihood method, in which degrees of dysplasia were fixed effects and individuals were random effects (151

RESULTS

All IPF lung samples showed major features of an advanced form of UIP with evident fibrotic architecture remodelling, particularly in the lower lobes. Careful morphological analysis detected peripheral foci of infiltrating carcinoma in three cases: one adenocarcinoma-mixed type (stage IIB) and two squamous histotypes (both stage IA). High-grade dysplasia foci, mainly squamous type, were found in seven cases. Fibrosis extension and profibrogenic cytokine TGF-b expression showed a mean value_SD (median value; range) of 34.8±7.8% (33.6%; 21.3–60.6%) and 38.1±8.5% (38.5%; 20.5–54.0%), respectively.

Serpin B3/B4 and Ki-67 immunohistochemical findings

Immunostaining for Serpin B3/B4 was detected in the cytoplasm of many metaplastic alveolar epithelial cells in all IPF cases (positive cell percentage of 37.5±12.1%). As expected, squamous metaplastic cells showed stronger staining and higher Serpin B3/B4 values (72.9±26.5%) than cuboidal (32.5±12.6%) and bronchial (36.0±12.2%), ($p=0.0001$, Fig. 1A–C). The mean of Serpin B3 expression in their native lungs was higher than that detected in lung biopsies (41.8 versus 22.8%). Positive staining for Serpin B3/B4 was observed in neoplastic and dysplastic epithelial cells, mainly in squamous type. In particular, patients with high-grade dysplasia or infiltrating carcinoma showed more increased Serpin B3/B4 expression in metaplastic epithelial cells than patients without (46.0±11.9% versus 35.3±11.4%; $p=0.01$) (Fig. 1D). When Serpin B3/B4 levels were compared between patients with high grade dysplasia and CIS and those with infiltrating cancer, higher values of Serpin B3/B4 were seen in those with cancer, although the difference did not reach statistical significance (49.3% versus 44.3%) also because of low power. Ki-67 labelling index in metaplastic epithelial cells was 6.2±5.1% (5.0%; 0.4-9.2%) which was significantly higher than that of the control group (non-IPF DPLDs) (0.5±0.7%; 0%; 0–2%; $p<0.0006$). Squamous, cuboidal and bronchial metaplastic epithelial cells showed different mean values of Ki-67 labelling index, significantly higher in squamous type ($p<0.0001$; Fig. 1E). Consecutive serial sections and confocal microscopy analysis showed coexpression of Serpin B3/B4 and Ki-67 in many metaplastic epithelial cells (Fig. 2A–E).
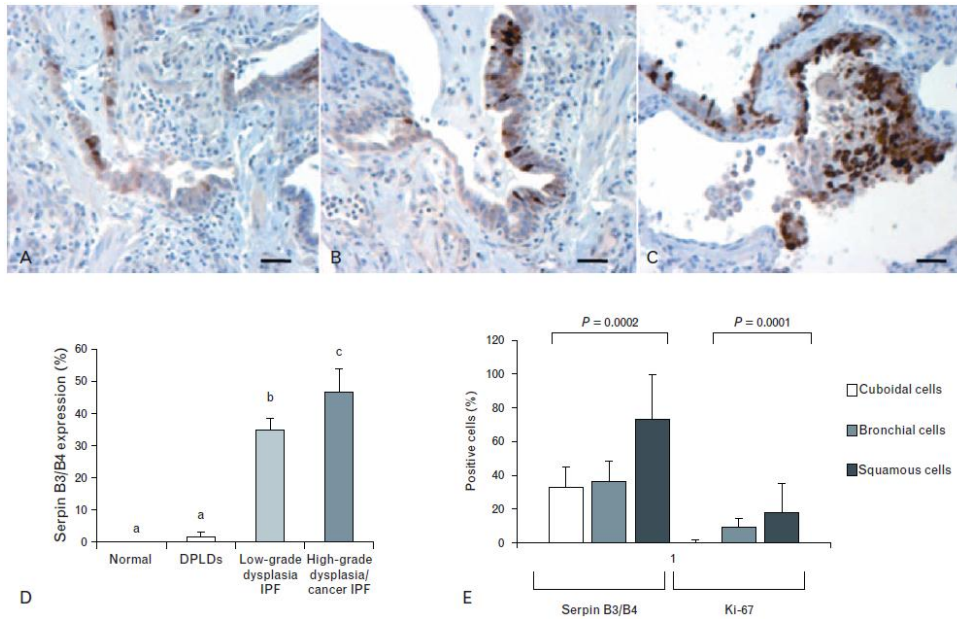
Fig. 1 Immunohistochemical detection of Serpin B3 and Ki-67 in lung samples. Immunohistochemistry for Serpin B3/B4 in an emblematic case of end-stage IPF. Strong cytoplasmic and/or nuclear immunoreactivity is found in the three metaplastic alveolar epithelial cell types: (A) cuboidal, (B) bronchial and (C) squamous. Scale bar 20mm. (D) Comparison of mean Serpin B3/B4 expression by type of sample lungs (p<0.0001). p value was performed by unbalanced one-way analysis of variance (GLM procedure). Vertical lines indicate 95%CI. Multiple comparisons were performed by Scheffe's test and statistical significance was indicated by different letters. (E) The histogram shows that squamous metaplastic cells have a stronger Serpin B3/B4 expression than cuboidal and bronchial cells (p<0.0001) and higher proliferative activity (Ki-67 labelling index) (p<0.0001) (p values were performed by multiple regression mixed models analysis. In the models, adjusted p for multiple comparisons were performed by means of Scheffe's test)
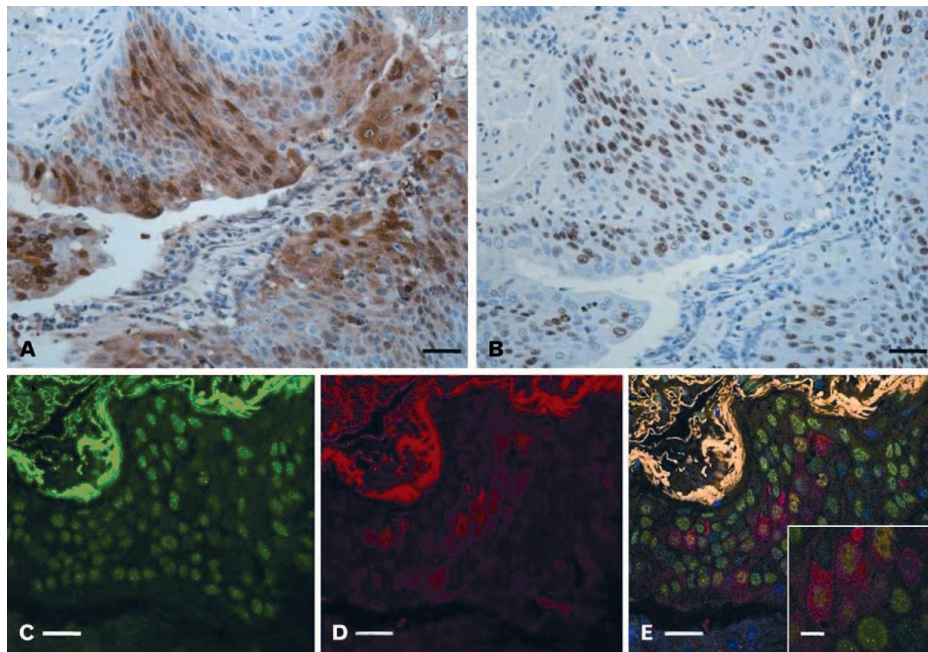
Fig. 2 Evaluation of Serpin B3/B4 and Ki-67 coexpression. (A) Immunohistochemistry of sequential serial sections for Serpin B3/B4 and (B) Ki-67 in end-stage IPF: note the strong positivity for both antibodies in the same cells. Scale bar¼10mm. (C) Immunofluorescence laser scanning microscopy analysis confirms the coexpression of Ki-67 (green) and (D) Serpin B3/B4 (red) (E) in the same metaplastic epithelial cells. Scale bar 10mm. Inset: higher magnification of double staining. Scale bar¼5mm.

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Serpin B3/B4 expression in relation to clinicopathological findings

The expression of Serpin B3/B4 was linearly and positively associated with age (r=0.33, p=0.02) (Fig. 3A). Patients with more impaired DLCO (as defined by a value lower than 20% predicted) showed a significantly higher expression of Serpin B3/B4 (41.4±8.8% vs 32.8±11.7%; p=0.04, Fig. 3B). The association between Serpin expression and DLCO levels was marginally significant (p=0.09) after including age at transplantation in the analysis as a possible confounder. A positive linear relationship was found to be statistically significant between Serpin B3/B4 expression and TGF-b value (Fig. 3C), as well as with Ki-67 labelling index (Fig. 3D).
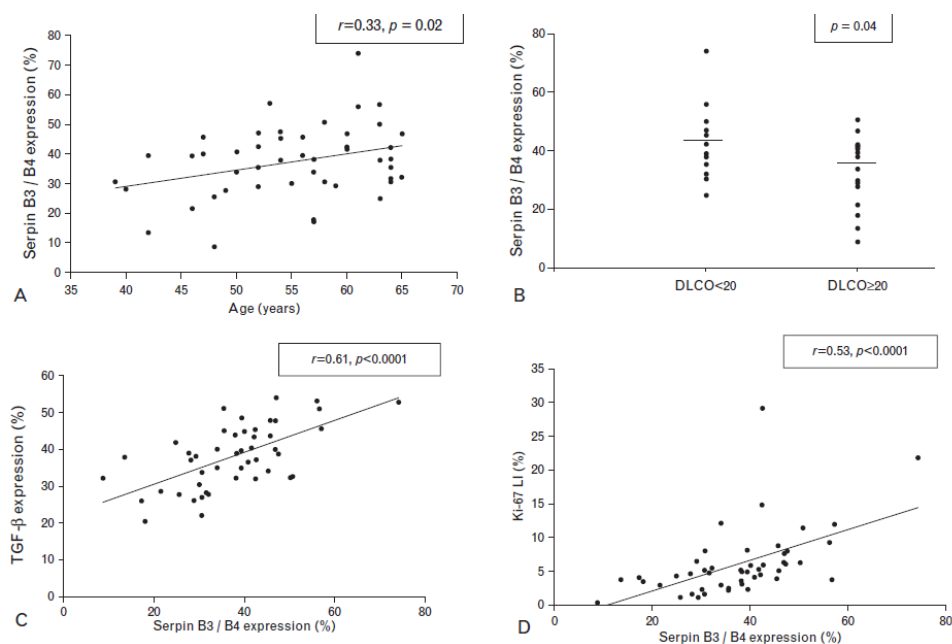

Fig. 3 Main clinico-pathological correlations. (A) Graphs show a direct relationship between Serpin B3/B4 and patient age (p=0.02). (B) Patients with DLCO <20% show higher Serpin B3/B4 expression than patients with DLCO _20 (p=0.04); DLCO was available only in 32 patients. (C) Serpin B3/B4 values were directly related to TGF-b expression (p<0.0001) as well as (D) Ki-67 labelling index (p=0.0001). Linearity of the relationships between Serpin B3/B4 values and other quantitative variables was tested by means of the Spearman non parametric regression coefficient.

The results of multivariate analysis indicated that only Serpin level is independently related to Ki-67 (p=0.0005).

Serpin B3/B4 molecular findings

Real-time RT-PCR showed that human Serpin B3 mRNA was expressed in all the examined lung samples of both IPF and control groups. In contrast, only IPF patients showed detectable threshold cycles for Serpin B4 mRNA. A wide deviation of Serpin B3 and B4 in IPF cases may be related to a different content of metaplastic epithelial cells present in the small frozen samples processed for molecular investigations.

DISCUSSION

In this study we observed an overexpression of Serpin B3/B4, particularly the isoform B4, that was detected in proliferating metaplastic epithelial cells of honeycomb areas which were more evident in IPF with high-grade dysplasia or associated lung cancer. Squamous metaplastic epithelial cells showed significantly higher Serpin B3/B4 expression and an increased proliferative index compared to the expression in the other metaplastic cells. Previous studies have reported a high incidence of lung cancer in IPF patients with more extensive squamous metaplasia and more frequent squamous histotype cancer (152). These data, together with our findings, support the idea that squamous cell metaplasia may be considered the major unstable epithelial phenotype (152,153,154-56,70,157). Serpin B3/B4 has been found overexpressed in patients with squamous cell carcinoma type of different organs (158,159) including lung cancer (157). A few previous studies reported a prevalent Serpin B4 isoform in dysplastic and malignant processes of squamous type (159,160).

The exact molecular mechanisms through which clade B Serpin, in particular Serpin B4, can drive this process is still not well known. In vitro studies have demonstrated that Serpin B4 inhibits cathepsin G and mast cell chymase (161) and attenuates apoptosis of tumour cells (162-165). The overexpression of Serpin B3/B4, particularly the isoform B4, in our IPF patients may be an important factor in the resistance of injured epithelial cells to apoptosis, thus leading to progressive lesions at high risk of malignant transformation. It is of interest that Serpin B3/B4 levels were more progressively increased from IPF at diagnosis as shown in our previous study (166) to end-stage and to end-stage IPF with cancer.

A direct relationship was found between Serpin B3/B4 and TGF-b expression and this validates our previous findings in a smaller number of IPF patients affected by less severe disease (166). Different works, in vitro and in vivo (both human and animal) have demonstrated a strict influence between Serpin B3 and TGF-b (166,167). The serpins have been shown to render cells more resistant to apoptotic cell death and proapoptotic effect of TGF-b might represent a counter-balancing mechanism leading to accumulation of fibrosis as a secondary effect. More recently, epithelial mesenchymal transition, typically ascribed for TGF-b (168) has also been related to Serpin B3 (169). The study has provided evidence that the antiprotease activity of Serpin could be implicated in the TGFb induction. The high level of TGF-b detected in

our advanced IPF patients may be mainly due to an enhanced number of proliferative metaplastic epithelial cells, which have been demonstrated to be an important source of this cytokine in lung fibrosis (166,167-173).

In our study increased tissue Serpin B3/B4 expression has a major influence on epithelial proliferation compared to other potential risk factors for cancer such as age and smoking status. The observation that smoking has no significant influence on Serpin secretion and epithelial proliferation is confirmed by an irrelevant Serpin expression and low epithelial proliferation rate detected in non-IPF DPLDs, in particular in heavy smokers (histiocytosis and desquamative interstitial pneumonitis). Although the patients included in the present study were all affected by end-stage disease, higher Serpin B3/B4 levels were observed in patients with more impaired DLCO. However, when multivariate statistical analysis is performed, only Serpin B3 was related to cell proliferation and this emphasises the important impact of Serpin on epithelial instability more than other clinical/functional parameters considered in the present study.

CONCLUSIONS

In conclusion, the investigation of tissue Serpin B3/B4 expression seems to be useful for more precise prognostic stratification of IPF patients with the aim of identifying those with higher epithelial instability and increased susceptibility to lung cancer. Serpin B3 and B4 are mainly retained in the cytosol but there are now several works that have shown an active or passive release into the circulation (174).

Similar pathogenetic mechanisms have recently been detected in two progressive fibrotic diseases (cirrhosis and IPF), both characterized by epithelial damage and neoplastic transformation. Additional lung cancers detected in explanted lungs, as those found in our cases, are usually unsuspected findings which have a significant impact on lung transplantation management and short-term survival (175). Further analysis of the SCCA-immunoglobulin M complex will lead to finding a potential biomarker for selection and monitoring of IPF patients as candidates for lung transplantation, particularly when single lung procedures are planned.

AIM OF THE SECOND STUDY

The aim of this second study was to investigate the relevance of respiratory viruses in patients with IPF, specially focusing on cases with PH. For this purpose IPF patients and control subjects including other non-IPF diffuse parenchymal lung diseases (DPLDs) were studied. Viral molecular data were correlated with mean pulmonary arterial pressure (mPAP) and arterial remodelling. Different clinical and morphological variables (including TGF-β expression) were studied by univariate and multivariate analysis at time of transplant and in the early post-transplant period.

A mouse model was used to examine whether murine gammaherpesvirus-68 (MHV-68), genetically and biologically similar to EBV, contributes to the development of vessel remodelling other than pulmonary fibrosis.

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MATHERIALS AND METHODS

Study population

Native lungs from 55 IPF patients who underwent lung transplantation (LT) between September 1998 and February 2010 in our Centre were studied (39 males and 16 females; mean age: 55.2 ± 9.2 yrs, 33 single vs 22 bilateral LT). The control group consisted of 22 native lungs from other non-IPF -DPLDs- (8 males and 14 females; mean age: 44 ± 11.4 yrs, 11 lymphangioleiomyomatosis, 4 Langerhans-cell histiocytosis, 2 sarcoidosis, 2 hypersensitivity pneumonitis 1 non-specific interstitial pneumonia, 1 desquamative interstitial pneumonia, 1 scleroderma lung fibrosis) and 19 normal lungs (13 non implanted donor lungs and 6 autopsy cases). Prior to LT after the diagnosis of IPF, the majority received oral prednisone while a minority were treated with prednisone and azathioprine.

Each patient underwent pulmonary function testing, high resolution computed tomography and right heart catheterization. These tests were performed in all patients at the time of waiting list inclusion and before LT. For the present study clinical/functional parameters collected at the time of LT were considered.

The diagnosis of interstitial lung disease was based on the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus Classification System (84). The clinical history was collected, with special emphasis on age at onset

of symptoms, smoking history, occupational exposure, co-morbidities and therapy (medical and non medical).

Written informed consent was obtained from all subjects. The work was approved by the Institutional Ethics Committee. Relevant clinical data of the IPF and non-IPF DPLD population are summarized in Table 1.

Table 1 Clinical data of the IPF and non-IPF DPLD population

	IPF patients (n=55)	DPLDs (n=22)
Age at transplantation (years)	55.1 (7.9)	44.1 (12.6)
Type of transplantation (bilateral)	22 (40.0%)	16 (72.7%)
Men	39 (70.9%)	9 (40.2%)
BMI	27.1 (4.0)	23.4 (5.2)
Smoking status		
never smoked	9 (16.4%)	10 (45.4%)
previous smoker	46 (83.6%)	7 (31.8%)
History of environmental exposure	3 (5.5%)	0
Co-morbidities		
Gastritis	5 (9.1%)	1 (4.5%)
Diabetes	6 (10.9%)	0
CHD	4 (7.3%)	0
Obesity	13 (23.6%)	2 (9.1%)
FEV1 (% of predicted value)	47.9 (16.2)	39.2 (25.3)
FVC (% of predicted value)	45.6 (14.5)	49.0 (22.4)
VC (% of predicted value)	43.4 (14.3)	51.0 (21.0)
TLC (% of predicted value)	53.4 (15.4)	71.4 (20.9)
DLco (% of predicted value)	26.0 (17.0)	23.5 (20.5)
mPAP	24.0 (8.9)	28.8 (15.4)

Data are number of patients (%) or mean (SE). IPF: idiopathic pulmonary fibrosis; DPLDs: Diffuse parenchymal lung diseases; BMI: body mass index; CHD: coronary heart disease.

Molecular viral detection

Nucleic acids were extracted from fresh lung tissues sampled from different areas (~1 mg of tissue) of all 96 cases (55 IPF patients and 41 control subjects) using a modified RNAzol method (176). Reverse transcriptase (RT)-polymerase chain reaction (PCR), PCR or nested-PCR were used to detect principal respiratory viral genomes: adenovirus, cytomegalovirus (CMV), EBV, rhinovirus, influenzavirus A and B, metapneumovirus, herpes virus (HHV)-6, HHV-7, HHV-8, parvovirus (PV) B19,

parainfluenzavirus 1 and 3 and respiratory syncytial virus (as heminested PCR) as previously reported (177). Samples were considered as true positive when the reproducibility of PCR analysis was verified a second time (177).

Since EBV was identified with high frequency in IPF patients, RNA-in situ hybridization-ISH was performed to identify the viral target cells.

Analysis of fibrosis extension, TGF-β expression and vessel remodelling

The extent of fibrosis was evaluated in Masson's trichrome stained sections analyzing 10 random fields using computer-assisted morphometric software (Image ProPlus® 5.1), as previously reported (178). The immunohistochemical detection and quantification of the profibrotic cytokine TGF-β (mouse monoclonal anti-TGF-β NovoCastra, Newcastle, UK), was performed as described before: immunostaining scores were based on the products of percentage positive cells multiplied by stain intensity (0 = negative, 1= weak, 2 = moderate, 3 = strong) in three different high power fields. Control sections were stained without the primary antibody, without primary and secondary antibodies, or with normal sera to control for background reactivity (178).

Arterial thickening was quantified using computer-assisted morphometric software on elastic-Van Gieson (EVG) stained lung sections focusing on muscular arteries of an average diameter of 300 µm (range: 100-500 µm). In particular, medial thickening (MT) was evaluated as previously described in at least 5 arterial sections. The same approach was used to measure intimal thickening (IT): MT% = (2 x medial layer thickening/external diameter) x 100; IT% = (2 x intimal layer thickening/external diameter) x 100 (179). For Ki-67 immunohistochemistry the sections were treated with normal serum (Immunotech, Marseille, France) and incubated for 60 min with the primary monoclonal antibody anti-Ki67 (MIB-1, Gene Tex, Irvine, CA) at a dilution of 1:100. Ki-67 expression positivity were evaluated only in blood vessel cell components. All evaluations were performed blindly.

Animal model

Female, 5 to 8 week old CD-1 mice [Hsd:ICR (CD1)] were purchased from Harlan Laboratories, UK and housed at the University of Liverpool under specific pathogen-free conditions. Mice were intranasally infected with 4 x 10^5 PFU MHV-68 and euthanized 7 days (n=3), 14 days (n=6) and 23 days (n=3) post infection (post infection). Uninfected mice (n=4) served as controls.

Immediately after death, lungs were collected and parts frozen at -80□C for RNA extraction: others were fixed in 10% buffered formalin and routinely embedded in paraffin wax.

Analysis of fibrosis extension, TGF-β expression and vessel remodelling

Consecutive paraffin-embedded sections were stained with haematoxylin and eosin for histology, with Masson's trichrome stain for fibrosis extension and with EVG for vessel remodelling, both morphometrically measured as above. The evaluation of arterial thickening focused on pulmonary arteries with an average diameter of 82 μm ranging from 43 to 100 μm. The assessment of vascular remodelling included an evaluation of proliferation of vessel cell components using Ki-67 immunostaining, as described above. Immunohistochemistry was also employed for the detection of MHV-68 antigen, to highlight lung macrophages (expression of lysozyme) and to demonstrate TGF-b expression (Genetex, Irvane, CA). Viral tRNA was demonstrated by RNA-ISH (180).

Statistical analysis

Data were analyzed using the SAS statistical software version 9.1.3 (SAS Institute, Cary, NC, USA). For quantitative variables the results are expressed as mean values and standard deviation if normally distributed, otherwise as median, Q1-Q3. The normality of distribution of quantitative variables was tested by means of Shapiro-Wilk statistics. For quantitative characteristics, differences between subjects with and without viral infection were evaluated by using the Mann-Whitney test. The prevalence of specific conditions was expressed as a percentage, and differences between groups were evaluated using the squared test and exact Fisher's test, as adequate.

Unadjusted and adjusted relationships between virus and clinical/morphological features were tested with general linear models (GLM procedure). In all analyses, a two-tail level for significance was set at 0.05.

Study population

Viral data, tissue and vessel remodelling

Patients with IPF showed a higher frequency of viral infection than control cases (40% vs 7.3%; *p*=0.0003). Normal lungs were all negative. Herpes viruses were the only detected genomes in IPF and EBV resulted as the most frequent, present in more than half of IPF patients. EBV was never detected in control cases (DPLDs and normal lungs). Gene sequencing of all amplicons showed a high homology (from 95% to 99%) with human viral genome sequences. RNA-ISH for EBV (EBER) identified viral RNA in 40% of EBV-PCR positive IPF cases. The positivity was detected in alveolar epithelial cells other than within monocytes/macrophages (Figure 1).
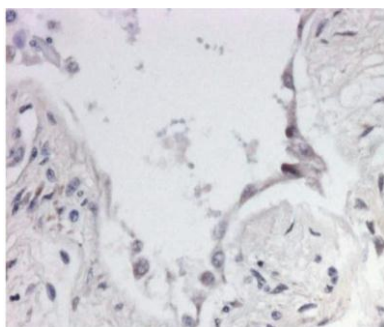

Figure 1: In situ hybridization for EBV. using an EBV-encoded RNA (EBER) probe shows EBER expression (arrows) in alveolar epithelial cells (EBER transcripts well seen in the nuclei of two alveolar epithelial cells) (arrows) . The positivity was mainly detected in alveolar epithelial cells more than within monocytes/macrophages. Real time PCR showed a high number of EBV genome copies (mean±SD:1085000±120208 copies/µl DNA).

Real time PCR showed a high number of EBV genome copies (mean±SD:1085000±120208 copies/µl DNA).   The extent of fibrosis in IPF lungs showed a mean of 36.7±12.3% (range: 14.6%-65.3%) and was significantly higher than in the DPLD control group (36.7±12.3% vs 15.4±15.4%, p<0.0001). Normal lungs from donors showed no evidence of pathological remodelling.

TGF-β expression in IPF lungs was mainly detected in macrophages (median, Q1-Q3: 100, 20-210) and metaplastic alveolar epithelial cells (120, 70-210). Median TGF-β scores in the alveolar epithelium were significantly higher in IPF patients than in the DPLD group (120, 70-210 vs 0, 0-0, p<0.0001). In normal lungs, TGF-β expression was restricted to only scattered intraalveolar macrophages. Arterial remodelling in IPF cases showed a median total thickness score of 43.8% (Q1-Q3: 35.7-53.1%) with IT and MT scores of 17.7% (Q1-Q3: 13.5-24%) and 26.2% (Q1-Q3: 21.8-28.9%)

respectively. These values were significantly lower in the DPLD group (total thickness: 37.9% vs 43.8%, *p*=0.03; IT: 12.3% vs 17.7%, *p*=0.005). Normal lungs did not show arterial remodelling (Table 2).

Table 2: Viral genome frequency and pathological data in IPF and control cases

	IPF patients (55)	Control cases (41)*	*p-values*
Virus positive cases	22/55 (40%)	3/41 (7.3%)	*0.0003*
EBV	13/22 (59.1%)	0	
HHV-6	7/22 (31.8%)	1/3 (33.3%)**	
CMV	4/22 (18.2%)	2/3 (66.7%)**	
PVB19	0	1/3 (33.3%)**	
Fibrotic extension, mean % (SD)	36.7 (12.3)	15.4 (15.4)***	*<0.0001*
Medial arterial remodelling, median % (Q1-Q3)	26.2 (21.8-28.9)	22.4 (18.9-27.5)***	*ns*
Intimal arterial remodelling, median % (Q1-Q3)	17.7 (13.5-24.0)	12.3 (9.0-17.0) ***	*0.005*
Total arterial remodelling, median % (Q1-Q3)	43.8 (35.7-53.1)	37.9 (31.7-44.6) ***	*0.03*
Macrophagic TGF-ß score,median % (Q1-Q3)	100 (20-210)	50 (10-180) ***	*ns*
Alveolar epithelial TGF-β score, median % (Q1-Q3)	120 (70-210)	0 (0-0)***	*<0.0001*

Correlation between viral molecular data and clinical/morphological parameters

Virus-positive IPF cases showed increased mPAP (28.6±10.9 mmHg vs 21.2±6.0 mmHg, *p*=0.01) and worse performance in the 6MWT (175.2±100 vs 300.5±138.8, *p*=0.002) than virus negative cases (Figure 2 A,B). The statistical value of these associations (mPAP and 6MWT) was still evident when EBV positive IPF patients were compared with both other virus positive and negative IPF cases (p<0.05 for both).
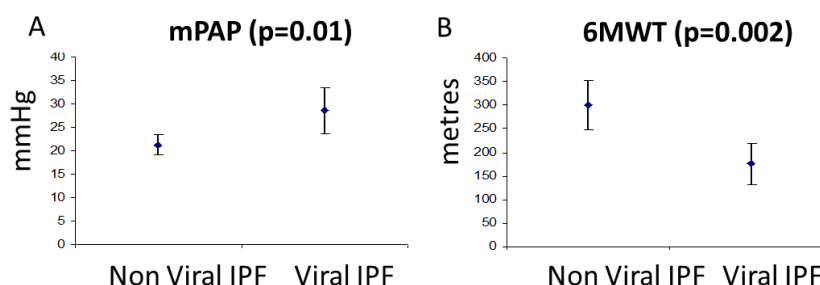

Figure 2. Mean pulmonary arterial pressure (mPAP) values. Significant higher mean value of mPAP (A) and worse 6MWT (B) in virus positive compared to virus negative cases.

Virus positive IPF cases showed a higher total thickening of muscular arteries (50.3%, 43.8-58.8% vs 39.5%, 34.7-45.7%, *p*=0.002) than virus negative cases. The intimal layer was most severely affected (21.8%, 17.2-26.8% vs 15.5%, 12.6-19.1%; *p*=0.004) (Figure 3 A,B,C,D).

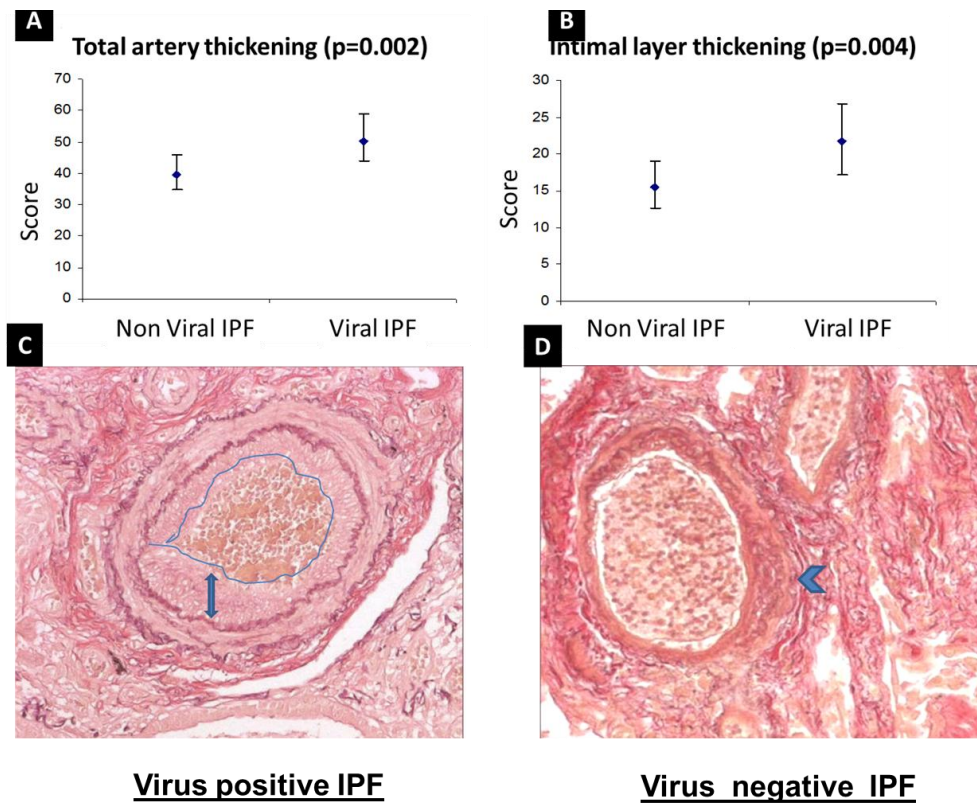**Virus positive IPF**   **Virus negative IPF**

Figure 3. Vascular remodelling in IPF lung tissue. Significantly increased arterial thickening (A) particularly of the intimal layer (B) is seen in virus positive cases. Elastic Van Gieson stained sections showed increased wall thickening (increased elastic and collagen fibers) especially of the intimal layer (arrow) in virus positive (C) compared to virus negative (D). Bar scale: 100 µm. L= lumen

Strong Ki-67 positivity was observed in both endothelial cells (CD31 positive, data not shown) and smooth muscle cells (smooth muscle actin positive, data not shown). Vessel cell proliferation was particularly seen in pulmonary arteries in proximity to metaplastic alveolar epithelial cells or macrophages. TGF-β scores were higher in virus-positive cases, although this was only statistically significant when epithelial expression was considered (score: 195, 140-210 vs 100, 40-120; *p*=0.002) (Figure 4 A,B,C,). The statistical value of these parameters (vessel remodelling and TGF-β

expression) was still evident when EBV positive IPF patients were compared with both other virus positive and negative IPF cases (p<0.05 for all).

metaplastic alveolar epithelial cells
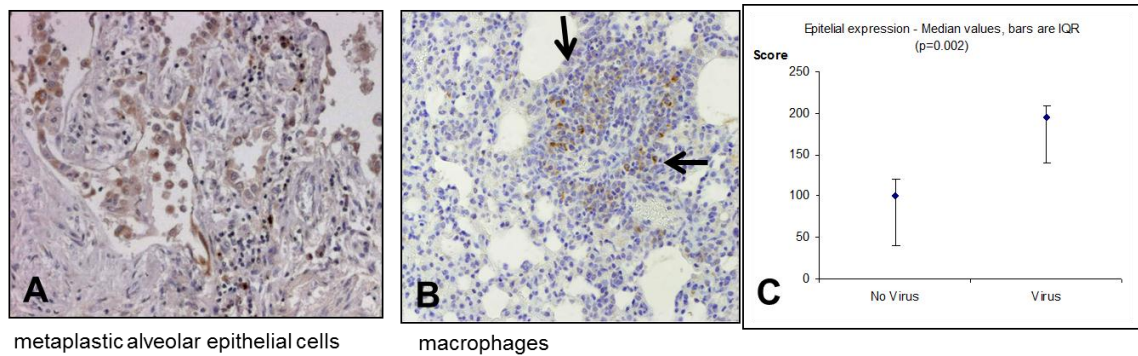
macrophages

Figure 4. TGF-β expression in IPF lung tissue. Extensive TGF-β immunostaining well seen in metaplastic alveolar epithelial cells (A) and macrophages (B). A significantly increased TGF-β median score value (bars are IQR) of epithelial cells is seen in virus positive cases and virus negative cases. Bar scale: 5 μm.

A summary of all main clinicopathological features in relation to virus infection among IPF patients is shown in the Table 3.

Viral presence was significantly associated with higher mPAP (p=0.03) after adjusting for other related covariates and intermediate factors (i.e.; age, sex, duration of disease, smoking history, and fibrosis extension). Moreover, viral presence was an independent marker of significantly poorer performance in the 6MWT (p=0.008) using the GLM adjusting for covariates (i.e. VC, FVC, DLCO and fibrosis extension).

Table 3. Clinicopathological features in relation to virus infection among IPF patients.

	virus positive (n=22)	virus negative (n=33)	P values
Age at diagnosis (years)	50.5 (7.8)	50.7 (8.1)	ns
Age at transplantation (years)	54.7 (8.6)	55.3 (7.6)	ns
Men	16 (72.7%)	23 (69.7%)	ns
BMI*	28.3 (3.9)	26.4 (4.1)	ns
Smoking status			ns
never smoked	6 (27.2%)	3 (9.0%)	ns
previous smoker	16 (72.8%)	30 (91.0%)	ns
pack/years	23.5 (30.2)	22.1 (19.3)	ns
Time from symptoms to transplantation (months)	61.5 (35.0)	59.3 (32.0)	ns
Family history of IPF	0	0	-
FEV1	49.2 (16.3)	47.1 (16.4)	ns
FVC	44.6 (14.5)	45.8 (14.3)	ns
VC	43.8 (14.2)	43.2 (14.6)	ns
TLC	52.7 (15.6)	53.9 (15.8)	ns
DLco	21.3 (12.7)	28.6 (18.7)	ns
Mean PAP (mmHg)	28.6 (10.9)	21.2 (6.0)	*0.01*
Distance of 6-min-walk (m)	175.2 (100)	300.5 (138.8)	*0.002*
Use of supplemental oxygen	21 (95.4%)	29 (87.8%)	ns
Use of prednisolone or equivalent	16 (72.7%)	22 (66.7%)	ns
Fibrotic extension	34.8 (8.8)	38.1 (14.3)	ns
Median arterial remodelling	27.8 (5.9)	24.7 (4.8)	ns
Intimal arterial remodelling	23.2 (7.5)	17.2 (7.9)	*0.004*
Total arterial remodelling	60.0 (10.6)	41.6 (10.8)	*0.002*
Epithelial TGF-β‡ expression	169.5 (71.3)	99.9 (64.5)	*0.002*
Macrophagic TGF-β expression	140.0 (106.1)	99.7 (87.0)	ns

MHV-68 infected CD1 mice

Tissue and vessel remodelling

Analysis of MHV-68 DNA load showed, as previously demonstrated, that viral infection peaked on day 7 post infection (181).

The histological examination of uninfected control animals did not identify any pathological changes. On day 7 post infection, all infected animals exhibited increased,

macrophage-dominated interstitial cellularity, mild type II pneumocyte hyperplasia and perivascular inflammatory infiltration. The latter infiltrates were associated with slight patchy collagen deposition. Scattered individual macrophages and metaplastic alveolar epithelial cells were found to express MHV-68 antigen. Viral latency, represented by the expression of viral tRNA, was detected in type II pneumocytes and alveolar macrophages. On day 14 post infection, mild multifocal, almost diffuse fibrosis of alveolar septa was observed with a median of 26% (range: 17-38%). In some animals, this was associated with random patches of collagen deposition. Numerous macrophages were seen within the interstitium and often around arteries. In addition, some type II pneumocytes exhibited TGF-β expression.

Arteries were assessed for vessel remodelling and compared for the thickening scores. In uninfected control mice, the average score was 16.5. Seven and 14 days post infection, it was 18 and 24.5 respectively (Figure 5 A,B,C,D). The extent of fibrosis was slightly more marked in mice with vessel wall thickening. On day 23 post infection both the parenchymal and vessel remodelling were less evident.
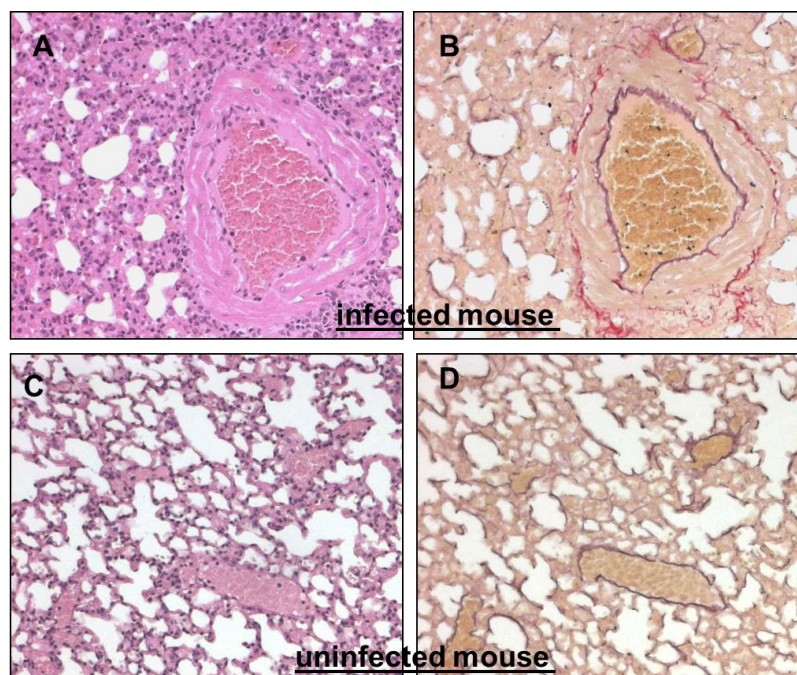

Figure 5. Vascular remodelling in lung tissue of MHV-68 infected CD1mice. Haematoxylin-Eosin (A, C) and Masson' trichrome (B, D) stained sections: marked arterial thickening is seen in a MHV-68 infected mouse (A and B, scale bars: 20 µm) in comparison to an uninfected mouse (C and D). Bar scale: 20 µm. A: small artery; V: venule.

DISCUSSION

In the present study different respiratory viruses were investigated in lungs from a large cohort of IPF patients and the control group (non IPF DPLDs and normal lungs) displaying the prevalent role of herpes viruses. The work highlights the broad impact of herpesviral infection, particularly EBV, in the disease emphasizing its association with a more severe disease phenotype as IPF with associated higher mPAP and worse 6MWT. A multiple logistic regression analysis demonstrated that herpesviral infection was independently associated with more marked vessel remodelling, higher value of PAP and worse performance in the 6MWT. Of particular note is that neither clinical data (smoking history, age at diagnosis and at transplant, duration of the disease, BMI, lung volumes and hypoxemic respiratory failure) nor morphological changes (fibrosis extension) had more significant impact than viral infection. Of particular note is that the presence of viral infection had also a negative influence on the early graft function. Only one previous study performed on 24 IPF patients reported more rapid disease progression in EBV positive cases. The majority of viral cases died from respiratory failure at a mean of 41 months follow up. In the study PH was not specifically considered (182). The reported frequency of herpes viruses in IPF lungs ranges from 30 to 100%. These differences may be related to technical sensitivity, disease heterogeneity or selection of patients. The presence of herpes viruses detected in our cases may even be underestimated due the fact that the molecular investigation was performed on tissue samples from end-stage diseases. EBV, the most frequent herpes virus detected in IPF, has a well known epitheliotropism other than lymphotropism. Several works have detected EBV in alveolar epithelial cells of IPF lungs, thus confirming the concept that these cells represent the principal viral target in IPF (183-188) The role of herpes viruses, important contributing factors for the development of pulmonary fibrosis, has been emphasized in different experimental models and the TGF–β signalling pathway seems to play a crucial role in the profibrogenetic action.

To the best of our knowledge no attempt has been made to specifically investigate the influence of herpes viruses on arterial remodelling and PH in IPF patients.

Several types of viruses, particularly those of the herpes family, have been found to be associated with vessel remodelling, development of atherosclerosis and clinical features of hypertension (189-190). Vasculotropism of gamma herpes viruses (such as HHV-8, EHV-5 and MHV68) has been demonstrated in lung parenchyma of patients and animals (horses) with PH even if a causal relationship still remains quite debated (190-192). Up to today, there is little evidence of a "direct" role of viral agents in the pathogenesis of PH. Even when more significant arterial intimal thickness could more convincingly suggest a direct viral endothelial injury, in our work EBV was never detected in endothelial cells of IPF cases. The presence of chemokines and cytokines, viral protein components, and increased expression of growth and transcriptional factors released at the site of infection could contribute to further recruitment of inflammatory cells and proliferation of smooth muscle and endothelial cells. An interesting finding of our study was the most frequent distribution of remodelled vessels with proliferating components in proximity to metaplastic epithelial cells and/or macrophages, both cell types considered as principal targets of EBV. Injured epithelial or endothelial cells involved in tissue and vessel remodelling are considered an important source of growth factors and mediators, among which TGF-β plays a key role. Although the function of TGF in vascular cell growth in *vivo* has not been well defined, in vitro and experimental studies have demonstrated an important influence of this cytokine in muscle/fibroblast proliferation, endothelial-mesenchymal transition, and extracellular matrix production of intimal and medial layers (193). In our study significantly higher TGF-β levels detected in our viral IPF cases as well as in MHV-68 infected mice suggest an indirect influence of viral infection on vessel remodelling through this cytokine even if TGF-β expression was not significantly related to arterial thickening. Similar data were found by Farkas L. et al. in a different experimental model of pulmonary fibrosis (194). The authors detected high levels of active TGF-β in areas with increased fibrogenesis and pulmonary artery remodelling. At day 14, this was significantly associated with pulmonary hypertension.

The demonstration of a direct causal relationship between herpesvirus infection and vessel remodelling/PH in IPF would require longitudinal studies of the same patients, an impossible task with lung tissue but attainable with bronchoalveolar lavage or peripheral blood samples. However this limitation has been partially overcome in the present study using a laboratory MHV-68 infected mouse model. Indeed, in these animals 2 weeks after infection significant arterial remodelling and increased TGF-β

expression was seen, as those observed in clinical lung specimens from IPF patients with high mPAP.

CONCLUSION

In conclusion, our results demonstrated a different phenotype of virus-positive IPF patients. In particular virus-positive IPF cases showed more pronounced vessel remodelling and a higher mPAP, a complication that severely affects negatively prognosis of the disease. While there is large mechanistic evidence of epithelial herpesvirus-associated alveolar injury, the effect of these viruses on the pulmonary vasculature in IPF merits investigation. A deeper knowledge of viral-induced pathways in endothelial cells could give new insights for a targeted therapeutic approach of this important complication in the subgroup of patients (virus positive cases). In this context, the high degree of similarity between MHV-68 infection of CD-1 mice and virus positive IPF indicates that this is an excellent model with which to study pathogenesis and interventions.

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