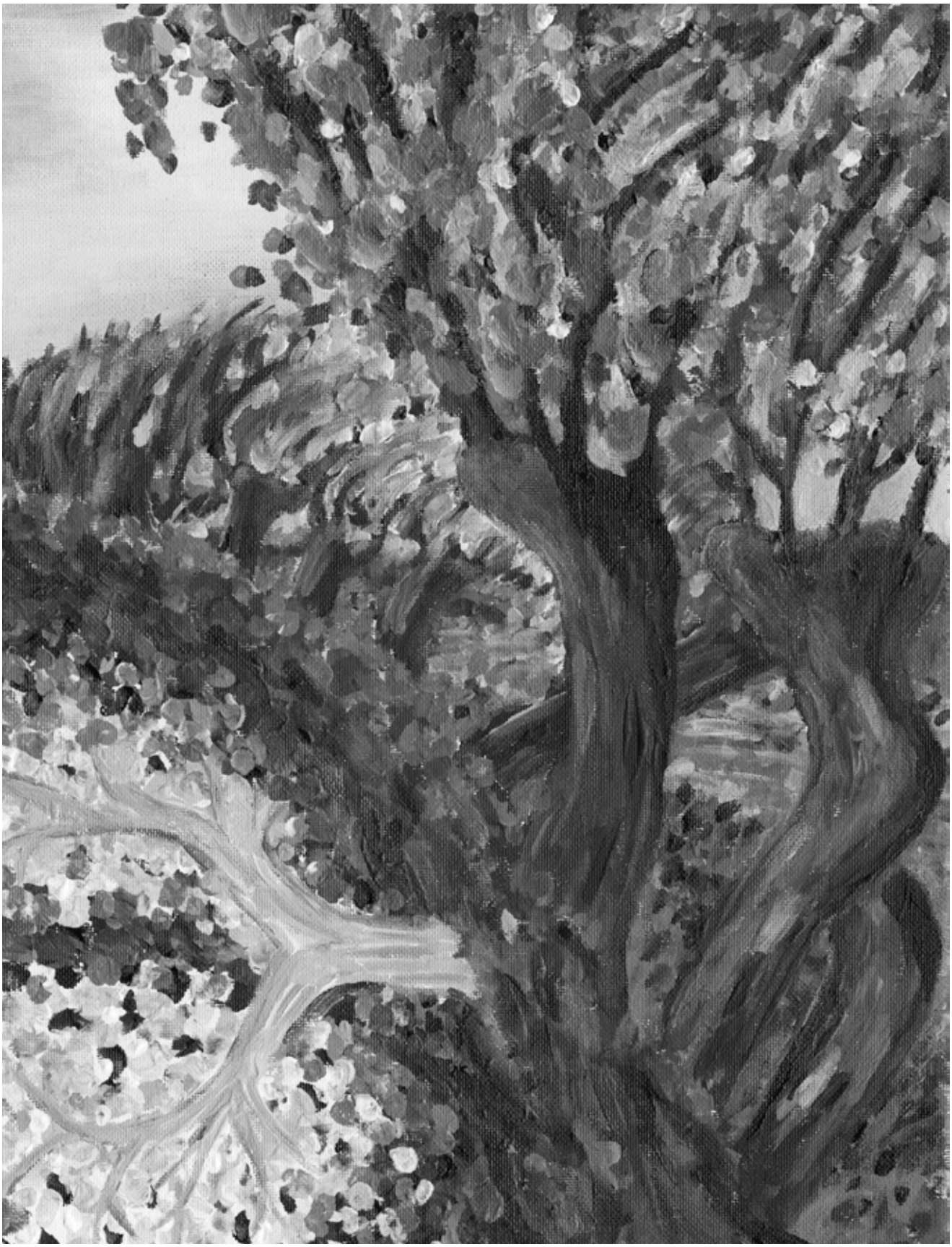

PROGNOSTIC BIOMARKERS in Idiopathic Pulmonary Fibrosis

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Prognostic biomarkers in idiopathic pulmonary fibrosis
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Prognostic Biomarkers in Idiopathic Pulmonary Fibrosis

Prognostische biomerkers voor idiopathische longfibrose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 17 september 2013 des ochtends te 10.30 uur

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1

GENERAL INTRODUCTION

IDIOPATHIC PULMONARY FIBROSIS

Definition

Idiopathic pulmonary fibrosis (IPF) is the most common of 7 forms of idiopathic interstitial pneumonias (IIP), as described by the ATS/ ERS/JRS/ALAT in 2011.[1] Other types of IIP include desquamative interstitial pneumonia (DIP), respiratory bronchiolitis interstitial lung disease (RB-ILD), non-specific interstitial pneumonia (NSIP), acute interstitial pneumonia (AIP), cryptogenic organising pneumonia (COP) and lymphocytic interstitial pneumonia (LIP).

IPF was formerly known as cryptogenic fibrosing alveolitis (CFA). This entity comprises the whole family of idiopathic interstitial pneumonias that shared the clinical features of shortness of breath, diffuse pulmonary infiltrates and varying degrees of inflammation and fibrosis on biopsy.[2] Former studies included different forms of interstitial pneumonias under the term of IPF. In 2002, the ATS/ ERS defined a statement in which the clinical, radiological and pathological manifestations of IPF and the other six interstitial pneumonias are extensively described. Because these conditions are rare and few physicians have substantial experience there was a need for a uniform guideline. Patients were commonly misclassified as having an idiopathic interstitial pneumonia, while there was an association with collagen vascular diseases or drug exposure. Further, IPF and the other interstitial pneumonias generally have a different prognosis and response to therapy, therefore the distinction of IPF from other forms of IIP is highly important.

Nowadays the diagnosis of IPF is strictly bound to the definition of the ATS/ ERS/JRS/ALAT: a chronic progressive fibrosing interstitial pneumonia of unknown cause, occurring primarily in adults, limited to the lungs and associated with the histological and/ or radiological usual interstitial pneumonia (UIP) pattern. The definition requires exclusion of other forms of IIP and interstitial lung diseases associated with environment, systemic disease or medication.[1] While the ATS/ERS statement in 2002 highlighted the distinction of IPF from the other IIPs, the newest statement includes recommendations to empower clinicians to make appropriate decisions regarding diagnosis and treatment.

Epidemiology

A population-based study for IPF in the United States revealed a prevalence of 42.7 cases per 100,000.[3] The prevalence of IPF was largely age-dependent and varied between 4.0 per 100,000 persons aged 18 to 34 years to 227.2 per 100,000 among those who were 75 years or older. The incidence of IPF was estimated at 16.3 per 100,000 persons. However, one should keep in mind that the definition in this study was

based on the ICD-9 diagnosis code, which is less accurate than the ATS/ ERS criteria. European studies show variable results. In Finland the prevalence was estimated at 16 – 18 per 100,000,[4] in Norway 23.9 per 100,000[5] while the incidence of IPF was estimated to be even lower in Spain[6] (7.6 per 100,000) and Belgium[7] (1 per 100,000).

Diagnosis

Clinical features

Dyspnea and a non-productive cough are the most common clinical features. Generally, IPF presents around the age of 60 and is more common in men.[8] A detailed occupational history is important as several occupational factors are associated with IPF. Exposures such as farming, livestock, hairdressing, birds, stone polishing and wood and stone dust are significantly more common in IPF patients than in healthy controls.[9] Further, a history of cigarette smoking is also associated with an increased risk for the development of IPF.[10] Physical examination often reveals fine bibasilar inspiratory crackles (Velcro rales) and in 25 to 50% digital clubbing is present.[11] Lung function parameters usually show a restrictive pattern with a reduced diffusion capacity, although in the early phase pulmonary function tests and chest radiographs may be normal.

BAL

Bronchoalveolar lavage fluid (BALf) may contain increased amounts of neutrophils and eosinophils. Lymphocytosis is not a typical UIP feature and in case of lymphocytosis one should think of other causes such as NSIP or hypersensitivity pneumonitis (HP).[12] The prognostic value of BAL is questionable. Lymphocytosis has been associated with better survival, and eosinophilia and neutrophilia have been associated with diminished survival.[13-15] However, current studies do not agree on a prognostic value of BALf cellular profiles. Since there is no consensus about the prognostic role of BAL, the clinical value of BALf cellular profiles to predict survival in IPF is limited. The value of BAL is currently restricted to the exclusion of other diagnoses.

Radiology

UIP is characterized on HRCT by the presence of honeycombing, reticular opacities and traction bronchiectasis.[1,16] Ground glass attenuation may be present, but is not a typical UIP feature. Often architectural distortion is present due to extensive fibrosis. A characteristic UIP pattern is distributed mostly peripheral and basal, although often patchy. Honeycombing on HRCT usually correlates with honeycombing on lung biopsy and ground glass attenuation may correlate with inflammatory mononuclear cell infiltrates.[17] If honeycombing is absent, but other radiologic features meet the criteria for UIP, the radiologic pattern is characterized as possible UIP, and surgical lung biopsy is needed to make a definite diagnosis (table 1).[1]

Histology

In case of a possible UIP pattern on HRCT, a surgical lung biopsy is required for accurate diagnosis. Key histologic features of the UIP pattern on surgical lung biopsy are architectural destruction, fibrosis often with honeycombing, scattered fibroblast foci, a patchy distribution and involvement of the periphery of the acinus or lobule. There is a heterogeneous distribution, with alternating areas of normal lung, fibrosis and honeycombing. The peripheral subpleural parenchyma is most severely affected. If a biopsy does not show a definite UIP pattern, the biopsy may be termed as nonclassifiable fibrosis. However, in the absence of an alternative condition, such biopsies may be consistent with IPF after careful multidisciplinary discussion (table 2 and 3).

Table 1 HRCT criteria for a radiologic UIP pattern.[1]

UIP pattern (all 4)	**Possible UIP (all 3)**	**Inconsistent with UIP (any of the 7)**
Subpleural, basal predominance	Subpleural, basal predominance	Upper or mid lobe predominance
Reticular abnormalities	Reticular abnormalities	Peribronchovascular predominance
Honeycombing with or without traction bronchiectasis	Absence of features inconsistent with UIP	Extensive groundglass attenuation
Absence of features inconsistent with UIP		Profuse micronodules
		Discrete cysts
		Diffuse mosaic attenuation/ airtrapping
		Consolidation in bronchopulmonary segments/ lobes

Table 2 Histopathological criteria for UIP pattern.[1]

UIP pattern (all 4)	Probable UIP pattern	Possible UIP pattern (all 3)	Not UIP pattern (any of 6)
Evidence of marked fibrosis/ architectural distortion, ± honeycombing in a predominantly subpleural distribution	Evidence of marked fibrosis/ architectural distortion, ± honeycombing	Patchy or diffuse involvement of lung parenchyma by fibrosis, with or without interstitial inflammation	Hyaline membranes
Patchy involvement by fibrosis	Absence of either patchy involvement or fibroblast foci, but not both	Absence of other criteria for UIP	Organizing pneumonia
Fibroblast foci	Absence of features against UIP	Absence of features against UIP	granulomas
Absence of features against UIP	OR		Marked interstitial inflammatory cell infiltrate
	Honeycomb changes only (end-stage fibrotic lung disease)		Predominantly airway centred changes
			Other features suggestive of an alternative diagnosis

Table 3 Combination of HRCT and lung biopsy for the diagnosis of IPF

HRCT pattern	Lung biopsy pattern (when performed)	Diagnosis of IPF?
UIP	UIP Probable UIP Possible UIP Non-classifiable fibrosis	Yes
Possible UIP	Not UIP	No
Possible UIP	UIP Probable UIP	Yes
Possible UIP	Possible UIP Non-classifiable fibrosis	Probable UIP
Possible UIP	Not UIP	No
Inconsistent with UIP	UIP	Possible UIP
Inconsistent with UIP	Probable UIP Possible UIP Non-classifiable fibrosis Not UIP	No

Pathogenesis

The hypothesis of the pathogenesis of IPF has changed over time. Originally, one thought that parenchymal fibrosis was mainly the result of an ongoing chronic inflammatory process.[18] An unidentified trigger was thought to cause a cycle of inflammation and injury, ultimately leading to fibrosis. The assumption was that interrupting the inflammatory cascade would prevent fibrosis, which explains the initial thought to use anti-inflammatory therapy, such as corticosteroids in the treatment of IPF. However, recent insights show that anti-inflammatory therapy with corticosteroids and/ or azathioprine do not improve the natural evolution of IPF.[1] Together with the lack of inflammatory infiltrates on surgical lung biopsies this led to a new hypothesis. Nowadays, the hypothesis is that a still unidentified stimulus causes repeated episodes of acute lung injury. A lack of proper re-epithelialization and unregulated proliferation of fibroblasts leads to aberrant wound healing and ultimately fibrosis.

Normally, the alveolar basement membrane is lined with alveolar epithelial cells (AEC).[19] Ninety-five percent is covered by squamous type I AECs, which are responsible for gas exchange. The remaining 5 % consists of the cuboidal type II AECs. Type II cells secrete surfactant, function as antigen presenting cells and are progenitor cells that can regenerate the alveolar epithelium after injury. Biopsies often show a denuded basement membrane, which indicates increase death of type I and II AECs or a lack of the proliferative capacity of the type 2 AEC, or a combination of both.[20] A consistent histological feature in lung biopsies is the greatly reduced number of type I AECs. Type I AECs appear more susceptible to injury than AEC type II cells, leaving AECs type II cells behind, which are able to re-epithelialize the denuded basement membrane.[21]

Denudation of the basement membrane initiates the release of epithelial growth factors, such as keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF).[22] KGF and HGF both promote proliferation of AECs, resulting in local expansion of the amount of type II AECs on the alveolar basement membrane. In addition, AECs also synthesize growth factors and cytokines that activate fibroblasts, such as TGF-β. TGF-β induces the synthesis of extracellular matrix molecules, and is a potent inducer of a process called epithelial-mesenchymal transition (EMT), a process whereby fully differentiated epithelial cells undergo transition to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts.[23] Other factors that are secreted by type II AECs and stimulate the activation and proliferation of fibroblasts are platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-α) and endothelin-1 (ET1).[20]

In IPF, a locally disturbed coagulation is also thought to contribute to the accumulation of extracellular matrix. A local pro-coagulant environment promotes fibrosis. The plasminogen activation system is critical to normal wound healing. Plasminogen is responsible for the degradation of fibrin clots. Plasminogen activity

is negatively regulated by plasminogen activator inhibitor1 (PAI-1), which is produced by AECs. AECs are able to modulate intra-alveolar coagulation through upregulation of tissue factor (TF) after injury.[24] In IPF there is an increased amount of PAI-1 in bronchoalveolar lavage fluid, together with increased amounts of TF.[25] This results in an increased anti-fibrinolytic and pro-coagulant environment.

Prognosis

Before 2002, IPF was part of a heterogeneous group of interstitial pneumonias and prognosis was different compared to prognosis nowadays. Until then, the mean survival of cryptogenic fibrosing alveolitis was described to be 3.2 to 6 years,[26-29] based on few studies with and without lung biopsies, sometimes in the presence of systemic disease. Bjoraker and colleagues were one of the first who described the differences in the pattern on surgical lung biopsy in relation to prognosis.[30] They described 104 patients with cryptogenic fibrosing alveolitis who had undergone a surgical lung biopsy. The median survival of patients who had a UIP pattern on biopsy was 2.8 years, which was consistently shorter than patients with other forms of histologic patterns of interstitial pneumonia.

After 2002, the definition of IPF became more strict and consequently, estimated survival time worsened. Using narrow criteria (UIP on a surgical lung biopsy specimen or a definite UIP pattern on HRCT), median survival was 3.5 years[31] according to Fernandez Perez et al.

The rate of progression varies between individual patients and clinically three different forms of progression can be described; slowly progressive IPF, rapidly progressive IPF and periods with relative stability interposed by periods of rapid acceleration. The slowly progressive form of IPF is characterized by gradual decline in lung function and worsening of dyspnea without any periods of acceleration. The mean rate of decline in FVC is between 130 to 210 ml FVC per year.[32] In the above mentioned study of Fernandez Perez et al,[31] only 10 out of 47 IPF patients had a slowly progressive respiratory decline without any evidence for acute respiratory worsening that required hospitalization.

Patients with rapidly progressive IPF were characterized by Selman et al. A subset of patients has a short duration of symptoms before first presentation, with rapid progression to death, in contrast to patients who experienced symptoms for a few years before presentation. They divided 114 IPF patients in 26 rapid progressors (< 6 months of symptoms before presentation) and 88 slowly progressors (> 24 months of symptoms before presentation). Median survival in the rapid progressors was 13.5 months, compared to 90 months in the slow progressors. No differences in age, lung functional alterations, oxygen saturation, extent of HRCT changes and BAL cell profile

were found between the groups, but there were significantly more males and smokers in the group of rapid progressors. The rapid progressors displayed a different gene expression profile compared to the slow progressors. Boon et al.[33] analysed lung biopsies of 6 rapid progressors (defined as > 10% decline in predicted FVC and >15% decline in predicted DLCO in 12 months) and 6 slow progressors (< 10% decline in predicted FVC and < 15% decline in predicted DLCO in 12months). Similarly, a distinct molecular signature was found at the time of diagnosis in the rapid progressors compared to the slow progressors.

Periods of acute respiratory worsening are often referred to as acute exacerbations. The definition of acute exacerbation is a rapid deterioration (< 1 month) in symptoms, lung function and radiology, in the absence of known causes of deterioration, such as infection, heart failure, pulmonary embolism or other known causes.[34, 35] Risk factors for acute exacerbations are concomitant emphysema and low DLCO.[36] Mortality rates are generally high but may vary between 22 and 100%.[34]

Martinez and colleagues[37] described 168 patients in the placebo group of a trial evaluating interferon-γ in order to analyse the clinical course of patients with mild to moderate IPF. One third of the patients had a total of 95 hospital admissions in the 76 weeks of observation. The most commonly reason for hospitalization (33%) was presumed infection. In the observation period 36 patients (21%) died. Death was considered to be IPF related in 89% of the patients; progression of IPF was the primary cause of death in 56% of the patients. This is comparable to other studies, most studies state that progression of IPF is the most important cause of death, followed by infection, heart failure, bronchogenic carcinoma and pulmonary embolism. [38, 39]

Treatment

A lot of trials have been performed in IPF patients, but none of them improved survival. However, some treatment strategies have shown some benefit. Pirfenidone may decrease the rate of decline in VC and may increase the progression free survival time over one year.[40] Another promising treatment is nintedanib (BIBF 1120), this is a triple tyrosin kinase inhibitor and a potent antagonist of growth factors such as platelet-derived growth factor, vascular endothelial growth factor and basic fibroblast growth factor. It is currently evaluated in phase III studies as a potential IPF therapy. Preclinical data demonstrated that BIBF 1120 is able to slow the progression of lung fibrosis and to improve the disease outcome.[41]

Since the amount of clinical trials in IPF is still growing and mostly resulted in no effect or maybe little effect in a subgroup of IPF patients, the ATS/ ERS/ JRS/ ALAT defined a statement in which recommendations are made against certain treatments. There is a strong recommendation against corticosteroid monotherapy, colchicine,

cyclosporine A, combined corticosteroid and immune-modulator therapy, interferon-γ, bosentan and etanercept. Weak recommendations against the following therapies have been stated: combined acetylcysteine, azathioprine and prednisone, acetylcysteine monotherapy, anticoagulation and pirfenidone. This means that these therapies should not be used in the majority of patients, but can be reasonable in a minority.[1] After this statement two studies were published which also strongly discouraged acetylcysteine, azathioprine and prednisone[42] and warfarin,[43] leaving only acetylcysteine monotherapy and pirfenidone behind as potential therapies. Considerations are to refer to a specialized centre for lung transplantation when appropriate, participation in clinical trials, treat gastro-esophagal reflux disease if present, long-term oxygen therapy when resting hypoxemia is present, or just supportive care. It is important not to harm patients with side-effects of treatment which did not prove any benefit.[1]

IPF is after COPD the second most frequent disease for which lung transplantation is performed and within the interstitial lung diseases the most common among referrals for lung transplantation.[44] IPF patients have the highest waiting list mortality compared to other diseases.[45,46] This pleads for early referral to a transplantation centre.[47]

Biomarkers

Biomarkers can act as surrogates for clinically meaningful outcomes. Research on biomarkers in IPF focusses on two different clinical utilities. Diagnostic biomarkers reflect the presence of IPF, and can ideally discriminate IPF from other interstitial pneumonias. Prognostic biomarkers however, reflect the disease severity or progressiveness of the disease and add prognostic information about mortality. Currently there are no validated biomarkers that are routinely used in the clinical care of IPF patients.

Krebs von den Lungen-6 (KL-6) is a mucin-like glycoprotein that is expressed at the extracellular surface of alveolar type II cells and bronchial epithelial cells. KL-6 acts a chemotactic factor that promotes migration and proliferation for fibroblasts.[48] Both BALf and serum KL-6 concentrations are elevated in IPF patients and are correlated to each other, which may reflect the increased production of KL-6 by the lungs.[49] In relation to other interstitial lung diseases, serum KL-6 levels in IPF are significantly elevated.[50] Increased serum KL-6 was associated with worse survival, and serum KL-6 levels were independently associated with survival in a multivariate analysis.[51]

Surfactant proteins also have diagnostic and prognostic abilities. Surfactant proteins decrease surface tension in the alveoli, but also play a role in the host defense mechanisms against pathogens. Both surfactant protein-A (SP-A) and surfactant protein –D (SP-D) can distinguish IPF patients from other interstitial lung diseases.[50] Serum SP-A levels are significantly increased in IPF patients and are independently associated with survival.[52-54] Serum SP-D were as well elevated in IPF patients and

associated with survival.[52, 53]

YKL-40 is a chitinase-like protein that regulates cell proliferation and survival. It is not specific for IPF and is also increased in fibrotic liver disease, sarcoidosis, COPD and asthma.[55] YKL-40 has mitogenic effect on lung fibroblasts and induces alveolar macrophages to release profibrotic and pro-inflammatory cytokines.[56] Immunohistochemical staining has demonstrated that YKL-40 localizes to lung fibroblasts and alveolar macrophages adjacent to fibrotic areas in PF patients.[56] YKL-40 levels are elevated in BALf and serum of IPF patients and serum levels are negatively correlated to DLco.[56] Moreover, increased serum YKL-40 levels (> 79 ng/ml) were associated with worse survival.[57]

Matrix metalloproteinases are a structurally and functionally related family of proteases involved in the breakdown of extracellular matrix components.[58] BALf matrix metalloproteinase 7 (MMP7) levels are increased in IPF, however this is not specific as levels are not significantly different from patients with other interstitial lung diseases.[59,60] BALf and serum MMP7 levels were negatively correlated with forced vital capacity (FVC) and DLco. In a large study, Richards et al. demonstrated that plasma levels of MMP7 were independently associated with increased mortality.[61]

CC chemokine ligand 18 (CCL-18) is a chemokine that is expressed at high levels in the lung. CCL18 is derived from alveolar macrophages and attracts lymphocytes to the lung.[62] In IPF patients, CCL18 measured in serum and BALf is significantly increased and is negatively correlated with total lung capacity (TLC) and DLco.[63] In a study from Prasse et al. baseline serum CCL18 was correlated to changes in TLC and FVC over a six month period.[64] Multivariate analysis revealed that a high serum CCL18 (> 150 ng/ml) was independently associated with survival in IPF patients.[64]

Genetic background

Familial IPF

The majority of patients with IPF present at an age older than 55 years, but especially patients with a familial form of IPF may present at a younger age. Familial pulmonary fibrosis (FPF) is defined as the presence of at least two first-degree members of a biological family having one form of IIP.[65] [4] The first registration of familial IPF is from Sandoz in 1907, who described 18-year-old twin sisters, who both died from slowly progressive respiratory failure. Their post-mortem exam showed end-stage honeycombing.[66] Multiple other studies confirmed familial clustering of IPF later on. Two to nineteen percent of IPF patients have been reported to have at least one first-degree family member with some form of IIP.[65] Familial cases appeared to be younger, but were otherwise not distinct from sporadic IPF.

Surfactant protein mutations have been implicated in the development of IPF.

In 2001, Amin et al described a, 11-year old girl with progressive lung disease, whose mother and half sister also suffered from interstitial lung disease at a young age.[67] In all three family members, levels of surfactant protein C were below detection limits in bronchoalveolar lavage fluid, while surfactant protein A and D were decreased. Sequence analysis of the protein C gene, however did not reveal an apparent mutation. Later on, Thomas and colleagues[68] described 11 adults and three children having lung disease similar to IPF. In three affected family members, a heterozygous leucine to glutamine substitution was found in the c-terminal of region of the protein. This region appears to be critical for intracellular trafficking and folding of the protein. However, the mutation was also found in two unaffected family members, suggesting incomplete penetrance. In our cohort of patients with IIP in the St Antonius hospital, 10% of the IIP patients (n = 229) had a form of FPF and within the FPF group, 25% could be explained by a mutation in SFTPC.[69]

In addition to the association between SFTPC mutations and FPF, other genes encoding for surfactant proteins were investigated. Wang et al performed whole genome linkage analysis in 29 family members having pulmonary fibrosis, bronchoalveolar cell carcinoma or undefined lung disease. They identified two mutations in SFTPA2, the gene encoding for SP-A2, one the two different isoforms of SP-A. The underlying mechanism which causes fibrosis may be similar to the mutations in SFTPC; the accumulation of unfolded surfactant protein may lead to endoplasmatic reticulum stress in alveolar epithelial cells, causing apoptosis.[70]

In 2007, Armanios et al. described mutations in genes encoding telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) to be responsible for 8 % of the cases of FPF.[71] Mutations in these genes resulted in telomere shortening that leads to apoptosis, including the alveolar epithelial cells. In the study performed by Cronkhite et al.[72], even up to 37% of the familial cases and 25% from the sporadic cases had significantly shortened telomeres. These patients were selected not having a TERT or TERC mutation, making telomere shortening a plausible underlying mechanism in IPF, even in patients without mutations.

Genetic predisposition

Although SFTPC, TERC and TERT mutations form a genetic basis for some familial cases of IPF, they are not associated with all cases of familial IPF. In addition, the majority of patients with IPF are sporadic cases, without a family history. Therefore, the development of IPF is likely to be determined by multiple genetic factors that each contributes to a modest effect on predisposition to this disease. Candidate genes that have been analysed for this disease are primarily involved in inflammatory responses. Although many candidate genes for a key role in IPF pathogenesis can be proposed,

only limited numbers have demonstrated confirmed associations.

A great number of studies evaluated the association between idiopathic pulmonary fibrosis and cytokine or cytokine receptor polymorphisms. Polymorphisms in IL1A, IL1B, IL1RN, IL6, IL8, IL8RA, IL8RB and TNF have been examined.[73-77] Only a few positive associations have been demonstrated. In cohorts of English and Italian IPF patients, the presence of single base variations at position +2018 in the IL1RN gene conferred a significant increased odds ratio for the presence of pulmonary fibrosis.[75] However, other studies of polymorphisms in this gene did not demonstrate the association with pulmonary fibrosis.[73, 77] Two separate investigations have demonstrated an increased frequency of a guanine to adenine substitution at position – 308 in the TNF-α gene. One study in Australia included 22 patients and 140 controls[77] and another investigation included a cohort of English and Italian IPF patients.[75] Both observed a significant association of this polymorphism with IPF, but a different study including 72 IPF patients did not demonstrate a significant difference in allele frequencies.[76] Thus, it is not entirely clear if alterations on the TNF gene are associated with IPF. Further, one study demonstrated an increased frequency of an IL6 promoter intron 4 polymorphism in an English population,[76] and another reported an increased frequency of a polymorphism involving a C to G substitution in exon 33 of the CR1 gene in patients with IPF.[78] However, both associations could not be confirmed by other investigators.[79]

A promotor polymorphism in the MUC5 gene was found to be associated with IPF.[80] This SNP was also associated with the predisposition to familial interstitial pneumonias (34% versus 9% in healthy controls), but the minor allele of this polymorphism was also increased in IPF patients (38%). MUC5B expression was increased in IPF patients, and the variant allele was associated with upregulation of MUC5B expression in unaffected subjects. These findings were replicated in two independent cohorts.[81]

A genome-wide association study identified an association of a genetic variation in the TERT gene with susceptibility to IPF.[82] This SNP in intron 2 of the TERT gene encodes a reverse transcriptase that is a component of a telomerase.

Genetics and prognosis

Most studies about genetics in IPF describe the association between genetic variability and susceptibility to IPF, only a few studies investigated the association of genetic variability to disease severity or prognosis.

Molina-Molina et al[83]. investigated the association between the G-6A polymorphism of the AGT gene with IPF development, severity and progression. Angiotensin II (ANG II) induces alveolar epithelial cell apoptosis, enhances fibroblast proliferation and lung collagen production, and increases transforming growth factor (TGF)-β_1 synthesis. The

G-6A polymorphism of the angiotensinogen gene is associated with IPF progression but not with disease predisposition. The presence of the A allele was strongly associated with increased alveolar arterial oxygen tension difference during follow-up, and thus a prognostic worse phenotype, however the presence of the A allele was not significantly associated with other changes in pulmonary function tests during follow-up.

Further, Xue et al.[84] hypothesized an abnormal adaptive immune response in IPF and thus investigated HLA allele frequencies. They evaluated the HLA Class II allele frequencies of IPF patients and found over-representation of HLA-DRB1*15 in a cohort of 79 IPF subjects who had undergone lung transplantation compared to the normal reference population. IPF patients with DRB1*1501 tended to have decreased DL_{CO} compared to the subjects who lacked this allele.

Genetics and biomarkers

The association between genetic variability and biomarkers has not been investigated in IPF patients, but we know from other diseases that certain SNPs can influence the concentration of biomarkers. For example, angiotensin converting enzyme (ACE) is used in the diagnosis and follow-up of sarcoidosis. ACE activity is influenced by the ACE I/D polymorphism. As a consequence, ignoring genotype may result in 8.5% misclassification of 'elevated' versus 'normal' ACE or vice versa.[85] Thus, for the interpretation of levels of biomarkers, it is important to know the impact of genetic variation on biomarker levels.

Surfactant protein D (SP-D) is one of the biomarkers under investigation in IPF. SP-D is mainly synthesized in type II pneumocytes and contributes to the function of surfactant in the alveoli.[86, 87] In IPF patients, serum SP-D is elevated and related to disease extent and progression. [52, 53] Genetic variation in the gene encoding for SP-D results in significantly different serum SP-D levels in healthy controls. Serum SP-D levels are under influence of the Met11Thr polymorphism; subjects carrying the Met variant (T allele) have higher serum SP-D levels. Constitutional SP-D serum levels are predominantly under control of genetic factors and the Met11Thr polymorphism determines half of this genetic component.[88]

Krebs von den Lungen – 6 (KL-6) is another potential biomarker in IPF. KL-6 is a lung specific antigen on mucin (MUC)1. High concentrations of KL-6 are present in bronchoalveolar lavage fluid (BALf) and serum of IPF patients and correlate with prognosis.[89, 90] Serum KL-6 levels are dependent on the functional adenosine to guanine mucin-1 (MUC1) gene polymorphism. Serum KL-6 levels were higher in subjects carrying the G allele of the 568 A to G polymorphism. A similar effect was observed in sarcoidosis patients, despite the 3-fold increase of overall KL-6 levels compared to healthy subjects.[91]

Moreover, YKL-40 is a serum biomarker in diseases with fibrosis, inflammation and tissue remodeling and is increased in serum and BAL from IPF patients. Comparable to the above mentioned biomarkers, serumYKL-40 levels are also under influence of genetic variation in the corresponding encoding gene.[92] The - 329 polymorphism was associated with both serum and BALF YKL-40 levels in IPF patients.[57] Thus, for the interpretation of biomarkers, it can be useful to investigate the influence of SNPs on serum levels and to take into account that values can be misclassified as high or low partly due to genetic variation.

AIM

IPF patients have an extremely poor prognosis, this is due to the facts that there is still no satisfying treatment available for IPF and the high waiting list mortality. Since it is an uncommon disease which is slowly progressive, both patient and doctors delay occur, with delayed referral to a specialized centre. One of the future goals is to create greater awareness among doctors in community-based hospitals to refer immediately when IPF is suspected. Moreover, we need tools to give priority to IPF patients on the lung transplantation waiting list. Since one third of the IPF patients dies while on the waiting list[46] and mortality in IPF patients is still the highest among all diseases, the current scoring system is apparently not sufficient for IPF patients in the Netherlands.

The debate about the pathogenesis of IPF is still not closed, inflammation or not, that's the question. Studies in which the pathogenesis of IPF is investigated show increased expression of proteins which are involved in inflammation, fibrogenesis, coagulation and apoptosis in lung tissue, BALF or serum. One can hypothesize that these proteins might serve as a biomarker for the severity or prognosis of IPF. A biomarker is a molecule that indicates an alteration in physiology from normal. There are different types of biomarkers, and the discrimination between diagnostic and prognostic features of a biomarker is important. A diagnostic biomarker must be sensitive and specific, a prognostic biomarker must predict the progressiveness of the disease. In order to give IPF patients priority on the lung transplantation waiting list we need a biomarker which predicts the rate of progression. The discrimination between slow or rapid progressive disease at the time of diagnosis might help in this. Further, since a great proportion of patients (10-50%) develops an acute exacerbation[34] which is often fatal, prediction of acute exacerbations would contribute to adequate prognostication. This thesis focuses on the need for a prognostic biomarker. The perfect prognostic biomarker for IPF is not invasive, easily reproducible, and predicts decline and prognosis.[93]

The above considerations were the motivation to start research in this field. In the following chapters an overview is given of potential prognostic biomarkers in IPF.

OUTLINE OF THE THESIS

Chapter 2 gives an overview from studies describing molecular and non-molecular markers that can predict prognosis in IPF, according to the definition as stated by the ATS/ ERS in 2002.

In **chapter 3,** the cohort which forms the basis for further research is extensively described. A cohort of IPF patients has not been described in the Netherlands since the ATS/ ERS statement, and since further research would be based on this cohort, a comparison is made between this cohort and IPF patients in other studies.

Chapter 4 evaluates the value of surfactant protein-D (SP-D) to predict prognosis in IPF patients. SP-D levels in serum and the Met11Thr polymorphism are determined in IPF patients and healthy controls. In IPF patients serum SP-D levels were related to prognosis.

In **chapter 5** the potential of endothelin-1(ET-1) as a prognostic biomarker in IPF is described. In order to gain insight into the role of ET-1 in the pathogenesis of IPF and to evaluate the potential of ET-1 as a biomarker in IPF, we measured ET-1 in serum and bronchoalveolar lavage fluid (BALf) of IPF patients and healthy controls and related this to clinical parameters.

Chapter 6 describes the susceptibility and disease modifying effects of genetic variations in the IL1RN and IL1B gene in IPF. The changed balance between IL-1Ra and IL-1β in serum and BALf in IPF patients compared to healthy controls is evaluated.

In **chapter 7** the influence of SNPs in the CCL18 gene on CCL18 expression and survival is evaluated. Serum CCL18 and CCL18 expression in PBMCs from healthy controls were related to genotype of the CCL18 polymorphism. CCL18 levels in IPF patients were under influence of the CCL18 polymorphism and were related to survival.

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PREDICTING PROGNOSIS IN IDIOPATHIC PULMONARY FIBROSIS

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a parenchymal lung disease characterized by progressive interstitial fibrosis. In 2002, the ATS/ERS published new criteria that significantly changed the definition of IPF, resulting in a more homogeneous group of patients. IPF has a poor prognosis with a median of 2.5 –3.5 years, but varying from a few months to a decade. In order to predict survival at diagnosis or during follow-up, a considerable number of studies were conducted identifying promising prognostic biomarkers. However, many had been performed before the new ATS/ERS consensus and included patients who would not meet current IPF criteria. This review provides an overview of prognostic markers of survival in IPF after the ATS/ERS consensus statement in 2002. Molecular biomarkers in serum, especially so-called pneumoproteins are relatively easy to obtain and have been independently replicated as predictors of prognosis. Cellular constituents of bronchoalveolar lavage (BAL) have been investigated as predictors of survival, but results remain contradictory. Further, a robust marker of prognosis is the change in lung function over time. However, calculating change in lung function is usually only possible over a 6 -12 months period, and is therefore not useful at first presentation. The extent of fibrosis on HRCT scan and the number of fibroblast foci on lung biopsy can be measured at presentation and correlate with prognosis, but the applicability of these markers is being hampered by the lack of user- and patient friendliness. In conclusion, a number of biomarkers are potential candidates for an individualised prognosis of IPF, of which so-called pneumoproteins appear most promising and should be a major focus of future research.

INTRODUCTION

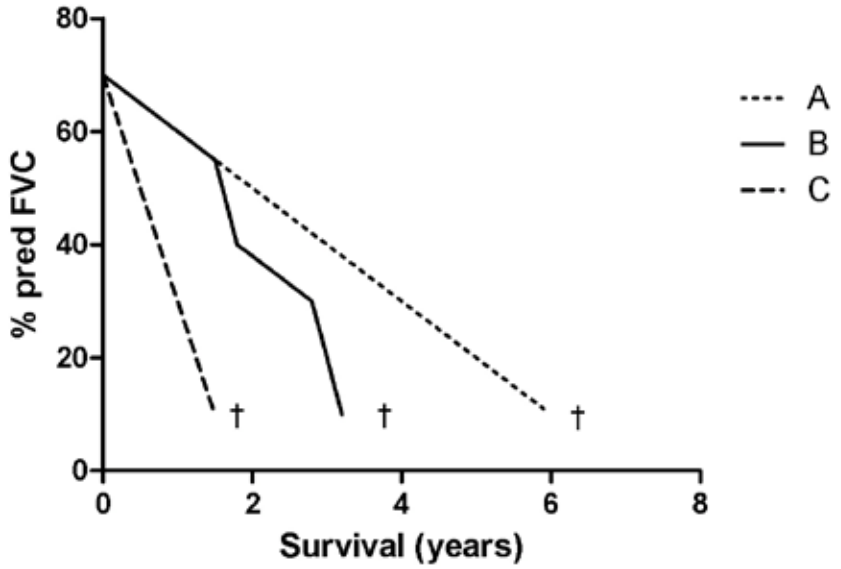
Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease of unknown etiology. It is the most common of seven entities of interstitial pneumonia, as described by the American Thoracic Society/ European Respiratory Society consensus statement in 2002.[1] Clinically, IPF is characterized by dyspnea and worsening of lung function. The disease course of IPF is unpredictable, but in general mean life expectancy varies between 2.4 and 4.2 years.[2-5] However, survival from an individual patient may vary from a few months to almost a decade. Despite extensive basic research and several clinical trials, no therapy has yet been proven to prolong survival.[6] Therefore, optimal timing of referral for lung transplantation is crucial and dependent on accurately predicting survival for an individual patient.

The clinical history of IPF has been studied in detail in the placebo arm of a clinical trial. The investigators noted frequent hospitalizations for respiratory disorders and although pulmonary function parameters such as FVC changed little during 72 weeks of follow-up, acute clinical deterioration preceded death in half of the patients who died of IPF.[7] This indicated that the incidence of acute exacerbations, which are defined as subjective worsening over 1 month, new radiographic infiltrates, the absence of an identifiable etiology, and acute alveolar injury on biopsy,[8] form a substantial part of the IPF-related deaths.

The natural history of IPF is difficult to predict. Patients with apparently similar stages of disease severity may come to a pulmonary physician, but one may demonstrate rapidly progressive disease with a survival of only one or two years, while another may show a survival time of more than 6 years. Hypothetically it appears as if there are at least three different patterns of disease behaviour and survival,[7, 9] which are depicted in figure 1. Pattern A is characterized by a slowly progressive decline in lung function; pattern B is characterized by one or more episodes of rapid deterioration, the last one with fatal outcome; and pattern C is characterized by a devastating rate of deterioration from the first presentation of disease with survival of less than 1 to 2 years. Ideally, pulmonary physicians caring for these patients would have the availability of biomarkers for an early differentiation between these patterns.

A great number of studies have focused on the identification of determinants of prognosis in IPF. However, many of these studies have been conducted before the ATS/ ERS consensus statement, which highlighted the importance of distinguishing usual interstitial pneumonia (UIP) from non-specific interstitial pneumonia (NSIP), and other types of interstitial pneumonia.[1] Especially NSIP and UIP appear to be different entities in terms of treatment and prognosis. Before 2002, patients with idiopathic interstitial pneumonias that would now be categorised as NSIP, were often lumped

Figure 1 Hypothetical clinical courses of IPF.
A: slowly progressive IPF;
B: slowly progressive with two episodes of rapid deterioration;
C: rapidly progressive IPF.



together with IPF/UIP patients, and this would certainly have influenced the results of these studies.

In an attempt to provide an up to date overview of molecular and non-molecular markers that can predict prognosis in IPF, one needs to consider the studies that only included IPF patients according to the new definition. The aim of this review is to provide such an overview of determinants of prognosis in definite IPF patients. A table which summarizes all relevant studies evaluating prognostic biomarkers is available in the appendix.

Baseline characteristics that influence survival

The incidence of IPF is lower in females[10] and it is has been found that the female sex is associated with a better survival.[4] Recently, Han et al.[11] demonstrated that males indeed showed a greater rate of disease progression. The survival benefit for the female sex persisted after adjustment for relative change in desaturation and percentage predicted FVC. The influence of age at the time of diagnosis on survival has also been described but is less convincing. Some studies reported an unfavourable prognosis in case of an age older than 50 years, but these studies were all from before the ATS/ERS consensus statement,[12-14] In recent studies, however, age did not turn out to be a significant factor in multivariate analysis.[15, 16] In fact, as NSIP patients are generally younger than IPF patients,[17, 18] misdiagnosis could possibly account for the survival differences in former studies.

A relationship between body mass index (BMI) and mortality has recently been demonstrated by Alakhas and colleagues.[19] They studied 197 patients with IPF that were categorized into three groups according to BMI: < 25, 25-30, and > 30. Median survival was 3.6, 3.8 and 5.8 years respectively. Although the exact underlying mechanism remains to be elucidated, it is an interesting finding that higher BMI is associated with better survival. Unfortunately, BMI > 30 is regarded as a contraindication for lung transplantation.[20]

Predictive markers in blood

In 1999, Hermans and Bernard extensively described lung epithelium-specific proteins and their applications as biomarkers in serum.[21] These so-called pneumoproteins, are thought to occur in the bloodstream due to increased permeability of the alveolar-capillary membrane and increased secretion by regenerating alveolar type II cells. Krebs von den Lungen 6 (KL-6) is a lung specific antigen on mucin (MUC)1 that also displays chemotactic activity for human fibroblasts.[22] High concentrations of KL-6 are present in bronchoalveolar lavage fluid (BALf) and serum of IPF patients. A Japanese group found that initial serum KL-6 levels could predict long-term survival in IPF patients. A total of 27 IPF patients were followed up during at least 3 years. At the optimal cut-off level of 1000 U/ml, patients were categorized as having low or high serum levels of KL-6. Although the study involved only a small cohort of IPF patients, a significant difference in survival was observed between patients with low and high serum levels of KL-6 levels.[23]

Surfactant proteins are produced by type II pneumocytes and contribute to surfactant homeostasis and local immune defence. Surfactant proteins A (SP-A) and D (SP-D) can be detected in serum and are elevated in patients with IPF, pulmonary alveolar proteinosis (PAP) and interstitial pneumonia associated with collagen diseases.[24-26] The prognostic value of SP-A and SP-D was first described by Takahashi et al. in 2000.[27] Kinder et al. independently confirmed these results in a large and well-characterised cohort of IPF patients.[15] They found an association between serum levels of SP-A and SP-D and mortality in 82 patients with biopsy-proven IPF. In the first year after the diagnosis, each increase of 49 ng/ml in concentration of serum SP-A was associated with a 3.3 fold increase of mortality risk, after controlling for age, gender, smoking, lung function parameters and BALf neutrophil percentage. The association of serum SP-D with survival was less obvious, but showed a trend towards significance. Adding serum SP-A and SP-D levels to a statistical model for IPF prediction demonstrated a significant improvement compared to clinical characteristics only.

CC chemokine ligand 18 (CCL-18) is a chemokine that is expressed at high levels in the lung. It is produced by macrophages and attracts lymphocytes to the lung. CCL-

18 also stimulates fibroblast proliferation and collagen production.[28] Baseline serum CCL18 concentrations in IPF patients are associated with the change in TLC and FVC at 6 month follow up, and a significant higher mortality was observed in the group who had serum CCL-18 levels > 150 ng/ml. Thus, serum CCL-18 levels were found to be highly predictive for the change in lung function parameters and survival.[16]

Recently, another interesting finding was the occurrence of fibrocytes in blood as indicators of prognosis. Fibrocytes are circulating mesenchymal progenitor cells which are involved in tissue repair and fibrosis. Fibrocytes were significantly elevated in IPF patients compared to healthy controls and ARDS patients, with a further elevation in patients with an acute exacerbation. Fibrocyte numbers were not correlated with lung function impairment or radiological extent of disease, but they were an independent predictor of mortality within 2 years of follow-up.[29]

Interestingly, changes of markers of oxidative stress in exhaled breath condensate, sputum and serum have been found in IPF patients. IPF patients seem to have lower anti-oxidant capacity and higher levels of reactive oxygen species than healthy controls. The role of anti-oxidants and reactive oxygen species seems promising, both in the pathophysiology of the disease, as well as markers reflecting disease severity. This has extensively been reviewed[30-34] and seems to be a promising direction for new diagnostic and prognostic markers, however associations with mortality have not been described yet. Future studies are necessary to determine the prognostic value of these markers.

Predictive markers in BALf

The relationship between cell types in bronchoalveolar lavage fluid (BALf) and the clinical course of patients with IPF has been the subject of several studies. The first studies showed that increased numbers of eosinophils were associated with increased mortality and that lymphocytosis was associated with a better prognosis.[35-37] However, these studies were all conducted before the new ATS/ ERS classification of IIPs in 2002.[1] After the new classification, three studies on this subject with a considerable number of patients were conducted. Ryu et al. included 87 pathologically confirmed UIP and 35 NSIP patients in their study.[17] They found that UIP patients had a higher number of neutrophils (7%) compared to NSIP (3%) and that lymphocyte count was higher in NSIP patients (29%) compared to UIP (5.5%). The pathologic diagnosis of NSIP seemed to be the best predictor of longer survival. When only UIP patients were included in the analysis, lymphocytosis was the only predictor of longer survival. Of note, Ohshimo and colleagues recently described that increased lymphocytes in BALf in patients with suspected IPF are indicative of an alternative diagnosis, i.e. chronic extrinsic allergic alveolitis and idiopathic NSIP, which underlines the importance of

disease homogeneity in the search for predictive markers for IPF in BALf.[38] The predictive value of lymphocytosis could not be confirmed by Kinder et al. who included 156 biopsy-proven IPF patients and did not find an association between lymphocytosis or eosinophils and survival.[39] Interestingly, they found that BALf neutrophil percentage was the only independent predictor of death and that this relation was most prominent in the first years of follow-up and attenuated over time. Another study, from Veeraghaven et al. did not find any association between cellular profiles in BALf and prognosis at all.[40]

Next to cellular components of BALf, other proteins in BALf can also be informative in the context of estimating prognosis. Matrix metalloproteinases (MMPs) degrade all of the extracellular matrix components of the interstitium and may play a role in abnormal alveolar permeability. MMP-8 and MMP-9 levels in BALf were significantly elevated in those patients who showed rapid lung function decline compared to patients who showed slow deterioration. Patients who died during 3-year follow-up showed higher MMP-8 and MMP-9 levels compared to those who did not die, but BALf levels did not predict survival time.[41] MMPs are also detectable in serum, but have not been described to predict survival yet.[42, 43]

Pulmonary function parameters

Lung function impairment at diagnosis is indicative of the severity of the disease, but does not necessarily reflect the progressiveness of the underlying pathological process. It requires at least two measurements with a substantial time interval, usually 6-12 months, to collect this information. Change in lung function parameters over time has therefore been proven to be a better predictor of survival than baseline values at the time of diagnosis. Collard et al. evaluated the predictive value of changes in clinical and physiologic variables over time for survival in 81 patients with biopsy-proven IPF.[44] Six and 12- month changes in dyspnea score, total lung capacity (TLC), forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), diffusing capacity of carbon monoxide (DL_{CO}), partial pressure of arterial oxygen (p_aO_2) and oxygen saturation (s_aO_2) were predictive of survival time even after adjustment for baseline values. That changes in these variables predict survival, suggests that the rate of progression is independent of the initial degree of severity.[44, 45] Interestingly, the change in FVC over time appeared to be superior in predicting prognosis compared to the histological pattern. After 12 months of follow-up, the distinction between biopsy-proven IPF and another idiopathic interstitial pneumonia, namely NSIP, provided no additional prognostic information, once serial pulmonary function trends had been taken into account.[46, 47]

Cardiopulmonary exercise testing (CPET), especially V_{O2max}, integrates

pulmonary function with cardiovascular and neuromuscular function[48] and has been shown to be significantly related to mortality in IPF. In a retrospective analysis of 117 IPF patients, V_{O2max} did not predict survival when examined as a continuous variable, but a threshold of 8.3ml/min/kg was associated with an increased risk of mortality.[49] The importance of reduced oxygen uptake as a predictor of prognosis also follows from the clinical radiographic and physiologic (CRP) score. A decrease in p_aO_2 during CPET is one of the constituents of the CRP score and contributes for 10.5% to this CRP score that estimates survival. The CRP score is derived from a cohort of 238 biopsy-proven IPF patients and integrates smoking status, clubbing, extent of radiographic profusion, pulmonary hypertension, TLC (% pred) and p_aO_2 at maximal exercise.[50]

The six minute walk test (6MWT) is relatively easy to perform: the patient is instructed to walk as fast and as far as possible in 6 minutes. Desaturation < 88% during the test has been shown to be a strong predictor of mortality. Biopsy-proven IPF patients who desaturate during a 6MWT had an increased risk of dying during a median follow-up time of 3 years.[51, 52] Further, the 6 minute walk distance (6MWD) also showed to be highly predictive of mortality. Lederer et al. investigated waiting list mortality in IPF patients listed for transplantation. They found that a lower 6MWD was associated with increased mortality. A cut-off value of 207 m was used to identify patients at a high risk of mortality and showed to be a better predictor than the change in percent predicted FVC at 6 months.[53]

The composite physiology index (CPI) is a score that consists of different pulmonary function tests.[54] It combines the values of FVC, FEV_1 and DL_{CO} and as it includes FEV_1, the confounding effect of emphysema is hereby of minimal value. The CPI was derived in one group of 106 IPF patients by fitting pulmonary function tests against disease extent on HRCT and tested in a second group of 106 IPF patients. The CPI correlated more strongly with disease extent on HRCT and survival than individual pulmonary function variables. However, the score has to be calculated from other parameters, and is therefore not easy applicable in everyday clinical practice.

Imaging

HRCT

Flaherty and colleagues have evaluated the influence of HRCT appearance on survival in patients with idiopathic interstitial pneumonia.[18] They divided HRCT scans from patients with histological UIP (n=73) or histological NSIP (n=23) into 5 categories: definite UIP, probable UIP, indeterminate, probable NSIP, or definite NSIP. Patients with an HRCT that was diagnosed as definite or probable UIP had a shorter survival than those with an indeterminate HRCT or definite or probable NSIP. Patients with histological UIP but without the corresponding HRCT diagnosis of probable or definite

UIP showed a better survival than patients with the corresponding UIP pattern on HRCT. Thus, patients with a typical UIP pattern on HRCT scan have the highest risk of mortality. A subsequent study from Lynch et al. described 315 IPF patients, who were included in a randomized controlled study evaluating interferon (IFN) -γ.[55] Lung function parameters and HRCT features were studied in relation to mortality. A higher extent of fibrosis on HRCT was found to be an independent predictor of mortality in the multivariate analysis. A recent study by Best and colleagues confirmed this finding.[56] They included 167 IPF patients who underwent HRCT scanning at enrolment and in 95 cases also at 12 months follow-up. A greater extent of fibrosis at baseline as well as an increase of fibrosis during one year were both significant predictors of survival. Not only disease extent, but also disease pattern was identified as a predictor of prognosis. Akira and colleagues studied HRCT data of 58 IPF patients before and at the time of an acute exacerbation.[57] New areas of parenchymal ground glass opacification that spread rapidly throughout the lung were pathologically correlated with diffuse alveolar damage (DAD). This diffuse pattern was associated with worse survival compared to patients with a multifocal and peripheral pattern.

Molecular imaging

On HRCT scan, one can not differentiate between established fibrosis and lesions that exist of actively proliferating fibroblasts. HRCT pictures give information on lung density, but can not visualize the actual activity of the fibrotic process, *i.e.* fibrogenesis. Imaging of fibrogenesis could be very useful in the prediction of disease progression in IPF and other fibrotic interstitial lung diseases. Molecular imaging techniques, using radio-labelled markers to detect disease activity are relatively novel and promising techniques in this respect. Umeda and colleagues have described the use of dual-time-point ^{18}F-FDG PET to asses disease progression in IIP patients.[58] Fifty IIP patients (of whom 21 IPF, 18 NSIP and 11 COP) underwent one scan at 60 minutes and a second scan at 180 minutes after ^{18}F-FDG injection. The retention index (percent difference between the first and second scan) in IPF and NSIP patients was significantly greater in patients who showed lung function deterioration after one year, compared to patients without deterioration. Further, ^{18}F-proline PET has been shown to be a reliable marker for fibrosis formation in animal studies. This technique was recently also tested in IPF patients, but the results showed only low uptake in the lungs. Therefore ^{18}F-proline PET does not seem to be a promising biomarker for the imaging of fibrogenesis in IPF, but better radioligands may appear on the horizon in the near future.[59]

Another imaging technique to visualize fibroblast activity may be the ^{111}In-ocreotide-scintigraphy. Octreotide is a somatostatin-analog with strong affinity for the somatostatin receptor subtype 2, and inhibits fibroblast activity. Lebtahi and colleagues

evaluated the expression of somatostatin receptors in patients with IPF and pulmonary fibrosis associated with systemic sclerosis, and healthy controls.[60] They found an increased uptake in both patient groups. Furthermore the degree of uptake correlated with deterioration of lung function parameters over time, and BALf cellularity.

Histology

The relevance of distinguishing UIP from other interstitial pneumonias was first pointed out by Bjoraker and colleagues.[61] They reviewed the lung biopsy material of 104 patients with a diagnosis of IPF before the ATS/ERS consensus paper on the classification of IIPs in 2002, and related pathologic diagnosis to survival. Patients with a UIP pattern on lung biopsy had a median survival of 2.8 years, which was significantly worse than those with NSIP, DIP, BOOP and other interstitial pneumonias. In 2004, Monaghan and colleagues investigated 64 patients in whom multiple biopsies at different locations were performed that showed either a pattern of UIP or NSIP.[62] Patients were categorized in three groups: concordant UIP-UIP (n = 25), discordant UIP-NSIP (n = 8) and concordant NSIP-NSIP (n = 31). Patients with discordant UIP-NSIP showed clinical behaviour similar to those with concordant UIP-UIP and should thus be regarded as having UIP, in the context of prognosis and therapeutic management.

A typical finding in the histopathology of UIP is a fibroblast focus. The presence of these aggregates of actively proliferating myofibroblasts indicates that fibrosis is actively ongoing rather than representing the residuum of a process that occurred in the past. King et al. studied 87 patients with UIP confirmed on lung biopsy.[63] The extent and degree of histological features such as fibroblast foci, alveolar space cellularity and alveolar wall fibrosis were graded by independent pathologists and related to survival. The number of fibroblast foci present in a UIP biopsy predicted survival. Thus, the ongoing process of damage and aberrant epithelial repair is more important in the pathway to end-stage fibrosis than alveolitis. Moreover, the number of fibroblast foci correlated with the decline in FVC and DL_{CO}.[64] Enomoto et al. confirmed this and added to these findings by using a more objective method to score the extent of fibroblast foci.[65] Instead of counting fibroblast foci in a selected area by 2 or more independent pathologists, they used a camera and image analysis software. This quantitative scoring method was less observer-dependent and still showed a significant relation of the degree of fibroblast foci with survival. However, Hanak et al. were unable to find any association between the number of fibroblast foci and survival.[66] In their study, patients with accelerating IPF were excluded in order to investigate if the number of fibroblast foci was informative in the stable IPF patient. Additionally, they randomly selected the areas to count the number of fibroblast foci, including areas with normal lung tissue or honeycombing.

Another histopathological feature in necroscopic lung tissue of IPF patients is diffuse alveolar damage (DAD). DAD may point to common preterminal events in the critically ill patients, such as shock, intravascular coagulation, sepsis or oxygen toxicity.[67] Acute exacerbations of IPF may also be caused by DAD. Tiitto et al investigated whether the number of fibroblast foci was related to DAD at necroscopic lung samples.[68] Although the amount of fibroblast foci was indeed increased in the subjects with worst survival, no relation could be demonstrated between fibroblast foci and DAD.

Pulmonary hypertension

The presence of pulmonary hypertension in IPF patients has important prognostic implications. Nadrous and colleagues investigated 88 IPF patients who underwent transthoracic echocardiography. They found that the systolic pulmonary artery pressure (SPAP) inversely correlated with DL_{CO} and that patients with SPAP > 50 mm Hg had a significantly worse survival compared to patients with SPAP < 50 mm Hg.[69] Song et al. added to these findings by including both echocardiography and brain natriuretic peptide (BNP) in their study.[70] Using SPAP of 40 mm Hg as a threshold for pulmonary hypertension, patients with pulmonary hypertension had a significantly worse mean survival (10.8 months) compared to patients with SPAP < 40 mm Hg (23.7 months). Further, an elevated level of BNP appeared to be an independent predictor of prognosis on multivariate analysis. Recently, in a cohort of 110 IPF patients, the presence of emphysema, pulmonary hypertension and pulmonary function were evaluated in relation to mortality. Patients with emphysema showed higher mortality rates than patients without emphysema. Further, a Cox regression model showed that FVC < 50 % predicted and SPAP > 75 mm Hg (by echocardiography) were the most important predictors of mortality.[71]

However, the golden standard for the measurement of pulmonary hypertension is right heart catheterization. Nathan et al. reported a cohort of 110 IPF who underwent both right heart catheterization and echocardiography.[72] In only 40 %, echocardiography accurately reflected the SPAP as measured by right heart catheterization. Pulmonary hypertension is common in IPF patients. Shorr et al investigated 2,525 IPF patients who were registered at the lung transplant registry for USA between 1995 and 2004 and had undergone right heart catheterization. Forty-six percent of these patients had a mean pulmonary artery pressure (mPAP) of > 25 mm Hg and 9 percent had severe pulmonary hypertension with a mPAP of > 40 mm Hg.[73] Lettieri et al. found that pulmonary hypertension (mPAP > 25 mm Hg) was present in 31.6 % of their cohort of 79 IPF patients undergoing pretransplantation right heart catheterization. Patients with pulmonary hypertension appeared to have a lower $DL_{CO,}$ were more likely to require supplemental oxygen and had a poor 6MWT performance. One-year

mortality rates in patients with pulmonary hypertension were significantly higher in patients with pulmonary hypertension (28%) compared to patients without pulmonary hypertension (5.5%).[74] Thus, pulmonary hypertension is common in advanced cases of IPF. These patients may warrant more aggressive management or early referral for lung transplantation.

CONCLUSIONS

This review focuses on prognostic factors for IPF that were found after the establishment of international criteria for a clinical diagnosis of this disease, and the reclassification of IIPs in 2002. As such, it summarizes the results of the studies that included patients with IPF according to the newest definition. The key findings are summarized in Table 1, and presented with cut-off values for different parameters. Although far from perfect, these parameters are currently the best tools to help the clinician to distinguish patients who show the rapidly progressive variant of IPF (pattern C, as mentioned in figure 1) from patients who show a slowly progressive clinical course (pattern A). Despite these useful prognostic determinants, it is still impossible to predict whether a patient will develop an acute exacerbation (pattern B).

Especially the research of biomarkers in serum is promising, and might lead to the identification of better markers for a prediction of survival in IPF in the near future. Serologic biomarkers are attractive because they are easily accessible, and may have the ability to reflect change of disease course more accurately than pulmonary function test or HRCT. Ideally, serum biomarkers could even detect disease progression before this becomes clinically apparent. This would be a valuable addition to current determinants of disease progression and prognosis in follow-up. The use of so-called pneumoproteins as prognostic indicators has already been the issue of several studies. A growing body of evidence supports the application of KL-6, SP-A and SP-D as predictors of mortality in IPF. The association between increased levels of pneumoproteins and mortality has now been independently confirmed by different investigators, which adds substantial validity. However, further prospective studies are needed before widespread acceptation will occur.

The identification of new biomarkers is important, and also techniques like microarrays and gene expression profiling may become more and more important. Selman et al. compared gene expression profiles in lung samples from 4 IPF patients with rapid progression and 4 patients with slow progression.[75] Rapidly progressing IPF patients strongly expressed genes involved in morphogenesis, cancer, oxidative stress, apoptosis, cell proliferation and genes from fibroblasts and smooth muscle cells. Around

30% of the differentially expressed genes were downregulated in the rapid progressor lungs, including genes related to signal transducer activity, and epithelial receptors. This kind of innovative research is necessary to shed a new light on biomarkers for disease progression and outcome.

Table 1 Summary of used determinants and cut-off levels to predict survival.

Determinant	Favourable	Unfavourable	ref
Sex	Female	Male	4, 11, 47
BMI	> 30	< 25	19
Serum KL-6	< 1000 U/ml	> 1000 U/ml	23
Serum SP-A	<123 ng/ml	> 123 ng/ml	15
Serum CCL-18	< 150 ng/ml	> 150 ng/ml	16
Serum fibrocytes	< 5%	> 5%	29
BAL neutrophils	< 3%	> 3%	39
DL_{CO} (% pred)	> 35%	< 35%	46
Change dyspnea score (6 mo)	> 2 pt increase	> 2 pt decline	44
Change FVC (12 mo)	< 10%	> 10%	14, 44, 45, 47
Change A-a gradient (6 mo)	> 5 mm Hg decrease	> 5 mm Hg increase	44
V_{O2max}	> 8.3 ml/kg/min	< 8.3 ml/kg/min	49
6MWT (desaturation)	> 88%	< 88%	51, 52
6MWD (meters)	> 207	< 207	53
CPI	Low	High	54
Fibrosis score on HRCT	Low	High	55, 56
Alveolar opacity pattern on HRCT	Peripheral	Diffuse	57
^{18}F-FDG PET Retention index	< 0%	> 0%	58
Fibroblast foci score on biopsy	Low	High	63-65
Systolic PAP (echocardiography)	< 40 mm Hg	> 40 mm Hg	70
	< 50 mm Hg	> 50 mm Hg	69
	< 75 mm Hg	> 75 mm Hg	71
Mean PAP (right heart catheterization)	< 25 mm Hg	> 25 mm Hg	74

BMI: *body mass index;*
KL-6: *Krebs von den Lungen-6;*
SP: *surfactant protein;*
CCL-18: *CC-chemokine ligand 18;*
BAL: *bronchoalveolar lavage;*
DL_{CO}: *diffusion capacity for carbon monoxide;*
FVC: *forced vital capacity;*
V_{O2max}: *maximal O_2 uptake;*
6-MWT: *6-minute walking test;*
6MWD: *6-minute walking distance;*
CPI: *composite physiology index;*
HRCT: *high-resolution computed tomography;*
PAP: *pulmonary artery pressure*

APPENDIX

Table 2 Summary of studies concerning prognostic determinants in IPF, performed after the 2002 consensus statement on IIPs

	Year	Outcome	N	N_{UIP}	design	Follow-up time	ref
Serum	2006	High KL-6 levels predict shorter survival	27	16	retro	36 mo	23
	2009	SP-A is a stronger predictor of mortality than SP-D	82	82	retro	Median 36 mo	15
	2009	Higher mortality in patients with high CCL18	72	20	pro	24 mo	16
	2009	Circulating fibrocyte numbers predict early mortality	51	17	retro	24 mo	29
BAL	2003	No prognostic value of BAL findings	35	35	retro	Median 38 mo	40
	2007	Lymphocytosis is associated with better survival	87	87	retro	Median 21 mo	17
	2008	Increased BAL neutrophil percentage predicts early mortality	156	156	retro	Median 30 mo	39
PFT	2003	12-month changes in dyspnea score, TLC, FVC, p_aO_2, s_aO_2 and A-a gradient were predictive of survival time	81	81	pro	6 mo (n= 81) 12 mo (n = 51)	44
	2003	6-month change in FVC predicts mortality	80	80	retro	Median 58 mo	45
	2003	Changes in DL_{CO}, CPI, FVC, FEV_1 were more predictive than histological diagnosis	61 (IPF) 43 (NSIP)	61	retro	Median 32 mo	46
	2003	Desaturation during 6-MWT was associated with increased mortality	83	83	retro	Median 35 mo	51
	2003	CPI is strongly linked to mortality	212	36	retro	Median 28 mo	54
	2005	6-month changes in FVC, DL_{CO} and sex were independent prognostic factors.	131 (IPF) 48 (NSIP)	131	retro	Median 24 mo	47
	2006	Lower 6MWD was associated with an increased mortality	454	NM	retro	Median 4 mo	53
	2006	Predictive ability of serial changes in PFT varied when patients were stratified by the presence of desaturation < 88%	197	146	retro	NM	52
	2009	Patients with baseline maximal oxygen uptake less than 8.3 ml/kg/min had an increased risk of death	117	75	retro	NM	49
Imaging	2003	A typical UIP pattern on HRCT predicts high mortality	73 (UIP) 23 (NSIP)	73	retro	Median 37 mo	18
	2005	Extent of reticulation and honeycombing is an independent predictor of mortality	315	205	pro	14 mo	55
	2008	Disease extent on HRCT predicts mortality and serial imaging can show disease progression	167	NM	retro	Median 18 mo	56

	2008	Greater disease extent and diffuse opacification pattern on HRCT predict worse survival	58	29	retro	Median 35 mo	[57]
	2009	Dual point ^{18}F-FDG PET predicts deterioration of lung function parameters after 1 year of follow-up	21	9	pro	12 mo	[58]
Histology	2002	Increased numbers of FF were linked to mortality	53	53	retro	Median 24 mo	[64]
	2004	Discordant UIP and NSIP on multiple biopsies should be considered as UIP	25 (UIP) 8 (UIP&NSIP) 31 (NSIP)		retro	60 mo	[62]
	2006	Quantitative scoring of FF accurately predicts mortality	16	16	retro	NM	[65]
	2006	The number of FF is associated with poor survival but not with DAD. FF can not predict acute exacerbation	64	64	retro	NM	[68]
	2008	A higher number of FF is not associated with survival	43	43	retro	Median 19 mo	[66]
Pulmonary Hypertension	2005	Survival in patients with systolic PAP of > 50 mm Hg (echocardiography) was significantly worse.	88	17	retro	36 mo	[69]
	2006	Mortality rates were higher in patients with mean PAP > 25 mm Hg (right heart catheterization)	79	79	retro	NM	[74]
	2009	Both increased BNP and systolic PAP of > 40 mm Hg (echocardiography) are predictive of poor survival	131	69	retro	Median 10 mo	[70]
	2009	Emphysema, FVC < 50 % and SPAP > 75 mm Hg (echocardiography) were associated with increased mortality.	110	42	retro	NM	[71]

N: *number of patients;*
N_{UIP}: *Number of patients with biopsy-proven usual interstitial pneumonia;*
KL-6: *Krebs von den Lungen-6;*
retro: *retrospective;*
mo: *months;*
SP: *surfactant protein;*
CCL18: *CC-chemokine ligand 18;*
pro: *prospective;*
BAL: *bronchoalveolar lavage;*
PFT: *pulmonary function tests;*
TLC: *total lung capacity;*
FVC: *forced vital capacity;*
p_aO_2: *partial pressure of oxygen;*
s_aO_2: *oxygen saturation;*
IPF: *idiopathic pulmonary fibrosis;*
NSIP: *non-specific interstitial pneumonia;*
6-MWT: *6-minute walking test;*
CPI: *composite physiology index;*
DL_{CO}: *diffusion capacity for carbon monoxide;*
6MWD: *6-minute walking distance;*
NM: *not mentioned;*
HRCT: *high-resolution computed tomography;*
FF: *fibroblast foci;*
DAD: *diffuse alveolar damage;*
PAP: *pulmonary artery pressure;*
BNP: *brain natriuretic peptide*

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3

IDIOPATHIC PULMONARY FIBROSIS; DESCRIPTION OF A DUTCH COHORT

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ABSTRACT

Aim - Idiopathic pulmonary fibrosis (IPF) is a rapidly progressive disease, and belongs to the idiopathic interstitial pneumonias (IIP). This study aims to describe the clinical characteristics, diagnosis, and prognosis of a population of Dutch patients with IPF.

Methods - Retrospective cohort study: records were retrieved of 113 patients with a diagnosis of IPF according to the new diagnostic criteria as defined by ATS/ ERS in 2002.

Results - Mean age at the time of presentation was 61.9 (SD 12.7) years and a strong male predominance was observed (90 men vs 23 women). Common clinical features at presentation were dyspnea, cough, clubbing, and basilar fine crackles. Lung function tests revealed restrictive impairment and a reduced diffusing capacity. In 72% of cases the diagnosis was histologically confirmed by open lung biopsy, showing a pattern of usual interstitial pneumonia. The majority of patients received corticosteroids, either alone or in combination with cytotoxic agents such as azathioprine or cyclophosphamide. After screening, 28 patients were eligible for lung transplantation. Of them 12 patients underwent a lung transplantation, 9 died and 7 are still on the waiting list. Overall survival was poor (median 3.9 years)

Conclusion - IPF is a rapidly progressive disease with only a marginal response to medication and a poor prognosis. It is of great importance to differentiate IPF from other fibrotic lung diseases and to refer to a specialist center, especially in case of possible lung transplant candidates or inclusion in therapeutic trials.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of the idiopathic interstitial pneumonias (IIPs), a collection of heterogeneous interstitial lung diseases, characterized by inflammation and fibrosis of the lung parenchyma. Since IIPs are uncommon, they may frequently be misdiagnosed. In order to standardize the classification of IIPs, in 2002 the American Thoracic Society (ATS) and European Respiratory Society (ERS) determined uniform diagnostic criteria for IIPs.[1]

IPF is a chronic fibrosing interstitial pneumonia, of which the lung biopsy shows a pattern of a usual interstitial pneumonia (UIP).[1] The disease course is rapidly progressive and the cause is unknown. The current hypothesis is that a particular stimulus causes repetitive damage to alveolar epithelial cells. Repair might be accompanied by excessive proliferation and differentiation of fibroblasts which ultimately leads to fibrosis. The exact stimuli are still unclear, but exposure to stone and metal dust, birds and livestock are suggested.[2] Besides environmental factors there seems to be a basis for a genetic cause. An estimated 0.5 to 20% of patients had a familial form of IPF.[3, 4]

A patient with a UIP pattern on lung biopsy meets the IPF criteria if (1) other causes such as medications, exhibitions and collagen diseases are excluded, (2) there is a UIP pattern present on high resolution computed tomography (HRCT), (3) abnormal pulmonary function parameters showing restriction and/or abnormalities in diffusion capacity. If a lung biopsy is not available, the diagnosis of IPF can be made on the basis of a number of clinical and radiological criteria listed in table 1.[1]

Since the introduction of uniform criteria by the ATS/ERS, there has not been described a cohort of IPF patients in the Netherlands. In the following text, an overview is given of the clinical presentation, diagnosis, therapy and survival of patients with IPF, diagnosed at the St Antonius Hospital in Nieuwegein and UMC Utrecht. Our aim was to give an illustration of this cohort with respect to IPF patients described in international literature and to create greater awareness of this disease with very poor prognosis.

Table 1 ATS/ ERS criteria for diagnosis of idiopathic pulmonary fibrosis in absence of a surgical lung biopsy.[1]

Major criteria
Exclusion of other known causes of ILD such as certain drug toxicities, environmental exposures, and connective tissue diseases
Abnormal pulmonary function studies that include evidence of restriction (reduced VC, often with an increased FEV1/ FVC ratio) and impaired gas exchange (increased $P(A\text{-}a)O_2$, decreased Pa_{O2} with rest or exercise or decreased DL_{CO})
Bibasilar reticular abnormalities with minimal groundglass opacities on HRCT scans
Transbronchial lung biopsy or BAL showing no features to support an alternative diagnosis
Minor criteria
Age > 50 yr
Insidious onset of otherwise unexplained dyspnea on exertion
Duration of illness > 3 months
Bibasilar, inspiratory crackles (dry or "Velcro"- type in quality)

BAL = Bronchoalveolar lavage;
DL_{CO} = diffusing capacity of the lung for carbon monoxide;
HRCT = high-resolution computerized tomography;
ILD = interstitial lung disease;
$P(A\text{-}a)O_2$ = alveolar-arterial pressure difference for O_2;
VC = Vital capacity.

METHODS

Patient selection

Retrospectively, data were collected from all IPF patients between 1998 and 2007 in the St Antonius Hospital and UMC Utrecht. In the St Antonius Hospital patients with interstitial lung diseases were registered in a specially designed research database. In UMC Utrecht, the diagnosis registration system was used to search for IPF patients. Patients were included after revision of the diagnosis, according to the current ATS/ ERS criteria.[1]

Response to medication

Response to medication was recorded as improvement, worsening or stable lung function. An improvement was defined when at least two of the three following criteria were met: 1) reduction in symptoms 2) reduction of UIP features on HRCT, 3) ≥ 10% improvement in VC (or 200 mL increase) and/ or ≥ 15% improvement in DI_{CO} (or ≥

3 mL/ min/ mm Hg). Deterioration was defined as two of the three following criteria were met: 1) worsening of symptoms, 2) increase in UIP features on HRCT, 3) ≥ 10% deterioration in VC (or 200 mL decrease) and/ or ≥ 15% deterioration in DI_{CO} or ≥ 3 mL/ min/ mm Hg. In the remaining cases there was a stable situation.

Statistics

Patient characteristics were described using means and standard deviations. Data with a large distribution were described as median with interquartile ranges. Survival curves were made using the Kaplan Meier method in GraphPad Prism 4 (Graphpad Software, California, USA).

RESULTS

A total of 113 patients were enrolled who met the criteria for IPF: 89 from the St Antonius Hospital and 24 from UMC Utrecht. Mean age at diagnosis was 61.9 (SD 12.7) years. The majority of patients were men (80%). Almost half of the patients (46%) were exposed to a certain environmental factor, of which metal dust (10%), birds (15%) and asbestos (9%) were the most common. Sixty-six percent of patients had a history of smoking, with a median of 17 packyears (IQR 8.5 – 30) per person.

Nineteen patients (17%) had a form of familial pulmonary fibrosis, which is an IPF patients with a first-degree family member with an IIP.[4, 5] Clinical presentation of familial IPF did not differ from sporadic IPF. However, the age at diagnosis was significantly lower in the familial IPF group: 52.3 (SD 14.8) vs 63.4 (SD 11.3) years (p = 0.023).

Signs and symptoms

Dyspnea and cough were the most common symptoms at presentation: 88% and 76% respectively. These symptoms were median 11 months present (IQR 3.7 – 35.3) before the first visit to the pulmonologist. At physical examination 91% had bibasilar crackles and 55% had clubbing.

Diagnosis

The diagnosis of IPF was established in a multidisciplinar team of clinicians, radiologists and pathologists. A lung biopsy showing a UIP pattern confirmed in 72% the diagnosis of IPF. In the remaining patients, the diagnosis IPF was based on clinical and radiological criteria, as presented in table 1.

At the time of presentation all patients underwent lung function tests (table 2). Patients presented with a restrictive lung function and a decreased diffusion capacity. A bronchoalveolar lavage (BAL) was performed in 60 patients (53%). BAL fluid showed increased percentages of neutrophils and eosinophils (table 3).

Table 2 Characteristics of IPF patients at the time of diagnosis

	n = 113
Sex (M/ V)	90/ 23
Age (years)	61,9 (SD 12,7)
Smoking	
Current smokers	3 (2,7%)
Non-smokers	38 (33,6%)
Ex-smokers	72 (63,7%)
Expositions	
Metal dust	11 (10%)
Birds	17 (15%)
Asbestos	10 (9%)
Lung function parameters	
FEV1 (% pred)	74,8 (SD 23,1)
FVC (% pred)	60,9 (SD 19,6)
RV (% pred)	61,7 (SD 18,2)
TLC (% pred)	64,4 (SD 15,1)
DI_{CO} (% pred)	43,6 (SD 16,5)
DI_{CO} /Va (% pred)	72,3 (SD 21,1)

FEV1 = forced expiratory volume in one second,
FVC = forced vital capacity,
RV = residual volume,
TLC = total lung capacity,
DI_{CO} = diffusing capacity of the lung for carbon monoxide,
Va = alveolar volume

Table 3 Cellular profiles in bronchoalveolar lavage fluid in IPF patients, with reference values[28]

	Smokers		**Non-smokers**	
	IPF patients	**Reference values**	**IPF patients**	**Reference values**
% macrophages	72,5 (SD 18,3)	90 - 96%	73,7 (SD 19,8)	85 - 92%
% lymphocytes	10,7 (SD 12,0)	2 - 7%	9,9 (SD 8,8)	7 - 13%
% neutrophils	9,3 (SD 11,7)	0 - 2%	8,7 (SD 11,9)	0 - 2%
% eosinophils	6,7 (SD 7,1)	0 - 1%	7,8 (SD 12,6)	0 - 1%
CD4/ CD8 ratio	2,7 (SD 3,2)	1,0 - 3,3	2,5 (SD 1,1)	1,0 - 3,3

Treatment

After diagnosis, in 101 patients (90%) drug treatment was initiated. In 84% corticosteroids were started, in 56% combined with immune suppressives such as azathioprine or cyclophosphamide. Fifty-six percent of patients also received acetylcysteine. Six

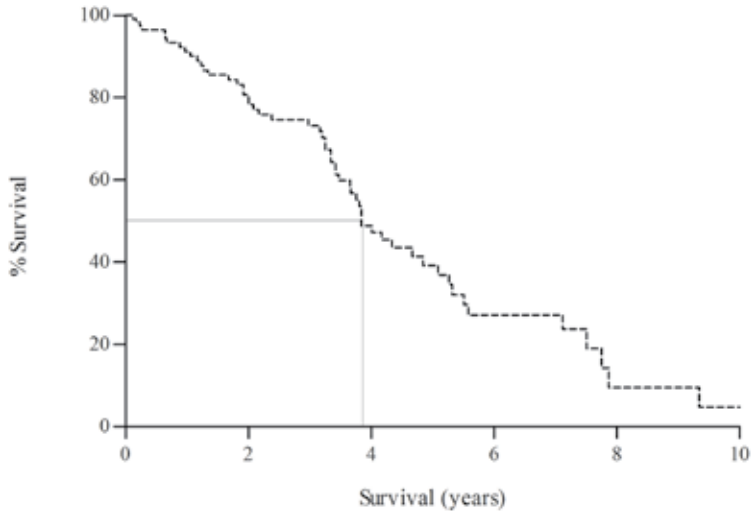
months after starting medication, only 1 patient showed improvement. In 33% the situation remained stable and in 65% there was worsening of the disease. In 12 patients (10%) no therapy was initiated and a policy of watchful waiting was permitted, mainly because of comorbidities.

Survival

Until November 2007, 72 of the 113 patients died (diagnosis between 1998 and 2007). Forty-nine patients (68%) died of respiratory failure associated with IPF, including 9 with a rapidly progressive form of IPF.[6] Eight patients died of lung cancer which was diagnosed after the diagnosis of IPF, 7 to a cause not related to IPF and from 8 patients the cause of death was unknown.

Survival of the IPF patients is shown in a survival curve (Figure 1). Censoring occurred when the patient was transplanted or if the patient was still alive before the end of follow-up. Due to death and censoring, the group of IPF patients becomes smaller during follow-up. The median survival was 3.9 years.

Figure 1 Survival curve of IPF patients (n = 113), with a median survival of 3,9 years.



Transplantation

Initially there were 38 patients eligible for screening for lung transplantation. After the pre-transplant screening 28 patients were selected for the waiting list. So far 12 of these patients underwent a transplantation, nine died on the waiting list and 7 are still waiting for transplantation. Of the patients who were transplanted, three died. The duration from diagnosis to transplantation ranged from 0.4 to 7.8 years, with a median of 2.3 years.

DISCUSSION

This study provides an overview of the clinical presentation of IPF. IPF is a disease that presents at an older age and predominantly occurs in men. In a minority of IPF patients in this study, there is a familial form of IPF (17%). The diagnosis of IPF is a decision made on the combination of data acquired by HRCT scan, bronchoalveolar lavage and a lung biopsy. Despite drug therapy, the prognosis is poor, in this study with a median survival of 3.9 years.

Comparison with other cohorts of IPF patients

Comparison of the above cohort with other cohorts of IPF patients shows that the clinical presentation with respect to age, lung function parameters and therapy is similar.[7-12] In our cohort, however, the proportion of men (80%) is larger than in other studies (50% - 62%).[12-14] It is unclear why this difference exists. A possible explanation may lie in the labor participation of women. The Netherlands is among the four European countries where the labor participation in women is the lowest.[15] As a result, women may be less than men in contact with substances that are potentially harmful to the alveolar epithelium.

Familial IPF in this cohort is present in 17% of the IPF patients. This is higher than can be expected on the basis of current literature. However, a large proportion of patients in the St Antonius Hospital has been referred by another pulmonologist, sometimes specifically with the question whether there is familial IPF. In addition, young patients who are eligible for transplantation were preferentially referred to a transplantation center, and the dominantly inherited familial form of IPF presents often at a younger age. This lower age is in other studies also reported in familial IPF. The clinical presentation differs not essentially from the non-familial form of IPF.3,[16]

A UIP pattern at lung biopsy to confirm the diagnosis was present in 72% of the patients. This proportion is bigger than in large cohorts of IPF patients in the UK and United States (41% to 58%)[10, 7, 13] It is essential to differentiate IPF from other IIPs because IPF hardly responds to medication and carries a worse prognosis than the other IIPs. There appears to be a difference between community-based physicians compared to those in specialized centers when obtaining an IIP diagnosis. Doctors in community hospitals were more likely to assign a final diagnosis of IPF compared with specialized centers.[17] Given the large difference in treatment and prognosis, patients with IIPs should be referred to centers with expertise this field.

To date there are no proven effective drug therapies that can positively influence the prognosis of IPF. Corticosteroids are widely prescribed, but the effect is disputable.[18] Adding azathioprin[19] or cyclophosphamide[20] may have a positive effect on

survival, but other studies could not confirm this.[21] Finally we mention in this context, N-acetylcysteine. This is an antioxidant with potential anti-fibrotic properties. One study showed that N-acetylcysteine in combination with corticosteroids and azathioprine decreases lung function decline.[22] Hopefully future research may discover new drugs that can inhibit the progressive course of IPF.

IPF is a rapidly progressive disease, in our population with a median survival of approximately 4 years. This corresponds to the survival in other cohorts: 2.8 to 4.2 years.[9, 12, 13, 23] Lung transplantation for patients under 65 years eventually becomes the only therapeutic option. After COPD, IPF is the most frequent disease for which lung transplantation is performed.[24] Unfortunately, IPF patients have the highest mortality among patients on the waiting list.[25] Therefore it is important to refer IPF patients who are eligible for transplantation or may be included in experimental trials to a specialized center.[26]

Epidemiology

In the above text a comparison with the international literature is illustrated. Clinical presentation, diagnosis and prognosis in this cohort is comparable to other cohorts of IPF patients, but epidemiological data are lacking. Since it is a rare disease for which national or regional registration is missing, we can not take the epidemiology into consideration. Improved survival from IPF is dependent on better understanding of the epidemiology of the disease and only few institutions have significant numbers to provide this information. To assess the incidence of IPF in the Netherlands and in other European country it is important to register patients with this disease nationally and internationally.[27]

CONCLUSION

IPF presents at older age and is a disease which predominantly affects men. IPF responds poorly to medical treatment and in our population patients have a median survival of only 3.9 years. The clinical presentation, diagnosis and prognosis is consistent with other cohorts of IPF patients in literature.

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4

SURFACTANT PROTEIN-D PREDICTS SURVIVAL IN PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS

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ABSTRACT

Background - Idiopathic pulmonary fibrosis is a progressive interstitial lung disease with a high mortality rate. As lung transplantation is the only therapeutic option, it is important to predict survival.

Objective - This study evaluates the clinical value of surfactant protein-D as a marker of prognosis in patients with idiopathic pulmonary fibrosis.

Design - Surfactant protein-D was measured in serum of 72 patients and 305 healthy controls. The optimal cut-off level to define unfavourable prognosis was determined using a ROC analysis. A Cox's proportional Hazards model was used to evaluate variables that were significant predictors of survival.

Results - Serum levels of surfactant protein-D were significantly higher in patients than in controls. ROC analysis showed 460 ng/ml to be the optimal cut-off level to discriminate survivor from non-survivors after 1 year. Patients with high levels (> 460 ng/ml) had a median survival time of 13 months, compared to 67 months in the group with low levels (< 460 ng/ml). Surfactant protein-D showed to be a significant predictor of prognosis, even when corrected for age, sex, smoking, and lung function.

Conclusion - The measurement of surfactant protein-D in serum of patients with idiopathic pulmonary fibrosis might be a clinically relevant tool to predict survival.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lung parenchyma. Clinically, it is characterized by dyspnea and worsening of lung function. The clinical course of IPF is unpredictable and median survival time varies between 2.4 and 4.2 years.[1-4] To date, no therapy has been proven to prolong survival.[5] Lung transplantation seems to be the only option for those who meet the appropriate criteria. Unfortunately, IPF patients have the highest mortality rate on the transplant waiting list.[6] Optimal timing of referral for lung transplantation is therefore crucial and dependent on predicting survival. In this respect it is of great importance to study new biomarkers that can predict survival.

Lung-specific secretory proteins, also referred to as pneumoproteins, are potential biomarkers to asses disease severity and progression in interstitial lung disease.[7] These proteins are secreted by the respiratory tract epithelium and their occurrence in serum is probably due to leakage through the lung parenchyma. Surfactant protein (SP)-D is mainly synthesized in type II pneumocytes, but it is also detected in Clara cells and in other extrapulmonary epithelial cells.[8] SP-D contributes to the function of surfactant in the alveoli.[9] SP-A and SP-D are part of the innate immune system and regulate the functions of innate immune cells, such as macrophages. They also modulate the adaptive immune response by interacting with antigen-presenting cells and T cells, thereby linking innate and adaptive immunity.[10]

SP-D has been studied as a marker in patients with bird fanciers' lung and sarcoidosis and has been shown to be associated with lung function impairment and disease severity.[11, 12] In IPF patients, serum SP-D is elevated compared to sarcoidosis, beryllium disease and healthy controls.[13] In a series of Japanese IPF patients, concentrations of SP-D were significantly increased and related to disease extent and progression.[14] Although previous studies have shown that serum SP-D is a potentially useful marker, there is still insufficient information to use serum SP-D in clinical practise as a biomarker to predict prognosis.

Different single nucleotide polymorphisms (SNPs) of the SP-D gene (SFTPD) have been described previously. The rs721917 SNP results in an alteration of the codon corresponding to amino acid 11, where a methionine (Met) is exchanged for a threonine (Thr). The Met11Thr polymorphism results in significantly different serum SP-D levels in healthy controls. The Met variant (T allele) is associated with higher serum SP-D levels. Constitutional SP-D serum levels are approximately 80% under control of genetic factors and the Met11Thr polymorphism determines half of this genetic component.[15] Because the value of some markers can improve when serum levels were corrected for genotype[16, 17], it might be important to assess the relationship

between serum SP-D levels in IPF patients and the Met11Thr polymorphism.

As SP-D in serum is a potential biomarker in interstitial lung diseases and high levels might indicate worse prognosis, it was our aim to evaluate the clinical value of SP-D measurements in IPF at the time of diagnosis in order to find a cut-off level for defining unfavourable prognosis.

METHODS

Patients and healthy controls

Patients with IPF presenting at the Department of Pulmonology of the St. Antonius Hospital in Nieuwegein between 1998 and 2007 were retrospectively included in this study. Medical records were retrieved and patients were included according to current ATS/ ERS guidelines; a histologic or radiologic pattern typical of usual interstitial pneumonia (UIP).[18] Diagnoses made before 2002 were reviewed by a clinician and only included when current ATS/ ERS criteria were met. Other causes of UIP (drugs, collagen vascular diseases) were ruled out. Serum and BALf were collected from all ILD patients, and were systematically enrolled in our database used for scientific research. Serum samples of 72 IPF patients were available at the time of diagnosis. Bronchoalveolar lavage fluid (BALf) was available from 54 IPF patients and was obtained using fibreoptic bronchoscopy according to a previously described method.[19] Serum and BALf samples were stored at - 80°C until analysis. Serum from healthy controls was obtained from 305 self-reported healthy employees of the St. Antonius Hospital. Bronchoalveolar lavage was performed in 30 healthy controls. The study protocol was approved by the Ethical Committee of the St. Antonius Hospital, and all subject gave their written informed consent.

Pulmonary function tests

Pulmonary function tests were performed according to ERS recommendations.[20] Vital capacity (VC), forced expiratory volume in 1 second (FEV_1) and diffusing capacity for carbon monoxide ($D_{L_{co}}$) were measured with a Jaeger System. All values were expressed as percentage of predicted value. The interval between pulmonary function testing and collection of the serum and BAL samples was less than three months.

Analysis of SP-D and genotyping of the polymorphism

The concentrations of SP-D in serum and bronchoalveolar lavage fluid (BALf) were detected by monoclonal anti-human SP-D antibody using a commercially available

enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Biovendor; Heidelberg, Germany). Serum was diluted to a concentration of 1:11 and bronchoalveolar lavage 1:40, the detection limit was 1.2 ng/ml. The Met11Thr polymorphism in the surfactant protein-D gene, corresponding to rs721917, was analysed with a custom Illumina goldengate bead SNP assay using sequence specific primers. The assay was performed in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, CA, USA)

Statistical analysis

Data were expressed as median and interquartile ranges (IQR). Differences in serum or BALf concentrations between independent groups were analysed using a Mann-Whitney U test. Differences between more than two groups were analysed using one-way analysis of variance (ANOVA). The relationship between markers in serum and BALf and clinical data was assessed using Spearman's correlation coefficients. To find the optimal cut-off level to discriminate survivors from non-survivors after one year, receiver operating curves (ROC) were used. The Kaplan-Meier method was used to describe survival time and the log-rank test to evaluate statistical significance between groups. Transplants and non-IPF deaths were censored. To determine the patients status and cause of death we retrieved medical records and if not conclusive we contacted the patients general practitioner. A considerable part of our cohort would, due to age restrictions, not meet the criteria to undergo lung transplantation. Therefore, a subanalysis of the group of patients with age < 65 years was performed. A Cox's proportional Hazards model was used to determine covariates that influence survival. Statistical analysis was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc; San Diego, CA, USA). Statistical significance was considered at a value of $p < 0.05$.

RESULTS

Clinical characteristics

Seventy-two IPF patients (56 male and 16 female, mean age 62.9 years [SD 12.9]) were included in the study. Fifteen IPF patients were treated with low-dose oral corticosteroids at the time of serum and/or BAL sampling. In 50 IPF patients (69%) the histological diagnosis of UIP was confirmed by open lung biopsy. Table 1 shows the clinical characteristics of patients and controls.

Table 1 Characteristics of patients and healthy controls

	IPF Patients	Healthy controls
Number of subjects	72	305
Sex M/F	56/ 16*	115/ 190
Age, yr (mean, SD)	62.9 (12.9)*	40.4 (11.7)
Smoking status		
Smoker	3	61
Non-smoker	19	192
Ex-smoker	50	52
Lung function, (median, IQR)		
% pred FEV_1	77 (64 - 95)	-
% pred VC	75 (60 - 87)	-
% pred $D_{L_{co}}$	43 (33 - 56)	-

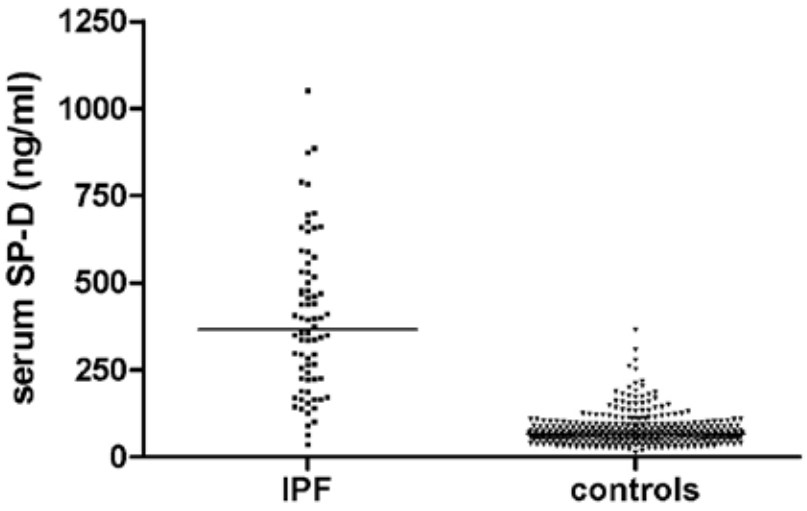
**p < 0.05 compared to healthy controls*

Table 2 BALf and serum SP-D levels in IPF patients and healthy controls

SP-D levels (median, IQR)	IPF patients	Healthy controls
BALf	n = 54	n = 30
	385 (290– 530)*	504 (357 - 734)
Serum	n = 72	n = 305
	365 (226 – 527)†	57.5 (41.2 – 77.5)

**p < 0.05 compared to healthy controls. †p < 0.0001 compared to healthy controls.*

Figure 1 Serum SP-D levels in 72 IPF patients and 305 healthy controls (HC).



SP-D levels

Median serum and BALf levels of SP-D in patients and healthy controls are shown in table 2. The median serum SP-D level in healthy controls was 57.5 ng/ml (IQR 41.2 – 77.5). There was no difference in serum SP-D levels between men and women. Serum SP-D levels were weakly correlated with age ($r = 0.18$ $p < 0.05$). In healthy controls, no influence of smoking was seen in relation to serum SP-D levels (data not shown). Serum SP-D levels in IPF patients were significantly higher (365 ng/ml, IQR 226 – 527) than in healthy controls (57.5 ng/ml, IQR 41.2 – 77.5) ($p < 0.0001$), but SP-D in BALf of IPF patients (385 ng/ml, IQR 290 – 530) was significantly lower than in controls (504 ng/ml, IQR 357 – 734). Serum and BALf levels in IPF patients were not significantly different between smokers (serum: 353 ng/ml, 227 – 506; BALf: 380 ng/ml, IQR 246 – 519) and non-smokers (serum: 357 ng/ml, IQR 164 – 530; BALf: 322 ng/ml, IQR 195 – 504). Figure 1 shows serum SP-D levels at the time of diagnosis in IPF patients and healthy controls. In patients and healthy controls, serum levels of SP-D did not correlate with BALf levels. Serum SP-D levels in IPF patients were correlated with $D_{L_{CO}}$ ($r = -0.315$, $p = 0.04$). There were no correlations between serum SP-D and any other lung function parameters, BALf parameters or age. Furthermore, SP-D in BALf did not show a correlation with lung function, BALf parameters, survival or age either.

Met11Thr polymorphism and serum SP-D levels

The distribution of the SFTPD genotype in patients and healthy controls was in Hardy-Weinberg equilibrium. There was no significantly different allele frequency in IPF patients (T 56%; C 44%) compared to healthy controls (T 58%; C 42%). Similarly, no significant difference was seen in genotype counts: IPF patients TT 17 (30%), CT 30 (53%), CC 10 (17%); healthy controls TT 99 (32%), CT 157 (52%) and CC 49 (16%) respectively. Figure 2 and 3 show the influence of the Met11Thr polymorphism and corresponding serum SP-D levels in healthy controls and IPF patients. Significant differences in serum SP-D levels were found within the population of healthy controls when groups were formed according to their genotype: TT 71.7 ng/ml (IQR 52.5 – 97.7), CT 66.6 ng/ml (IQR 50.8 – 88.3), and CC 53.0 ng/ml (IQR 33.1 – 83.0), ANOVA: $p = 0.002$. When serum SP-D levels of IPF patients were grouped according to genotype, no significant difference between these groups was observed, ANOVA: $p = 0.9$.

Figure 2 Scatterplot illustrating the association between the Met11Thr polymorphism in the SFTPD gene and serum SP-D levels in healthy controls (n = 305). Horizontal bars represent median values. ANOVA: p = 0.002.

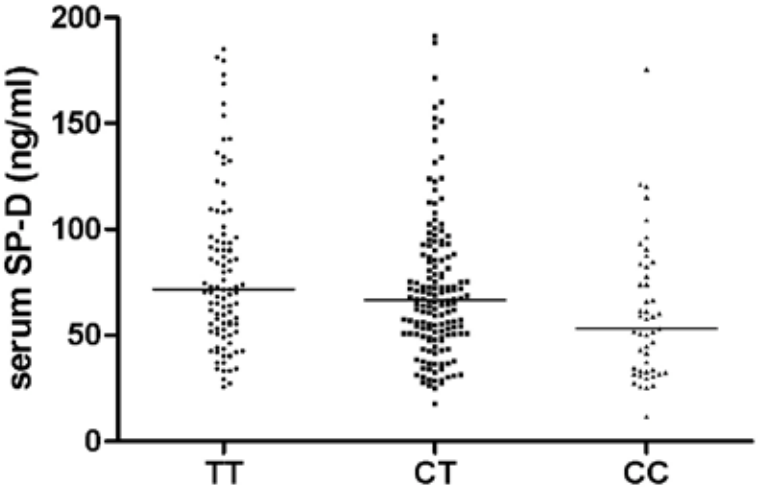
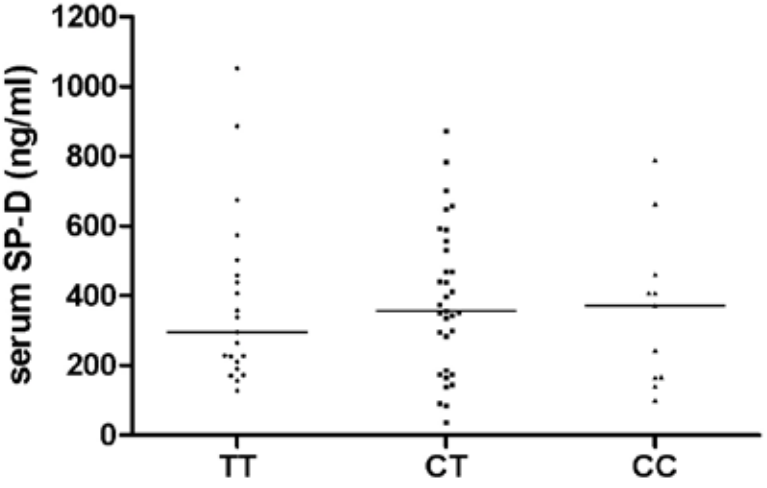


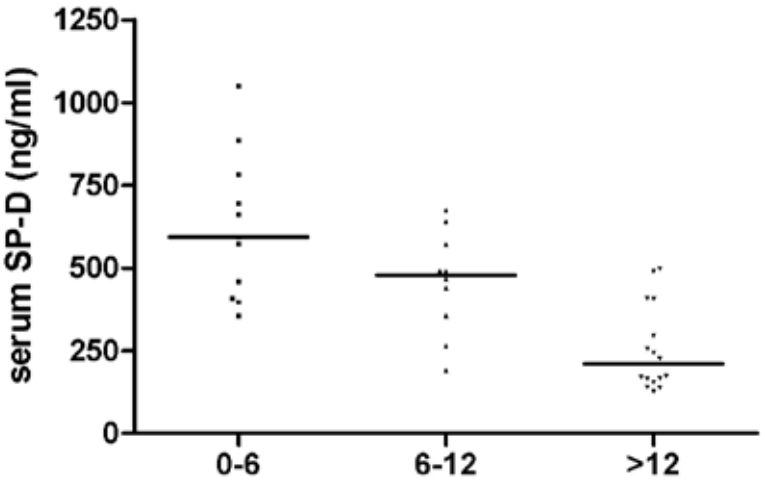
Figure 3 Scatterplot illustrating the absence of association between the Met11Thr polymorphism in the SFTPD gene and serum SP-D levels in IPF patients (n = 57). Horizontal bars represent median values. ANOVA: p = 0.9.



Survival

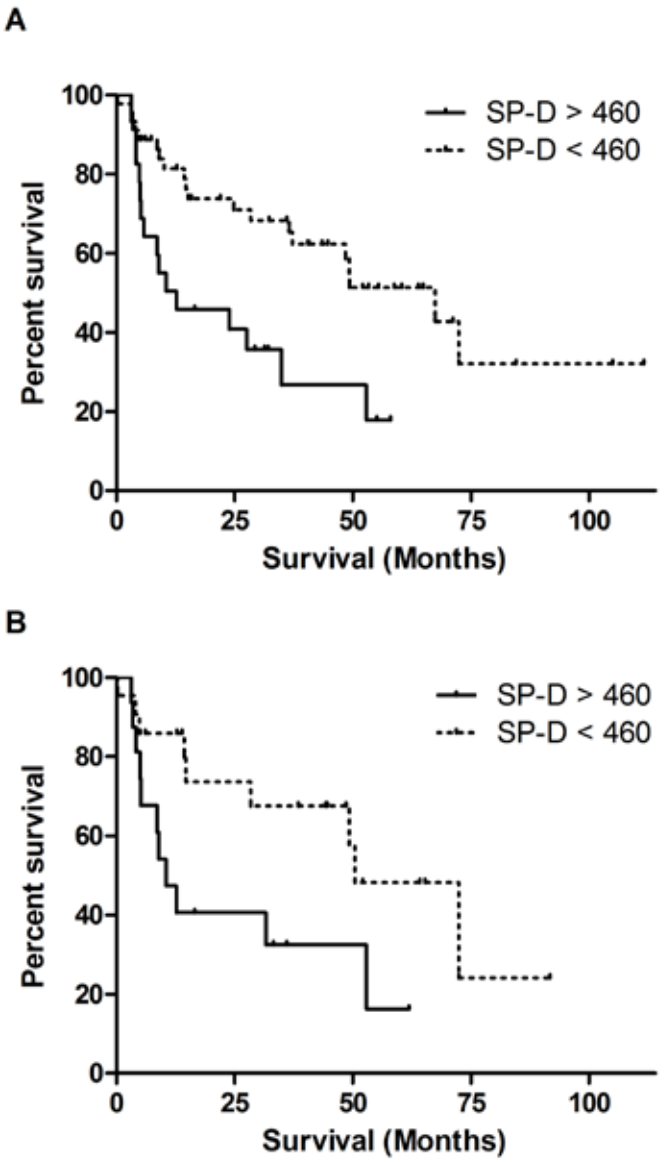
The median follow-up period for IPF patients was 39 months (range: 1 – 114). Within the study period 48 from the total of 71 IPF patients died, one patient was lost to follow-up. The cause of death was respiratory failure due to progressive IPF (n = 37), lung carcinoma (n = 4), pneumonia (n = 5), and pulmonary embolism (n = 1). One patient died from an extrapulmonary cause and two patients underwent lung transplantation, those cases were censored in the survival analysis. Figure 4 illustrates that patients with a survival less than 6 months have significantly higher serum SP-D levels than patients who lived longer (survival < 6 months: 661 ng/ml [IQR 573 – 886]; survival 6 –12 months: 465 ng/ml [IQR 265 – 567]; survival > 12 months: 250 ng/ml [IQR 163 – 370]), ANOVA: $p < 0.0001$.

Figure 4 Scatterplot illustrating serum SP-D levels in patients who died from progressive IPF (n=37). Patients were categorized according to survival time. ANOVA: $p < 0.0001$



To find an optimal cut-off level for serum SP-D to discriminate survivors from non-survivors after one year, ROC curves were used. According to the ROC curves, the optimal cut-off level for SP-D was 460 ng/ml (sensitivity 0.625, specificity 0.783, AUC 0.690). Patients were divided into two groups according to the cut-off level of 460 ng/ml. Median survival in the low SP-D group (n = 47) was 67 months (SE 7.5) compared to a median of 13 (SE 12) months in the high SP-D group (n = 25, Log-rank test p = 0.001, figure 5A). Patients within the group of serum SP-D levels > 460 ng/ml did not show significant differences in age, duration of symptoms until diagnosis, smoking behaviour, lung function parameters or therapy compared to the group with serum levels < 460 ng/ml (data not shown). A subgroup analysis was performed for patients with age under 65 years and a similar result could be shown. Median survival in the low SP-D group (n = 22) was 50 months (SE 9.6) compared to a median of 11 (SE 1.8) months in the high SP-D group (n = 17, Log-rank test p = 0.02, figure 5B).

Figure 5 **A.** Kaplan – Meier curve showing a median survival of 13 months in IPF patients with high serum SP-D levels (> 460 ng/ml, n = 25) and a median survival of 67 months in patients with low (< 460 ng/ml, n = 47) serum SP-D levels. The difference between the two curves is statistically significant, p = 0.001.
B. Kaplan-Meier curve for the subgroup of patients with age < 65 years. Patients with SP-D levels > 460 ng/ml (n = 17) have a median survival of 11 months, compared to 50 months in the group of patients with low SP-D levels (n = 22), p = 0.02.



Cox proportional hazards models were used to examine the influence of serum SP-D levels on survival while adjusting for known predictors of prognosis such as age[21], smoking status[13, 21], VC[22, 23], $D_{L_{co}}$[23, 24] and BAL fluid neutrophilia[25]. In the univariate analysis (table 3), both $D_{L_{co}}$ and SP-D levels were significantly related to survival. In the multivariate analysis only SP-D levels were associated with increased mortality, Hazard ratio 3.22 (95% CI 1.33 – 7.81), p = 0.01 (table 4).

Table 3 Univariate Cox's proportional Hazards model, describing hazard ratios of covariates in relation to survival.

Covariate	Hazard ratio	CI	p-value
Age	1.01	0.98 – 1.04	0.32
Smoking	0.89	0.57 – 3.00	0.53
VC	1.08	0.70 – 2.23	0.40
% neutrophils in BALf	1.03	0.99 – 1.06	0.14
$D_{L_{co}}$	0.05	0.01– 0.717	0.05
SP-D (<460 vs ³ 460 ng/ml)	3.01	1.55 – 5.87	<0.01

Table 4 Multivariate Cox's proportional Hazards model, describing hazard ratios of covariates in relation to survival.

Covariate	Hazard ratio	CI	p-value
Age	1.04	0.99 – 1.08	0.38
Smoking	0.78	0.21 – 2.82	0.77
VC	0.98	0.95 – 1.02	0.41
% neutrophils in BALf	1.03	0.97 – 1.03	0.33
$D_{L_{co}}$	0.14	0.01– 2.02	0.15
SP-D (< 460 vs ³ 460 ng/ml)	3.22	1.33 – 7.81	0.01

CI: Confidence interval; *$D_{L_{co}}$: Diffusion capacity for carbon monoxide;*
VC: Vital capacity; *SP-D: Surfactant protein-D*

DISCUSSION

The present study showed that SP-D in serum can predict mortality in IPF patients, and that the value of SP-D remains stable after adjustment for known predictors of mortality. A serum SP-D level higher than 460 ng/ml indicates a significantly worse prognosis compared to levels lower than 460 ng/ml. This cut-off value can be useful in clinical practice. It might help in estimating survival time, which is important for optimal timing of referral for lung transplantation in selected candidates. Furthermore, the study showed that the Met11Thr polymorphism influences serum SP-D levels in healthy controls, but not in IPF patients.

Part of our results are in agreement with data from Takahashi et al.[14] Our study, however, adds to these findings by providing a cut-off levels for prognosis and shows a clear relationship of high serum SP-D levels and short survival. This can facilitate interpretation of serum SP-D levels in clinical practice, and helps the identification of patients with the worst prognosis. The Kaplan-Meier curve (fig. 5) shows us that the difference between the two lines is mainly caused by the rapid decline in the first 12 months in the group with SP-D levels higher than 460 ng/ml. After 12 months, the two

lines run parallel. This means that high serum SP-D levels predict a rapid deterioration and that high serum SP-D levels mainly predict short-term survival (i.e. < 12 months). In figure 4 this is supported by the fact that patients with a shorter survival time show higher SP-D levels. Furthermore, we performed a Cox proportional Hazards model to evaluate serum SP-D while adjusting for patient characteristics and lung function parameters. Even after adjustment, serum SP-D levels at the cut-off value of 460 ng/ml remain a significant predictor of mortality. This strengthens the recommendation of using SP-D in clinical practice as a new marker for prognosis in IPF.

The source of increased serum concentrations of SP-D has to be further elucidated but it seems likely that it is at least in part the result of increased alveolar-capillary permeability.[7] It is also assumed that it correlates with the total amount of damaged epithelium in the alveolar compartment. In contrast, lower SP-D levels in BALf were found compared to controls, and this might be related to the replacement of alveolar epithelial cells by scar tissue. As alveolar type II epithelial cells are the major producers of SP-D, a reduced number of these cells could lead to decreased amounts of SP-D in BALf. However, this does not explain the lack of correlation between serum and BALf SP-D levels. Fujii et al.[26] suggested that the leakage from the alveolar to the vascular compartment is superior to the secretion of SP-D into the alveolar lining fluid. Whether an increased local clearance of SP-D by alveolar macrophages or other alveolar cells might also play a role is unclear.

Although increased serum SP-D levels are most likely the result from increased secretion and/or leakage of these molecules across the alveolar-capillary membrane, it can not be ruled out that there are other cells in the circulation that secrete SP-D.[27] For example, it has recently been reported that SP-D is expressed in vascular smooth muscle cells, and plays a role in the local regulation of inflammatory processes and innate host defense.[28] Therefore, increased serum SP-D levels in IPF might also partly be due to SP-D released by vascular endothelial cells, and reflect a local inflammatory response involving the vascular endothelium.

In healthy controls, Sorensen et al. illustrated that the Met11Thr polymorphism accounted for marked differences in serum SP-D levels.[15] We confirmed their findings, and in addition showed that in IPF patients serum SP-D levels were independent from the Met11Thr polymorphism. A possible explanation could be that functional effects of SNPs on protein levels in healthy controls may become less prominent in pathological conditions.

One of the limitations of this study is that it is a retrospective study. A prospectively conducted study is needed to determine whether serum SP-D levels rise as lung function declines. Currently, a prospective study with serial measurements of serum SP-D and lung function is being conducted in our centre. As such, we can validate our

results and test the cut-off level in a prospective manner.

In summary, SP-D is a marker that can be easily determined in serum and has been proven to be a prognostic marker in IPF patients. This study adds clinically useful cut-off levels that could identify patients with a significantly worse prognosis. This prognostic value of SP-D persists after adjustment for known predictors of mortality. Taken all previously published studies into account, we encourage the implication of routine measurement of SP-D at the time of diagnosis in IPF patients.

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5

POTENTIAL ROLE OF ET-1 IN PULMONARY FIBROSIS. FROM THE BENCH TO THE CLINIC.

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INTRODUCTION

Idiopathic pulmonary fibrosis is a rapidly progressive interstitial lung disease with unknown etiology. Treatment options remain disappointing and survival from diagnosis is less than four years.[1-4] Lung transplantation seems to be the only option for those who meet the transplantation criteria, but unfortunately the waiting list mortality is high in IPF patients.[5] Historically, IPF was thought to result from chronic inflammation. However, in early stages of IPF no inflammation was seen on lung biopsy and anti-inflammatory treatment did not prove any benefit. The present concept is that repeated episodes of lung injury lead to an aberrant wound healing response with unregulated proliferation of fibroblasts.[6] A better understanding of the determinants of abnormal wound repair in IPF is necessary to find new treatment options for IPF.

Endothelin-1 (ET-1) is a potent vasoconstrictor, a mitogen, and is involved in the development of fibrosis.[7] The first studies on ET-1 and fibrosis involve the influence of ET-1 on fibroblast chemotaxis. ET-1 appeared to be one of the major determinants of chemotaxis for pulmonary artery fibroblasts in vitro.[8, 9] Only recently it was described that ET-1 plays a crucial role in epithelial-mesenchymal transition (EMT), whereby normal epithelial cells transform to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts.[10] TGF-β is activated by ET-1 and influences alveolar epithelial cells to lose an epithelial marker (pro-surfactant protein B) and gain the mesenchymal marker alpha smooth muscle actin.[11] Moreover, ET-1 is known to stimulate fibroblast replication, contraction, collagen synthesis and decreases collagen degradation. [12-14]

There is a growing body of evidence that endothelin-1 is involved in the pathogenesis of IPF. Increased expression of ET-1 is seen in airway epithelium, proliferating type II pneumocytes and endothelial and inflammatory cells in IPF patients.[15] In sera increased levels of ET-1 are observed[16] and also in BAL increased levels of ET-1 were described in a heterogeneous group of patients with interstitial lung diseases.[17] In order to gain insight in the role of ET-1 in the pathogenesis of IPF and to evaluate the potential of ET-1 as a biomarker in IPF, we measured ET-1 in serum and bronchoalveolar lavage fluid (BALf) of IPF patients and healthy controls and related this to clinical parameters.

METHODS

Patients and healthy controls

Patients with IPF presenting at the Department of Pulmonology of the St. Antonius Hospital in Nieuwegein between 1998 and 2007 were retrospectively included in this

study. Medical records were retrieved and patients were included according to current ATS/ ERS guidelines; a histologic or radiologic pattern typical of usual interstitial pneumonia (UIP).[18] Diagnoses made before 2002 were reviewed by an expert clinician (JG, JvdB) and only included when current ATS/ ERS criteria were met. Other causes of UIP (drugs, collagen vascular diseases) were ruled out. Serum and BALf were collected from all ILD patients, and were systematically enrolled in our database used for scientific research. Serum samples of 71 IPF patients were available at the time of diagnosis. Bronchoalveolar lavage fluid (BALf) was available from 54 IPF patients and was obtained using fibreoptic bronchoscopy according to a previously described method.[19] Serum and BALf samples were stored at - 80°C until analysis. Bronchoalveolar lavage was performed in 30 healthy controls. The study protocol was approved by the Ethical Committee of the St. Antonius Hospital, and all subject gave their written informed consent.

Pulmonary function tests

Pulmonary function tests were performed according to ERS recommendations.[20] Vital capacity (VC), forced expiratory volume in 1 second (FEV_1) and diffusing capacity for carbon monoxide ($D_{L_{co}}$) were measured with a Jaeger System. All values were expressed as percentage of predicted value. The interval between pulmonary function testing and collection of the serum and BAL samples was less than three months. Furthermore, from most patients multiple pulmonary function tests were available, this varied according to the duration of follow-up. To assess progressiveness of the disease, the change in pulmonary function parameters during time was calculated. The change in pulmonary function tests was described as delta VC, delta $D_{L_{co}}$, and delta FEV_1.

Endothelin-1 levels

Serum and BALf levels of ET-1 were determined using a commercially available human ET-1 immunoassay (R& D systems, MN, USA) according to the manufacturer's protocol. The lower limit of detection was 0.34pg/mL

Statistical analysis

Data were expressed as median and interquartile ranges (IQR). Differences in serum or BALf concentrations between independent groups were analysed using a Mann-Whitney U test. For analysis of correlation, log-transformation was used to reach near normal distribution before Pearson's correlation was applied. To find the optimal cut-off level to discriminate survivors from non-survivors after one year, receiver operating curves (ROC) were used. The Kaplan-Meier method was used to describe survival time

and the log-rank test to evaluate statistical significance between groups. Transplants and non-IPF deaths were censored. Statistical analysis was performed using SPSS 15.0 (SPSS Inc; Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc; San Diego, CA, USA). Statistical significance was considered at a value of $p < 0.05$.

RESULTS

Seventy-one IPF patients (55 male, 16 female, mean age 62.9 years [SD 12.9]) and 30 healthy controls (18 male, 12 female, mean age 21.4 years [SD 2.1])were included. From the 71 IPF patients who donated serum, 54 patients underwent BAL. In 50 IPF patients (70%) the histological diagnosis of UIP was confirmed by open lung biopsy. Table 1 shows the clinical characteristics of patients and controls.

Table 1 Characteristics of IPF patients and healthy controls

	IPF Patients	**Healthy controls**
Number of subjects	71	30
Sex M/F	55/ 16*	18/ 12
Age, yr (mean, SD)	62.9 (12.9)*	21.4 (2.1)
Smoking status*		
Smoker	3	19
Non-smoker	18	11
Ex-smoker	50	0
BALf cellular profile (median, IQR)		
Total cell count ($\cdot 10^6$)	20.9 (13.1 - 31.0)*	12.0 (7.0-22.1)
% macrophages	75.8 (63.1 - 86.3)*	89.2 (81.6 – 94.4)
% neutrophils	5.9 (2.7 – 12.7)*	1.4 (0.7 – 2.8)
% lymphocytes	8.3 (3.5 – 14.6)	8.7 (2.4 -13.2)
% eosinophils	4.0 (1,9 – 8.9)*	0.3 (0.1 – 0.6)
Lung function, (median, IQR)		
% pred FEV_1	77 (64 - 94)*	105.5 (99.6 – 110.9)
% pred VC	76 (60 - 87)*	108.6 (102.7 – 116.5)
% pred $D_{L_{co}}$	43 (33 - 54)	-

**p < 0.05 compared to healthy controls.*

ET-1 levels in serum and BALf

The median serum ET-1 level in IPF (1.15pg/ml, IQR 0.92 – 1.42) was significantly increased compared to healthy controls (0.85 pg/ml, IQR 0.70 – 0.95), $p < 0.0001$.

Interestingly, ET-1 levels in BALf were significantly decreased in IPF (1.33 pg/ml, IQR 0.81- 1.91) compared to healthy controls (2.10 pg/ml, IQR 1.43 – 3.13), p = 0.0005. If corrected for the BALf albumin concentration the difference is even more pronounced. BALf ET-1/ albumin levels for IPF (16.7, IQR 8.7 – 33.1) were significantly decreased compared to healthy controls, (63.4, IQR 40.9 - 84.5) p < 0.0001, figure 1.

In IPF patients there was an effect of age on BALf ET-1 levels; BALf ET-1 levels were negatively correlated to age (r= -0.36, p = 0.007), however in healthy controls there was no correlation to age (r= -0.139, p = 0.48, Figure 2). There were 8 patients who used low dose oral corticosteroids, ET-1 levels in serum and BALf were not statistically different in this group. Smoking status did not affect serum or BALf ET-1 levels either.

Figure 1 A. Scatter dot plot showing serum concentrations of endothelin-1 (ET-1) in IPF patients and healthy controls. The bars represent median values with interquartile ranges.
B . Scatter dot plot showing ET-1 levels in BALf.
C . Scatter dot plot showing ET-1 levels corrected for albumin concentration in BALf.

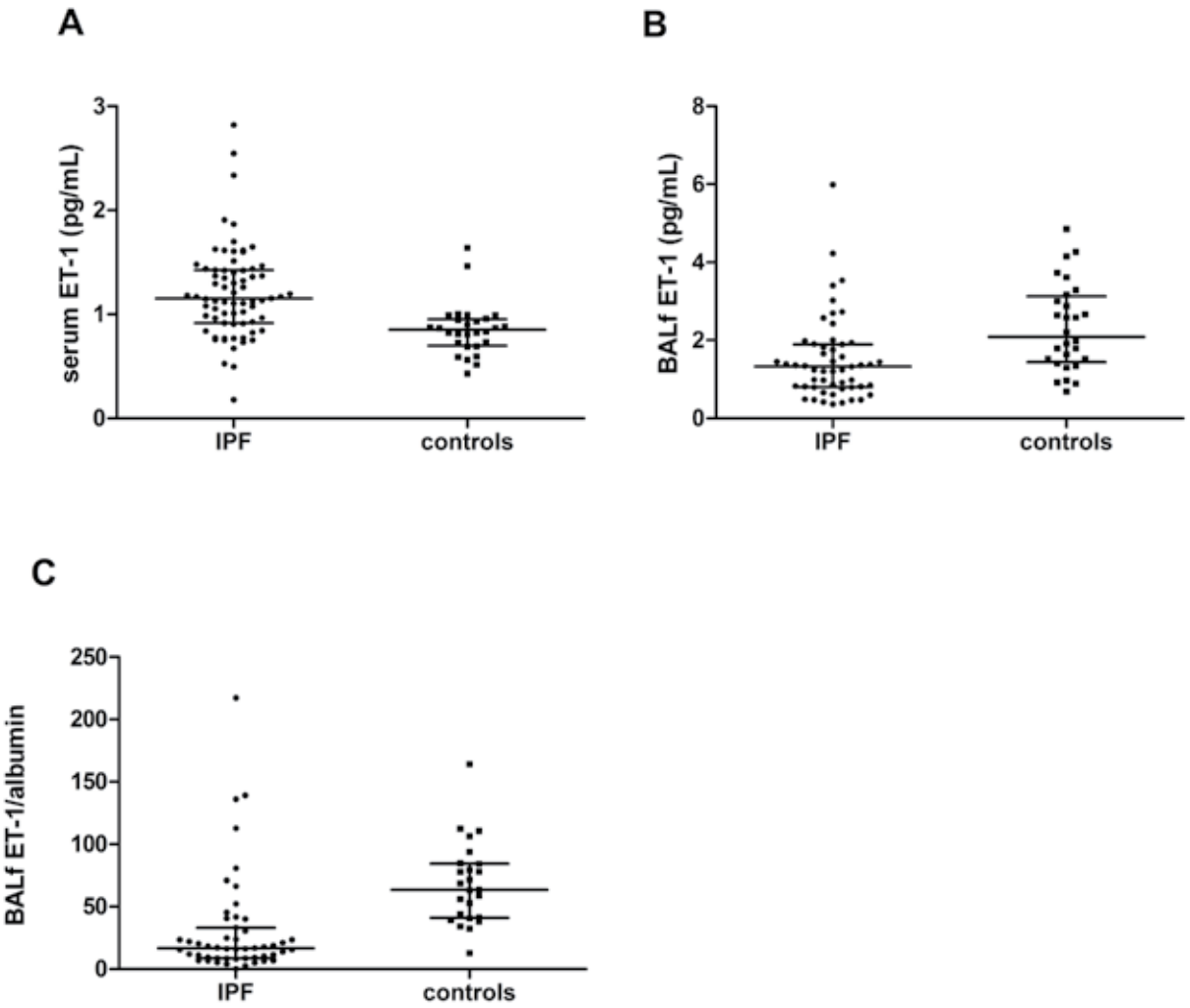
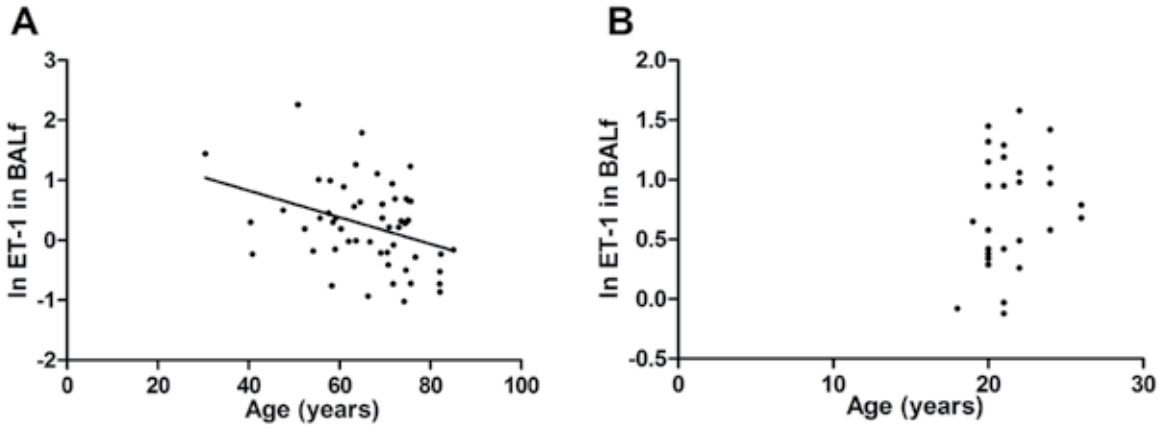


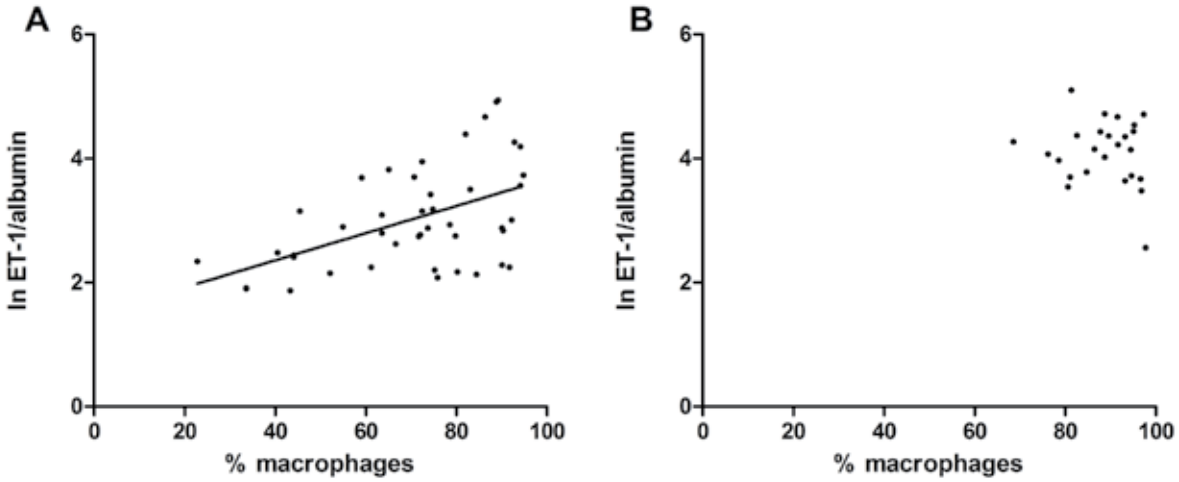
Figure 2 Scatter dot plot showing the correlation between log-transformed values of ET-1 in BALf and age at the time of BAL in IPF patients (A, r = -0.37, p = 0.007) and healthy controls (B, r = 0.21, p = 0.08)



Relation to cellular profiles in BALf

BALf levels of ET-1 and ET-1/ albumin were in IPF patients correlated to the percentage of macrophages; r = 0.34, p = 0.01 and r = 0.50, p <0.001 respectively, but not to lymphocytes, neutrophils or eosinophils. In healthy controls, there was no correlation between ET-1 or ET-1/ albumin and macrophages or other cells. (Figure 3)

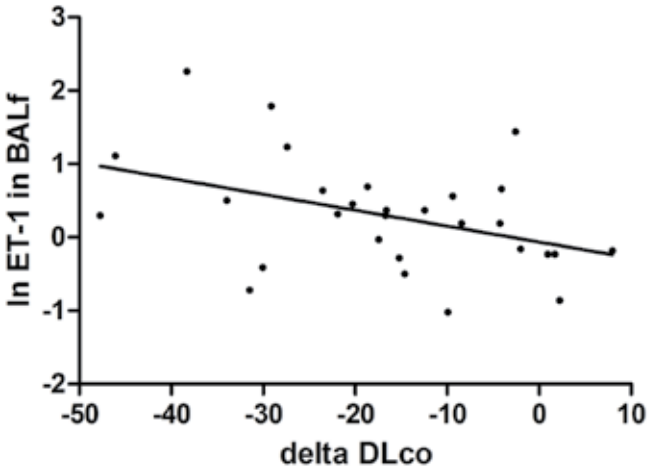
Figure 3 Scatter dot plot showing the correlation between log-transformed values of ET-1 corrected for albumin in BALf with the percentage of macrophages in IPF patients (A, r = 0.50, p = 0.0005) and healthy controls (B, r = -0.16, p = 0.45)



Relation to clinical parameters

Serum and BALf levels of ET-1 were not correlated to static lung function parameters such as DL_{CO}, FEV_1 or TLC (Pearson's correlation, not significant). However, BALf ET-1 was negatively correlated to the decrease in DL_{CO} during follow-up (delta DL_{CO}); Pearson's correlation: r = -0.41, p = 0.02 (Figure 4). Serum ET-1 was not correlated to delta DL_{CO}, FEV_1 or TLC.

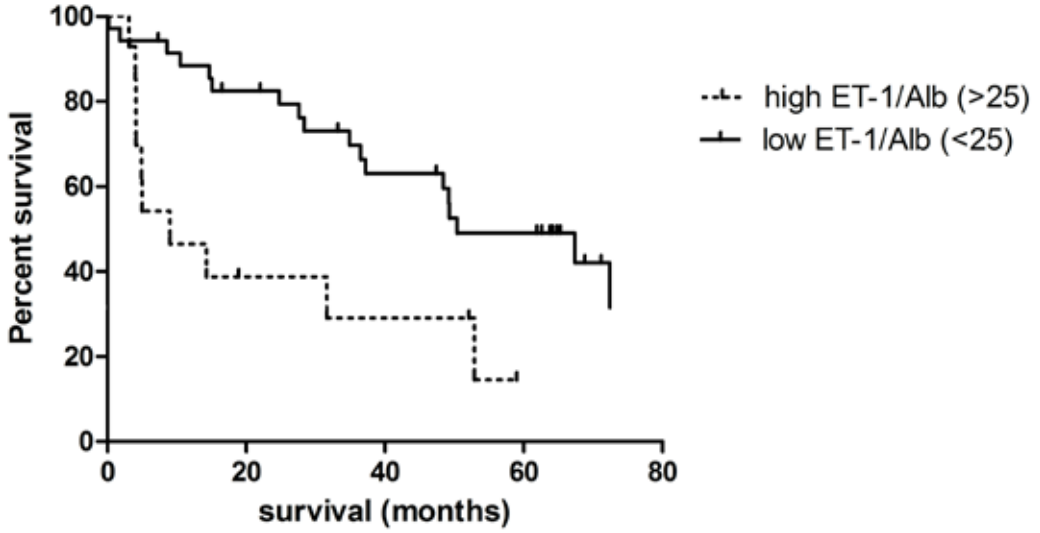
Figure 4 Scatter dot plot showing the correlation between log-transformed values of ET-1 corrected for albumin in BALf with the change in DL_{CO}, r = -0.41, p = 0.03.



Survival

The median follow-up period for IPF patients was 39 months (range 1-114). Within the study period 48 of the 70 IPF patients died, one patient was lost to follow-up. The cause of death was respiratory failure due to progressive IPF (n=37), lung carcinoma (n=4), pneumonia (n=5) and pulmonary embolism (n = 1). One patients died from an extrapulmonary cause and two patients underwent lung transplantation, those cases were censored in the survival analysis. Because only BALf ET-1/Alb was correlated to survival, the survival analysis only includes the patients who underwent BAL, this accounted for 54 patients in total. To find an optimal cut-off level for BALf ET-1/Albumin to discriminate survivors from non-survivors, ROC curves were used. According to the ROC curve, the optimal cut-off level was 25. Patients were divided into two groups according to the cut-off level of 25. Median survival in the low BALf ET-1/Alb group (n = 37) was 50.4 (SE 11.8) months, compared to a median of 9 (SE 5.6) months in the high ET-1/Alb group (n = 17), (p = 0.006, Log Rank test, Figure 5).

Figure 5 Kaplan-Meier curve showing the difference in survival time between patients with high BALf ET-1 corrected for albumin (>25) vs low ET-1 corrected for albumin (<25). Median survival was 9.0 months vs 50.4 months, p = 0.006.



DISCUSSION

In this study we showed that increased ET-1 levels were present in serum of IPF patients compared to healthy controls, but remarkably in BALf ET-1 levels were decreased. BALf ET-1 levels correlated to the percentage of macrophages in IPF patients, which was not observed in controls. High BALf ET-1 levels corresponded to progressiveness of the disease, as was showed by a greater decline in DL_{CO} and a worse prognosis compared to those who had low BALf levels of ET-1.

Increased levels of ET-1 in serum or plasma from IPF patients were previously described by Uguccioni[16] and Simler.[21] Immunohistochemical analysis of lung biopsies from patients with IPF showed increased expression of ET-converting enzyme, ET-1 and its biologically inactive precursor big ET-1.[15, 16] In IPF not only the expression was increased in airway epithelium, proliferating type II pneumocytes and in inflammatory and endothelial cells, also ET-1 expression correlated with disease activity, as characterized by the presence of inflammatory cells and granulation tissue.[15] These findings suggest that ET-1 may contribute to the pathogenesis of IPF.

A new observation in this study is that IPF patients have a significant decrease in BALf ET-1 levels compared to healthy controls. Another publication in which BALf ET-1 levels are measured in IPF patients showed increased ET-1 levels and no significantly different ET-1/albumin levels in BALf compared to controls. However, this study included only 9

IPF patients and the control group consisted of a heterogeneous group of 19 patients who had no interstitial lung disease, but tumors (n=6), fever of unknown origin (n=2) or no proven pulmonary disease (n=11).[17] One may speculate about an increased leakage through the damaged interstitium which overrides the secretion of ET-1 by epithelial cells and macrophages. Moreover, when extensive fibrosis is present, the absolute number of cells that produce ET-1 may become less than in the healthy lung. As we do not have a clear explanation for this finding, we encourage further studies on this subject.

We found a correlation between the percentage of macrophages and ET-1 levels in BALf in IPF patients but not in controls. Interestingly, Shahar et al described that macrophages in IPF patients secrete ET-1, whereas this was not observed in alveolar macrophages from controls.[12] Macrophages play a profibrotic role in IPF by the release of fibronectin, which acts as a chemotactic and proliferative agent for fibroblasts.[22, 23] Macrophages in IPF display an alternatively activated phenotype. There seem to be two different types of macrophages: the classically activated macrophage is activated by LPS or IFN-γ, the alternative pathway by IL-4 or glucocorticoid. The alternatively activated macrophages increase proliferation and collagen synthesis of fibroblasts, while classically activated macrophages do not.[24] The alternatively activated macrophage may well explain the release of ET-1 in IPF patients, while there is no secretion of ET-1 in healthy controls.

In animal models an increased expression was described of ET-1 mainly in macrophages and alveolar epithelial cells after the instillation of bleomycin. This resulted in an increase in collagen deposition.[25] Further, a reduction in bleomycin-induced fibrosis was observed after treatment with bosentan.[26] In another animal model, transgenic mouse overexpressing the human ET-1 gene did not develop significant pulmonary hypertension, but progressive pulmonary fibrosis and recruitment of inflammatory cells.[27] In this perspective one expected that bosentan in human IPF would influence the course of IPF, however it has recently been shown that bosentan did not affect time to worsening or death due to IPF.[28]

Our finding that BALf levels of ET-1 correspond with the progressiveness of IPF may rise the question whether ET-1 will be a good biomarker for the prognosis of IPF. The need for a useful biomarker in IPF has emerged from the short survival and high waiting list mortality, which forces us to search for tools that may predict survival more accurately than the current waiting list allocation score. Powerful markers for survival eventually may give IPF patients priority on the waiting list. A useful prognostic biomarker needs to be minimally invasive, reproducible and sufficiently specific in predicting prognosis.[29] Although several biomarkers for the prognosis of IPF have been under investigation, there is still no perfect biomarker available and routinely used in

clinical practise.[30] ET-1 is involved in the pathogenesis of IPF, but ET-1 in serum is not correlated to parameters of disease progressiveness. ET-1 in BALf however, is related to the decline in DL_{CO} and survival, which thus is a marker for progressiveness. The increased levels of ET-1 in BALf in patients who have the worst prognosis gives us insight into the role of ET-1 in disease progression, but since bronchoalveolar lavage is an invasive method, it is not very likely that ET-1 in BALf will be used in clinical practise to predict prognosis.

A limitation of this study is that patients and controls were not well matched for age. A relation was found between age and ET-1 levels in BALf in IPF patients, but not in healthy controls. We do not know if part of the difference in ET-1 levels between IPF patients and controls may lie in the difference in age. This illustrates the difficulty in recruiting healthy elderly controls.

The standardization of BAL procedure is difficult, resulting in variable retrieval and thus concentrations of the recovered molecules. As variability in retrieval was equal between IPF patients and healthy controls, this would have little consequences when comparing median levels of ET-1 between patients and controls. However, when correlating individual BALf ET-1 concentrations to outcome measurements such as lung function parameters or survival, there may be more error due to dilution. As an attempt to circumvent this problem, ET-1 concentrations were related to the albumin concentration. It is a common used correction in patients with interstitial lung disease [17,31,32], but since albumin is increased in interstitial lung disease, it is still controversial.[33-35]

This study confirms the present concept that ET-1 is implicated in the pathogenesis of IPF. Increased levels of serum ET-1 and a strong correlation of ET-1 with macrophages seem to reflect the increased production of ET-1 in IPF by macrophages, proliferating type II pneumocytes and endothelial cells. However, a new question arises from our findings that BALf levels of ET-1 are significantly decreased compared to healthy controls, an observation that needs further investigation. The relation of increased BALf ET-1 levels with more progressive disease and a worse prognosis implicates a determining role for ET-1 in the course of IPF.

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6

GENETIC VARIABILITY IN THE IL1RN GENE AND THE BALANCE BETWEEN IL1-RA AND IL-1B IN IPF

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ABSTRACT

Introduction - Idiopathic pulmonary fibrosis (IPF) is a rapidly progressive interstitial lung disease of unknown etiology. Interleukin (IL) -1β plays an important role in inflammation and has been associated with fibrotic remodelling. We investigated the balance between IL-1β and interleukin-1 receptor antagonist (IL-1Ra) in bronchoalveolar lavage fluid (BALf) and serum as well as the influence of genetic variability in the IL1B and IL1RN gene on disease susceptibility and cytokine levels.

Materials and Methods - In 77 IPF patients and 349 healthy controls, single nucleotide polymorphisms (SNPs) in the IL1RN and IL1B gene were determined. Serum and BALf IL-1Ra and IL-1β levels were measured using a multiplex suspension bead array system and were correlated with genotypes.

Results - Both in serum and BALf a significantly decreased IL-1Ra/ IL-1β ratio was found in IPF patients compared to healthy controls. In the IL1RN gene, one SNP was associated with both the susceptibility to IPF and reduced IL-1Ra/ IL-1β ratios in BALf.

Discussion - Our results show that genetic variability in the IL1RN gene may play a role in the pathogenesis of IPF and that this role may be more important than until recently thought. The imbalance between IL-1Ra and IL-1β might contribute to a pro-inflammatory and pro-fibrotic environment in their lungs.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease, of unknown etiology, and is characterized by an extremely poor prognosis of 2 – 4 years after diagnosis. [1-3] The pathogenetic mechanisms underlying IPF are incompletely understood. The disease is characterized by abnormal repair and airway remodelling and is associated with increased pro-inflammatory and pro-fibrotic signals. Previous research has shown that IL-1 cytokines are involved in the development of fibrosis[4]

The IL-1 family consists of three structurally related proteins, of which two are agonists (IL-1α and IL-1β) and the third, interleukin receptor antagonist (IL-1Ra), is a competitive antagonist. IL-1Ra is the inhibitor of these IL-1 agonists and acts by competitively binding to IL-1 receptors without eliciting signal transduction[5] Interleukin-1 beta (IL-1β) is produced by activated macrophages and epithelial cells, inducing production of other cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6).

Polymorphisms in the interleukin-1 receptor antagonist gene (IL1RN) and tumor necrosis factor alpha gene (TNF) have been associated with susceptibility to IPF[6, 7] Several studies suggest that IL-1β and IL-1Ra play a critical role in bleomycin-induced fibrosis in mice. Fibrosis is induced by IL-1β and neutralization of IL-1β by antibodies or specific blockage of the receptor IL-1R1 reduces the development of fibrosis.[8] In normal homeostasis, IL-1Ra production by alveolar macrophages is higher than the production of IL-1β. However, decrease in the ratio of IL-1Ra to IL-1β favours the augmentation of the pro-fibrotic function of IL-1β.[9]

The aim of this study was to investigate both the predisposition and disease modifying effects of genetic variations in the IL1B and IL1RN gene and corresponding pro-inflammatory cytokine levels in serum and bronchoalveolar lavage fluid (BALf) in a cohort of IPF patients.

METHODS

Patients and healthy controls

Patients with IPF presenting at the Department of Pulmonology of the St Antonius Hospital in Nieuwegein between 1998 and 2007 were included in this study. From that time serum, BALf and DNA were collected from all ILD patients presented at our department after informed consent was given. These patients were enrolled in our database for scientific research. Retrospectively, the diagnosis of IPF was reviewed and validated using current ATS/ERS guidelines. Diagnoses made before 2002 were

reviewed by an experienced clinician (JvdB, JG), and patients were only included when current ATS/ ERS criteria were met. Other causes of UIP (drugs, collagen vascular diseases) were ruled out. Seventy-seven IPF patients (mean age 60.8 years [SD 13.6], 58 males, 19 females) were included in the present study and donated DNA. In 54 of 77 cases also serum and BALf samples were available at the time of diagnosis. At the time of serum sampling 8 patients received low dose oral corticosteroids. In 58 cases the diagnosis of UIP was confirmed on lung biopsy (75%). BALf was collected as previously described.[10] Samples were stored at – 80°C until analysis. Median lung function parameters at the time of diagnosis were as follows: FVC 75.7 % predicted (IQR 61.7 – 87.3), DL_{CO} 42.5 % predicted (IQR 33.1 – 55.6)

The control group consisted of 349 healthy Caucasian volunteers (mean age 39.2 years [SD 12.4], 139 males, 210 females). In 36 cases of the control group, BAL was performed and in those controls cytokine levels in serum and BALf were measured. The study protocol was approved by the Ethical Committee of the St. Antonius Hospital and all subjects gave written informed consent.

Genotyping

Three haplotype tagging single nucleotide polymorphisms (SNPs) for each gene were selected using the Tagger program for the gene region of IL1B and IL1RN ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted under the pairwise tagging option, with a minimum allele frequency of 25% and a high Illumina design score. The algorithm was set to select tags that would cover the Caucasian HapMap panel with an r^2 of 0.8 or greater.[11] Furthermore, for both genes one additional custom SNP was selected on the basis of previously published association studies or presumed functionality. The following single nucleotide polymorphisms were genotyped in the IL1B gene; rs1143627 (tag), rs1143634 (tag), rs1143643 (tag) and rs1799916 (custom); IL1RN: rs11677397 (custom), rs2637988 (tag), rs408392 (tag), rs397211 (tag). DNA was extracted from whole blood samples and SNP typing was conducted using a custom Illumina goldengate bead SNP assay in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, USA).

Cytokine levels

Serum and BALf levels of IL-1β and IL-1Ra were determined using a multiplex suspension bead array system according to the manufacturer's protocol (Bio-Rad Laboratories, CA, USA). Data analysis was performed using the Bioplex 100 system and Bioplex Manager software version 4.1 (Bio-Rad Laboratories, CA, USA). The lower limit of detection was 0.3 pg/ml for IL-1β and 2.2 pg/ml for IL-1Ra. Since the variation in BALf retrieval in healthy controls was not significantly different from retrieval in IPF patients, we did not correct for that.

Statistical analysis

Genotype frequencies were tested for Hardy-Weinberg equilibrium (http://ihg2.helmholtz-muenchen.de/ihg/snps.html). Genotype and allele frequencies in the IPF group were compared with the control population using chi-square test. Haplotypes and linkage disequilibrium (LD) were calculated (Haploview 4.1, Broad Institute of MIT and Harvard, USA). Serum and BALf data were expressed as median and interquartile ranges (IQR). Differences in serum or BALf concentrations between patients and controls were analysed using a Mann-Whitney U test. For analysis of correlation, log-transformation was used to reach near normal distribution. The correlation between cytokines in BALf and clinical data was assessed using Pearson's correlation coefficients. The differences between cytokine levels in different genotypes were assessed with the Kruskal-Wallis test. Statistical analysis was performed using SPSS 15.0 (SPSS Inc; Chicago, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc; San Diego, USA). Statistical significance was considered at a value of $p < 0.05$.

RESULTS

IL-1 levels in serum and BALf

Serum levels of IL-1β in IPF patients were significantly increased compared to healthy controls while serum levels of IL-1Ra were decreased (table 1). Furthermore, BALf levels of both IL-1β and IL-1Ra were significantly increased in IPF patients compared to healthy controls. In the IPF group there were 8 patients receiving low dose corticosteroids, the median serum and BALf IL-1Ra levels were significantly higher in the patients who were on corticosteroids; serum IL-1Ra 284.3 (IQR 202.3 – 515.3) vs 214.3 (IQR 175.6 – 255.2), p = 0.006; BALf IL-1 Ra 152.9 (IQR 67.2 – 622.3) vs 74.0 (IQR 37.0 – 121.4), p = 0.026. IL-1β levels were not affected by corticosteroids.

Table 1 Serum levels in IPF patients and healthy controls

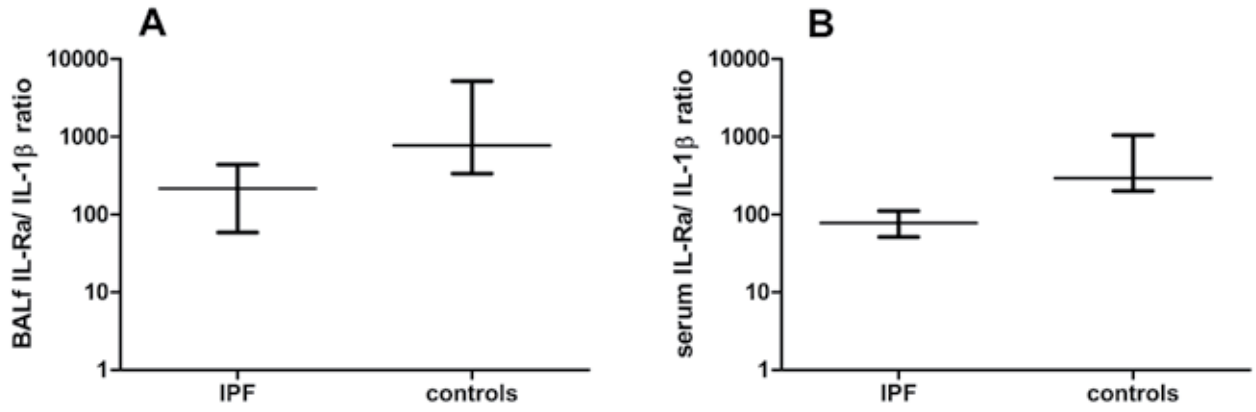
	IPF Patients N = 54	**Healthy controls** N = 36
Serum levels (median, IQR)		
IL-1β (pg/ml)	3.2 (2.3 – 4.1)*	1.4 (0.6 – 2.1)
IL-1Ra (pg/ml)	224.6 (179.3 – 312.0)*	406.7 (309.5 – 690.7)
BALf levels (median, IQR)		
IL-1β (pg/ml)	0.6 (0.3 – 2.7)*	< 0.3
IL-1Ra (pg/ml)	87.2 (43.1 – 138.1)*	36.4 (26.3 – 48.3)

**p < 0.05 compared to healthy controls.*

As IL-1Ra inhibits the physiological activities of IL-1β by occupying the IL-1 receptor, we evaluated IL-1Ra in relation to IL-1β through calculation of the IL-1Ra/ IL-1β ratio. IPF patients showed a 3.5 -fold decrease in IL-1Ra/ IL-1β ratio in BALf (215.7; IQR 58.6 – 437.9) compared to healthy controls (771.4; IQR 337.4 – 5210.0), $p < 0.0001$. A similar decrease in IL-1Ra/ IL-1β ratio was found in serum from patients (77.9; IQR 51.5 – 110.9) compared to healthy controls (293.5; IQR 201.1 – 1054.0), $p < 0.0001$ (figure 1).

The IL-1Ra/ IL-1β ratio in serum was significantly affected by the use of corticosteroids, the 8 patients who were on corticosteroids had a significantly higher IL-1Ra/ IL-1β ratio: 101.7 (IQR 77.2 – 143.4) vs 71.5 (IQR 51.0 – 102.2), $p = 0.01$. In BALf there was no significant difference.

Figure 1 BALf (A) and serum (B) IL-1Ra/ IL- 1β ratios in patients with idiopathic pulmonary fibrosis (IPF) and healthy controls, both $p < 0.0001$. Data are shown as median with interquartile ranges.



Polymorphisms in cytokine genes

Table 2 summarizes allelic and genotype frequencies in IPF patients and controls. Both populations were in Hardy-Weinberg equilibrium for all genotypes. One SNP in the IL1RN gene was associated with IPF. The frequency of the rs2637988 allele 2 (G) in the IL1RN gene was increased in the IPF group (47%), compared to the controls (38%), $p = 0.04$. The best fitting genetic model was a risk conferred by the carriage of allele 2 compared to non-carriers; OR 1.95 (95% CI 1.11 – 3.42; $p = 0.02$). Frequency of the rs408392 allele 2 (T) was increased in IPF patients and showed a trend towards significance; allele 2 occurred in 32% of the IPF patients compared to 26% in controls, $p = 0.09$. For carriage of allele 2 versus non-carriers, the OR was 1.58 (95% CI 0.96 - 2.60, $p = 0.07$). There was significant linkage disequilibrium between the two SNPs; D' = 0.94, $r^2 = 0.46$. Additionally, haplotype frequencies were calculated. Haplotype analysis was of no superior value compared to single SNP analysis.

Table 2 Genotype and allele frequencies of the IL1B and IL1RN polymorphisms in IPF patients and healthy controls.

		IPF (n = 77)				Controls (n = 349)			
	Major/ minor allele	**Genotype frequency**			**Minor allele**	**Genotype frequency**			**Minor allele carriership**
		1.1	1.2	2.2	2	1.1	1.2	2.2	2
IL1B									
rs1143627	T/C	36 (28)	48 (37)	16 (12)	64 (49)	44 (153)	45 (158)	11 (38)	56 (196)
rs1143634	C/T	53 (41)	39 (30)	8 (6)	47 (36)	56 (197)	36 (125)	8 (27)	44 (152)
rs1143643	G/A	55 (42)	35 (27)	10 (8)	45 (35)	44 (152)	46 (161)	10 (36)	56 (197)
rs1799916	T/G	100 (77)	0 (0)	0 (0)	0 (0)	100 (349)	0 (0)	0 (0)	0 (0)
IL1RN									
rs11677397	C/T	49 (38)	43 (33)	8 (6)	51 (39)	56 (196)	37 (129)	7 (24)	44 (153)
rs2637988*	A/G	25 (19)	56 (43)	19 (15)	75 (58)	39 (136)	45 (158)	16 (55)	61 (213)
rs397211	T/C	45 (35)	45 (35)	9 (7)	55 (42)	51 (178)	40 (140)	9 (31)	49 (171)
rs408392†	G/T	44 (34)	47 (36)	9 (7)	56 (43)	56 (194)	37 (130)	7 (25)	44 (155)

*Genotype frequencies and minor allele carriership are shown in percentages, absolute numbers are shown in parenthesis. 1 = major allele, 2 = minor allele. *minor allele carriership IPF versus healthy controls, p = 0.02 †minor allele carriership IPF versus healthy controls, p = 0.07*

The polymorphisms in the IL1RN and IL1B genes did not significantly influence BALf or serum IL1-Ra or IL1-β levels in IPF patients and healthy controls. However, differences were seen between genotypes of the rs2637988 polymorphism and the BALf IL-1Ra/ IL1-β ratio; AA 1856 (IQR 1421- 3730), AG 223.7 (IQR 84.6 – 384.9), GG 29.3 (IQR 6.95 – 130), p = 0.005 (figure 2). A less significant effect was found when genotypes of the rs408392 polymorphism were compared (p = 0.09). Other SNPs were not associated with the IL-1Ra/ IL-1β ratio in serum or BALf.

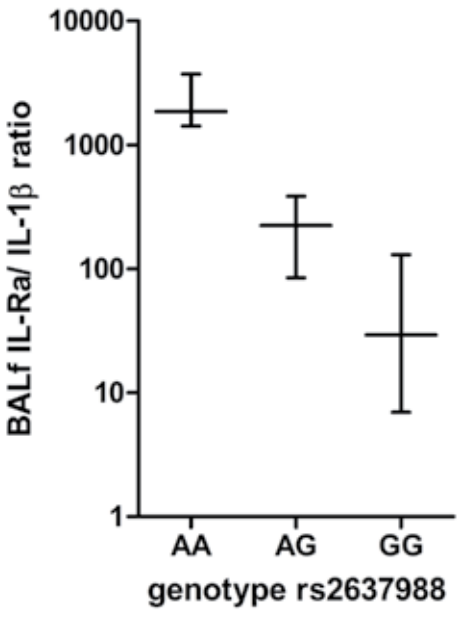


Figure 2 BALf IL-1Ra/ IL-1β ratio in IPF patients according to genotype of the rs2637988 polymorphism, AA (n = 19), AG (n = 43), GG (n = 15). Data are shown as median with interquartile ranges. BALf IL-1Ra/ IL-1β ratios are dependent on the rs2637988 polymorphism, p = 0.005 (Kruskal Wallis test)

Cellular profiles in BALf

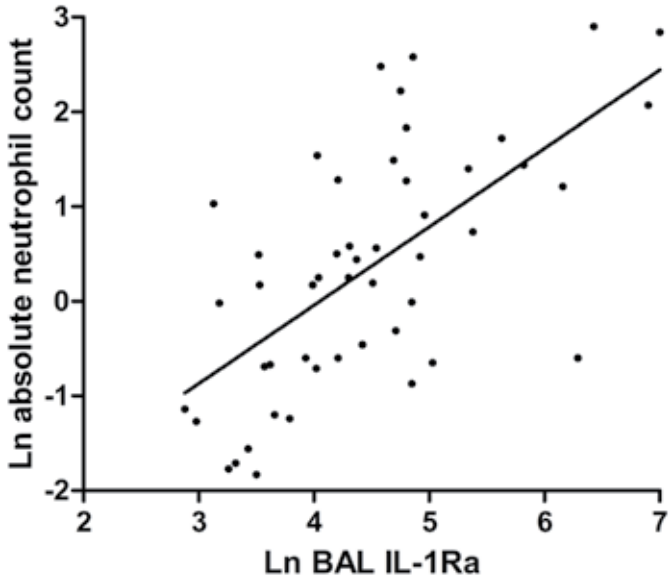
The total cell count and absolute numbers of macrophages, lymphocytes, neutrophils and eosinophils in BALf were significantly increased in IPF patients compared to healthy controls (all $p < 0.001$; table 3). The relationship between BALf cellular profiles and IL-1β and IL-1Ra is shown to illustrate the relevance in clinical perspective**.** In healthy controls, there was no correlation between BALf IL-1β levels or IL-1Ra and absolute neutrophil counts. However, in IPF patients absolute neutrophil counts were correlated to both BALf levels of IL-1β ($r = 0.32$, $p = 0.05$) and IL-1Ra ($r = 0.65$, $p < 0.001$), fig 3.

Table 3 Cellular profiles in BALf in IPF patients and healthy controls

	IPF Patients **N = 54**	**Healthy controls** **N = 36**
Cellular profiles in BALf (median, IQR)		
Total cell count ($\times 10^6$)	20.9 (13.1 – 30.1)*	11.8 (7.2 – 20.3)
Macrophages ($\times 10^6$)	17.1 (12.7 – 25.2)*	6.4 (6.1 – 18.8)
Lymphocytes ($\times 10^6$)	1.6 (0.6 – 3.6)*	0.9 (0.4 – 1.3)
Neutrophils ($\times 10^6$)	1.3 (0.5 – 3.8)*	0.2 (0.1 – 0.3)
Eosinophils ($\times 10^6$)	0.8 (0.4 – 2.7)*	0.03 (0.01 – 0.08)

**p < 0.05 compared to healthy controls.*

Figure 3 Scatter plot illustrating the correlation between absolute neutrophil count in IPF patients with IL-1Ra in BALf. Values on the X and Y-axis represent log-transformed values. $r = 0.65$, $p < 0.001$.



DISCUSSION

Disease development in IPF is thought to result from repetitive injury to epithelial cells and an abnormal fibrotic response. Pro-inflammatory mediators, like IL-1β, are known to promote fibrosis, but can be regulated by the receptor antagonist IL-1Ra. In the present study, we found that the ratio between IL-1Ra and IL-1β was decreased in both serum and BALf of IPF patients compared to healthy controls. Furthermore, we showed that one SNP in IL1RN, rs2637988, associated with susceptibility to IPF and with the IL-1Ra/IL-1β ratio in BALf.

A predisposing effect of genetic variation in IL1RN was previously described by Whyte et al. who found an increased risk of fibrosing alveolitis in an Italian and a British population.[6] They investigated the IL1RN+2018 SNP, which in the Caucasian Hapmap panel is in complete linkage disequilibrium with our tag rs408392 ($r^2 = 1$). In our study, rs408392 was not the most significantly associated SNP, although carriership of allele 2 of rs408392 was more common in patients with IPF (p=0.07). In other studies the variable number of tandem repeats (VNTR) in intron 2 of IL1RN was investigated and found to be in linkage disequilibrium with the IL1RN+2018 SNP. However, both a small Australian[7] and an independent Czech cohort[12] did not reveal any association between the VNTR and IPF susceptibility.[13] Functional effects of IL1RN+2018 alleles have been demonstrated by Carter et al. They showed that IL1RN+2018 allele 2, not only correlated with the susceptibility to ulcerative colitis, but also to a significantly decreased ratio between the protein and mRNA content of IL-1Ra and total IL-1 in the colonic mucosa.[14]

Although we found the same trend as reported in the Italian and British cohorts, our data suggests that carriership of the G allele of IL1RN rs2637988 is more strongly associated with IPF. Carriership of the G-allele is higher in IPF patients (75%) compared to controls (61%), p = 0.02. In addition, we showed that IPF patients carrying the rs2637988 G-allele had a significantly lower IL-1Ra/ IL-1β ratio in BALf, suggesting a relative shortage of IL-1Ra compared to IL-1β. This implies that presence of the G allele has a pathogenic role in IPF.

The balance between IL-1 and IL-1Ra seems crucial in inflammatory diseases.[15-18] Although IPF is not primarily an inflammatory disease, IPF is characterized by high levels of inflammatory parameters. The balance between IL-1 and IL-1Ra has rarely been studied in IPF, but extensively in inflammatory diseases. In inflammatory bowel disease, changes in the IL-1Ra/IL-1β ratio have also been studied. Protein levels in the colonic mucosa of IL-1Ra, IL-1α and IL-1β were higher than in controls, but the ratio between IL-1Ra and total IL-1 was significantly decreased.[14,19] Similarly, it was found that the protein and gene transcript ratio between IL-1Ra and IL-1β in cultured alveolar

macrophages of patients with interstitial lung diseases were significantly lower in comparison with healthy controls.[9] We found that IL-1Ra levels in BALf of IPF patients were increased, but this was not enough to equal the vast increase in local IL-1β. Altogether this resulted in a 3.5 fold decrease in the IL-1Ra/IL-1β ratio in IPF patients compared to healthy controls.

In animal studies it has been shown that alterations in the balance between IL-1β and IL-1Ra cause the development of lung fibrosis. Mice with bleomycin-induced fibrosis have an upregulated expression of IL-1β mRNA after instillation of bleomycin[20], and addition of recombinant IL-1β induces fibrotic remodelling.[8] Overexpression of IL-1β in rat lungs after intratracheal administration of bleomycin was associated with severe progressive tissue fibrosis in the lung, characterized by the presence of myofibroblasts, fibroblast foci, and significant extracellular accumulations of collagen and fibronectin.[4] Other studies showed that administration of exogenous IL-1Ra prevented or even reversed the generation of pulmonary and synovial fibrosis.[21-23] The pathogenetic processes in bleomycin-induced fibrosis are just a model for IPF and results can not be extrapolated to human IPF. However, in patients with acute myocardial infarction, there is evidence that IL-1 blockade with IL-1Ra suppresses the inflammatory response and positively affects tissue remodelling.[24]

IL-1 ligands such as IL-1α, IL-1β and IL-1Ra all bind to the IL-1 receptor (IL-1R1). Mice lacking the IL-1R1 receptor showed significantly reduced cellular infiltrates, alveolar wall destruction, and collagen deposition. Moreover, blockade of the IL-1R1 receptor by exogenous IL-Ra (anakinra) dramatically reduced neutrophil influx and the formation of bleomycin-induced fibrosis in mice.[8] Altogether, IL-1 seems to be a critical cytokine and may possibly be a therapeutical target in IPF.

There are different hypotheses about the role of inflammation and thus pro-inflammatory cytokines like IL-1β in the role of pulmonary fibrosis. Historically, the hypothesis was that inflammation in response to an unknown agent was the key process in IPF, ultimately resulting in fibrosis. The current concept is that IPF is a result of repeated episodes of lung injury, with a minor role for inflammation. This concept states that inflammation in IPF could be a consequence of the architectural remodelling, rather than a cause. The increased parameters of inflammation such as neutrophilia in BALf may be a reflection of remodelling and traction bronchiectasis due to fibrosis.[25] However, this does not exclude a role for inflammation in an earlier stage of the disease. An interesting paper in this context is the study of Flaherty et al[26] in which the co-existence of UIP and NSIP has been described in a considerable amount of patients who had multiple lung biopsies, demonstrating the presence of chronic inflammation and fibrosis next to each other. This pleads for a hypothesis in which UIP and NSIP are two different entities in one continuum. Before discarding the

role of inflammation in the pathogenesis of IPF, we first need to understand the natural history of UIP.[27] Our hypothesis states the association of a SNP in the IL1RN gene with IPF predisposition, this suggests a role for IL-1 in the beginning of the pathogenetic process.

The present study is one of the more expanded studies evaluating IL-1Ra and IL-1β cytokine polymorphisms and corresponding protein levels in IPF. However, a limitation of this study is that the number of IPF patients is relatively small for genetic associations. On the other hand, the results are in line with previously published literature.[6,28] Although our data suggests no effect of age or gender on the IL-1Ra/ IL-1 β ratio (results not shown), more studies are needed to confirm the role of a decreased ratio in IPF. Another point that needs attention is that the rs2637988 polymorphism influenced the IL-1Ra/IL-1β ratio of but not the individual cytokine levels. The cytokine values of IL-1Ra and IL-1β were not significantly influenced, but a mild trend is present. Carriers of the G allele had a slightly lower BALf IL-1Ra level (p=0.21) and a higher BALf IL-1β level (p = 0.16). Although both not significant, when the ratio is calculated this effect is enhanced. A hypothetical explanation is that the balance between pro- and anti-inflammatory cytokines is of more biological importance than the absolute concentrations of IL-1Ra and IL-1β. Carter et al.[14] showed that carriage of the IL1RN+2018 allele 2 was associated with a reduced colonic IL-1Ra protein level and a reduced IL-1Ra/ total IL-1 ratio. It is likely that in our population a similar effect is present, however our population might not be big enough to illustrate this with significant results, this should be replicated in a larger cohort.

In conclusion, this study showed that variation in the IL1RN associates with susceptibility to IPF. The subsequent imbalance between IL-1β and IL-1Ra might have a significant pathogenetic effect in IPF patients. Better understanding of the role of these mediators in the context of disease susceptibility and progression is important as it may help us to find rational for newly available therapies.

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7

GENETIC VARIATION IN CCL18 GENE INFLUENCES CCL18 EXPRESSION AND CORRELATES WITH SURVIVAL IN IPF

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ABSTRACT

Rationale - IPF is a progressive fibrotic disease, characterized by fibroblast proliferation and extracellular matrix deposition. CC-chemokine ligand 18 (CCL18) upregulates the production of collagen by lung fibroblasts and is a promising biomarker in IPF.

Objectives - To evaluate the influence of single nucleotide polymorphisms (SNPs) in the CCL18 gene on CCL18 expression and survival in IPF patients.

Methods - Serum CCL18 levels and four SNPs in the CCL18 gene were analysed in 77 IPF patients and 349 healthy volunteers. CCL18 mRNA expression was analysed in peripheral blood mononuclear cells (PBMCs) from 18 healthy subjects. We related mRNA expression and serum CCL18 levels to genotypes. Further, survival data from IPF patients were analysed for dependency on serum CCL18 levels and genotypes.

Measurements and Main Results - IPF patients demonstrated significantly higher CCL18 serum levels than healthy controls ($p<0.0001$). Both in IPF patients and in healthy controls, serum CCL18 levels were influenced by genotype of the rs2015086 C>T polymorphism, resulting in the highest levels for individuals carrying the C allele. Constitutive CCL18 mRNA expression in PBMCs was significantly increased in individuals carrying the C-allele and correlated with serum CCL18 levels. In IPF patients, high serum levels correlated with decreased survival ($p = 0.02$). Patients carrying the CT genotype showed a significantly worse survival than patients with the TT genotype ($p=0.01$).

Conclusions - Genetic variability in the CCL18 gene accounts for significant differences in CCL18 mRNA expression and serum levels and showed to have a modifying role in the course of IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lung parenchyma, characterized by fibroblast proliferation and extracellular matrix deposition. The prognosis remains poor, and trials for the treatment of IPF mostly yielded only minor advances or even negative results.[1-5] Median survival time for patients with IPF varies between 2.5 and 4 years.[6-8] There is substantial inter-individual difference in the clinical course of the disease, ranging from rapid decline from time of diagnosis to periods of relative stability for many years before decline. To predict the disease course, an increasing number of studies investigated the use of several biomarkers in IPF.[9-13]

CC-chemokine ligand 18 (CCL18) is a promising biomarker for IPF. Serum and bronchoalveolar lavage fluid (BALF) levels of patients with idiopathic interstitial pneumonias showed a significant elevation of CCL18 compared to healthy controls.[14] Further, in patients with systemic sclerosis, elevated serum CCL18 levels sensitively reflected pulmonary fibrosis activity.[15] Recently, a clear relationship has been demonstrated between elevated serum levels of CCL18 and a diminished survival in IPF patients.[16]

CCL18 is predominantly expressed by alveolar macrophages and occurs at relatively high levels in human lung tissue.[17] In response to CCL18, lung fibroblasts from healthy adults showed increased expression of collagen mRNA.[18] Furthermore, it was shown that alveolar macrophages from patients with pulmonary fibrosis display an alternatively activated phenotype, which up-regulates the production of collagen by lung fibroblasts via the production of CCL18.[19] As fibroblast contact and exposure to collagen increases spontaneous CCL18 production by alveolar macrophages, a positive feedback loop was suggested that may perpetuate fibrosis.

The gene encoding CCL18 is small, positioned at the q arm of chromosome 17 and consist of 3 exons. A considerable number of SNPs are present in the region. In line with these findings, we hypothesized that genetic variation in the CCL18 gene might be associated with increased CCL18 expression, and may predispose to an unfavourable prognosis in subjects with IPF.

METHODS

Patients and healthy controls

Seventy-seven IPF patients (58 male, 19 female, median 61.4 age years [IQR 54.1–71.6]) were included in this study (table 1). IPF patients were diagnosed at the Department

of Pulmonology of the St. Antonius Hospital in Nieuwegein between 1998 and 2007. Diagnoses made before 2002 were reviewed by expert clinicians (JG, JvdB) and only included when current ATS/ ERS criteria were met.[20] Lung biopsy was performed in 58 patients (75%) and revealed a pathological pattern of usual interstitial pneumonia (UIP). Follow-up was scheduled according to the patients' condition. Stable patients visited our clinic at least twice a year, deteriorating patients up to six times a year. To determine survival status and cause of death we retrieved medical records and, if not conclusive, we contacted the patient's general practitioner.

DNA was collected from 349 healthy subjects (139 male, 210 female, median age 39.4 years, [IQR 28.3 – 49.1]). All controls and all but three patients were of Caucasian descent. The study protocol was approved by the Ethical Committee of the St. Antonius Hospital and all subjects gave written informed consent.

Table 1 Baseline characteristics IPF patients.

	IPF
	N = 77
Age - yr	
Median (IQR)	61.4 (54.1–71.6)
Male sex – no. (%)	58 (75)
Smoking	
Non-smoker – no. (%)	18 (24)
Smoker – no. (%)	3 (4)
Ex-smokers – no. (%)	56 (73)
Lung function	
FVC, % pred, median (IQR)	75.7 (61.7 – 87.3)
$DL_{CO,}$ % pred, median (IQR)	42.5 (33.1-55.6)

Material

Serum and DNA were systematically collected from all ILD patients who visited our outpatient clinic, after written informed consent was obtained. Serum, DNA and patient characteristics were enrolled in a scientific database. Ultimately, seventy-seven IPF patients donated DNA, and from 64 patients serum was available at the time of diagnosis. Characteristics of patients who donated serum were not significantly different from the total group of IPF patients. At the time of serum sampling, 8 patients received low dose corticosteroids. From the total group of 349 healthy controls, 204 healthy controls were selected to measure serum CCL18 concentrations according to genotype of rs2015086 with a preference for the minor allele. Thus, all serum samples of patients with the CC and CT genotype were analysed, and in addition randomly

selected samples from patients with TT were analysed. Within two hours from sampling, blood samples were centrifuged for 10 minutes at 2200 rpm, serum was transferred to a new tube and stored at -20°C. Every two months stored samples were moved to –80°C until analysis.

CCL18 levels

Levels of CCL18 were analysed using a monoplex suspension bead array system. CCL18 antibodies (R&D systems, Minneapolis, MN, USA) were coupled to fluorescent carboxylated beads (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.[21] Data analysis was performed using the Bioplex 100 system and Bioplex Manager software version 4.1 (Bio-Rad Laboratories, CA, USA). The lower limit of detection was 0.9 pg/ml.

Genotyping

Two SNPs with presumed functionality in the promoter region were genotyped (rs712040, rs2015086). To cover all genetic variability in the CCL18 gene, additionally two haplotype tagging SNPs (rs712042, rs712044) were selected using the Tagger program for the genomic region of CCL18 ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted using the pair wise tagging option, a minimum allele frequency setting of 10% and a high Illumina design score. The algorithm was set to select tags that would cover the Caucasian HapMap panel with an r^2 of 0.8 or greater.[22] DNA was extracted from whole blood samples and SNP typing was conducted using a custom Illumina goldengate bead SNP assay. The assay was performed in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, CA, USA).

RNA expression analysis

Peripheral Blood Mononuclear Cells (PBMCs) from 18 healthy donors were isolated from heparinized venous blood using Ficoll-Paque density gradient centrifugation and cryopreserved until further analysis. After carefully thawing, the expression of CCL18 mRNA was analysed by quantitative RT-PCR amplification. Total RNA was isolated from PBMC using de RNeasy microkit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. 0.2 µg RNA was used for first-strand cDNA synthesis using the i-script cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). The obtained cDNA was diluted 1/10 with water of which 4 µl was used for amplification in a reaction volume of 20 µl. Primer sets were purchased from Sigma. The PCR was performed with the RT^2 Real-Time^{TM} SYBR Green PCR master mix (SA-Biosciences, Frederick, USA) according to the manufacturer's protocol. Samples were amplified using a biorad MyiQ real time PCR detection system for 40 cycles (10 sec at 95˚C, 20 sec at 55˚C and

25 sec at 72˚C. The copy number of the CCL18 was normalized by the housekeeping gene β-actin, and is presented as the number of transcripts per 1 copy of β-actin.[23]

Statistical analysis

Genotypes were tested for Hardy–Weinberg equilibrium using the website http://ihg2.helmholtz-muenchen.de/ihg/snps.html. Linkage disequilibrium (r^2) was calculated using the computer program Haploview (Haploview 4.1, Broad Institute of MIT and Harvard, USA). Haplotypes were determined using Phase v2.1.[24] Data are presented as medians and interquartile ranges (IQR). Statistical comparisons were made with the use of the Mann–Whitney-U test for two groups or Kruskal-Wallis for more than two groups. To define the optimal cut-off point with the highest predictive accuracy for one year survival we performed multiple receiver operating curves (ROC) for distinct CCL18 serum concentrations. The optimal cut-off point was used to stratify patients in the survival analysis. The Kaplan-Meier method was used to describe survival time and the log-rank test to evaluate statistical significance between groups. For analysis of correlation, log-transformation was used to reach near normal distribution. The correlation between mRNA expression and serum levels was assessed using Pearson's correlation coefficients. Statistical analysis was performed using SPSS 16.0 (SPSS Inc; Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc; San Diego, CA, USA). Statistical significance was considered at a value of $p < 0.05$.

RESULTS

Genotypes and allele carrier frequencies

Genotypes and allele carrier frequencies in IPF patients and controls are summarized in table 2. Healthy controls and IPF patients were in Hardy-Weinberg equilibrium for all polymorphisms. Comparison of the SNPs in the CCL18 gene revealed no significant differences in allele frequencies between IPF patients and controls. The following SNPs showed strong linkage disequilibrium (LD), rs712040, rs712042 and rs2015086; $0.76 < r^2 < 0.90$ (figure 1). Additionally, based on 4 SNPs, only 3 haplotypes were constructed with a frequency > 5%. Haplotype frequencies were not significantly different between IPF patients and healthy controls (data not shown). Due to strong LD between individual SNPs, subsequent data will only be given for rs2015086.

Table 2 Allele carrier and genotype frequencies in IPF patients and healthy controls.

Polymorphism	Allele and genotype	IPF n = 77	Healthy controls n = 349
rs712040	C	16 (10%)	86 (12%)
	T	138 (90%)	612 (88%)
	CC	0 (0%)	7 (2%)
	CT	16 (21%)	72 (21%)
	TT	61 (79%)	270 (77%)
rs2015086*	C	18 (12%)	101 (15%)
	T	136 (88%)	593 (85%)
	CC	0 (0%)	10 (3%)
	CT	18 (23%)	81 (23%)
	TT	59 (77%)	256 (74%)
rs712042	A	136 (88%)	597 (86%)
	G	18 (12%)	101 (14%)
	AA	59 (77%)	258 (74%)
	AG	18 (23%)	81 (23%)
	GG	0 (0%)	10 (3%)
rs712044	A	97 (63%)	480 (69%)
	G	57 (37%)	218 (31%)
	AA	33 (43%)	172 (49%)
	AG	31 (40%)	136 (39%)
	GG	13 (17%)	41 (12%)

**healthy controls: n = 347 due to unreliable genotyping in 2 controls.*

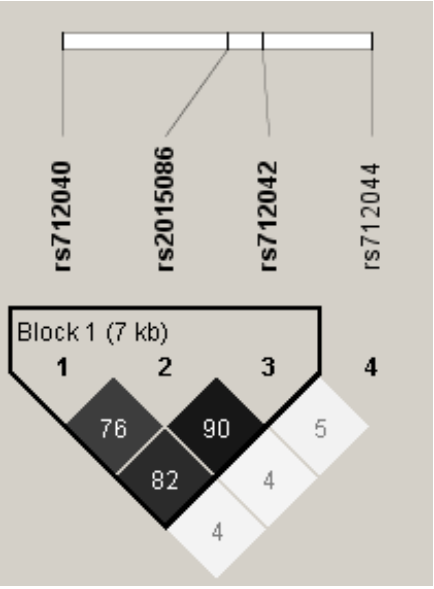


Figure 1 Linkage Disequilibrium (LD) Map of 4 SNPs in the CCL18 gene. The dark squares represent high r^2 values and the triangle represents a haplotype block.

CCL18 genotypes and serum levels

Serum CCL18 levels were significantly higher in IPF patients (645 ng/ml [IQR 393 – 847]) than in healthy controls (185 ng/ml [IQR 123-272]), p<0.0001, (figure 2). Serum CCL18 levels in eight patients who received low dose corticosteroids were not significantly different from IPF patients who did not receive therapy at the moment of serum sampling (data not shown). In healthy controls, significant differences in CCL18 serum levels were observed between the carriers and non-carriers of the C-allele of the rs2015086 polymorphism; TT 151 ng/ml (IQR 109-224), CT + CC 239 ng/ml (IQR 152-328), (p<0.0001) (figure 3A). There was no correlation between serum CCL18 levels and age in healthy controls (r = 0.06, p = 0.36) and IPF patients (r = 0.17, p = 0.16)

In IPF patients, pronounced differences in CCL18 serum levels were observed between genotypes of the rs2015086 polymorphism; TT 585 ng/ml (IQR 340 –793) and CT 817 ng/ml (IQR 681 – 1278), p = 0.002. (figure 3B).

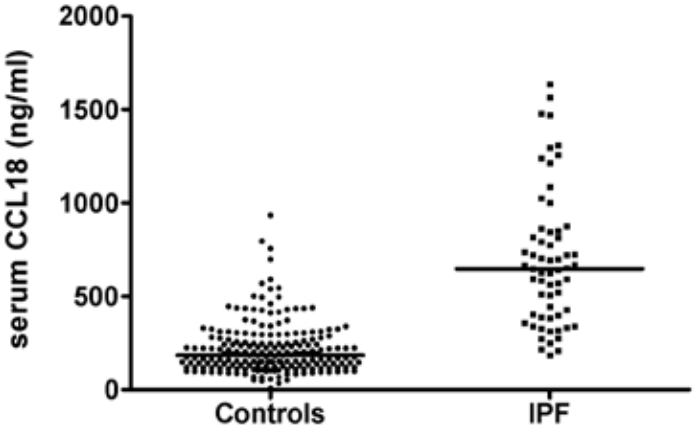


Figure 2 Scatterplot showing CCL18 serum levels for healthy controls and IPF patients, Mann Whitney U-test: p<0.0001

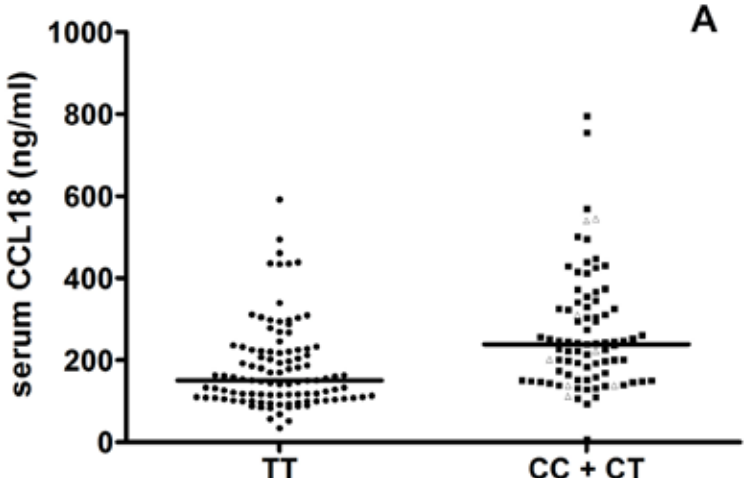
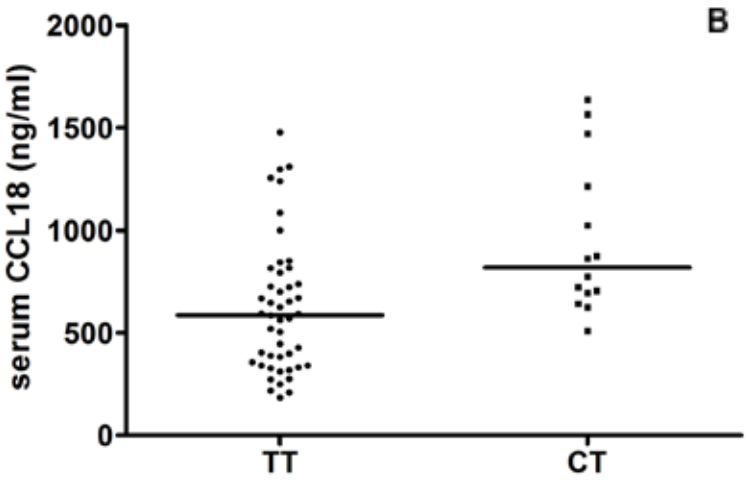


Figure 3 Scatterplot showing serum CCL18 levels according to the rs2015086 genotype.

3A Genotypes TT (●) vs CT (■) and CC (∆) in healthy controls, Kruskal-Wallis: p<0.0001;

3B Genotypes TT vs CT in IPF patients, Mann-Whitney U test: p = 0.002.

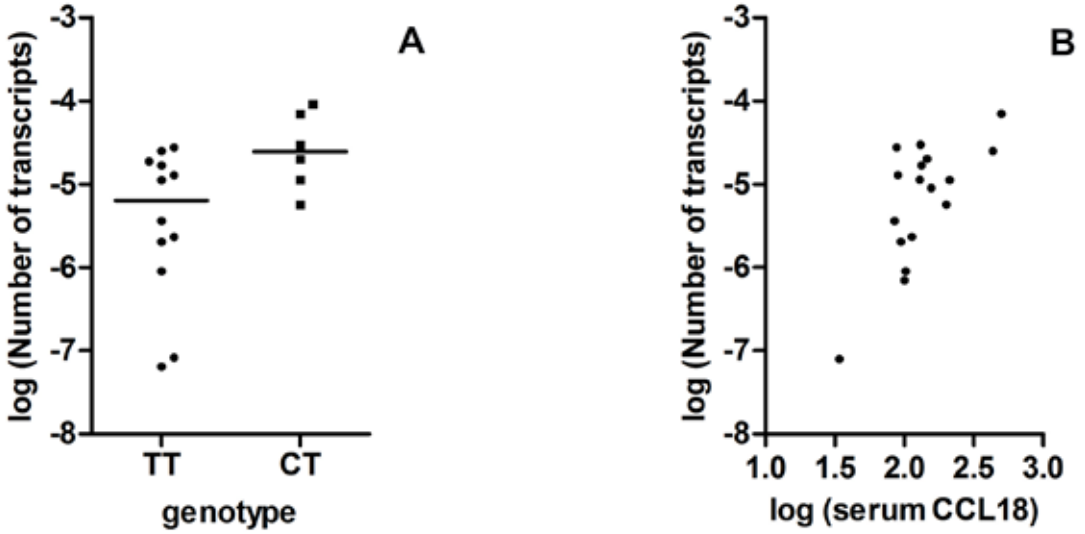


CCL18 genotypes and mRNA expression

The expression of CCL18 mRNA in PBMCs was analysed in 18 healthy controls. Six subjects had genotype CT for the rs2015086 SNP and 12 subjects had TT. Subjects with the CT genotype had a 4-fold higher gene expression ($3.0 \cdot 10^{-5}$; IQR $1.8 \cdot 10^{-5}$ - $7.7 \cdot 10^{-5}$) than subjects with TT ($7.4 \cdot 10^{-6}$; IQR $1.1 \cdot 10^{-6}$ -$1.8 \cdot 10^{-5}$, p = 0.007), figure 4A. CCL18 mRNA expression correlated significantly with serum CCL18 levels (r = 0.73, p = 0.002), figure 4B.

Figure 4 A. Scatterplot showing the mRNA expression of CCL18 in PBMCs from 18 healthy controls, expressed as the number of CCL18 transcripts per copy of β-actin according to genotype (TT: n = 12; CT: n = 6), p = 0.007.

B. Scatterplot showing the correlation between serum CCL18 levels and the number of CCL18 mRNA transcripts per copy of β-actin. Values on the X and Y-axis represent log-transformed values, Pearson's correlation: r = 0.73, p = 0.002.



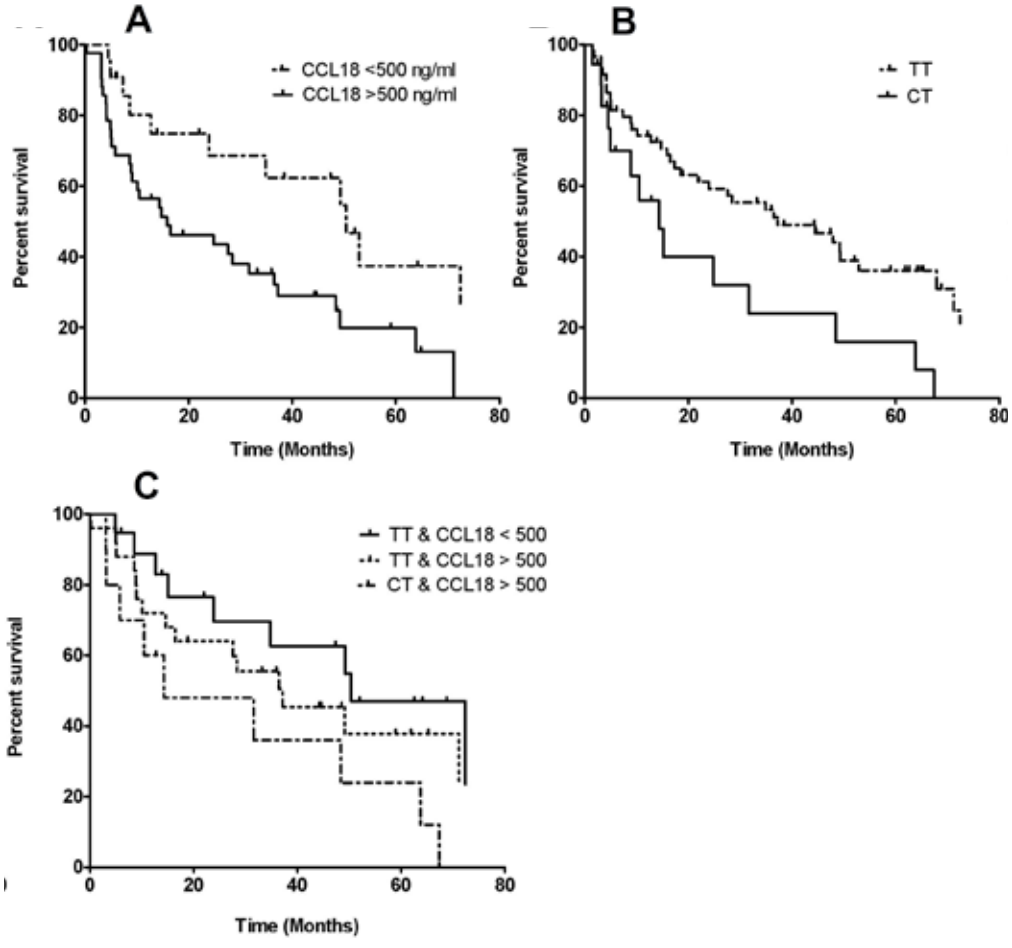
Survival in IPF patients

Median survival in the IPF group was 35.0 months (95% CI 21.1 – 48.7) Within the study period 50 out of 77 IPF patients died, and one patient was lost to follow-up. ROC analyses were performed to identify the optimal serum CCL18 cut-off point for defining an unfavourable prognosis. The highest Area under the Curve (AuC) was calculated for a serum CCL18 concentration of 500 ng/ml (AuC = 0.72). According to this cut-off level, patients were categorized as having high (serum CCL18 > 500 ng/ml) or low levels (serum CCL18 < 500 ng/ml). Median survival in the group with low serum CCL18 levels was 50.4 months (95% CI 31.9 – 68.9) and 27.6 months (95% CI 8.1 – 47.0) in the group with high serum CCL18 levels, (p = 0.02). (Figure 5A)

Figure 5 **A**. Kaplan-Meier curve showing a significant difference in survival between IPF patients with high serum CCL18 levels (> 500 ng/ml) and low CCL18 levels (< 500 ng/ml), log-rank test: p = 0.02.

B. Kaplan-Meier curve showing a significant difference in survival between IPF patients with the rs2015086 CT and TT genotype, log-rank test: p = 0.01.

C. Kaplan-Meier curve showing the difference between three groups: low CCL18 and genotype TT; high CCL18 and genotype TT; high CCL18 and genotype CT, log-rank test: p = 0.03.



Survival was also analysed for dependency on CCL18 genotype using Kaplan-Meier curves. Patients with the rs2015086 CT genotype showed a significantly worse survival than patients with the TT genotype: CT median 14.3 months (95% CI 0.0 – 35.9), vs TT median 37.2 months (15.4 – 58.9), p = 0.01 (figure 5B). Patients were censored from the survival analysis if alive (n = 15) or transplanted (n = 11). Censored patients were genotyped CT (n = 4) and TT (n = 22). At presentation, patients with the rs2015086 CT genotype did not show any significant differences in demographics or lung function parameters compared to patients with the TT genotype, as shown in table 3.

Table 3 Characteristics of IPF patients with the CT and TT genotype of the rs2015086 polymorphism.

	CT (n = 18)	**TT (n = 59)**	**p-value**
Age – yr			
Median (IQR)	61.3 (53.1 – 74.8)	62.8 (52.4 – 73.6)	0.9
Male sex – no. (%)	11 (61)	47(79)	0.2
Lung function			
VC, % pred, median (IQR)	73.7 (51.7 – 88.1)	75.7 (62.5 – 89.2)	0.7
$DL_{CO,}$% pred (SD),median (IQR)	40.4 (32.0 – 46.4)	47.4 (31.2 – 60.2)	0.3
$FEV_{1,}$% pred (SD),median (IQR)	70.9 (56.9 – 95.2)	74.9 (64.2 – 97.0)	0.6

Survival rates were also analyzed in three groups based on a combination of the level of serum CCL18 and genotype: low CCL18 (< 500 ng/ml) and genotype TT, median survival 50.4 months (95% CI 25.4 – 75.4); high CCL18 (> 500 ng/ml) and genotype TT, median survival 37.2 months (95% CI 13.1 – 61.3) ; and high CCL18 (> 500 ng/ml) and genotype CT, median survival 14.3 months (95% CI 1.4 – 27.2), p = 0.03 (figure 5C). There were no patients with low CCL18 and genotype CT.

DISCUSSION

We showed that the rs2015086 C/T polymorphism contributes to inter-individual differences in healthy controls, with individuals carrying the C allele having the highest CCL18 mRNA and protein expression. A similar genotypic effect on serum CCL18 levels was observed in patients with IPF, even though mean serum levels showed a 3.5 –fold increase compared to healthy controls. Both elevated serum CCL18 levels and genotypes were related to a significantly diminished long-term survival in IPF. Patients with the worst survival on the basis of high serum CCL18 levels could be subdivided into intermediate and worse survival according to genotype.

Serum CCL18 concentrations reflect pulmonary fibrotic activity in patients with

IIPs and systemic sclerosis with pulmonary involvement.[14, 15] Recently, Prasse et al. demonstrated that increased serum CCL18 levels were associated with increased short-term mortality in IPF patients.[16] In our study, we independently confirmed these results and added to this finding the predictive value of serum CCL18 for long-term survival. Further, we showed that serum CCL18 levels were genotype dependent. Subjects with the CT genotype display higher constitutive serum CCL18 levels. The genotype of rs2015086 caused a four-fold higher mRNA expression in PBMCs from healthy controls. Interestingly, Hägg et al. recently described that patients with carotic artery plaques and the CT genotype of rs2015086 had a three-fold higher gene expression level in macrophages than subjects with the TT genotype.[25] This is in the same order of magnitude as our results and, with that, both the genotype-mRNA correlation and the protein-survival correlation have been demonstrated twice independently.

We showed the association between the rs2015086 polymorphism and CCL18 serum levels, in both controls and patients. Besides that, one may question whether higher constitutive CCL18 levels predispose to fibrotic disease. In order to investigate whether carriage of the C-allele predisposes to IPF we compared allele frequencies in cases and controls, and showed that groups were in Hardy-Weinberg equilibrium and that allelic frequencies were not significantly different. We had 80% power to detect an OR ≥ 2.1 under a dominant gene model. The absence of an association shows that carriage of the C-allele does not significantly predispose to IPF, however, due to limited sample size, small predisposing effects may still exist

Alveolar macrophages are the main source of CCL18 in the lung and show an alternatively activated phenotype in IPF.[19] Fibroblast contact and exposure to collagen increases CCL18 production by alveolar macrophages and these macrophages up-regulate collagen production by lung fibroblasts via the production of CCL18. As such, we hypothesize that the origin of the association between increased serum CCL18 levels and mortality in IPF patients is in part based on genetic variation in the CCL18 gene.

In the search of a biomarker to predict prognosis in IPF, a great number of studies have focused on proteins in serum and BALF. This study is the first to show a genetic association with disease course in IPF. With a median survival of 2 to 5 years after diagnosis[7, 26] and no therapeutic options, predicting survival becomes increasingly important, especially in the case of potential lung transplantation candidates. A challenge in IPF is finding disease markers that predict long-term survival. In many studies where a cut-off value is used to differentiate patients with an unfavourable prognosis, the effect of a discriminative value becomes less prominent during follow-up.[11, 12, 16] As we showed in figure 5 and table 3, the effect of genotype remained significant during a considerable follow-up time and is independent from commonly used disease

parameters such as lung function. Genotyping IPF patients for the rs2015086 SNP in the CCL18 gene may therefore add substantial information in the interpretation of serum CCL18 levels to predict the disease course.

This study has some limitations. Although the total number of patients is comparable to the study from Prasse et al,[16] the number of patients with genotype CT in the IPF group is rather small. In the light of the impact of the finding that genetic differences influence survival in IPF patients, this needs to be replicated in a large independent cohort.

Furthermore, we can not rule out whether there are other genes in the vicinity of the CCL18 gene which could be of pathophysiologic relevance. In the human genome, CCL18 can be found close to CCL3 and CCL4, in a 47-kb interval with substantial linkage disequilibrium.[27] It has been found that protein levels of CCL3 and CCL4 are upregulated in lavage fluid of IPF patients.[28] Therefore, it might be possible that CCL18 rs2015086 is in linkage disequilibrium with polymorphisms in CCL3 and CCL4 that could influence protein expression and disease development.

As serum CCL18 levels are increased in IPF and influence the disease course in IPF, it can be hypothesized that the rs2015086 polymorphism may show similar effects in other fibrotic lung diseases. CCL18 expression is increased in patients with systemic sclerosis and in hypersensitivity pneumonitis.[15, 29] Morbidity and mortality in these diseases are mainly caused by pulmonary fibrosis. Both diseases show a subset of patients who develop a phenotype in which progressive pulmonary fibrosis is the major cause of death. Further research is needed to investigate whether genetic variation in the CCL18 gene influences serum levels and disease course in systemic sclerosis and hypersensitivity pneumonitis.

IPF patients with the CT genotype may be disadvantaged in terms of higher CCL18 levels and diminished prognosis. Interrupting the positive feedback loop by blocking CCL18 could be an interesting subject for future therapeutic research. IPF is a relentlessly progressive disease and there is still no evidence for a therapy that can improve survival.[1] As increased CCL18 levels stimulate fibroblasts to produce collagen, inhibiting CCL18 activity may directly inhibit fibrogenesis. Patients with the CT genotype may especially benefit from CCL18 blockade as they show the highest serum CCL18 levels.

In conclusion, we showed that genetic variability in the CCL18 gene accounts for significant differences in CCL18 mRNA expression and serum levels and showed to have a modifying role in the course of IPF. Our findings emphasize the value of serum CCL18 as a prognostic marker for IPF and show that future studies concerning CCL18 should take into account that mRNA and protein expression are influenced by genetic polymorphisms in the CCL18 gene.

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8

SUMMARY AND GENERAL DISCUSSION

SUMMARY

This thesis describes the search for a prognostic biomarker in idiopathic pulmonary fibrosis (IPF). In chapter 2 current knowledge about prognostic determinants in IPF and in chapter 3 our study population is described. In chapter 4, 5, 6, and 7 several potential biomarkers, and the influence of their encoding genes on protein level, disease susceptibility and/or progression are described, and discussed in the context of the pathogenesis of IPF.

IPF is a chronic, relentlessly progressive fibrosing disease of the lung, with limited survival ranging between a few months to several years from diagnosis. Individual disease course may vary from slowly progressive, to one or more rapid accelerations, or to a devastating rate of progression from onset. The clinical need to early identify these different courses of diseases, and to predict survival has stimulated investigators for many years to search for prognostic markers. However, a lot of these studies have been conducted before the ATS/ERS statement in 2002, which was a cornerstone document in the differentiation of IPF from NSIP and other interstitial pneumonias.[1] In **chapter 2** an overview is given from studies describing molecular and non-molecular markers that can predict prognosis in IPF, according to the definition as stated by the ATS/ERS in 2002. The change in VC or DL_{CO} is the most robust determinant of prognosis used in daily practise, but since this needs a time interval of 6 or 12 months, it can not be used as a predictor at first presentation. Results of studies evaluating cellular constituents of BAL fluid remain contradictory and are therefore not very useful to predict prognosis. The extent of fibrosis on HRCT scan and the number of fibroblast foci on lung biopsy can be measured at presentation and correlate with prognosis, but the applicability of these markers is being hampered by the lack of user- and patient friendliness. Most promising in this context seem serological biomarkers because they are non-invasive, easily reproducible and may have the ability to reflect subtle changes more accurately than pulmonary function tests or HRCT. Ideally serological markers may indicate deterioration before this becomes clinically apparent. A promising group of biomarkers in this respect are so called pneumoproteins. Pneumoproteins are proteins that are present in the alveolar lining fluid and increase in serum due to increased permeability of the alveolar-capillary membrane and increased secretion by regenerating alveolar type II cells. These proteins are therefore interesting focus for further research.

In **chapter 3**, the cohort which formed the basis for further research was extensively described. A cohort of IPF patients has never been described in the Netherlands since the ATS/ ERS statement, and since further research would be based on this cohort, a comparison was made between this cohort and IPF patients in other

studies. Patient characteristics such as age at the time of diagnosis, lung function and BALf parameters in this cohort were comparable to cohorts described in international literature. A slightly altered men to women ratio was observed in our cohort, with more men than in other cohorts. This might be explained by the low labor participation in women in the Netherlands, hereby resulting in a lower exposure to potential harmful particles for the alveolar epithelium. Another important observation was the large proportion of patients with familial IPF in our cohort. This can be explained by the fact that patients were sometimes specifically referred with the question whether there could be a familial form of IPF. Further it was found that our IPF population had a median survival of approximately 4 years, which result was consistent with survival shown in international IPF cohorts after the 2002 ATS/ERS statement.

In **chapter 4** the value of surfactant protein-D (SP-D) to predict prognosis was evaluated. SP-D is mainly synthesized by type II pneumocytes and occurs in serum due to leakage through the lung parenchyma. SP-D levels in healthy controls are under influence of the Met11Thr polymorphism (rs721917), and since the value of some biomarkers may improve when corrected for genotype, we also determined the influence of the Met11Thr polymorphism on serum SP-D levels in IPF patients. Serum SP-D levels were significantly increased in IPF patients compared to controls, but were not influenced by the Met11Thr polymorphism. A serum SP-D level of > 460 ng/ml indicated a significantly worse prognosis than levels lower than 460 ng/ml and remained stable as a predictor of prognosis after adjustment for known predictors of mortality. Therefore determining serum SP-D might help in estimating survival time, which is important for optimal timing of referral for lung transplantation.

In **chapter 5** the potential of endothelin- 1(ET-1) as a prognostic biomarker in IPF is described. ET-1 is known to influence fibroblast chemotaxis, collagen production and epithelial-mesenchymal transition. Increased serum levels of ET-1 in serum was found, but remarkably decreased levels of ET-1 in BALf compared to healthy controls. This unexpected finding led to the speculation that increased leakage through the damaged interstitium overrides the secretion of ET-1 by epithelial cells and macrophages. No clear explanation for this finding was found, so this will finding will require further investigation. Furthermore, a positive correlation was found between the percentage of macrophages in BALf and ET-1 in IPF patients, but not in healthy controls, reflecting the increased ET-1 production in IPF by macrophages. Serum ET-1 did not correlate to parameters of disease progression and as such can not be used as a predictor of prognosis. However, in BALf, increased levels of ET-1 were related to the change in DL_{CO} and high levels were predictive of short survival. The implication of the use of ET-1 as a biomarker for prognosis in not very likely since BAL is an invasive procedure, however the relation between increased BALf ET-1 levels and worse prognosis indicate

a determining role for ET-1 in the course of IPF.

Chapter 6 describes the susceptibility and disease modifying effects of genetic variations in the IL1RN and IL1B gene in IPF and the changed balance between IL-1Ra and IL-1β. IL-1 cytokines play an important role in fibrosis in animal models of bleomycin induced fibrosis. In IPF this role has yet to be determined, but an association between a SNP in the IL1RN gene and the susceptibility to IPF was previously described. Serum levels of IL-1β in IPF patients were significantly increased compared to healthy controls while serum levels of IL-1Ra were decreased. Furthermore, BALf levels of both IL-1β and IL-1Ra were significantly increased in IPF patients compared to healthy controls. As IL-1Ra inhibits the physiological activities of IL-1β by occupying the IL-1 receptor, we calculated the IL-1Ra/ IL-1β ratio. A 3.5-fold decrease was observed in both serum and BALf of IPF patients compared to healthy controls, resulting in a relative shortage of IL-1Ra and thus a pro-inflammatory environment. Furthermore, we confirmed the previously described SNP in the IL1RN gene to be associated with the susceptibility to IPF and added to this finding that another SNP in the IL1RN gene(rs2637988) was more strongly associated with IPF and also influenced the IL-1Ra/ IL-1β ratio. We conclude that genetic variation in the IL1RN gene and the subsequent imbalance between IL1Ra and IL1β may have significant effects on the pathogenesis of IPF.

In **chapter 7** the influence of SNPs in the CCL18 gene on CCL18 expression and survival was evaluated. CCL18 upregulates the production of collagen by lung fibroblasts and is a promising biomarker for IPF. IPF patients demonstrated increased serum CCL18 levels. Both in IPF and healthy controls, the rs2015086 C>T polymorphism significantly influenced CCL18 levels, resulting in the highest levels for individuals carrying the C allele. This polymorphism also demonstrated differences in CCL18 expression in PBMCs from healthy controls. Furthermore, high serum CCL18 levels correlated with decreased survival and patients carrying the CT genotype showed a significantly worse survival than patients with the TT genotype. Our findings emphasize the value of serum CCL18 as a prognostic marker for IPF and show that future studies concerning CCL18 should take into account that mRNA and protein expression are influenced by genetic polymorphisms in the CCL18 gene.

GENERAL DISCUSSION

Pathogenesis of IPF

Changing hypothesis about the pathogenesis of IPF

IPF is a disease of unknown etiology and the concept of the pathogenesis of IPF has changed over the years. The proteins that have been investigated in this thesis all play different roles in the pathogenesis. Surfactant protein D (SP-D) is present in alveolar epithelial lining fluid and is produced by type II pneumocytes. Since SP-D is not directly involved in the pathogenesis of IPF, its presence in serum should reflect damage of the alveolar epithelial cells. CCL-18 and Endothelin-1 (ET-1) are both macrophage-derived biomarkers. CCL-18 induces the production of collagen by fibroblasts and ET-1 is involved in fibroblast chemotaxis. The role of IL1-β, a potent pro-inflammatory cytokine and IL1-Ra, an anti-inflammatory cytokine in IPF is less clear. The disputable role of inflammation formed the background of chapter 6. The first ideas about the pathogenesis originated from pathological studies in the 1980, which demonstrated structural abnormalities in lung tissue from biopsies. A thickened basement membrane and loss of type 1 alveolar cells were described, leading to exposure of the denudated basement membrane.[2] It was hypothesized that multiple insults of unknown origin lead to a damaged endothelium, epithelium and basement membrane. The loss of normal basement membrane integrity results in the inability to reepithelialize the basement membrane. As a response to that injury, invasion of inflammatory cells takes place such as macrophages, neutrophils and fibroblasts. This process ultimately leads to the formation of fibroblast foci, composed of fibroblasts and extracellular matrix proteins.[3] The key point in this hypothesis was that the cycle of dysregulated repair after injury and inflammation of the basement leads to chronic inflammation, ultimately leading to pulmonary fibrosis.

In 2001 Selman et al[4] 'revolutionary' stated that pulmonary fibrosis results from epithelial injury and abnormal wound repair in the *absence* of chronic inflammation. The inflammation component in lung biopsies is often mild and earlier stages do not show more inflammation than later stages.[5] Further, interstitial lung diseases in which inflammation is a prominent feature of early disease such as hypersensitivity pneumonitis often do not progress to fibrosis. Moreover despite long-term anti-inflammatory therapy, IPF is always progressive and leads to death without any response to therapy. They proposed that IPF represents a form of abnormal wound healing, characterized by fibroblast migration and proliferation and decreased myofibroblast apoptosis.

Alveolar epithelial injury

Selman describes in the new hypothesis that the process starts with alveolar epithelial

injury. Multiple microscopic sites of ongoing alveolar epithelial injury are followed by the activation of fibroblasts and the formation of fibroblast foci, which ultimately leads to fibrosis. The phase of epithelial injury is characterized by loss of alveolar epithelial cells and in other areas hyperplasia of type II pneumocytes occurs. The type II pneumocyte is a source of several cytokines and growth factors such as TGF-β, TNF-α, PDGF and endothelin-1. Further, the type II pneumocytes produce surfactant proteins, which may act as a biomarker for the epithelial damage. In chapter 4 surfactant protein D (SP-D) is described, which is mainly present in alveolar type II cells, but also in Clara cells. The exact role of SP-D in the lung is not entirely clear. There is evidence that SP-D plays a role in host defence by binding to bacteria, viruses and fungi, hereby facilitating uptake by phagocytes. However SP-D rarely has surfactant-active properties.[6] The fact that SP-D does not play a leading role in the pathogenesis of IPF and is not increased in BAL fluid of IPF patients, makes it an ideal serological marker for the occurred damage to the interstitium. These properties are not solely indicative for interstitial damage in IPF, also other types of fibrosis display increased serum levels of SP-D. In patients with pulmonary fibrosis due to systemic sclerosis, hypersensitivity pneumonitis, fibrotic sarcoidosis, cystic fibrosis, pulmonary alveolar proteinosis and in patients with inflammation such as pneumonia or ARDS also high serum levels of SP-D are detected. Comparing SP-D levels within these groups shows that SP-D levels in pneumonia are the highest, followed by PAP, IPF, collagen diseases and then by ARDS, CF and sarcoidosis.[7] Although not specific for interstitial damage in IPF, SP-D is still a promising biomarker for the prognosis of IPF. Evidence is accumulating that serum SP-D correlates with parameters of disease progression and survival[8-11] and that patients with an acute exacerbation show increased SP-D levels compared to patients with stable IPF.[12] In the case of lung transplantation pre- and post-operative levels of SP-D nicely reflect the underlying pathologic processes. Among subjects with IPF undergoing bilateral transplantation, SP-D levels decline rapidly postoperatively. In contrast, SP-D levels in subjects undergoing single lung transplant for IPF remained significantly higher than those undergoing bilateral transplantation. Single lung allograft recipients without primary graft dysfunction show higher postoperative SP-D levels than bilateral allograft recipients with primary graft dysfunction, indicating that SP-D reflects pulmonary fibrosis in the native lungs, rather than primary graft dysfuntion.[13] Thus, the use of SP-D as a biomarker in IPF is founded on the thought that it reflects the damage to the alveolar epithelial cell, which is the key process in the pathogenesis of IPF.

A similar molecule that reflects alveolar epithelial damage is Krebs von den Lungen 6 (KL-6). KL-6 is a mucin-like protein expressed at the extracellular surface alveolar type II pneumocytes and bronchial epithelial cells. It acts as a chemotactic

factor that promotes migration, proliferation and survival of lung fibroblasts[14] and is significantly elevated in serum from IPF patients compared to healthy volunteers.[15] KL-6 is not only reflecting epithelial damage by direct leakage through the interstitium, but it also reflects disease activity. KL-6 levels in BALf are significantly correlated to serum levels, these results indicate that increased levels of serum KL-6 reflect the production levels of KL-6 derived from damaged or regenerating Type II pneumocytes in the lower respiratory tract.[16] Serum KL-6 has also been linked to survival, IPF patients with high (> 1000 U/ml) levels had worse survival than patients with low serum KL-6 levels.[17] Serial measurements of serum KL-6 with increasing values marked worsened survival compared to those who showed decreasing levels.[18] However, since these studies have been performed at the time that IPF patients were routinely treated with immunosuppressive therapy, it would be valuable to re-examine the serial KL-6 samples from those who were not on treatment.

The role of inflammation

In response to alveolar epithelial damage, fibroblast foci occur, leading to the formation of fibrosis. In Selmans new hypothesis, there is no leading role for inflammation. Inflammatory cells such as neutrophils are present in the blood stream and are recruited to the site of injury, in the case of IPF impaired wound healing as reflected by the formation of traction bronchiectasis and architectural distortion, but the precise cause-effect relationship remains unclear. Proponents of the theory that inflammation does play an important role, state that IPF, or more accurate UIP and NSIP may be two diseases in one continuum, rather than two different diseases. A considerable number of patients with multiple lung biopsies show both UIP and NSIP patterns.[19] This interlobar variability of idiopathic interstitial pneumonias suggests that there are two different disease processes possible in one patient, or that NSIP and UIP represent different stages of one disease. As NSIP, in contrast to UIP, is indeed characterized by marked inflammation, NSIP may be an early stage of UIP according to this theory.[20] In chapter 6 we state that the disturbed balance between an important pro-inflammatory cytokine, IL-1β and an anti-inflammatory cytokine, IL-1Ra, may contribute to a pro-inflammatory environment in IPF. We found decreased IL-1Ra/IL-1β ratios both in serum and BALf in IPF patients compared to healthy controls, which again questions the role of inflammation in the pathogenetic process. The inflammatory response could be a key process in the development of fibrosis, or just a reflection of the tissue remodelling and in other words just a paraphenomenon. This study in chapter 6 does not answer the question whether inflammation is a cause or a consequence, but the genetic association between a single nucleotide polymorphism in de the IL1RN gene and IPF predisposition may point slightly into the direction of a role for inflammation in the initial

phase of the disease. In a meta-analysis in which the results of chapter 6 are included next to four other studies, a convincing association was found between the genetic variation in the IL1RN gene and the susceptibility to IPF. Since the polymorphisms in the IL1RN gene influence IL-1Ra mRNA expression, it seems likely that low levels of IL-1Ra contribute to the development of IPF. This finding is in contrast with the new hypothesis about the development of IPF and may reconsider the value of the old hypothesis.

Increased and decreased apoptosis

The next phase in the formation of fibrosis is increased apoptosis of alveolar epithelial cells and decreased apoptosis of myofibroblasts, resulting in inefficient reepithelialization. A growing body of evidence has suggested a role for apoptosis of alveolar epithelial cells in IPF. Uhal et al.[21] demonstrated the apoptosis of alveolar epithelial cells was adjacent to underlying myofibroblasts. Apoptosis was usually found in areas of normal appearing alveoli in patients with IPF.[22] More recently, it was found that expression of apoptotic markers (p53, p21, bax, caspase-3) were increased and anti-apoptotic markers (bcl-2) were reduced in alveolar epithelial cells in IPF.[23] Thus, apoptosis of alveolar epithelial cells leads to disrupted basement membrane integrity and recruitment of fibroblasts. It has been suggested that oxidative stress may be associated with the apoptosis of alveolar epithelial cells in IPF patients. Several studies have demonstrated increased levels of oxidative stress products (hydrogen peroxide, myeloperoxidase, lipid peroxidation products, and nitric oxide)[24, 25] and decreased antioxidant protection (glutathione, superoxide dismutase)[26, 27] in IPF. Telomerase activity is also involved in the pathogenesis of apoptosis of alveolar epithelial cells. Telomerase is a ribonucleoprotein enzyme that adds telomere repeats to the ends of linear chromosomes. It consists of a catalytic component, telomerase reverse transcriptase (hTERT), and an RNA template (TERC). Telomerase expression and activity were essential to the proliferation and repair of alveolar epithelial cells.[28] Telomere shortening can cause DNA damage that leads to cell death and telomere/telomerase impairment is a main mechanism for cellular apoptosis.[29] Telomere activity has been demonstrated to be inversely associated with apoptosis of alveolar epithelial cells both in a bleomycin-induced model and in patients with IPF.[30] In addition to the above mechanisms, other reasons for apoptosis of alveolar epithelial cells have been suggested. Endoplasmic reticulum stress induced by mutation of surfactant protein C can lead to protein misfolding and activate the unfolded protein response, which may have an important role in the apoptosis of AECs in IPF.[31, 32]

After apoptosis of epithelial cells, the disrupted integrity of the epithelium, fibroblast over-activation and their resistance to apoptosis contribute to an abnormal

wound healing process. The hallmark pathological feature of IPF are fibroblast foci. It presents as a small focal area of a younger, myxoid-appearing matrix with aggregates of actively proliferating and collagen-producing myofibroblasts.[33] It is believed that the regions of fibroblast foci are the primary sites of ongoing injury and repair.[34] Myofibroblast cells, which are the major cells in the fibroblast foci, possess an ultrastructural phenotype intermediate between fibroblasts and smooth muscle cells.[35] It has been suggested that myofibroblast cells play important roles in tissue remodeling and fibrosis, because they are the primary cellular source of collagen and cytokines in IPF. The decreased rate of apoptosis in fibroblasts and myofibroblasts is thought to be a result of apoptosis resistance due to activation of different pathways. The WNT5A signaling mediated pathway has been suggested to take an important role in the apoptosis resistance of fibroblasts and myofibroblasts.[36] The WNT5A gene was significantly up-regulated in IPF fibroblasts compared with normal lung fibroblasts, and WNT5A significantly induced fibroblast proliferation and inhibited apoptosis. Besides, resistance of lung fibroblasts to Fas-mediated apoptosis has also been suggested. Moreover, a greater number of α-SMA-positive cells are present in IPF, which is considered to link with apoptosis resistance,[37] as studies have demonstrated myofibroblasts are more resistant to apoptosis than fibroblasts. Also, it is suggested that TGF-β1 can protect fibroblasts from apoptosis induced by plasminogen via the upregulation of plasminogen activator-1 and inhibition of plasminogen activation.[38] TGF-β1 can also inhibit myofibroblast apoptosis induced by IL-1β via suppressing an inducible nitric oxide synthase induction.[39]

The role of macrophages

Macrophages play an important role in the removal of apoptotic cells and BALf macrophages in IPF exhibit a decreased apoptotic rate compared to normal subjects.[40] Alveolar macrophages are the predominant inflammatory cells in alveolar epithelial lining fluid an may further contribute to the pathogenesis of pulmonary fibrosis by secreting cytokines, growth factors and extracellular matrix proteins. In chapter 5 and 7 two molecules are described that are secreted by macrophages in the pathologic condition of IPF, CCL18 and ET-1. CCL18 is primarily produced by macrophages, but also occasionally in dendritic cells and proliferating type II pneumocytes.[41] In healthy lungs ET-1 is only expressed in vascular endothelium and airway epithelium, but in IPF ET-1 is also strongly expressed by proliferating type II pneumocytes, macrophages and neutrophils.[42] In this context it is interesting to take a closer look to the role of the macrophage in the pathogenesis of IPF. Early studies already stated the profibrotic role of the macrophage in IPF. Macrophages of IPF patients produce fibronectin, a chemotactic agent for fibroblasts, at a rate 20 times higher than macrophages from healthy controls.[43] Alveolar macrophages of IPF patients did have glucocorticoid

receptors, but glucocorticoid therapy did not suppress release of fibronectin and alveolar macrophage derived growth factor.[44] Macrophages in IPF display an alternatively activated phenotype. There are two different types of macrophages: the classically activated macrophage is activated by LPS or IFN-γ, the alternatively activated macrophage by IL-4 or glucocorticoid. The alternatively activated macrophages increase proliferation and collagen synthesis of fibroblasts, while classically activated macrophages do not.[45] Crucially is that corticosteroids have been shown to induce the alternatively activated macrophage with pro-fibrotic phenotype.[46] Furthermore, the alternatively activated macrophages in IPF up-regulate the production of collagen by lung fibroblasts via the production of CCL18.[47] As fibroblast contact and exposure to collagen increases spontaneous CCL18 production by alveolar macrophages, a positive feedback loop was suggested that may perpetuate fibrosis. Interestingly, in mice the depletion of lung macrophages during fibrogenesis reduced the development of pulmonary fibrosis as measured by lung collagen, fibrosis score and markers such as Col1 and α-smooth muscle actin.[48] Since the current hypothesis about the pathogenesis of IPF does not focus on inflammation, the attention for the macrophage has been fading, but according to current evidence this seems unfair.

Limitations of the use of current animal models in IPF

Lots of ideas about the pathogenesis of IPF are derived from animal models. Both pathogenetic models as well as therapeutic models mostly are based on models in which pulmonary fibrosis is induced by intratracheally bleomycin, which is not equally comparable to human IPF. Over the years numerous therapeutic agents have been shown to inhibit fibrosis in animals, but none of these have shown a comparable effect in human IPF. The instillation of bleomycin intratracheally causes inflammation, with increased levels of IL-1, TNF-α, IL-6 and IFN-γ. This is followed by a fibrotic response with increased levels of TGF-β, fibronectin and procollagen-1. The transition from inflammation to fibrosis occurs around day 9 after bleomycin.[49] By far most studies about possible therapeutic targets for IPF have been done in the inflammatory phase of the response and are thus preventive in nature. A study from Moeller et al identified 221 studies describing benefit of antifibrotic regimens in bleomycin-induced fibrosis in the last 25 years, and only 10 of them were therapeutic trials which evaluated the use of an agent after the development of fibrosis.[50] In all 211 remaining papers the drug was given within 7 days of bleomycin application, and are thus anti-inflammatory and more preventive treatments than anti-fibrotic therapeutic treatments. In this respect future research in bleomycin-induced models of fibrosis should be more focused to the treatment of fibrosis, and extrapolation of results in bleomycin-induced fibrosis to human IPF should be assessed carefully.

More closely mimicking the pathogenesis of human IPF is an age-related model of lung fibrosis. Studies have shown that older mice are more susceptible than younger mice to bleomycin-induced injury and that aged male mice were more sensitive than aged female mice, which is comparable to human IPF.[51] Moreover, transgenic deletion of the genes for the receptor of advanced glycation end products (RAGE) or relaxin result in spontaneous age-related development of lung fibrosis[52, 53] and senescence-prone mice develop more severe fibrosis in response to bleomycin than do senescence-resistant mice.[54] The processes involved in fibrosis probably operate differently in aged lungs compared to young lungs and may more closely simulate the pathogenesis of fibrosis in human IPF.

In 2004, spontaneous IPF was identified in 16 domestic cats. The histopathology of IPF in cats consisted of interstitial fibrosis with fibroblast/myofibroblast foci, honeycombing and type II pneumocyte hyperplasia. Inflammation was not a prominent feature of the disease. Response to therapy (corticosteroids, antibiotics, bronchodilators, and diuretics) was poor, and most cats died within days to months. Cats with histologic changes compatible with UIP had signs that mimicked many of the clinical findings of human IPF.[55, 56] These findings may contribute to an animal model that is more in line with human IPF than the use of current models of bleomycin-induced fibrosis in mice.

Further, the use of epithelial lung organoids is a promising technique. Organoids are three-dimensionally arranged epithelial cells that closely resemble the organ they were isolated from. Classic two-dimensional cell culture systems are generally limited to investigate specific cellular or molecular responses and do not replicate the heterogenic origin of pulmonary fibrosis. Epithelial lung organoids could be used to investigate disease progression and test the efficacy/toxicity of experimental drugs. The intention is to establish organoids from biopsies of individual pulmonary fibrosis patients to functionally mimic the disease in vitro and study its etiology and treatment options.[57-59]

Prediction models

During time, several different scoring systems that predict survival have been developed. These prediction models are based on a combination of clinical and physiological findings and test results. A composite clinical-radiological-physiologic (CRP) scoring system was developed in IPF by King et al.[60, 61] They collected clinical, physiological and radiographic parameters from 238 patients with pathologically confirmed UIP and developed a scoring system, based on the features that were shown the best determinants of survival. Ultimately, age, smoking status, clubbing, the extent of interstitial opacities and pulmonary hypertension on chest radiography, percent predicted TLC and pO2 at maximal exercise were included in the scoring system. The

complete CRP score correlated with survival and with pathological features of IPF: fibrosis, cellularity, granulation, connective tissue and total pathologic derangement. However, this scoring system has not been adopted by clinicians, probably because some variables are not routinely measured in clinical practise. Moreover, the scoring system has not been externally validated in another cohort of IPF patients.

A more useful prediction system was described by du Bois et al,[62] who incorporated age, respiratory hospitalization, percent predicted FVC and 24-week change in FVC. They used data from two large clinical trials in patients with IPF (n = 1099) and developed a practical scoring system of mortality. Although more practical than the system developed by King, this system still needs validation in an external cohort.

The present thesis focuses on the use of different single biomarkers to predict prognosis. What needs further evaluation is the use of biomarkers in the context of prediction models together with other determinants of prognosis. The combination of a few powerful biomarkers may be more accurate to predict prognosis and excludes the influence of single outlier values that could lead to misinterpretation. However, for a model in which multiple biomarkers are included, a large cohort of patients is necessary. In our cohort, we lacked the power to develop a model that combines multiple biomarkers. Next to a model that includes different serum biomarkers, one could also think of a model in which physical or lung functional parameters are integrated along with serum biomarkers. The value of adding serum biomarkers in a prediction model was already proven by Kinder et al.[9] A model in which age, gender, race, smoking status, FVC, Dlco, and alveolar-arterial oxygen gradient were included predicted 1 year mortality in IPF with an area under the curve (AUC) of 0.79. When SP-A and SP-D were added, regression models showed significant improvement to an AUC of 0.89 ($p = 0.03$). In other diseases the prediction of prognosis is as well based on a combination of parameters rather than on one single parameter. In COPD for example the BODE index includes four determinants and the chance of surviving a pneumonia can easily be calculated by using the PSI score or CURB-65 index. Such a prediction model would be useful in IPF, however large cohorts are needed which is difficult in a rare disease like IPF.

Development of new biomarkers

The National Institute of Health defined the term biomarker as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention’.[63] Research on new biomarkers had largely been guided by experience and intuition. In our search towards new biomarkers we can

learn from the field of oncology. The National Cancer Institute developed a five stage approach to systemically discover and develop new biomarkers.[64] Phase 1 refers to the preclinical exploratory studies. Biomarkers are discovered through knowledge-based gene or protein profiling to distinguish diseased and normal tissue. In phase 2 an assay is established (DNA, RNA, protein or cell-based), which is validated for reproducibility and the essay should be evaluated for their clinical performance in terms of sensitivity and specificity. During phase 3 the investigator evaluates the sensitivity and specificity of the test for the detection of the disease which is further evaluated in phase 4. In phase 4 the biomarkers needs to be tested on a prospective cohort, in other words a positive test triggers an (often invasive) procedure. Phase 5 evaluates the overall benefits and risks. Although this represents the search towards a diagnostic biomarker, these phases can be slightly modified and translated into a search towards a prognostic biomarker. Phase 1 and 2 are equal for a prognostic biomarker. In phase 3 the progression of the disease should be evaluated instead of detection of the disease. In phase 4 the consequences of a prognostic biomarker should be prospectively evaluated, for example if the biomarker indicates short survival another therapeutic intervention should be initiated. Phase 5 is not different from the search towards a diagnostic biomarker.

A prognostic biomarker for IPF should accurately predict the progressiveness of the disease. Biomarkers studied so far mostly represent either presence of disease or progressiveness. There is much less need for a biomarker that predicts disease presence than a biomarker that predicts the progressiveness of the disease. Disease presence can be reasonably estimated by using HRCT or analysing the pattern at lung biopsy. When a confident pattern of UIP on HRCT and in the lung biopsy is present, it is unlikely that it responds to immunosuppressive therapy.[65] However, there is no such tool that predicts the rate of progression. Since there is significant heterogeneity in the rate of progression a biomarker is needed that predicts the rate of decline. In the search towards a prognostic biomarker, one should not be distracted by proteins that are simple by-products of fibrosis which are not informative about the rate of progression. The ideal marker should not be biased by the disease severity. Even in severe or extensive disease a prognostic biomarker should normalize in value if there is stable disease without disease progression.

Bringing a biomarker into clinical practise

The introduction of a new biomarker in clinical practise is only likely if it is supported by strong evidence and if results will improve patient management and outcome. Despite close to half a million publications since 1975 on biomarkers in Pubmed, only 1.5 proteins per year are added to the routine laboratory repertoire.[66] The proposed

introduction of a new biomarker requires assessment from different perspectives. From all perspectives, it is essential that implementation of a new test should be evidence-based, although other priorities may differ slightly. From the perspective of the healthcare provider, the test must be cost effective. Carefully designed cost-benefit studies must demonstrate that introducing the new biomarker improves the clinical pathway, reflecting fewer additional investigations. From a clinical perspective it must provide information that adds or replaces information available from existing tests and improves patients outcome. From a laboratory perspective, the biomarker needs to show stability in different matrices, the assay must be robust, precise and reproducible and it must be possible to incorporate the test into routine workflow.[67]

The journey of a protein biomarker from the bench to the clinic is long and challenging. The history of clinically useful biomarkers suggests that at least a decade is required for the transition of a marker from the bench to the bedside. For example prostate-specific antigen (PSA) was identified in 1970,[68] but only in 1987 the first definitive study proving its clinical utility was published.[69] And even after many years of clinical use of this test the appropriate clinical application and interpretation of PSA measurements remains controversial.

Prognostic biomarkers for IPF in clinical practise

From the investigated biomarkers in chapter 4, 5, 6, and 7, CCL18 and SP-D seem most promising. Both are easily accessible and reproducible, show robust correlations with survival and provide predictive information independent from lung function. Both biomarkers are increased in serum from patients with short survival compared to those who have longer survival. In studies like these, it is not difficult to demonstrate that a group of patients with short survival show a higher mean serum level of the biomarker compared to a group of patients that have longer survival, but the challenge is to find a biomarker that can predict the individual prognosis accurately enough and better than routine clinical parameters to act on the consequences. For the development and testing of such a prediction model a large cohort is necessary and a considerable time of follow-up. Recently, an individualized prediction rule for recurrent vascular events was published; based on a cohort of almost 6000 patients, it was possible to develop a model for prediction of 10-year recurrent vascular events, based on readily available clinical characteristics.[70] Research in IPF however, will always be hampered by the lack of enough patients for such prediction models.

However, from a clinical point of view, there is still a need for non-invasive prognostic biomarkers. The current data show that the biomarkers that have been investigated until now have the potential to aid clinical decision-making, but do not meet the requirements that are necessary to predict the prognosis of IPF in clinical practice.

External validation and prognostic evaluation in other IPF cohorts is necessary to bring the use of prognostic biomarkers one step closer to clinical implementation. Large multicenter studies in which the clinical usefulness is confirmed and expanded are still lacking. It would be helpful if serum samples from IPF patients who participated in large therapeutic trials could be analyzed for the described biomarkers. In this way, a well described prospective cohort could be analyzed and serial serum samples could be evaluated for the clinical usefulness of these biomarkers.

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NEDERLANDSE SAMENVATTING

PROGNOSTISCHE BIOMERKERS VOOR IDIOPATHISCHE LONGFIBROSE

Introductie

Idiopathische longfibrose (IPF) is een chronische fibroserende interstitiële longziekte met een snel en progressief ziektebeloop. IPF patiënten presenteren zich met progressieve kortademigheid en hoesten. De diagnose kan gesteld worden aan de hand van een aantal klinische criteria, een HRCT scan en een longbiopt met een karakteristiek UIP-patroon (usual interstitial pneumonia). De oorzaak van IPF is onbekend, maar er zijn wel aanwijzingen dat erfelijkheid een rol speelt. Een klein gedeelte van de IPF blijkt een mutatie te hebben in een gen waardoor de ziekte ontstaat en er zijn ook verschillende genetische variaties (polymorfismen) beschreven waardoor het risico op het ontwikkelen van IPF toeneemt. De mediane overleving bedraagt ongeveer 3 tot 4 jaar en tot op heden is er geen behandeling die de overleving verbetert. Voor patiënten die daarvoor in aanmerking komen is longtransplantatie de enige behandeloptie, maar helaas is de wachtlijst lang en onder de patiënten die op de wachtlijst staan is de sterfte van IPF-patiënten het hoogst. Dit zou geoptimaliseerd kunnen worden als we beter zouden kunnen voorspellen wat de overleving is van een patiënt met IPF.

In dit proefschrift worden een aantal prognostische biomerkers beschreven. Prognostische biomerkers zijn meetbare stoffen, in dit geval eiwitten, die de progressiviteit van de ziekte weergeven en die de overleving kunnen voorspellen. Tijdens de ontwikkeling van IPF zijn meerdere eiwitten betrokken die in verhoogde concentraties aanwezig zijn bij IPF patiënten ten opzichte van gezonde mensen, deze eiwitten lenen zich bij uitstek voor onderzoek naar prognostische biomerkers volgens het principe ‘hoe hoger de concentratie van de biomerker, hoe slechter de prognose’. In de volgende hoofdstukken worden verschillende potentiele biomerkers onderzocht met betrekking tot de prognose van IPF.

Hoofdstuk 2 geeft een overzicht van de determinanten die tot nu toe beschreven zijn als voorspellers van de prognose van IPF patiënten. In 2002 is de definitie van IPF door de ATS/ ERS opnieuw beschreven, waarin het belang uitgelegd word om onderscheid te maken tussen IPF en andere vormen van longfibrose zoals de niet-specifieke interstitiële pneumonie, welke een andere behandeling en prognose kent. Door alleen studies te beschouwen die gebaseerd zijn op de huidige definitie wordt een actueel overzicht gegeven van determinanten van prognose. De verandering in longfunctieparameters zoals vitale capaciteit (VC) of de diffusiecapaciteit (DLCO) gedurende 6 of 12 maanden is een robuuste meting die goed te gebruiken valt om de mate van progressiviteit van de ziekte te voorspellen, echter dit weet men pas na

6 of 12 maanden en is dus niet bruikbaar als voorspellend instrument op het moment van diagnose. De studies die de verhouding cellen verkregen bij bronchoalveolaire lavage (BAL) gebruiken als voorspeller van prognose spreken elkaar tegen en zijn dus niet bruikbaar in de praktijk. De uitgebreidheid van fibrose op een high resolution computed tomography (HRCT)- scan of in een longbiopt is een goede voorpeller, maar te patiëntonvriendelijk om te gebruiken in de dagelijkse praktijk. Het meest veelbelovend zijn biomerkers die meetbaar zijn in bloed omdat deze gemakkelijk te verkrijgen zijn, goed reproduceerbaar en kleine veranderingen kunnen detecteren, mogelijk al voordat dit klinisch merkbaar is. Zogenaamde pneumoproteinen zijn eiwitten die aanwezig zijn in de vloeistof in de alveolus, het longblaasje. Gedacht wordt dat door schade aan het interstitium, de ruimte tussen de bloedvaten en de alveoli, deze eiwitten van de long naar de bloedbaan lekken en als zodanig de mate van schade aan het interstitium weer kunnen geven. De potentie van deze biomerkers om de prognose van IPF te kunnen voorspellen is groot en zal verder onderzocht moeten worden.

Hoofdstuk 3 geeft een beschrijving van de populatie IPF patiënten, verzameld uit het St Antonius ziekenhuis Nieuwegein en het UMC Utrecht, welke de basis zal vormen voor verder onderzoek naar IPF. Sinds de ATS/ ERS criteria in 2002 is nog niet eerder een cohort IPF patiënten beschreven in Nederland. In dit hoofdstuk wordt deze populatie IPF patiënten vergeleken met andere groepen IPF patiënten uit de internationale literatuur. Patientkarakteristieken zoals leeftijd, celprofielen en longfunctie in bronchoalveolaire lavage en waren vergelijkbaar. In deze groep IPF patiënten waren er iets meer mannen dan internationaal gezien, een mogelijke verklaring hiervoor zou kunnen zijn dat het aandeel vrouwelijke arbeidspartipatie in Nederland laag is en dat vrouwen minder in aanraking komen met stoffen die schadelijk zijn voor het alveolaire epitheel. Verder is opvallend dat een relatief groot aandeel van dit cohort een familiaire vorm van IPF heeft, maar dit kan verklaard worden doordat patiënten vaak specifiek naar het St Antonius ziekenhuis in Nieuwegein worden verwezen met de vraag of sprake is van familiaire IPF. De gemiddelde overleving van de IPF patiënten in deze groep ligt rond de 4 jaar, dit is vergelijkbaar met de overleving elders.

In **hoofdstuk 4** wordt de waarde van surfactant proteïne D (SP-D) beschreven als biomerker voor de prognose van IPF. SP-D wordt gemaakt in epitheelcellen in de long (type II pneumocyten) en is in bloed aanwezig door lekkage door het beschadigde interstitium. De concentratie SP-D in bloed in gezonde mensen wordt beïnvloed door genetische variatie in het SP-D gen (het Met11Thr polymorfisme), dus ook dit polymorfisme werd bepaald om de invloed hiervan op de SP-D concentratie in IPF patiënten te beoordelen. De concentratie SP-D in bloed was significant hoger in IPF patiënten ten opzichte van controles, maar werden niet beïnvloed door het Mt11Thr polymorfisme. Patiënten met een concentratie SP-D hoger dan 460 ng/ml hadden

een slechtere prognose dan patiënten met een waarde lager dan 460 ng/ml. Deze voorspellende waarde bleef stabiel na correctie voor bekende voorspellers van prognose. Bepaling van SP-D in bloed kan dus helpen om de overleving te schatten va IPF patiënten, dit is belangrijk in het kader van timing voor longtransplantatie

In **hoofdstuk 5** wordt endotheline-1 (ET-1) bekeken als mogelijke prognostische biomerker voor IPF. Endotheline-1 trekt fibroblasten aan, stimuleert collageenproductie en is betrokken bij epitheliale-mesenchymale transitie. In bloed werden verhoogde concentraties ET-1 gevonden, maar in BAL vloeistof juist verlaagde concentraties. Dit is een onverwachte bevinding en leidt tot speculaties, mogelijk is de lekkage door het beschadigde interstitium groter dan de productie van ET-1 door de epitheelcellen, maar dit moet verder onderzocht worden. Verder werd er een positieve correlatie gevonden tussen ET-1 in BAL vloeistof en macrofagen, dit zal zeer waarschijnlijk de verhoogde ET-1 productie door macrofagen in IPF weergeven. De concentratie ET-1 invloed was niet gerelateerd aan de progressiviteit van de ziekte en zal niet gebruikt kunnen worden als prognostische biomerker. Echter, in BAL vloeistof was een hoge ET-1 concentratie wel duidelijk gecorreleerd met een verslechtering is diffusiecapaciteit en overleving. Aangezien BAL een invasieve procedure is, zal ET-1 in BAL niet goed bruikbaar zijn als biomerker in de praktijk, echter de relatie tussen ET-1 en prognose geeft wel meer inzicht in de belangrijke rol voor ET-1 in het beloop van IPF.

In **hoofdstuk 6** wordt zowel de relatie beschreven tussen genetische variatie in het IL1RN en IL1B gen en het risico op IPF als de verhouding tussen concentraties van de genproducten interleukine (IL)-1Ra en IL-1β. De IL-1 cytokines spelen een belangrijke rol in diermodellen voor fibrose. In IPF is het nog onduidelijk wat voor rol de IL-1 cytokines spelen, maar al eerder is een genetische variatie beschreven in het IL1RN gen, welke het risico op het ontstaan van IPF vergroot. De concentratie van IL-1β in IPF patienten was hoger dan in bloed van controles, de concentratie van IL-1Ra was in IPF patiënten verlaagd. De concentraties van IL-1β en IL-1Ra in BAL waren allebei verhoogd in IPF patiënten ten opzichte van controles. Aangezien IL-1Ra de activiteit van IL-1β remt, werd de IL-1Ra/ Il-1β ratio berekend. Zowel in bloed als in BAL vloeistof werd een verlaagde IL-1Ra/ IL-1β ratio gevonden welke een factor 3.5 keer lager lag dan bij gezonde controles. Dit geeft een relatief tekort aan IL-1Ra weer, wat resulteert in een pro-inflammatoire omgeving. De eerder gevonden relatie tussen genetische variatie in het IL1RN gen en het ontstaan van IPF werd bevestigd, maar ook werd een nieuwe genetische variatie gevonden welke sterker het ontstaan van IPF beïnvloedt. Deze genetische variatie was ook geassocieerd met een veranderde IL-1Ra/ IL-1β ratio. Concluderend lijkt het waarschijnlijk dat genetische variatie in het IL1RN gen en de resulterende verandering in de balans tussen IL-1Ra en IL-1β een rol speelt in de ontwikkeling van IPF.

In **hoofdstuk 7** wordt de invloed van genetische variatie in het CCL18 gen beschreven op CCL18 expressie en overleving van IPF patiënten. CCL18 verhoogt de productie van collageen door longfibroblasten en is een veelbelovende biomerker voor de prognose van IPF. IPF patiënten hadden een duidelijk verhoogde concentratie CCL18 in het bloed. Zowel in gezonde controles als in IPF patiënten bleek de concentratie CCL18 in bloed onder invloed van genetische variatie in hetCCL18 gen (het rs2015086 C>T polymorfisme), resulterend in de hoogste CCL18 waarden in mensen die het C allel droegen. Dit polymorfisme droeg ook bij aan verschillen in CCL18 expressie in mononucleaire cellen in het bloed van gezonde mensen. Verder was een hoog CCL18 niveau geassocieerd met een slechtere overleving en hadden patiënten met het CT genotype een slechtere overleving dan patiënten met het TT genotype. Deze bevindingen bevestigen de waarde van CCL18 als biomerker voor de prognose van IPF en laten zien dat er bij toekomstige studies naar CCL18 concentraties rekening gehouden moet worden met de invloed van het beschreven polymorfisme

Conclusie

In dit proefschrift zijn een aantal biomerkers onderzocht die kunnen helpen met het voorspellen van de prognose van patienten met IPF. De beschreven biomerkers spelen elk een andere rol in de pathogenese van IPF. De waarde van SP-D bestaat met name uit het gegeven dat het de mate van ‘lekkage’ door het interstitium reflecteert. De balans tussen IL-1β en IL-1Ra zegt iets over inflammatie. ET-1 en CCL-18 worden beiden door macrofagen geproduceerd, welke alternatief geactiveerd zijn en betrokken bij collageenproductie door fibroblasten. Van de beschreven biomerkers zijn SP-D en CCL-18 in serum het makkelijkst te bepalen en bestaat de sterkste associatie met prognose en overleving. Om biomerkers in de praktijk te gaan gebruiken is echter meer nodig. De moeilijkheid bij onderzoek naar IPF zit in het feit dan IPF een zeldzame ziekte is, waardoor het lastig is om grote patientenaantallen te verzamelen. De combinatie van enkele biomarkers zou nog beter de prognose van IPF kunnen voorspellen, maar hiervoor zijn grotere aantallen patienten nodig. Vooralsnog zijn de beschreven biomerkers behulpzaam in het ondersteunen van een schatting naar de prognose, maar nog niet krachtig genoeg om de besluitvorming te sturen of in die mate te beinvloeden dat het beleid erdoor veranderd.

LIST OF PUBLICATIONS

1. Korthagen NM, van Moorsel CH, **Barlo** NP, Kazemier KM, Ruven HJ, Grutters JC.Association between variations in cell cycle genes and idiopathic pulmonary fibrosis. *PLoS One. 2012;7(1):e30442*

2. Barlo NP, van Moorsel CH, Korthagen NM, Heron M, Rijkers GT, Ruven HJ, van den Bosch JM, Grutters JC. Genetic variability in the IL1RN gene and the balance between IL1-Ra and IL-1β in IPF. *Clin Exp Immunol. 2011 Dec;166(3):346-51.*

3. Korthagen NM, van Moorsel CH, **Barlo** NP, Ruven HJ, Kruit A, Heron M, van den Bosch JM, Grutters JC. Serum and BALF YKL-40 levels are predictors of survival in idiopathic pulmonary fibrosis. *Respir Med. 2011 Jan;105(1):106-13*

4. van Moorsel CH, van Oosterhout MF, **Barlo** NP, de Jong PA, van der Vis JJ, Ruven HJ, van Es HW, van den Bosch JM, Grutters JC. Surfactant protein C mutations are the basis of a significant portion of adult familial pulmonary fibrosis in a dutch cohort*. Am J Respir Crit Care Med. 2010 Dec 1;182(11):1419-25*

5. Barlo NP, van Moorsel CH, van den Bosch JM, Grutters JC. Predicting prognosis in idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis. 2010 Jul;27(2):85-95*

6. Barlo NP, van Moorsel CH, Kazemier KM, van den Bosch JM, Grutters JC. Potential role of endothelin-1 in pulmonary fibrosis: from the bench to the clinic. *Am J Respir Cell Mol Biol. 2010 May;42(5):633*

7. Barlo NP, van Moorsel CH, van den Bosch JM, van de Graaf EA, Kwakkel-van Erp JM, Grutters JC. Idiopathic pulmonary fibrosis: clinical description of a Dutch cohort *Ned Tijdschr Geneeskd.2009;153:B425.*

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9. Aldenhoven M, **Barlo NP**, Sanders C. Therapeutic strategies for epidemic Kaposi's sarcoma. *Int J STD AIDS 2006 Sep;17(9):571-8*

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CURRICULUM VITAE

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