Apoptotic and immunological markers in idiopathic pulmonary fibrosis:

evolving concepts of pathogenesis

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Apoptotische en immunologische merkers voor
idiopatische pulmonale fibrose:
Veranderende ideeën over de pathogenese.
(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 8 mei 2012 des middags te 12.45 uur

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Contents

CHAPTER 1

GENERAL INTRODUCTION

1. Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is part of the family of interstitial lung diseases (ILD), also referred to as diffuse parenchymal lung diseases (DPLD), that also includes sarcoidosis and hypersensitivity pneumonitis. ILD comprises a heterogeneous group of lung disorders that mainly affect the pulmonary interstitium, the connective tissue underneath the alveolar epithelium.

IPF is characterized by progressive and fatal fibrosis in the pulmonary interstitium. The aetiology of IPF is not well-understood and treatment options are limited. IPF mainly occurs in elderly Caucasian males, with 55%-80% of patients being men and an average age at diagnosis of 60-65 years old.[1,2] Familial occurrence is found in 2-19% of patients and these are generally younger at disease onset.[3,4] Patients usually present with slowly deteriorating exercise tolerance and often dry cough. In the majority of cases, inspiratory crackles and clubbing of the fingers and toes are found upon physical examination.

The median survival time after diagnosis is 2.5 to 3.8 years.[1,5-8] However, between individuals survival can vary from a few months to > 10 years. In most patients the disease has a steady progressive nature although the rate of progression is highly variable and acute exacerbations may occur (10-20%).[9] Acute exacerbations are defined as deteriorations in lung function within 4 weeks, without signs of infection, heart failure, pulmonary embolism or other identifiable cause.[9]

The annual incidence of IPF is estimated at 4.6 to 6.8 cases per 100 000 persons and may be rising.[2,6] The prevalence is estimated at 14 per 100 000 persons.[2] The most important risk factors are smoking and exposure to metal dust, although their exact role in disease aetiology remains unclear.

1.1 Diagnosis

As there are currently no diagnostic molecular markers for IPF, diagnosis is based on a combination of clinical and pathological findings.[10] Most patients present with a gradual worsening of dyspnea and a decrease in blood oxygen saturation during exercise. In early stages of disease, lung function tests show impaired gas exchange. This is usually followed by a decrease in total lung capacity, later in the disease. Diffusion capacity of the lung for carbon monoxide (DLCO) is considered a reliable parameter for the severity of IPF, and in some studies, DLCO at diagnosis has been associated with survival time.[11,12]

The typical pattern for IPF on high-resolution CT scan (HRCT) is classified as usual interstitial pneumonia (UIP). This pattern is characterized by basal and peripheral, and often patchy, presence of reticular opacities, traction bronchiectasis and honeycombing, although not all of these are always present.[10] Ground glass opacities may be present but should not be extensive.[10]

If radiographical or other clinical findings are inconclusive, a surgical lung biopsy may be neccessary for a confident diagnosis of IPF. Typical histological findings are also classified as UIP, and are hallmarked by a heterogeneous appearance with areas of subpleural and paraseptal fibrosis, scarring and honeycombing alongside relatively normal areas. The presence of fibroblast foci is required for diagnosis and inflammation is usually mild.[10,13]

UIP patterns on HRCT and lung biopsy can also occur secondary to other interstitial lung diseases such as hypersensitivity pneumonitis and connective tissue diseases. However, UIP secondary to these conditions is thought to be a distinct disease entity, with different clinical course and treatment options.[10] Therefore, thorough clinical examination to separate these UIP entities from IPF/UIP is of utmost importance.

Bronchoalveolar lavage (BAL) is helpful to exclude such alternate diagnoses, and can also be used to exclude infection and malignancy. In IPF patients, BAL fluid (BALF) cell counts typically show an increased number of neutrophils and eosinophils and low lymphocyte count, while the majority of cells are macrophages. The BALF also offers cellular and non-cellular materials from the bronchioloalveolar compartment that can be used experimentally in the search for new biomarkers.

1.2 IPF and complicating lung disorders

Many patients with IPF suffer from complicating lung problems, such as pulmonary arterial hypertension (PAH), which is associated with decreased survival time.[14] In some patients PAH can be successfully managed, which may improve quality of life.

In around one-third of IPF patients, emphysema is a complicating lung disorder, and this may have a negative impact on survival time.[15,16] It has been proposed that combined pulmonary fibrosis and emphysema (CPFE) could even be a distinct disease entity but this is a subject of ongoing debate.

IPF is further associated with an increased occurence of lung carcinomas.[17] It is unclear whether the risk of carcinoma development increases in response to the pathological changes in IPF, or if the two conditions share etiologic factors. In this respect, it is of note that cigarette smoking is a risk factor for both disorders.

1.3 Treatment

There is currently no medicinal treatment for IPF with a proven effect on survival. Anti-inflammatory medication such as steroids may relieve symptoms in some patients but do not influence disease progression or survival.[18,19] Recent trials with novel drugs such as bosentan, etanercept, imatinib and interferon-gamma have also reported no significant effect on disease progression or survival.[20-25] Pirfenidone as well as acetylcysteine (in combination with prednisone and azathioprine) may have some effect on lung function deterioration, but an effect on survival has not been demonstrated so far.[26-28] Several clinical trials are currently ongoing and will hopefully provide us with more effective drugs, such as BIBF1120. This tyrosine kinase inhibitor was recently demonstrated to reduce lung function decline, lower the incidence of acute exacerbations, and improve quality of life.[29]

The only non-medicinal therapy with a proven effect on survival is lung transplantation. However, this therapy is only available for a limited number of patients. Although there is no official age limit, transplant is generally not recommended to patients older than 65 years because of a steep increase in postoperative complications with age. Because of the late age of onset, not many IPF patients are eligible and mortality on the waitinglist is high. The post-transplantation 5-year survival is only about 44% although outcomes continue to improve due to better medical expertise.[30]

In the end-stage of disease, most patients are provided with oxygen supplementation. There is almost no role for mechanical ventilation, especially in patients with end-stage disease. It is hypothesized that mechanical ventilation may even be a risk factor that increases damage to the lung.[31-33] When no other treatment options remain available, palliative care with opiates such as morphine may be important to provide comfort to patients.

2. Pathogenesis

The alveolar epithelium is for 90% made up by flat thin type I alveolar epithelial cells (AEC) and between them a few type II alveolar epithelial cells that produce the proteins for the mucous lining of the alveoli (figure 1).

In IPF, scar tissue is found underneath a layer of type II alveolar epithelial cells, severely impairing gas exchange. The current hypothesis is that in IPF, repetitive epithelial injury induces inflammation and aberrant wound repair, resulting in remodelling of the alveolar interstitium.

In end stage IPF, the original alveolar structure has been completely replaced with fibroblasts and extracellular matrix components. The exact cause of this devastating fibroproliferative process remains unclear.

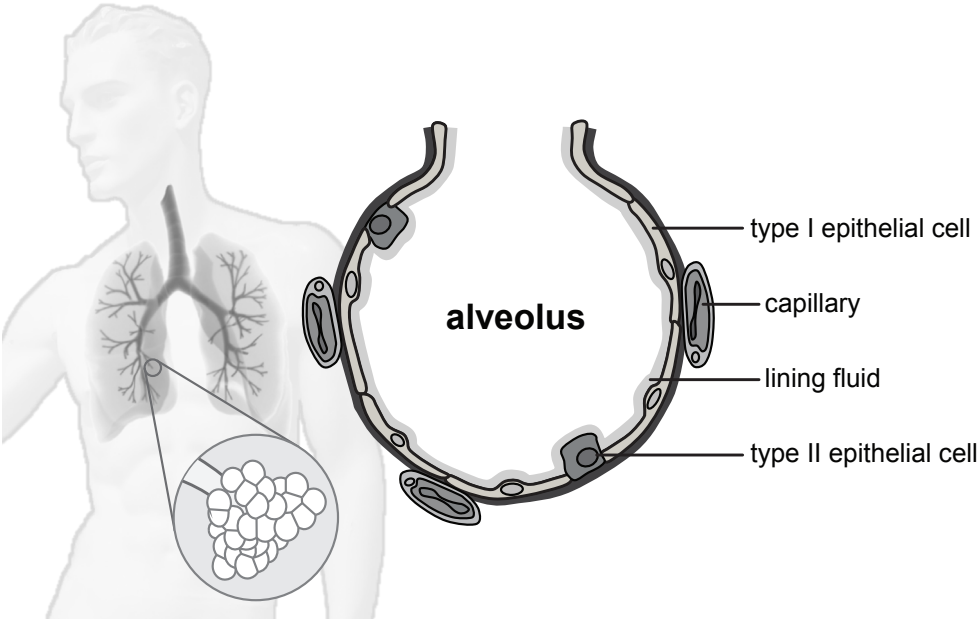

Figure 1. The alveoli are lined with type I epithelial cells that participate in gas exchange with the network of capillaries surrounding the alveoli. Cuboidal type II epithelial cells produce surfactant, a complex of lipids and proteins lining the alveolar surface.

2.1 Fibrogenesis

In IPF lungs, large amounts of fibroblasts are found and they can form clusters that are called fibroblast foci (figure 2).[13] The fibroblasts themselves also contribute to progressive scarring of the lung tissue through deposition of extracellular matrix components such as collagen. In IPF, the fibroblasts differentiate into myofibroblasts that are more contractile and have more profibrotic potential. The source of these myofibroblasts is a matter of debate. Resident fibroblasts are thought to contribute to the myofibroblast population although some research suggests that myofibroblasts are derived from epithelial cells through epithelial-mesenchymal transition.[34,35] Others suggest they are derived from the blood in the form of a precursor cell called the fibrocyte.[36] It is possible that all three sources contribute to the myofibroblast population in IPF.

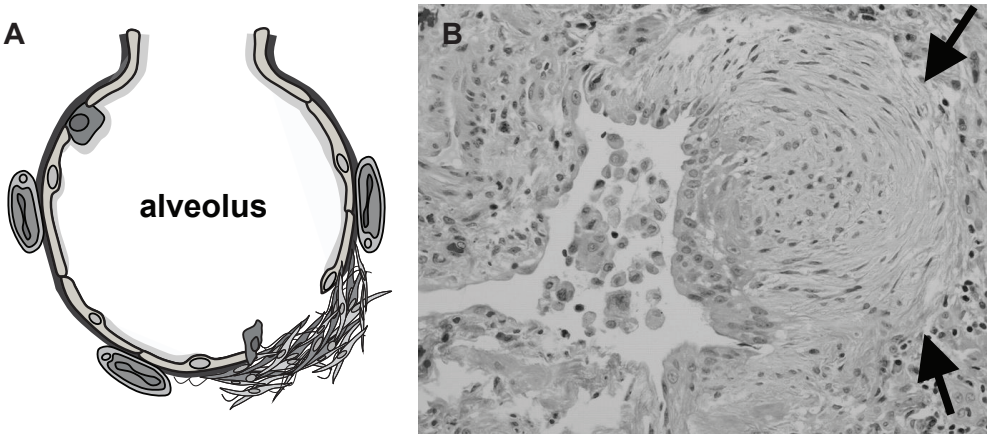

Figure 2. A) Schematic representation of a fibroblast focus. B) Fibroblast focus in a surgical lung biopsy from an IPF patient.

The exact trigger responsible for the abundant formation of fibrosis in IPF lungs is unclear, but it is unlikely that the fibroblasts themselves are the primary cause of IPF. It seems more likely that the alveolar epithelial cells play a key role by releasing chemotactic factors and mitogens that contribute to the migration, proliferation and differentiation of fibroblasts.[37] There is also accumulating evidence that a chronic inflammatory process is fundamental to the ongoing formation of fibrosis.[38]

2.2 Alveolar destruction

The fibrogenesis in IPF lungs is thought to begin with injury to the alveolar wall. Affected areas of IPF lung show a severe loss in type I alveolar epithelial cells and instead of a few isolated type II cells, there are rows of hyperplastic type II epithelial cells lining honeycomb cysts.[39] This indicates that the remaining type II cells have not lost the ability to proliferate and they might have been triggered by the wound healing process to re-epithelialize the alveoli. It is possible that they are unable to differentiate to type I cells while the renewal of the type I cell layer may be is essential to resolving the wound healing process. Alveolar type II cells are thought to maintain the alveolar surface and are essential for repair because they give rise to the gas exchanging alveolar type I cells. In IPF, increased numbers alveolar type II epithelial cells are undergoing programmed cell death, known as apoptosis. Most of these apoptotic cells are found near areas of remodeling.[40,41]
In normal tissue, apoptotic cells are rquicklyemoved by phagocytosis and are very difficult to visualize.[42] The fact that so many apoptotic cells are seen in IPF lungs may indicate that the response to apoptosis is impaired.[42] It is possible that the apoptotic cells do not display the normal signalling molecules that attract the phagocytes and facilitate recognition and clearance of apoptotic cells.[43,44] The phagocytes themselves may also be dysfunctional or their phenotype may be altered in IPF patients. However, another possibility is that the rate of apoptosis is so high that phagocytes can simply not keep up.[42]

The cause of this excessive apoptosis is unclear. In some familial cases of IPF, mutations in the surfactant protein C gene (*SFTPC*) have been identified.[4,45,46] This gene encodes the SP-C protein that is produced solely by alveolar type II cells. Mutations in the *SFTPC* gene cause a misfolding of the protein that induces apoptosis in alveolar type II cells.[45,46] It is thought that another cause of familial IPF may be rapid aging because of mutations in telomerase, the protein that regulates telomere lengthening.[47,48] When the telomeres on the ends of chromosomes become too short, the cell will go into apoptosis. In this way the telomeres limit the life-span of a cell.[49] In sporadic IPF cases telomere shortening may also play a role.[48,50] In these cases, chronic damage and repair processes may cause the cells to reach the end of their life-span sooner and become apoptotic.[51] In many patients DNA and cell damage may be caused by smoking or inhalation of metal dust.[51] Other causes remain to be discovered.

How the loss of epithelial cells may lead to IPF is unclear. The loss of too many stem cells may result in damage that simply can not be repaired by the ageing lung. Induction of apoptosis in the alveolar epithelium of mice causes lung inflammation and subsequently fibrosis.[52] Failure to clear apoptotic cells could cause IPF because the cells can release death signals such as reactive oxygen and proinflammatory signals that are involved in inflammatory lung disease.[53] Apoptotic cells that are not removed start to become necrotic, a process called secondary necrosis.[54] Release of ATP by secondary necrotic cells causes the activation of an inflammatory mechanism called the inflammasome and the release of interleukin-1β (IL-1β) that are thought to play an important role in the fibrotic process seen in IPF.[55] IL-1β is a cytokine, a small protein that regulates immune responses. However, it is unclear whether cell death and injury are responsible for the rampant circulation of a wide range of these inflammatory cytokines that is seen in IPF patients.

2.3 Immune response

Repetitive alveolar cell injury induces an inflammatory immune response in the lungs of IPF patients. This immune response is mirrored by many inflammatory cytokines that are elevated in the lung and blood of these patients. Originally, it was thought that an inflammatory process was the cause of IPF. However, anti-inflammatory medication has no effect on the progression of fibrosis in IPF and the typical white blood cells associated with inflammation are not elevated in IPF patients. Therefore, the inflammatory response was more recently thought to be a non-specific side-effect to the tissue damage and was no longer considered to be essential for IPF pathogenesis. However, new evidence suggests that some specific immune responses might still be fundamentally involved in IPF. Some immunological proteins, such as the cytokine TGF-β, CC-chemokines and metalloproteinases, have been found to mediate the fibrotic response and are considered to play a key role in IPF pathogenesis.[35] In addition, specific white blood cell subsets such as activated neutrophils or macrophages might enhance alveolar cell injury, and negatively influence normal repair.

2.3.1 Neutrophils

Elevated neutrophil counts (neutrophilia) are seen in the BAL of many IPF patients. They may have some prognostic value, but this remains controversial.[56-58] They are thought to be recruited to affected areas of the lung by chemoattractant factors such as IL-8, MCP-1 and MRP14.[59,60] Activated neutrophils release factors that under normal circumstances are a defence against extracellular pathogens, but can also increase the damage by causing lung injury, epithelial cell apoptosis and basement membrane loss.[61,62] Neutrophils are also thought to mediate the transition from acute to chronic inflammation that may precede fibrosis.[63]

2.3.2 Macrophages

Macrophages and the factors they produce are involved in all stages of the wound repair process.[64] However, it is becoming evident that macrophages can have different phenotypes, and that subgroups of macrophages are involved in different stages of wound repair. There is some uncertainty about the exact nature of the different macrophage subgroups, but the main concept involves two disparate types of macrophages known as M1 and M2 (Figure 3).

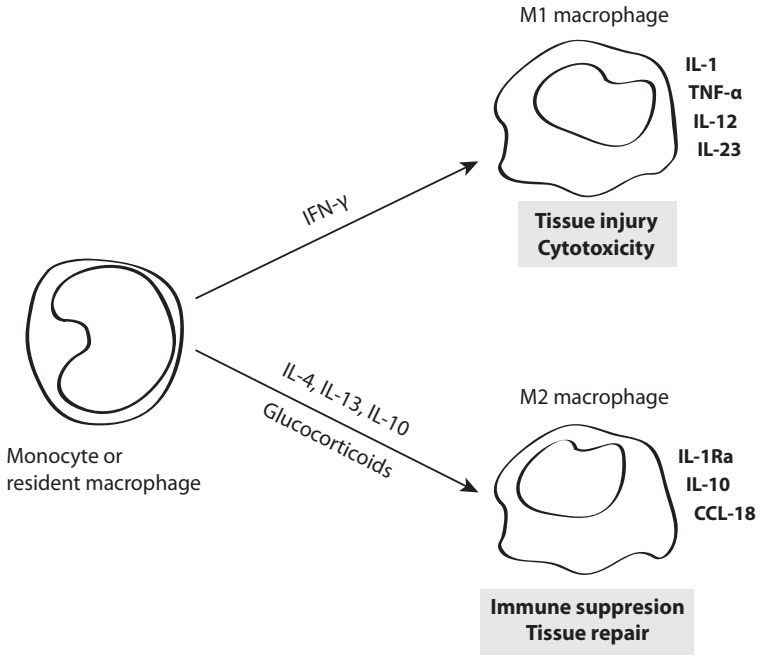

Figure 3. Schematic representation of macrophage differentiation to an M1 or M2 phenotype.

Following tissue damage, the initial response is pro-inflammatory. M1 macrophages are recruited and participate in the antimicrobial response by releasing reactive oxygen species, interleukin 1 (IL-1) and tumour necrosis factor (TNF). Although this is an important response to prevent infection after tissue damage it can also cause damage to adjacent tissues and may lead to chronic inflammation and disease.[64] M2 macrophages are thought to be important for resolving the wound repair process. They are anti-inflammatory and induce fibrosis by producing anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1Ra) and profibrotic cytokines such as TGF-β. Recent evidence suggests that M2 macrophages are overinduced in IPF and may predict survival.[65] M2 macrophages also produce CCL18, which has been associated with IPF survival.[66]

3. Markers of disease

Currently, there is no simple test available to diagnose IPF. Clinical findings such as lung function tests and HRCT are often considered sufficient, especially in typical cases of IPF. In other cases, lung biopsy will be needed. However, lung biopsy carries a certain risk of mortality (± 3%), and patients may have acute exacerbations after surgery. Due to differences in prognosis and treatment between IPF and other interstitial pneumonias (IP), a surgical biopsy can still be warranted. Finding molecular markers in blood and/or BAL fluid that help differentiate IPF from other IP entities would mean a major breakthrough in the diagnostic process of IPF.

Also, molecular markers that can be used to monitor progression of disease in IPF would be very helpful for disease management. This is important for the determination of prognosis and for optimal timing of therapeutic interventions, such as lung transplantation. Currently, the best available way to monitor the disease is by serial lung function testing and HRCT. Decreases in forced vital capacity (FVC) and diffusion capacity for carbon monoxide (DLCO) are observed in progressive disease. The rate of decline in lung function, in the absence of an alternative explanation, may have some prospective value.[13,65] However, obtaining accurate and timely lung function measurements during follow-up may be difficult in everyday clinical practice, and takes time (often 6-12 months). Molecular markers that can be used to monitor disease progression and can be determined at any stage of the disease, and at short-term intervals (e.g. 3 months), would provide an innovative tool in the clinical management of IPF.

4. Aim of this thesis

Many proteins involved in tissue damage, inflammation and fibrosis are elevated in both blood and lungs of IPF patients. To find out whether these proteins are specific markers for IPF, they will have to be compared to conventional clinical findings and be investigated in other, closely related, fibrosing interstitial lung diseases. In addition, genetic variations will be studied because they can influence protein levels, and might also play a role in disease susceptibility and clinical outcome.

In the search for molecular markers for diagnosis and prognosis, we focused especially on the immunological response to injury and on apoptosis, because of their emerging role in the pathogenesis of IPF. We hypothesized that the identification of such disease-specific proteins and genes might also contribute to new insights into the biological mechanisms involved in IPF.

5. Outline of the thesis

In **chapter 2**, single nucleotide polymorphisms in the genes encoding p53 and p21 are evaluated for their influence on IPF predisposition and prognosis. P53 and p21 are cell cycle regulators and determining factors in apoptosis.

In **chapter 3**, myeloid-related protein 14 (MRP14 / S100A9) levels in the broncho-alveolar lavage are compared between IPF patients, healthy controls and patients with different stages of sarcoidosis. MRP14 is thought to be elevated during chronic inflammation and may stimulate fibroblast growth.

In **chapter 4**, a meta-analysis was performed to investigate the role of the inter-leukin-1 receptor antagonist gene (*IL1RN*) in IPF. Genetic variations in *IL1RN* may be a risk factor for developing IPF.

In **chapter 5**, YKL-40 levels in the serum and BALF of IPF patients are evaluated for their potential as a survival marker. The -329 polymorphism in *CHI3L1*, the gene encoding YKL-40, and its effect on serum and BALF levels of YKL-40 is explored.

In **chapter 6**, the role of YKL-40 in disease is further explored. The change in serum YKL-40 level over time in IPF patients is monitored and serum YKL-40 levels are compared between IPF patients and other interstitial pneumonia patient groups.

In **chapter 7**, the potential source of YKL-40 is investigated in a short pilot study. *In vitro* studies were performed to determine whether YKL-40 is produced by alveolar macrophages in healthy individuals and in patients with interstitial lung diseases.

In **chapter 8**, the results are summarized and a general discussion is provided.

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CHAPTER 2

ASSOCIATION BETWEEN VARIATIONS IN CELL CYCLE GENES AND IDIOPATHIC PULMONARY FIBROSIS

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a devastating and progressive lung disease. Its aetiology is thought to involve damage to the epithelium and abnormal repair. Alveolar epithelial cells near areas of remodelling show an increased expression of proapoptotic molecules. Therefore, we investigated the role of genes involved in cell cycle control in IPF.

Genotypes for five single nucleotide polymorphisms (SNPs) in the tumour protein 53 (*TP53*) gene and four SNPs in cyclin-dependent kinase inhibitor 1A (*CDKN1A*), the gene encoding p21, were determined in 77 IPF patients and 353 controls. In peripheral blood mononuclear cells (PBMC) from 16 healthy controls mRNA expression of *TP53* and *CDKN1A* was determined.

rs12951053 and rs12602273, in *TP53*, were significantly associated with survival in IPF patients. Carriers of a minor allele had a 4-year survival of 22% versus 57% in the non-carrier group ($p = 0.006$). Rs2395655 and rs733590, in *CDKN1A*, were associated with an increased risk of developing IPF. In addition, the rs2395655 G allele correlated with progression of the disease as it increased the risk of a rapid decline in lung function. Functional experiments showed that rs733590 correlated significantly with *CDKN1A* mRNA expression levels in healthy controls.

This is the first study to show that genetic variations in the cell cycle genes encoding p53 and p21 are associated with IPF disease development and progression. These findings support the idea that cell cycle control plays a role in the pathology of IPF. Variations in *TP53* and *CDKN1A* can impair the response to cell damage and increase the loss of alveolar epithelial cells.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a severe and relentless lung disease that is characterized by fatal scarring of the lung parenchyma and progressive shortness of breath. IPF is a rare disease with a prevalence of 14 per 100 000 persons.[1] The annual incidence is estimated to be between 4.6 and 7.4 cases per 100 000 persons and about 5 million people are affected worldwide.[1-3] Moreover, the incidence continues to rise and IPF is now an important cause of respiratory mortality.[3]

The median survival time is only 2.5 to 3.5 years, but individual survival can vary from a few months to > 10 years.[2,4-6] Progression of the disease is mainly monitored by lung function testing, and some studies have found that lung function decline is associated with survival time.[7,8] However, it is unclear what causes this heterogeneity in survival time and whether it can be predicted by any other means.

The cause of IPF remains unknown but is thought to involve damage to the epithelium and abnormal repair. In 0.5-19 % of cases IPF is familial and is most likely caused by a single genetic mutation.[9] Apart from deleterious alleles, no rare risk variants have been found for IPF while a common risk variant in the *MUC5B* gene has recently been discovered to associate with both familial and sporadic IPF.[10] Susceptibility to IPF and progression of the disease is probably influenced by a combination of genetic variations that drive epithelial injury and abnormal wound healing processes.[11]

It has been suggested that IPF pathology has similarities to cancer and that it could be a neoproliferative disease.[12] Previous immunohistological examination of IPF lungs has revealed increased expression of proteins involved in cellular responses to injury and DNA damage, including p53 and p21.[13]

Tumour protein 53 (p53) is a key regulator of apoptosis. It is upregulated upon DNA damage and prevents damaged cells from becoming malignant by inducing growth arrest and cell death.[14,15] With increasing age, some cells can escape p53-induced cell death and the continued presence of these dysfunctional cells can lead to a decrease in tissue regeneration and repair as well as cancer.[16] Increased levels of p53 in the lungs of IPF patients are consistent with increased apoptosis.[13] Loss of alveolar epithelial cells by apoptosis can impair the regenerative capacity of the lung. P53-induced growth arrest is mediated by increased transcription of cyclin-dependent kinase inhibitor 1A (*CDKN1A*), the gene encoding p21.

The p21 protein (also known as Cip1, Sdi1, and Waf1) regulates cell cycle progression. Induction of this protein prevents proliferation and allows optimal DNA repair thereby reducing apoptosis and cancer risk.[17]

Genetic variations may play a role in IPF disease susceptibility and progression and could give important insights into disease aetiology.[11,18]

Materials and methods

Subjects

77 IPF patients who visited the Centre for Interstitial Lung Diseases at the St. Antonius Hospital, the Netherlands between November 1998 and 2007 were included in this study.

Diagnoses made before 2002 were reviewed by a clinician and patients were only included when the diagnosis met the current ATS/ERS guidelines.[6] Other causes of UIP (drugs, collagen vascular diseases) were ruled out. 58 males and 19 females (mean age 60.8 years {SD 13.6}) were included. In 58 cases the diagnosis of UIP was confirmed on lung biopsy (75%). From 64 patients lung function follow-up was available. In accordance with the method proposed by Egan *et al.*,[19] we defined a rapid decline in lung function as more than 15% decline in percent-predicted DLCO (diffusion capacity of the lung for carbon monoxide) or more than 10% decline in percent-predicted vital capacity (VC) over a one-year period. Length of follow-up for survival was up to 4 years and was based on hospital records. Patients that were still alive or transplanted were censored in the survival analysis. Clinical parameters at diagnosis were obtained from hospital records.

The control group consisted of 353 healthy volunteers (mean age 39.2 years {SD 12.4}, 139 males, 210 females). This included 313 self-reported healthy Caucasian employees of the St Antonius Hospital and 40 volunteers that underwent brochoalveolar lavage between January and October 2007.

The Ethical Committee of the St. Antonius Hospital approved the study protocol (R-05.08A). All subjects that met the inclusion criteria and gave written informed consent were included in this study.

Genotyping

DNA was extracted from whole blood samples and single nucleotide polymorphism (SNP) typing was conducted using a custom Illumina goldengate bead SNP assay in accordance with the manufacturer's recommendations (Illumina Inc; San Diego, USA). The tagger program[20] was used to select haplotype tagging SNPs (tagSNPs), that represent the genetic variation in a specific region. SNPs were selected using the CEU HapMap panel and covering the gene region plus 2500 basepairs upstream and downstream, based on NCBI build 35. We used an r^2 threshold for SNPs > 0.8 under the pairwise tagging options. Three tagSNPs in the p21 gene, *CDKN1A* located on chromosome 6p21.2, were selected based on a minor allele frequency (MAF) higher than 25% in the Caucasian population and one SNP (rs730506) was added because of its potential regulatory function on protein expression through localization on a transcription factor binding site.[21] For the p53 gene, *TP53* located on chromosome 17p13.1, three tagSNPs were selected by reducing the MAF with 5% increments until three tagSNPs could be selected with a MAF higher than 5%. Two potentially functional SNPs were added (rs16956880 and rs11575997) that could have an effect on splicing.[22]

Messenger RNA levels

We used thawed peripheral blood mononuclear cells (PBMC) from 16 healthy controls. The expression of *CDKN1A* and *TP53* mRNA was analysed by quantitative RT-PCR amplification as described previously.[23] Briefly, total RNA was isolated using an Rneasy microkit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. 0.2 µg RNA was used for first-strand cDNA synthesis with 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. The PCR was performed with the RT2 Real-Time™ 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 s at 95°C, 20 s at 61°C and 25 s at 72°C). The copy number of *CDKN1A* was normalized by the housekeeping gene β-actin (ACTB).

Statistics

SPSS 15 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism v. 3 (Graphpad software INC., San Diego, CA, USA) were used for statistical analysis. The Kaplan-Meier method with log-rank test was used to analyse whether any SNPs were associated with survival. Cox regression analysis with covariates was used to check for possible confounders. Pearson's goodness-of–fit Chi-square test and Fisher's exact test were used to test for deviation from Hardy-Weinberg equilibrium and for a difference in genotype and allele frequencies between patients and controls (as implemented online at http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Due to linkage disequilibrium between the SNPs (figure 1), the effective number of independent SNPs was 4.46, based on the method proposed by Li and Ji[24] (as implemented online at http://gump.qimr.edu.au/general/daleN/matSpD/).
The adjusted significance threshold was set at 0.05 / 4.46 = 0.011.

Results

TP53

Table 1. *TP53* genotype frequency in patients and controls

TP53	genotype	IPF	controls
rs12951053	AA	0.88 (68)	0.84 (295)
	AC	0.12 (9)	0.16 (56)
	CC	0.0 (0)	0.006 (2)
rs12602273	GG	0.86 (66)	0.85 (299)
	CG	0.14 (11)	0.15 (53)
	CC	0.0 (0)	0.003 (1)
rs2287497	GG	0.82 (63)	0.80 (281)
	AG	0.18 (14)	0.19 (68)
	AA	0.0 (0)	0.01 (4)
rs16956880	GG	1.0 (77)	1.0 (353)
rs11575997	CC	1.0 (77)	1.0 (353)

The values in parentheses are the number of individuals the frequency is based on. There were no significant differences between patients and controls

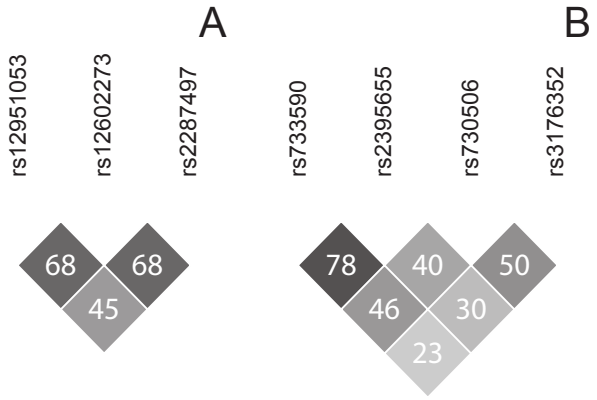

Figure 1. Linkage disequilibrium plot. Showing pairwise r^2 for SNPs in *TP53* (A) and *CDKN1A* (B)

Genotype and allele frequencies for *TP53* and *CDKN1A* SNPs did not deviate from Hardy-Weinberg equilibrium (table 1). There were no significant differences in genotype frequency between patients and controls in *TP53* SNPs. There was linkage disequilibrium between three SNPs (figure 1A): between rs12951053 and rs12602273 $D' = 0.85$ and $r^2 = 0.68$; between rs12951053 and rs2287497 $D' = 0.78$ and $r^2 = 0.45$; and between rs12602273 and rs2287497 $D' = 0.98$ and $r^2 = 0.68$.

Carriership of the minor alleles of rs12951053 (C) or rs12602273 (G) was significantly associated with shorter survival time, both individually and when the SNPs are combined (figure 2A). Carriers of the minor alleles had a 4-year survival of only 22% versus 57% in the non-carrier group (Kaplan-Meier, Log rank test $p = 0.006$). Cox regression analysis revealed that age, gender and lung function were no confounding factors. The hazard ratio for carriership of a *TP53* minor allele was 2.9 (95% CI 1.3 – 6.2, $p < 0.007$). Rs2287497 did not show any association with survival in our IPF cohort. The two SNPs in *TP53* that were associated with survival were tested for an association with lung function decline (table 2). Carriers of the rs12951053 C allele and carriers of the rs12602273 C allele were more likely to have a rapid decline in lung function, although the difference did not reach statistical significance. In the rs12951053 AC group 5 of 8 (63%) and in the AA group 15 of 50 patients (30%) had a rapid decline in lung function. Together these results suggest that carriership of the *TP53* rs12951053 C allele or rs12602273 C allele predisposes to a rapid progression of IPF.

No differences in *TP53* mRNA expression were observed between the *TP53* genotypes.

Table 2. Genotype and lung function decline

		genotype	rapid	non-rapid
TP53	rs12951053	AA	15	35
		AC	5	3
		CC	0	0
	rs12602273	GG	16	34
		CG	4	4
		CC	0	0
CDKN1A	rs733590	TT	3	13
		TC	10	17
		CC	7	8
	rs2395655*	AA	1	11
		AG	10	18
		GG	9	9

Number of IPF patients with non-rapid or rapid decline in % predicted vital capacity (> 10% in one year) or % predicted diffusion capacity (> 15% in one year).* Fisher's exact test with carriers of p21 rs2395655G vs. non-carriers resulted in p = 0.04.

CDKN1A

There was significant linkage disequilibrium between the four *CDKN1A* SNPs (figure 1B): between rs733590 and rs2395655 D' = 0.96 and r^2 = 0.78; between rs733590 and rs730506 D' = 0.99 and r^2 = 0.46; between rs733590 and rs3176352 D' = 0.62 and r^2 = 0.23; between rs2395655 and rs730506 D' = 1 and r^2 = 0.40; between rs2395655 and rs3176352 D' = 0.76 and r^2 = 0.30; and between rs730506 and rs3176352 D' = 0.81 and r^2 = 0.50.

All four of the SNPs in *CDKN1A* were associated with IPF (table 3). Only the association with rs733590 and rs2395655 remained significant at the adjusted threshold, and the association was strongest for rs2395655. Carriership of rs2395655 GG genotype in patients was almost twice as high as in controls (30% versus 16% respectively, p = 0.003). There is a high degree of linkage disequilibrium within the gene but haplotype analysis did not generate superior results (data not shown).

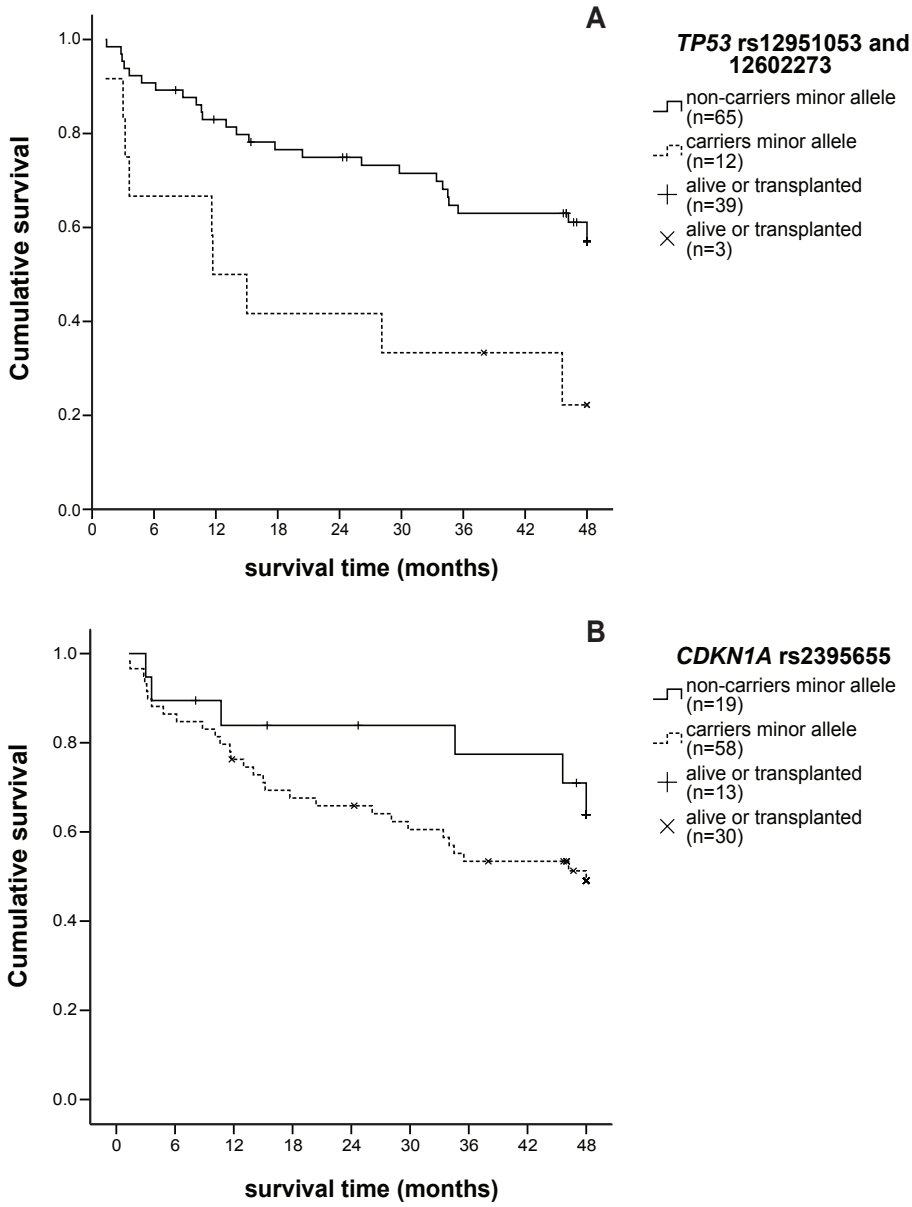

Figure 2. Kaplan-Meier analysis of survival in a cohort of IPF patients. A) Carriers of the *TP53* rs12951053 C or rs12602273 G alleles had significantly worse 4-year survival rate (p = 0.006). B) There was no significant difference in survival between carriers and non-carriers of the *CDKN1A* rs2395655 G allele (p = 0.2).

Survival in carriers of rs2395655 G allele was worse than in non-carriers, however, the difference did not reach statistical significance (p = 0.2, figure 2B).
Rs2395655 and rs733590 were tested for an association with lung function decline (table 2). The association between rs733590 and lung function decline did not reach statistical significance. Carriers of rs2395655 G allele were more likely to have a rapid decline in lung function (p = 0.04, table 2). Nineteen patients (41%) carrying the G allele had a rapid decline in lung function while only one patient (8%) with the AA genotype had a rapid decline. This did not remain significant after correction for multiple testing.

Table 3. *CDKN1A* genotype and carriership frequency in patients and controls.

CDKN1A	genotype	IPF	controls	carriership	IPF	controls	p-value
rs733590*	TT	0.31 (24)	0.39 (139)	T	0.74	0.86	CC p = 0.007
	TC	0.43 (33)	0.47 (166)	C	0.70	0.61	OR = 2.2
	CC	0.26 (20)	0.14 (48)				(1.23-4.03)
rs2395655*	AA	0.25 (19)	0.34 (120)	A	0.70	0.84	GG p = 0.003
	AG	0.45 (35)	0.50 (177)	G	0.76	0.66	OR = 2.3
	GG	0.30 (23)	0.16 (55)				(1.31-4.05)
rs730506	GG	0.49 (38)	0.59 (208)	G	0.89	0.96	CC p = 0.013
	CG	0.40 (31)	0.37 (132)	C	0.51	0.41	OR = 3.0
	CC	0.10 (8)	0.04 (13)				(1.21-7.58)
rs3176352	CC	0.39 (30)	0.54 (189)	C	0.87	0.94	CC p = 0.020
	CG	0.48 (37)	0.41 (143)	G	0.62	0.46	OR = 1.8
	GG	0.13 (10)	0.06 (21)				(1.09-3.00)

The values in parentheses are the number of individuals. P-values are based on the number of individuals with and without the specified genotype and are calculated using a Pearson's goodness-of-fit chi-square test. Odds ratio (OR) is shown with the 95% confidence interval in brackets. *After correction for multiple testing the association with rs733590 and rs2395655 remained significant.

CDKN1A mRNA expression in healthy controls was determined in relation to beta-actin (ACTB) expression and is shown in figure 3. *CDKN1A* mRNA levels were significantly higher in carriers of the rs733590 T allele, using an uncorrected t-test (p = 0.03 TT+CT vs CC). For carriers of the rs2395655 A allele, a similar trend was observed (p = 0.06).

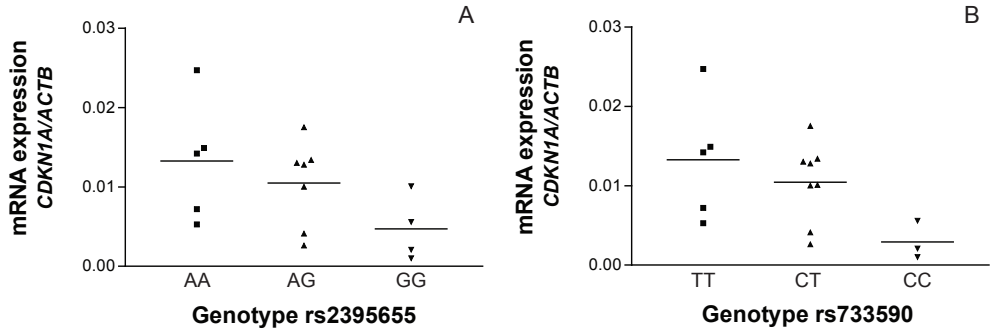

Figure 3. *CDKN1A* mRNA expression in healthy controls. *CDKN1A* mRNA levels were significantly different between rs733590 genotypes, p = 0.03 (TT+CT vs. CC). A similar trend was observed for rs2395655 genotypes (p = 0.06, AA+AG vs. GG).

Discussion

This study reports the novel finding that SNPs in *CDKN1A* predispose to IPF and that SNPs in both *TP53* and *CDKN1A* are associated with progression in IPF. The SNPs in *TP53* had a more pronounced effect on survival while rs2395655 in *CDKN1A* had a more pronounced effect on rapid lung function decline. Survival was significantly correlated with change in lung function in our cohort ($p = 8x10^{-9}$, results not shown) as is usually observed in IPF.[25] Together, our findings show that variations in cell cycle genes are involved in IPF.

Both p53 and p21 are vital cell cycle regulators after DNA damage and are determining factors in cell fate. Inhaled substances, like cigarette smoke, can cause DNA damage to lung cells and exposure to these substances have been associated with an increased risk of developing IPF.[26] In damaged cells, upregulation of p53 occurs and this induces growth arrest and apoptosis. It has been shown that in mice, injury to type II alveolar epithelial cells caused pulmonary fibrosis.[27] Alveolar type II cells are progenitors of type I cells and essential for alveolar repair after induced injury.[28] They react by immediate proliferation along the alveolar basement membrane. Previous studies have detected increased levels of proapoptotic molecules in hyperplastic alveolar epithelial cells in IPF patients.[13,29]

However, induction of p21 can rescue a cell from apoptosis by allowing DNA repair. In addition, p21 has been reported to be elevated during the differentiation of alveolar epithelial type II cells into type I cells.[30] We found that the *CDKN1A* allele that predisposed to IPF disease development was associated with decreased *CDKN1A* mRNA expression. This finding has to be further investigated in light of previous immunohistochemical findings that showed increased p21 levels in the lungs of IPF patients.[13] The upregulation of p21 in IPF lungs may occur later in disease or may be insufficient to prevent disease progression after injury. Forced expression of the transfected human of *CDKN1A* gene in mice resulted in decreased apoptosis, inflammation and fibrosis after bleomycin installation.[31] In addition, p21 attenuates epithelial mesenchymal transition, a process that contributes to the formation of fibroblast foci,[32] and it plays an important role in the prevention of cancer by inducing cell cycle arrest.[33] This dual functionality of p21 may therefore explain

both the remodelling in IPF as well as the increased incidence of carcinomas that is thought to occur in IPF patients.[34] Together this indicates that the absence of p21 causes a pro-fibrotic environment, while the induced presence of p21 results in a better healing process.

This study was part of hypothesis generating research, and therefore the findings will have to be validated in an independent cohort. The SNPs that were associated with IPF in this study are tagSNPs that represent the genetic variation in the gene region. It is likely that these SNPs are linked to a functional SNP in another part of the gene. The number of patients included in this study was limited, a problem that almost all studies with IPF patients face. For instance, an association was found between rs2395655 and lung function decline ($p = 0.04$). However, due to the correction for multiple testing, a cohort of at least 80 patients would be needed to reach the adjusted significance level of $p = 0.011$ with 80% power. Similarly, at least 46 individuals would be required to reach a power of 80% for the association between rs2395655 and *CDKN1A* mRNA expression. Another limitation of this study is the difference in age between patients and controls. However, no effect of age on genotype distribution was observed.

In conclusion, we found that the *TP53* rs12951053 and rs12602273 SNPs were significantly associated with survival in IPF patients and that *CDKN1A* SNPs rs2395655 and rs733590 were significantly associated with the risk of developing IPF. Furthermore, the *CDKN1A* SNPs were associated with a rapid decline in lung function and significantly decreased *CDKN1A* mRNA levels. This is the first study to show that genetic variation in the genes encoding p53 and p21 might play an important role in IPF. Further studies are needed to elucidate the role of cell cycle genes in IPF pathology.

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CHAPTER 3

MRP14 IS ELEVATED IN THE BRONCHOALVEOLAR LAVAGE FLUID OF FIBROSING INTERSTITIAL LUNG DISEASES

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Abstract

Pulmonary fibrosis is defined by an overgrowth of fibroblasts and extracellular matrix deposition and results in respiratory dysfunction that is often fatal. It is the end stage in many chronic inflammatory interstitial lung diseases (ILD) such as sarcoidosis and idiopathic pulmonary fibrosis (IPF). The migration inhibitory factor related proteins (MRP's) belong to the S100 family of calcium binding proteins and are highly expressed by neutrophils, macrophages and epithelial cells during chronic inflammation. MRP14 stimulates fibroblast proliferation *in vitro* and is expressed in granulomas from sarcoidosis patients. We hypothesized that MRP14 may be a biomarker for fibrotic interstitial lung diseases. The objective of this study was to investigate whether levels of MRP14 in the bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis and IPF correlate with clinical parameters. We used an ELISA to measure MRP14 in BALF of 74 sarcoidosis patients, 54 IPF patients and 19 controls. Mean BALF levels of MRP14 were significantly elevated in IPF ($p < 0.001$) and sarcoidosis ($p < 0.05$) patients compared to controls. MRP14 levels were linearly associated with sarcoidosis disease severity based on chest radiographic stage. Moreover, BALF MRP14 levels were inversely correlated with diffusion capacity and forced vital capacity in sarcoidosis patients. In IPF patients, a correlation with BALF neutrophil percentage was found. In conclusion, BALF MRP14 levels are elevated in IPF and sarcoidosis and are associated with disease severity in sarcoidosis. The results support the need for further studies into the role of MRP14 in the pathogenesis of lung fibrosis.

Introduction

Sarcoidosis and idiopathic pulmonary fibrosis (IPF) represent some of the most frequently occurring interstitial lung diseases (ILD). The aetiology of sarcoidosis and IPF remains unclear and lung biopsy is often required for diagnosis. Sarcoidosis is a multisystemic granulomatous disease that primarily affects the lung and lymphatic system of the body. It occurs most often in young and middle-aged adults and has an estimated mortality between 0.5 and 5%.[1] The cause of sarcoidosis is hypothesized to be an exaggerated cellular immune response to an unidentified antigen.[2] Pulmonary fibrosis occurs in 10-15% of sarcoidosis patients and is thought to be the result of chronic inflammation leading to the formation of scar tissue.[3] IPF is a rapidly progressing lung disease with a median survival of approximately 3 years.[4] The concept that IPF is inflammation driven has been replaced by the theory that epithelial damage causes aberrant wound healing, resulting in the accumulation of fibrosis in the lung.[5] There is currently no effective treatment available and lung transplantation remains the only option. IPF as well as pulmonary fibrosis in sarcoidosis are often characterized by an increased presence of neutrophils in the bronchoalveolar lavage fluid (BALF).[6,7] Many studies focus on the protein content of BALF, hoping to find disease biomarkers that aid in diagnosis, and give insight into disease aetiology.

The migration inhibitory factor related protein (MRP)-14 (also known as calgranulin B and S100A9) belongs to the S100 family of calcium-binding proteins. These proteins are highly expressed by neutrophils, but also by macrophages and on epithelial cells in active inflammatory disease. The S100 proteins are thought to play a role in inflammatory conditions and tumorigenesis.[8] MRP14 was initially thought to occur only as a heterodimer complex with MRP8 but recently MRP14 is more often found to act on its own.[9-12] It is expressed in healthy skin and lung, while MRP8 is undetectable in these tissues.[12] Although the exact function of MRP14 is not known, it may be associated with disease severity in chronic inflammatory diseases, and it was found to stimulate fibroblast proliferation *in vitro*.[11,13,14] MRP14 is expressed in affected tissue of gingivitis, rheumatoid arthritis, tuberculosis and sarcoidosis patients.[12,14,15] In sarcoidosis, MRP14 is expressed in epitheloid cells and giant cells composing the granuloma, whereas MRP8 is expressed only weakly or is even absent.[15] Using 2-D electrophoresis, Bargagli *et al.* recently found MRP14 to be differentially

expressed in the BALF of sarcoidosis and IPF patients.[16] But, it was not possible to quantitatively assess the relationship of MRP14 with patient characteristics.
In this study, we quantified BALF MRP14 levels in sarcoidosis and IPF patients using ELISA, and investigated whether MRP14 levels are associated with clinical parameters, and disease severity. This is the first step towards understanding the role of MRP14 in fibrosing interstitial lung diseases.

Materials and methods

Patients and controls

In this study, 74 sarcoidosis patients (54 male, 20 female) and 54 IPF patients (44 male, 10 female) were included retrospectively (table 1). IPF patients were diagnosed at the Department of Pulmonology of the St. Antonius Hospital Nieuwegein in the Netherlands and included when current ATS/ERS criteria were met.[4] All patients who underwent BAL within three months from diagnosis were included. Eight IPF patients were treated with low dose steroids at the time of diagnosis and BAL, the other IPF patients did not use immunosuppressants.
Sarcoidosis patients were diagnosed in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis.[17] Sarcoidosis patients were classified based on chest radiographic stages according to Scadding.[18] Stage I showed bilateral lymphadenopathy (12 patients), stage II lymphadenopathy with parenchymal abnormalities (11 patients), stage III showed no lymphadenopathy, but parenchymal abnormalities (19 patients), stage IV showed fibrosis (32 patients, 16 non-steroid users and 16 steroid users). We first selected patients that had BALF and a clear classifying chest radiograph at presentation and were not treated with steroids at that time (12/11/12/8 per stage I, II, III and IV, respectively). We subsequently added patients with BAL and classifying chest radiograph during follow-up; both steroid-positive (3 at stage III, and at 9 stage IV) and steroid negative patients (4 at stage III, and 13 at stage IV).
The control group consisted of 19 healthy subjects (9 male, 10 female) who underwent bronchoalveolar lavage.
The medical ethical committee of the St. Antonius Hospital in Nieuwegein approved this study and all subjects gave formal written informed consent.

Table 1. Characteristics of patients and controls

	Subjects (M/F)	Steroid treated (Y/N)	Age	% pred. DLCO	% pred. FVC	% pred. FEV1	% BALF Neutrophils
Controls	19 (9/10)	0/19	22 ± 2	NA	109 ± 11	106 ± 11	1.9 ± 1.8
IPF	54 (44/10)	8/46	66 ± 11	48 ± 16	80 ± 21	85 ± 22	10.1 ± 11.5
Sarcoidosis	74 (54/20)	12/62	43 ± 12	77 ± 14	90 ± 19	77 ± 25	4.2 ± 12.2
Stage I	12 (8/3)	0/12	38 ± 14	86 ± 11	104 ± 9	99 ± 12	1.3 ± 1.1
Stage II	11 (9/2)	0/11	41 ± 10	80 ± 15	96 ± 18	91 ± 22	1.3 ± 0.7
Stage III	19 (15/4)	3/16	42 ± 13	78 ± 14	102 ± 12	93 ± 18	2.0 ± 2.8
Stage IV	32 (25/7)	9/23	46 ± 10	64 ± 17	79 ± 18	60 ± 17	3.6 ± 5.7

Values are given as mean ± SD; NA = not available

Bronchoalveolar lavage

All patients underwent a bronchoalveolar lavage procedure as part of the diagnostic process. The bronchoscopy with bronchoalveolar lavage (BAL) was performed according to international accepted guidelines.[19,20] BAL was performed in the right middle lobe with a total volume of 200 ml saline (4 x 50 ml aliquots), which was returned in two separate fractions. The first fraction returned, after instilling 50 ml saline, was used for microbial culture. The following three aliquots were pooled in fraction II and used for cell analysis and ELISA.

Clinical parameters

Values for forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and diffusion capacity of the lungs for carbon monoxide (DLCO) were collected from all subjects that underwent lung function tests around the time of BAL. The parameters were expressed as a percentage of predicted values. The tests were performed according to international guidelines.[21]

Data on blood cell counts and CRP levels at the time of BAL as well as information on mortality and history of tobacco use was collected retrospectively.

ELISA

MRP14 ELISA (BMA biomedicals, Augst, Switzerland) was performed in accordance with manufacturers instructions. The manufacturer has developed this ELISA in such a way that it minimizes cross reactivity with the MRP8/14 heterodimer. The detection limit of the assay was 0.31 ng/ml. Samples that did not reach this limit were set at 50% of the detection limit. Samples equal to or lower than the negative control were set at zero.

Statistics

SPSS 15 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism v. 3 (Graphpad software INC., San Diego, CA, USA) were used for statistical analysis. ANOVA or student's t-test was used to test differences in BALF MRP14 levels between patient groups. Correlations with patients' characteristics were determined using Spearman's rho test. Linear regression was used to test for an association with pulmonary radiographic stage in sarcoidosis patients. A p-value < 0.05 was considered significant.

Results

MRP14 levels in patients and controls

Control and patient characteristics are shown in Table 1. Mean BALF MRP14 levels were significantly elevated in IPF patients ($p < 0.001$) and sarcoidosis patients ($p < 0.05$) compared to controls (Figure 1A). In addition, mean BALF MRP14 levels were higher in IPF patients than in sarcoidosis patients ($p < 0.01$). When the sarcoidosis patients were subdivided according to chest radographic stage, we found that the mean BALF MRP14 level was significantly elevated in stage IV sarcoidosis compared to controls ($p < 0.005$). When only sarcoidosis patients at presentation were included, the difference was also significant ($p < 0.01$). Interestingly, there appeared to be a linear association between BALF MRP14 levels and chest radiographic stage I, II, III, and IV ($r = 0.33$, $p < 0.005$, figure 1B). When using a t-test to compare stage I and stage IV sarcoidosis, the difference was also significant ($p < 0.05$). There was no difference in mean BAL MRP14 level between patients that were treated with oral steroids and those that were not.

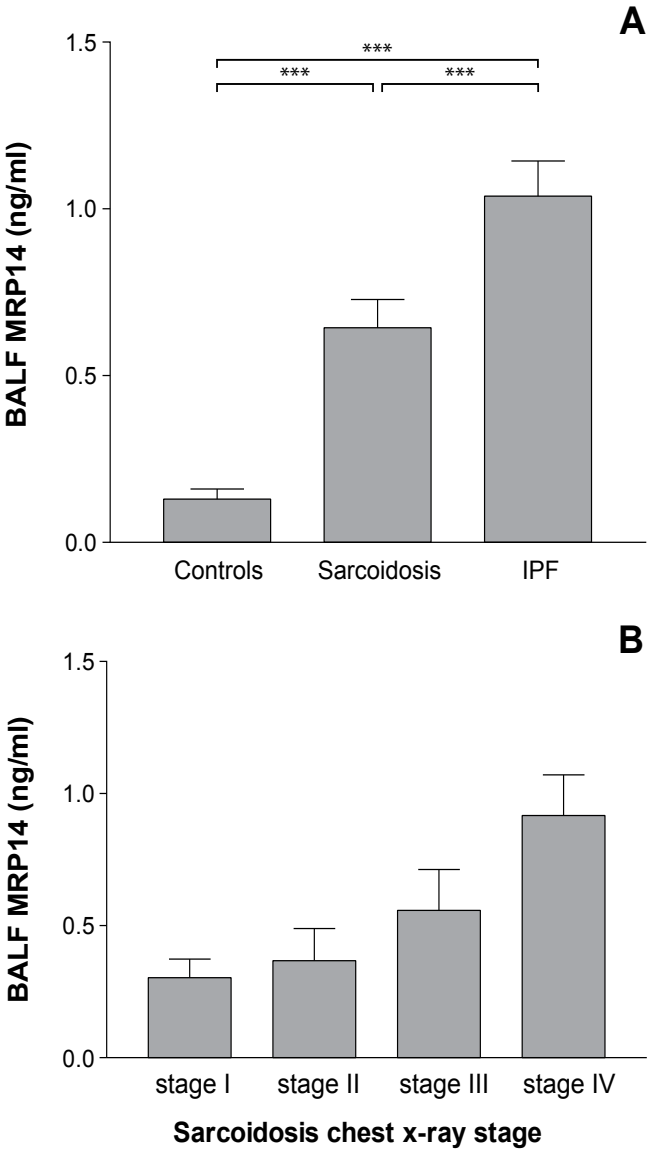

Figure 1. MRP14 levels in the BAL fluid of controls and patients. Mean and SEM are shown. A) Levels in patients with IPF and sarcoidosis compared to controls. B) Levels in patients with sarcoidosis per pulmonary stage. In comparison with controls, mean BALF MRP14 levels were significantly higher in patients with sarcoidosis stage IV ($p < 0.01$). BALF MRP14 levels were linearly associated with sarcoidosis pulmonary radiographic stage ($r = 0.33$, $p < 0.005$).
*$p < 0.05$, **$p < 0.01$ ***$p < 0.001$.

Correlation with clinical parameters

Higher BALF MRP14 levels were associated with lower percent predicted DLCO ($r = -0.49$, $p < 0.001$), lower percent predicted FVC ($r = -0.44$, $p < 0.005$), and lower percent predicted FEV1 ($r = -0.39$, $p < 0.01$) in sarcoidosis patients (Figure 2). However, lung function parameters were not correlated with BALF MRP14 levels in IPF patients. Interestingly, there was an association between BALF MRP14 levels and the percentage of neutrophils in BALF of IPF patients ($r = 0.33$, $p < 0.05$, Figure 3), but this association was not found in sarcoidosis patients.

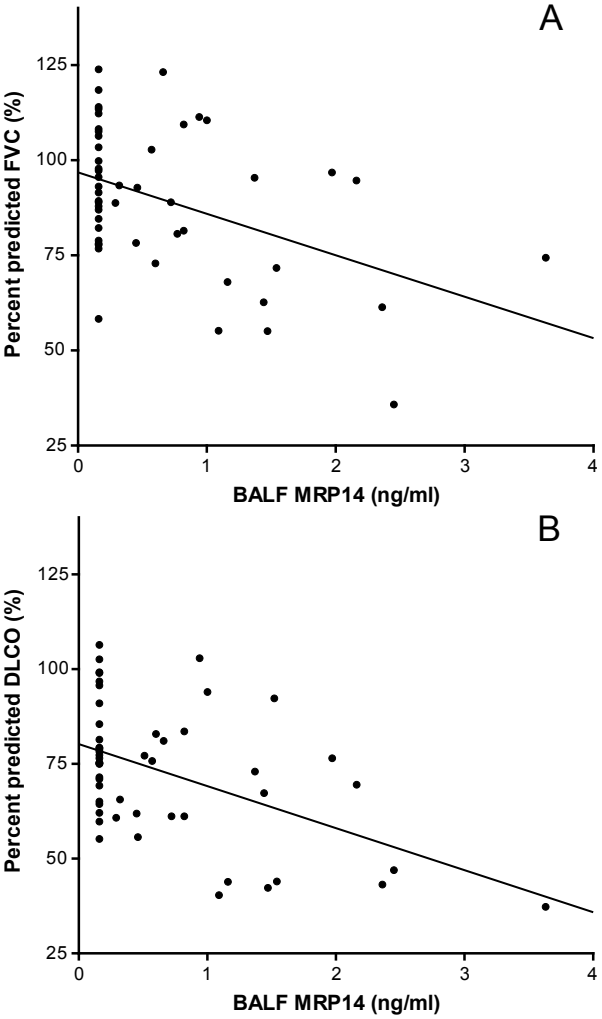

Figure 2. Correlation between lung function parameters and BAL MRP14 levels in sarcoidosis patients. A) Percent of predicted FVC, $r = -0.44$, $p < 0.005$. B) Percent of predicted DLCO, $r = 0.49$, $p < 0.001$.

BALF neutrophil percentage did show a weak correlation with sarcoidosis chest radiographic stage ($r = 0.21$, $p < 0.05$). We found no correlation between BALF MRP14 and macrophages or any other BALF cell types. Analysis of follow-up data from IPF patients did not reveal an association between BALF MRP14 levels and survival time. Smoking habits or gender did not affect BALF MRP14 levels in any patient group or controls. In addition, no correlation was found between BALF MRP14 and CRP levels in blood.

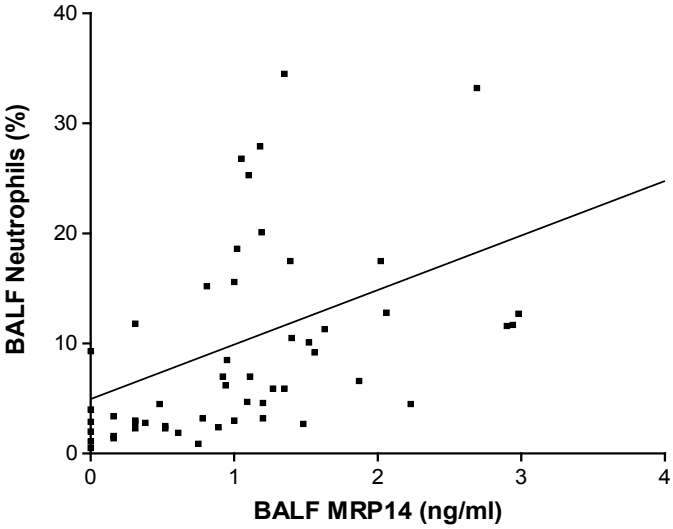

Figure 3. Correlation between BALF MRP14 levels and neutrophil percentage in IPF patients. $r = 0.33$, $p < 0.05$.

Discussion

The aim of the present study was to quantify BALF MRP14 levels in sarcoidosis and IPF, and investigate whether they are associated with clinical parameters and disease severity. We found that the mean level of BALF MRP14 was significantly elevated in both diseases compared to controls, with mean levels significantly higher in IPF patients than in sarcoidosis patients. In sarcoidosis, the highest BALF MRP14 levels were found in the fibrotic stage IV sarcoidosis patients with a linear association of increasing levels across the radiographic stages. High BALF MRP14 levels were also associated with poor diffusion capacity and restrictive lung function measures. So, our results demonstrate that BALF MRP14 levels are associated with pulmonary disease severity in sarcoidosis. We found no association between MRP14 levels and lung function in IPF. However, the observation that BALF MRP14 levels in IPF are higher than in sarcoidosis suggests they reflect the difference in severity between these diseases.

This is the first study to report BALF MRP14 levels measured by ELISA. Previously, Bargagli *et al.* showed that BALF MRP14 levels in IPF were higher than in controls, using 2d-gelelectrophoresis.[16] They found no association with sarcoidosis stage or lung function parameters, but this is most likely due to the relatively small number of patients included.

Our larger group of patients enabled us to investigate the relationship between clinical parameters and MRP14. To verify that the elevated levels of MRP14 measured in BALF is not a reflection of systemic inflammation; we established that there was no association with CRP levels. The neutrophilia in BALF, which is often found in IPF and pulmonary stage IV in sarcoidosis, could be responsible for the elevated MRP14 levels seen in patients. However, BALF MRP14 levels were much more strongly associated with pulmonary stage in sarcoidosis than the neutrophil percentage. This suggests MRP14 is a more specific biomarker for pulmonary disease severity in sarcoidosis than the amount of neutrophils in BALF. In addition, we did observe a correlation between MRP14 and BALF neutrophils in IPF patients, but it was small, and no such correlation was found in sarcoidosis patients. The lack of correlation with neutrophils in sarcoidosis indicates that high BALF MRP14 levels do not simply reflect the presence of neutrophils in the lung, although all the MRP proteins together make up approximately 50% of the neutrophils cytosolic protein content.[22] Previous reports on a possible chemoattractant role for MRP14 are ambiguous.

A study by Ryckman *et al.*[10] reported MRP8, MRP14 and the heterocomplex MRP-8/14 caused neutrophil chemotaxis *in vitro* and *in vivo*, and the same group also reported that antibodies against MRP14 blocked neutrophil recruitment.[23] However, other studies reported that MRP14 was not a chemoattractant for neutrophils and even repelled neutrophils.[24,25] Our data do not support a possible chemoattractant role for MRP14, but do not rule out the possibility that MRP14 is a chemoattractant for neutrophils under specific conditions, for instance in some IPF patients. An mRNA expression study in rabbits showed that after neutrophils migrate from the blood to inflammatory sites the mRNA expression of MRP14 rapidly increases.[26] In addition, neutrophilic MRP14 is phosphorylated and translocated to the membrane during human neutrophil activation.[27] This suggests that MRP14 levels during inflammatory reactions are not dependent on the number of neutrophils present but rather on their activity. Activated neutrophils can cause lung injury, epithelial cell apoptosis and basement membrane loss.[28,29] Neutrophils are also thought to mediate the transition from acute to chronic inflammation that may precede fibrosis.[30] Both neutrophils and macrophages have been reported to have an altered phenotype in the lungs of sarcoidosis patients.[31,32] It is possible that MRP14 is a marker for an activated subset of leukocytes. Further research is needed to reveal whether MRP14 expression is upregulated in neutrophils and alveolar macrophages in interstitial lung diseases.

It is intriguing to speculate about the exact role of MRP14. It may influence the functioning of leukocytes in several ways. For instance, a study by Newton and Hogg showed that MRP14 could be involved in the attachment of neutrophils to the endothelium, and could thus facilitate their migration.[24] MRP14 could also have a role in inhibiting the coagulation cascade during inflammatory disease.[33] Finally, MRP14 may directly influence the fibrotic process because its homodimer has been shown to induce proliferation of rat kidney fibroblasts *in vitro*.[11] All of these processes could be involved in the pathogenesis of fibrotic pulmonary sarcoidosis and IPF.
Further research is needed to identify why MRP14 levels are elevated in the lungs of fibrosis patients and to investigate whether MRP14 plays a role in disease aetiology. It would also be interesting to investigate whether the other S100 proteins, such as MRP8, the MRP8/14 heterodimer, and S100A12, play a similar role in ILD patients. These proteins are closely related although they do seem to have individual roles and can

have different expression patterns.[15,34,35] They are thought to be pro-inflammatory mediators and have been associated with several neoplastic disorders.[8] MRP8/14 is slightly elevated in the plasma of pulmonary sarcoidosis compared to controls but is was lower than in patients with mild tuberculosis.[36,37] The MRP-8/14 complex is involved in endothelial integrity loss and it stimulates IL-8 production by airway epithelial cells.[38,39] Therefore, it could also be a part of the remodelling process in IPF.[39] S100A12 has been found to be elevated in the BALF of ARDS patients.[40]

In conclusion, the S100 proteins are promising biomarkers in inflammation and cancer, and possibly, in lung diseases. The present study further explored the role of MRP14 in two predominant interstitial lung diseases. Our results confirm previous findings that BALF MRP14 levels are elevated in IPF. Furthermore, we show that BALF MRP14 levels are elevated in sarcoidosis, with highest levels in the fibrotic phenotype, and that they are associated with pulmonary disease severity. These results support the need for further study into the role of MRP14 in the aetiology of fibrosing interstitial lung diseases, and the application of this protein as a biomarker.

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CHAPTER 4

IL1RN GENETIC VARIATIONS AND RISK OF IPF: A META-ANALYSIS AND mRNA EXPRESSION STUDY

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Abstract

Background

Idiopathic pulmonary fibrosis (IPF) is a rare and devastating lung disease of unknown aetiology. Genetic variations in the *IL1RN* gene, encoding the interleukin-1 receptor antagonist (IL-1Ra), have been associated with IPF susceptibility. Several studies investigated the variable number tandem repeat (VNTR) or single nucleotide polymorphisms (SNPs) rs408392, rs419598 and rs2637988, with variable results. The aim of this study was to elucidate the influence of polymorphisms in *IL1RN* on IPF susceptibility and mRNA expression.

Methods

We performed a meta-analysis of the five case-control studies that investigated an *IL1RN* polymorphism in IPF in a Caucasian population. In addition, we investigated whether *IL1RN* mRNA expression was influenced by *IL1RN* polymorphisms.

Results

The VNTR, rs408392 and rs419598 were in tight linkage disequilibrium, with $D' > 0.99$. Furthermore, rs2637988 was in linkage disequilibrium with the VNTR ($D' = 0.90$). A haploblock of VNTR*2 and the minor alleles of rs408392and rs419598 was constructed. Meta-analysis revealed that this VNTR*2 haploblock is associated with IPF susceptibility both with an allelic model (odds ratio = 1.42, $p = 0.002$) and a carriership model (odds ratio = 1.60, $p = 0.002$). *IL1RN* mRNA expression was significantly influenced by rs2637988, with lower levels found in carriers of the (minor) GG genotype ($p < 0.001$)

Conclusions

From this meta-analysis we conclude that the VNTR*2 haploblock is associated with susceptibility to IPF. In addition, polymorphisms in *IL1RN* influence *IL1RN* mRNA expression, suggesting that lower levels of IL-1Ra predispose to developing IPF. Together these findings demonstrate that the cytokine IL-1Ra plays a role in IPF pathogenesis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a rapidly progressing lung disease with unknown cause and a median survival of only 2.5 to 3.5 years.[1-3] The disease is characterized by fibroblast growth, extracellular matrix deposition and remodelling of alveolar tissue, thereby disabling gas exchange across the alveolar epithelium.[4] IPF is a rare disease with a prevalence of 14 per 100 000 persons but the incidence continues to rise and it is now an important cause of respiratory mortality.[5, 6] Familial and ethnic clustering support the theory that genetic variations influence IPF disease susceptibility, and identification of the genes involved can increase understanding of this complex disease.[7] Futhermore, because no effective treatment for IPF is available at present, genetic analysis may also reveal a novel target for therapy.

Interleukin (IL)-1 is a proinflammatory and profibrotic cytokine that exists in two forms, IL-1α and IL-1β. Interleukin-1 receptor antagonist (IL-1Ra) is an inhibitor IL-1 by competitive binding to the IL-1 receptor. The *IL1RN* gene, coding for IL-1Ra protein, has been implicated in IPF susceptibility.[8] Genetic variations in *IL1RN*, *IL1A* and *IL1B* have been associated with ulcerative colitis, gastric cancer and rheumatoid arthritis.[9-11] Variations in these genes can modulate the effectiveness of IL-1 signalling and thereby predispose to disease.

Several polymorphisms in *IL1RN* have been investigated in IPF case-control studies. Whyte *et al.*, showed a significant association between IPF susceptibility and the single nucleotide polymorphism (SNP) rs419598 (also known as +2018) in two populations.[8] Others investigated a variable number tandem repeat (VNTR), rs408392 or rs2637988 and found that the genotype distributions of the VNTR and rs408392 were not significantly different between patients and controls.[12-14] However, in the largest of these studies, a trend was observed with rs408392, and rs2637988 was found to be significantly associated with IPF susceptibility.[14] In a rare disease like IPF, validation of genetic associations is hampered by the small sample size of available cohorts. To establish whether *IL1RN* is associated with the risk of IPF, we performed a meta-analysis of the five case-control studies. Although these five studies investigated different polymorphisms, they could be combined in a meta-analysis because the polymorphisms are in tight linkage disequilibrium.

At present it is unclear how polymorphisms in *IL1RN* predispose to disease. To compare the functional effects before disease onset, we determined genotype-dependent mRNA expression for the VNTR and rs2637988 in healthy controls.

Material and Methods

Study selection

For this meta-analysis we included all five case-control studies that investigated the association between polymorphisms in *IL1RN* and IPF in Caucasian populations (table 1). In the English population, rs419598 (chromosome position 113887207) was determined in 88 patients and 88 controls.[8] The same SNP, rs419598, was determined in the Italian population in 61 patients and 103 controls.[8] In the Czech population, the VNTR (chromosome position 113888106) was determined in 54 patients and 199 controls.[12] The VNTR was also determined in the Australian population in 22 patients and 140 controls, but only allele frequencies were given and no individual genotypes were available.[13] In the Dutch population, rs408392 (chromosome position 113887458) and rs2637988 (chromosome position 113876779) were determined in 77 patients and 349 controls.[14] In total, allele frequencies were available for 302 patients with IPF and 879 controls. Individual genotypes were available for 280 patients and 739 controls.

The VNTR (rs2234663) and rs408392 are located in intron 2 (identical to intron 3 in the extended gene encoding the intracellular isoform of IL-1Ra[15]); rs419598 is located at position +2018 in exon 2. rs2637988, is located upstream of *IL1RN*, near several transcription factor binding sites.

Table 1. Characteristics of the association studies on *IL1RN* and IPF

Study	Origin of cohort	Patients (n)	Controls (n)	Polymorphism	RAF patients	RAF controls	P-value (allelic association)
Whyte *et al.* 2000 a	Britain	88	88	rs419598	0.28	0.17	0.02
Whyte *et al.* 2000 b	Italy	61	103	rs419598	0.33	0.20	0.01
Hutyrova *et al.* 2002	Czech Rep.	54	199	VNTR	0.31	0.30	0.90
Riha *et al.* 2004	Australia	22	140	VNTR	0.23	0.25	0.77
Barlo *et al.* 2011	The Netherlands	77	349	rs408392 rs2637988	0.32 0.47	0.26 0.38	0.09 0.04

RAF = risk allele frequency; NA = not available

The studies were identified using a PUBMED search using the terms "polymorphism", "Genetic association", "pulmonary fibrosis" and "fibrosing alveolitis". Only studies on idiopathic pulmonary fibrosis (also called cryptogenic fibrosing alveolitis) were included. One study was not a case-control study but only reported associations with disease phenotype and was excluded.[16]

The populations included in this study were tested for allelic association with disease (table 1) using Pearson's goodness-of-fit Chi-square (as implemented online at http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). This could not be done for the Australian population because individual genotypes were not available, and therefore, we included the p-value as reported in their study in table 1.

Measurement of linkage disequilibrium

We investigated the linkage disequilibrium between the VNTR, rs419598, rs408392 and rs2637988. Because there is no HapMap data available for the VNTR we compared the genotypes for the VNTR, rs408392 and rs2637988 in the Dutch cohort of 349 healthy controls (table 1). Genotypes for rs408392 and rs2637988 were retrieved from the study by Barlo *et al.*[14] The VNTR polymorphism was determined by polymerase chain reaction (PCR). Primers for the VNTR region were sense 5'-ACTCATGGCCTTGTTCATT; antisense 5'- AAAACTAAAATCCCGAGGTC (Sigma-Aldrich, St. Louis, MO, USA). PCR products were run on a 1.5% agarose gel. A 25-basepair and a 200-basepair ladder were used to discern the number of 86-basepair repeats. The VNTR includes a variable number of repeats. The VNTR allele 2 (VNTR*2) is the shortest with only two repeats. The other alleles correspond to between three and six repeats.

Because rs419598 was not determined in the Dutch population, genotypes for the European HapMap population were retrieved for rs408392 and rs419598. We determined the linkage disequilibrium using the computer program Haploview 4.1 (Broad Institute of MIT and Harvard, USA).

Meta-analysis

For the meta-analysis we combined the case-control data from the VNTR, rs408392, and rs419598 (+2018) in *IL1RN* (table 2) from five populations. The case and control populations were tested for violation of Hardy-Weinberg equilibrium. The meta-analysis was performed using R software (version 2.9.2, Catmap package, version 1.6, www.r-project.org). Both a random effects (DerSimonian and Laird method) and

a fixed effects analysis (Mantel-Haenszel method) of the case-control data were performed. To test for heterogeneity, the Cochran's Q test and I^2 was used. In addition, a leave-one-out sensitivity analysis was performed. When all five populations were combined we could only use an allele-based model because individual genotypes were not available for the Australian population. To investigate whether the risk allele has a dominant or recessive influence on disease development in the population, both models were tested. Due to the absence of individual genotypes, the Australian population was removed and the remaining number of individuals carrying one or more risk alleles was used in the analysis.

Table 2. Data used in the meta-analysis

		Allele frequency model		Dominant model	
		Risk alleles/ Non risk alleles (n)		Risk carriers/ Non risk carriers (n)	
Study	Origin of cohort	Patients	Controls	Patients	Controls
Whyte *et al.* 2000 a	Britain	49/127	30/146	39/49	28/60
Whyte *et al.* 2000 b	Italy	40/82	42/164	35/26	37/66
Hutyrova *et al.* 2002	Czech Rep.	33/75	119/279	27/27	96/103
Riha *et al.* 2004	Australia	10/34	70/210	NA	NA
Barlo *et al.* 2011	The Netherlands	50/104	180/518	43/34	155/194

NA = not available; alleles refers to the number of alleles in the population; carriers refers to the number of individuals carrying the risk allele.

RNA expression

We used thawed peripheral blood mononuclear cells (PBMC) from 38 healthy controls (23 males and 15 females, mean age 22.5 years). The expression of *IL1RN* mRNA was analysed by quantitative RT-PCR amplification as described previously.[17] Briefly, total RNA was isolated using an Rneasy microkit (Qiagen, Venlo, the Netherlands) and cDNA was made using the I-script cDNA synthesis kit (Biorad, Veenendaal, the Netherlands). Primers used for expression analysis of *IL1RN* were, forward 5'-GAAGATGTGCCTGTCCTGTGTC and reverse 5'-CGCTTGTCCTGCTTTCTGTTC (Sigma-Aldrich, St. Louis, MO, USA). The copy numbers were normalized by the housekeeping gene β-actin (*ACTB*) (forward 5'-AGCCTCGCCTTTGCCGA reverse 5'-CTGGTGCCTGGGGCG). SPSS and GraphPad Prism software were used to test whether RNA expression differed per genotype. A difference with a $p < 0.05$ was considered statistically significant.

Results

Measurement of linkage disequilibrium

Analysis of European Hapmap population genotypes showed that the two biallelic SNPs rs408392 and rs419598 are in complete linkage disequilibrium (D' = 1). There was tight linkage disequilibrium between the VNTR and rs408392 in our Dutch control population (D' = 0.99, figure 1). We observed in the Dutch cohort, that in all individuals, the longer VNTR alleles with three or more repeats corresponded completely to the major allele of rs408392 and of rs419598, while the VNTR*2 allele corresponded to the minor alleles of these SNPs. Therefore, the VNTR, rs408392 and rs419598 are part of a haploblock and for analysis we combined the linked alleles: We named the combination of risk alleles "VNTR*2 haploblock" and this contained the VNTR*2 and the minor alleles of rs419598 and rs408392.

Linkage disequilibrium between rs2637988 and the VNTR (D' = 0.90) and between rs2637988 and rs408392 (D' = 0.94) was high (figure 1) but the difference in frequencies between the coupled alleles was substantial, and therefore rs2637988 was not included in the meta-analysis.

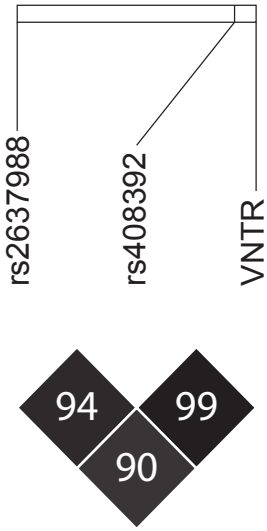

Figure 1. Linkage plot for *IL1RN* polymorphisms showing D'.

Meta-analysis

The data used for the meta-analysis are shown in table 2. Cases and controls were individuals of European ancestry from Britain, Italy, Czech Republic, Australia and the Netherlands. The genotypes from all populations were in Hardy-Weinberg equilibrium. Pooling of the populations resulted in a risk allele frequency of the VNTR*2 haploblock of 0.30 in patients (n = 302) and 0.25 in controls (n = 879).

A significant association between the VNTR*2 haploblock and IPF was observed using an allele frequency model with a fixed effects meta-analysis. The pooled odds ratio (OR) was 1.42 (95% confidence interval (CI) 1.14 - 1.76, p = 0.002, $\chi^2 = 9.93$) (figure 2). Sensitivity analysis showed that the association remained significant after sequential removal of each one of the studies. Cochran's Q test indicated there was

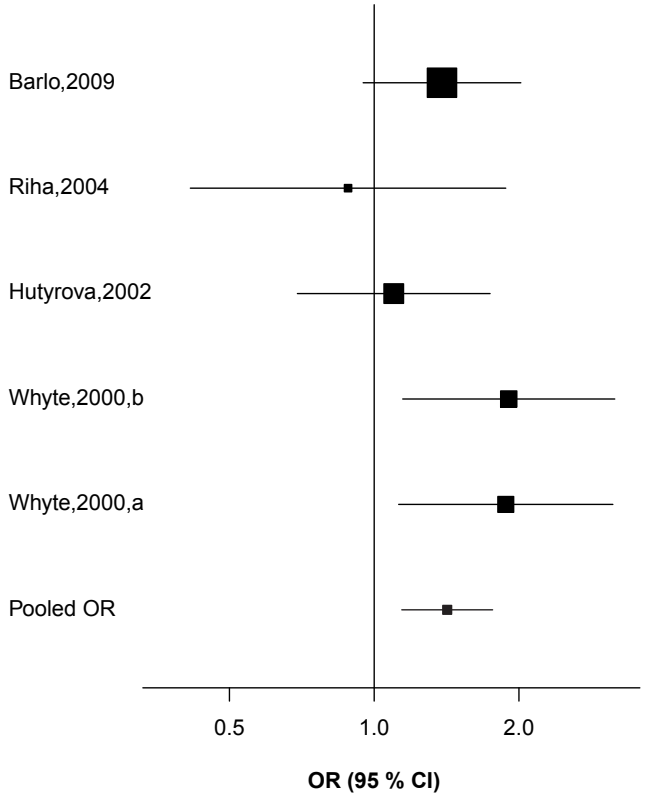

Figure 2. Fixed-effects meta-analysis with an allele frequency model of the effect of *IL1RN* on IPF susceptibility. Individual study odds ratios (ORs) are shown as well as the pooled OR for the VNTR*2 haploblock (VNTR, rs408392, rs419598). The pooled OR was 1.42 (CI 1.14 - 1.76, p = 0.002).

no significant variability/heterogeneity among the studies in this meta-analysis (χ^2 = 5,2 p = 0.27). The I^2 was 23%, indicating that inconsistency across studies was low. Although no heterogeneity was seen across studies, random effect models were run and ORs and CIs were almost identical to those seen in the fixed effects analysis. Under the dominant genetic model, comparing non-carriers to carriers of the VNTR*2 haploblock, the pooled OR was 1.60 (95% CI 1.20 - 2.14, p = 0.002, χ^2 = 10.01) under a fixed-effects assumption. The relative risk to develop IPF for an individual carrying the VNTR*2 haploblock was 1.29 (95% CI 1.05 - 1.57 p = 0.01) based on the four populations in this analysis. The recessive genetic model compares carriers of the VNTR*1 haploblock with homozygous carriers of VNTR*2 haploblock, and did not result in a significant association.

mRNA expression analysis

We analysed *IL1RN* mRNA expression in relation to *IL1RN* genotypes. A dosage effect tendency of the VNTR polymorphism on *IL1RN* mRNA expression is shown in figure 3, but this did not reach significance (p = 0.30). Rs2637988 A/G was significantly associated with a difference in *IL1RN* mRNA expression levels in healthy PBMC (figure 3). Significantly lower mRNA expression levels were found in control subjects with the minor GG genotype compared to controls with the AG genotype ($p < 0.05$) or the AA genotype ($p < 0.001$).

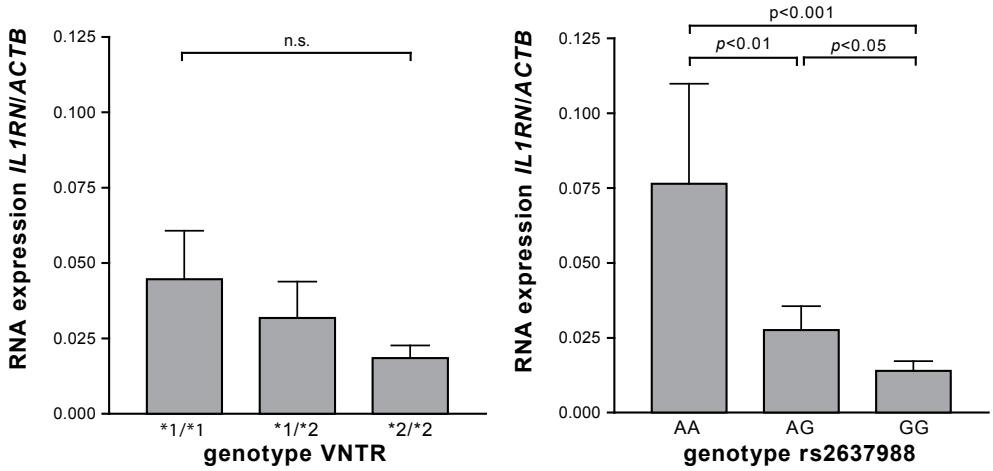

Figure 3. The effect of *IL1RN* genotype on mRNA expression. *IL1RN* mRNA expression in PBMC from healthy controls normalized to actin expression. There was no significant effect of *IL1RN* VNTR genotype on mRNA expression. A significant association between rs2637988 and mRNA expression was found. Error bars represent standard error of the mean. VNTR*1 corresponds to four repeats and VNTR*2 corresponds to two repeats of the VNTR polymorphism.

Discussion

This meta-analysis showed that variations in *IL1RN* are significantly associated with an increased risk of developing IPF. In our linkage analysis we found that there was tight linkage disequilibrium between the VNTR, rs408392 and rs419598, allowing the polymorphisms to be combined into a VNTR*2 haploblock for the meta-analysis of five IPF populations. The pooled odds ratio of the allele frequency model showed that the VNTR*2 haploblock was significantly associated with IPF. However, in the Italian and Dutch populations, the largest association was reported for carriership of the minor allele, corresponding to the VNTR*2 haploblock.[8,14] With a dominant genetic model, the meta-analysis showed an even stronger association with IPF than with the allelic model (OR = 1.60 vs. OR = 1.42). This confirms that carriership of the risk allele was most associated with IPF. Individuals that are homo- or heterozygous for the risk allele are at an increased risk of developing IPF. Calculation of the relative risk showed that carriership of the risk allele confers a 29% increase in the risk of developing IPF. Only individuals without a risk allele, the majority of the population, are protected from developing IPF.

The effects of IL-1 are mediated by two protein isoforms, IL-1α and IL-1β, who act through binding to the IL-1 receptor. Their effects are counterbalanced by the IL-1Ra protein that binds to the receptor but does not transduce any signal. The IL-1 effector response is determined by the balance between these proteins.[18-20] The ratio between IL1-Ra and IL-1 was found to be lower in serum from IPF patients and in macrophages from IPF lungs.[14,21] Previous studies have found that the IL-1Ra/IL-1 ratio is influenced by genetic variations in *IL1RN*.[14,22] Higher IL-1 protein levels were found in gastric mucosa from *Helicobacter pylori*-infected individuals that carried VNTR*2,[23] while lower IL-Ra protein levels were found in biopsies from ulcerative colitis patients with VNTR*2.[22] Together, this indicates that carriers of VNTR*2 have low levels of IL-1Ra but higher IL-1 levels. The VNTR is a variation in an 86-basepair repeat in an intron of the *IL1RN* gene. The change in mRNA length caused by the VNTR could have an effect on its processing and stability. Analysis of *IL1RN* mRNA expression in healthy controls showed that there was a suggestive effect of the VNTR polymorphism (and thus rs408392 and rs419598), but this did not reach significance. The dosage effect observed for the VNTR might be caused by linkage

to rs2637988, because rs2637988 is in nearly complete linkage disequilibrium with the VNTR. We found that rs2637988 GG was significantly associated with lower *IL1RN* mRNA expression. In addition, in the study by Barlo *et al.* the rs2637988 G allele was significantly associated with IPF susceptibility (OR = 1.95) whereas rs408392 showed only a trend towards significance.[14] Together, this indicates that the *IL1RN* rs2637988 polymorphism might have a greater effect on IPF disease development than the VNTR*2 haploblock. The study by Barlo *et al.* also showed that rs2637988 G was associated with a lower ratio of IL-1Ra to IL-1β.[14] Enhanced expression of IL-1β has been found in alveolar macrophages and pneumocytes in patients with acute pulmonary fibrotic diseases.[24] In animal models of IPF, IL-1β levels are elevated in mice with bleomycin-induced lung fibrosis.[25,26] Blocking the IL-1 receptor with IL-1Ra reduced bleomycin-induced inflammation and prevented fibrosis in these mice.[25,27] Transient overexpression of IL-1β caused acute lung injury resulting in pulmonary fibrosis in rats.[28] Thus, relatively low levels of IL-1Ra would fail to prevent the pro-fibrotic functions of IL-1 and this could play an important role in IPF disease aetiology. In light of these findings, treatment of IPF patients with an IL-1 antagonist, like Anakinra, should be considered. So far, treatment with IL-1 blocking agents has been safe and effective in rheumatoid arthritis, although the increased risk of respiratory infection may mean caution is called for.[29] In addition, it is possible that genetic variations in the *IL1A* and *IL1B* genes also influence the balance between IL-1 and IL-1Ra and could therefore play a role in IPF susceptibility. Future studies are needed to evaluate the role of polymorphisms in these genes and their interaction with each other.

In conclusion, our meta-analysis shows that polymorphisms associated with the *IL1RN* VNTR increase susceptibility to IPF. The *IL1RN* risk allele is associated with lower levels of IL-1Ra. After the recent association of the *MUC5B* gene with IPF,[30] this is the second largest IPF association study. The role of IL-1Ra in preventing fibrosis supports the notion that insufficiently expressed IL-1Ra can permit fibrogenesis to occur, thereby predisposing to IPF.

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CHAPTER 5

SERUM AND BALF YKL-40 LEVELS ARE PREDICTORS FOR SURVIVAL IN IDIOPATHIC PULMONARY FIBROSIS

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Abstract

Background

The chitinase-like protein YKL-40 is a serum biomarker in diseases with fibrosis, inflammation and tissue remodelling. Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease that is hallmarked by these processes.
The aim of this study was to investigate the potential of YKL-40 as a prognostic biomarker for survival in IPF patients.

Methods

Serum and bronchoalveolar lavage fluid (BALF) levels of YKL-40 at the time of diagnosis and a promoter polymorphism in *CHI3L1*, the gene encoding YKL-40, were determined in 85 IPF patients and 126 controls. The relationship between YKL-40 levels and clinical parameters was evaluated. Kaplan-Meier and Cox regression analyses were used to examine the association between YKL-40 levels and survival.

Results

Serum and BALF YKL-40 levels were significantly higher in patients than in healthy controls ($p < 0.001$). The –329 A/G polymorphism had a significant influence on BALF YKL-40 levels and the influence on serum YKL-40 levels showed a trend towards significance in IPF patients. IPF patients with high (> 79 ng/ml) serum or high BALF YKL-40 (> 17 ng/ml) levels had significantly shorter survival than those with low YKL-40 levels in serum or BALF. In patients with both low serum and low BALF YKL-40 levels no IPF-related mortality was observed. Cox regression modelling showed that there were no confounding factors.

Conclusions

The –329 polymorphism was associated with serum and BALF YKL-40 levels in IPF patients. High serum and BALF YKL-40 levels are associated with poor survival in IPF patients and could be useful prognostic markers for survival in IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a rapidly progressing disease that is characterized by remodelling of the lung parenchyma. It's aetiology remains unclear but tissue remodelling in IPF is thought to be caused by pneumocyte dysfunction, fibroblast proliferation, and chronic inflammation.[1-3] While the median survival time is only 2.5 to 3.5 years,[4] survival can vary between a few months and > 10 years. Although clinical trials show that some improvement in lung function deterioration can be achieved, there is currently no therapy available that has a proven long-term effect on survival.[5,6] Lung transplantation remains the only option for those who qualify, but mortality on the waiting list is high. To optimize the timing of referral for lung transplantation, predictors of survival are needed.

YKL-40 is already a very promising biomarker for survival in cancer.[7] It is a member of the highly conserved family of chitinases and chitinase-like proteins. Many of these proteins are thought to play a role in inflammatory conditions and asthma.[8] YKL-40 binds chitin but lacks actual chitinase activity. Its exact function remains unclear, but it has been shown to stimulate fibroblast growth.[9] In addition, YKL-40 is elevated in inflammatory conditions and could be involved in tissue remodeling.[10-15]

YKL-40 was recently shown to be elevated in the serum and lungs of patients with IPF and immunohistochemistry showed it to be expressed by alveolar epithelial cells and alveolar macrophages near fibrotic lesions in these patients.[16] Elevated YKL-40 levels are also associated with fibrotic lesions in several other diseases such as liver cirrhosis, Crohn's disease and systemic sclerosis.[17-19] In addition, serum YKL-40 levels are inversely correlated with lung function in asthma, pulmonary sarcoidosis and IPF patients.[16,20,21] Genetic variations in *CHI3L1*, the gene encoding YKL-40, have been associated with asthma susceptibility and the extend of liver fibrosis.[22,23] In healthy individuals serum YKL-40 levels are substantially influenced by polymorphisms in this gene.[21]

In our study, we measured pulmonary and circulating YKL-40 levels in a cohort of IPF patients and assessed whether YKL-40 concentrations were genotype dependent and could be used as a prognostic biomarker for survival in IPF.

Methods

Subjects

The medical ethical committee of the St. Antonius Hospital in Nieuwegein approved this study and all subjects gave formal written informed consent. Patients who visited the Centre for Interstitial Lung Diseases at the St. Antonius Hospital, the Netherlands between November 1998 and 2009 were included (Table 1). Diagnoses made before 2002 were reviewed by a clinician and patients were only included when the diagnosis met the criteria stated by the ATS/ERS in 2002.[4] Other causes of UIP (drugs, collagen vascular diseases) were ruled out and patients with proven familial disease were excluded. In 54 patients (64%) the diagnosis was confirmed by open lung biopsy and 22 patients were treated with low-dose oral steroids. At the time of diagnosis, patients were asked to donate blood for DNA extraction and for storage of serum samples. From 83 IPF patients serum samples were available that were obtained within 3 months after diagnosis. Bronchoalveolar lavage fluid (BALF) was available from 60 IPF patients. Bronchoalveolar lavage was performed in accordance with previously described methods,[24] and all patients underwent a lavage for diagnostic purposes. All samples were stored at –80°C until analysis.

Our control group comprised 83 healthy employees of the St. Antonius Hospital and an additional independent group of 43 healthy volunteers who underwent BAL.

Disease parameters

At presentation, IPF patients underwent lung function tests, providing values for forced expiratory volume in 1 second (FEV_1), vital capacity (VC), FEV_1/VC ratio, diffusion capacity of the lungs for carbon monoxide (D_{LCO}) and lung transfer coefficient (K_{CO}). Lung function parameters were available as absolute values and as percentage of predicted values.

Patients were asked about their smoking habits and smoking history and this was converted into a pack-year value. Haematological differentiation was performed using a cell counter (Coulter LH 750 Analyser, Beckman Coulter, Fullerton, CA, USA).

Length of follow-up for survival was up to 4 years and was based on hospital records. Patients that were still alive, that were transplanted or died from a cause unrelated to IPF were censored in the survival analysis.

YKL-40 protein detection

YKL-40 levels were determined by YKL-40 enzyme immunoassay (Quidel Corporation, San Diego, CA, USA), which was performed in accordance with the manufacturer's instructions. Twenty microliter of serum or BALF was used; either undiluted or diluted 1:5 when exceeding the standard curve range. The lower limit of quantitation of this ELISA is 15.6 ng/ml and the lower limit of detection is 5.4 ng/ml.

Genotyping

The –329 G > A promoter polymorphism, corresponding to rs10399931 was analysed using sequence-specific primers as previously described.[21] This polymorphism was shown to explain 23% of variation in serum YKL-40 levels in healthy controls.[21] Recent studies showed that a promoter polymorphism (rs4950928) at position -131 similarly influenced the serum level of YKL-40 in controls.[22,23] To investigate the linkage between the two sites we retrieved the genotypes from the Centre d'Etude du Polymorphisme Humain (CEPH) population from the dbSNP database (at http://www.ncbi.nlm.nih.gov/). Linkage Disequilibrium between rs4950928 (-131) and rs10399931 (-329) was calculated using Haploview 4.0 software (Broad Institute, MIT, Cambridge, MA, USA).[25]

Statistics

Serum and BALF YKL-40 levels are reported as median and inter quartile range (IQR) and were natural log (ln) transformed during statistical analysis to correct for non-Gaussian distributions. SPSS 15 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism v. 3 (Graphpad software INC., San Diego, CA, USA) were used for statistical analysis. Spearman's rho test was used to assess the correlation between serum YKL-40 concentrations and clinical parameters. The Kaplan-Meier method with log-rank test was used to analyse whether YKL-40 levels were associated with survival. The optimal cut-off point between the two survival groups was calculated with a ROC curve analysis. Cox regression analysis with covariates was used to check for possible confounders and to calculate a hazards ratio with 95% confidence interval (CI). Pearson's goodness-of–fit Chi-square test and Fisher's exact test were used to test for deviation from Hardy-Weinberg equilibrium and for a difference in genotype and allele frequencies between patients and controls (as implemented online at http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Differences with a p-value < 0.05 were considered statistically significant.

Results

Clinical characteristics of patients and controls are summarized in Table 1. Eighty-five patients with IPF (71 male and 14 female, mean age 65 ± 10 years) were included in this study.

Serum YKL-40 levels were significantly higher in IPF patients than in controls ($p < 0.0001$) (Table 1). In healthy controls, serum YKL-40 levels are genotype dependent ($p < 0.01$) and a trend towards significance ($p = 0.07$) was observed in IPF patients (Figure 1).

Table 1. Characteristics of patients and controls

	IPF patients	Serum controls	BALF controls
Number of subjects	85	83	43
Gender M/F	71/14	30/53	23/20
Age, yr	65 ± 10	56 ± 5	32 ± 16
Smoking			
Never	27	33	24
Former	52	34	2
Current	6	16	17
Packyear	18 ± 12	NA	NA
Diagnosis			
Pathological	43	NA	NA
Clinical	42	NA	NA
D_{LCO} % predicted	48 ± 18	NA	NA
FEV_1 % predicted	82 ± 23	NA	106 ± 11
FVC % predicted	78 ± 23	NA	110 ± 11
Blood neutrophils, %	8 ± 3	NA	56 ± 8
Blood monocytes, %	63 ± 13	NA	8 ± 2
Blood lymphocytes	24 ± 11	NA	32 ± 7
Blood eosinophils	4 ± 2	NA	3 ± 3
BALF macrophages, %	71 ± 18	NA	88 ± 10
BALF neutrophils %	10 ± 12	NA	2 ± 2
BALF YKL-40 level (Median, IQR)	12.3 (7.1-18.2)	NA	4.8 (2.7-8.8)
Serum YKL-40 level (Median, IQR)	109.4 (76.6 - 237.7)	46.2 (32.7 - 71.3)	38.1 (33.3 - 51.6)

Data are presented as mean ± SD unless otherwise indicated. NA = not available.

The median serum YKL-40 level in patients with the –329 AG genotype was 89.3 ng/ml (n = 31, IQR 71.5 - 202.5) compared to 128.0 ng/ml (n = 37, IQR 85.9 - 198.4) in patients with the GG genotype.

BALF YKL-40 levels were also significantly elevated in IPF patients compared to controls (P < 0.0001) (Table 1).

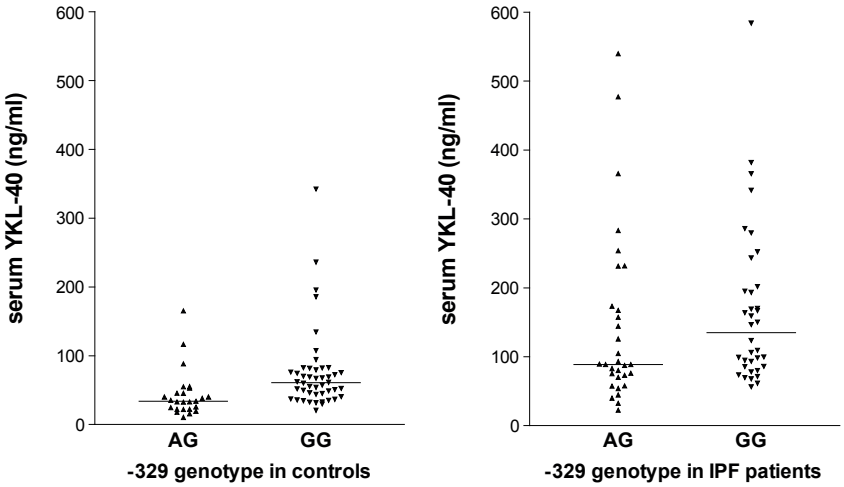

Figure 1. Serum YKL-40 levels in patients and healthy controls. In controls, serum YKL-40 levels were significantly higher (P < 0.01) in the GG group (n = 46) than in the AG group (n = 25). The effect of genotype on serum levels in patients was less pronounced (p = 0.07).

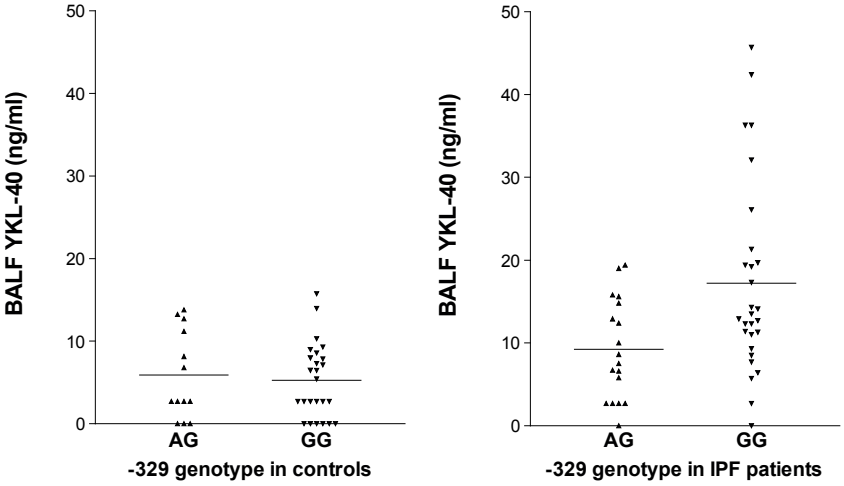

Figure 2. BALF YKL-40 levels in patients and controls. BALF YKL-40 levels were not associated with genotype in controls. In patients, BALF YKL-40 levels were significantly higher (p = 0.01) in the GG group (n = 28) than in the AG group (n = 18).

We did not find a relevant correlation between serum and BALF YKL-40 levels (Figure 3). The correlation between BALF YKL-40 or serum YKL-40 and lung function parameters was too small to consider relevant. We found no effect of smoking, steroid use or gender on serum or BALF YKL-40 levels.

Analysis of the genotype distribution in patients revealed that there was no association with disease. We also found no significant association between the *CHI3L1* –329 genotype and clinical parameters or survival. Linkage disequilibrium between the –329 and the –131 promoter polymorphisms in the CEPH-panel was high ($D' = 1$, $r^2 = 0.85$).

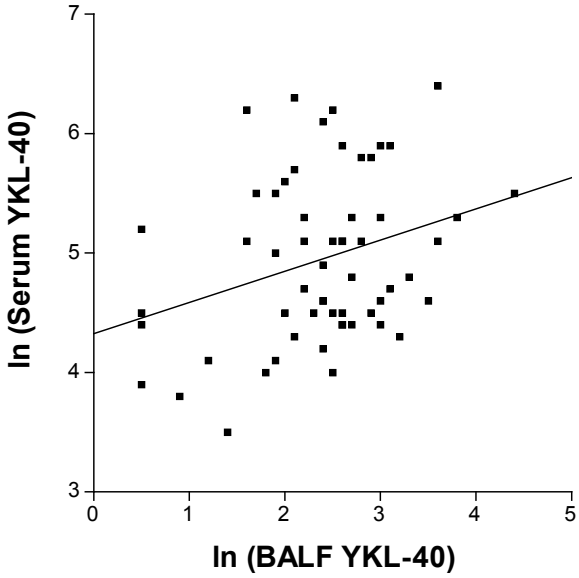

Figure 3. Correlation between Serum and BALF YKL-40 levels in IPF patients (n = 58). $r^2 = 0.09$, $p < 0.05$

Survival analysis: serum YKL-40

Of the 83 patients with serum, follow-up was available from 79 patients. The median follow-up was 28.6 months. 42 patients were still alive, six died from a cause unrelated to IPF and three had undergone lung transplantation. ROC-curve analysis showed that the optimum cut-off point for serum YKL-40 was 79 ng/ml, although there were several other cut-off points that also resulted in a significant difference on Kaplan-Meier curves. The 4-year survival rate in patients with serum YKL-40 level below 79 ng/ml was 86% versus 48% in the group with higher serum YKL-40 levels ($p < 0.01$, figure 4A).

Multivariate Cox regression analysis showed that age was also predictive for survival but was not a confounding factor in our analysis. After correction for age, gender, smoking, lung function and blood cell counts, the hazard ratio for serum YKL-40 (cut-off 79 ng/ml) was 10.9 (95% CI 1.9 - 63.8, $p < 0.01$).

Survival analysis: BALF YKL-40

Follow-up was available from 58 patients with BALF, of whom 31 patients were still alive, five died from a cause unrelated to IPF and two had undergone lung transplantation. ROC-curve analysis showed that the optimum cut-off point for BALF YKL-40 was 17 ng/ml. Patients with a BALF YKL-40 level below 17 ng/ml had a significantly better survival than patients with higher BALF YKL-40 levels ($p < 0.001$, Figure 4B). The percentage of neutrophils in BALF also showed a trend towards a significant association with survival ($p = 0.06$) but did not influence the association between BALF YKL-40 levels and survival. After correction for age, gender, smoking, lung function and BALF cell counts the hazard ratio for BALF YKL-40 (cut-off 17 ng/ml) was 3.0 (95% CI 1.1 – 8.4, $p < 0.05$).
Patients with the –329 GG genotype significantly more often had BALF YKL-40 levels above 17 ng/ml than patients with the AG genotype (11 of 28 patients versus 2 of 18 patients, respectively, $p < 0.05$). However, when we performed Kaplan-Meier analysis with the patients grouped according to genotype, the difference was not significant ($p = 0.5$).

Combining the cut-off points for serum and BALF YKL-40 levels improved the predictive value of the Kaplan-Meier analysis and made it possible to stratify patients in three groups ($p = 0.001$, Figure 4C). Ten patients had low serum YKL-40 levels and low BALF YKL-40 levels, i.e. values below their respective cut-off points. None of these 10 patients (0%) died from IPF during the follow-up period. Nine patients were still alive while one patient had died from a cause unrelated to IPF. In the intermediate group, either the serum or BALF YKL-40 level was low ($n = 31$). In this group, 10 patients (32%) died from IPF while 21 were censored. In contrast, 17 patients had both high serum and high BALF YKL-40 levels and of these 11 patients had died (65%).
Multivariate Cox regression analysis showed that the average hazard ratio after correction for age, gender, smoking, lung function blood and BALF cell counts was 4.8 (95% CI 1.5 – 15.3, $p < 0.01$).

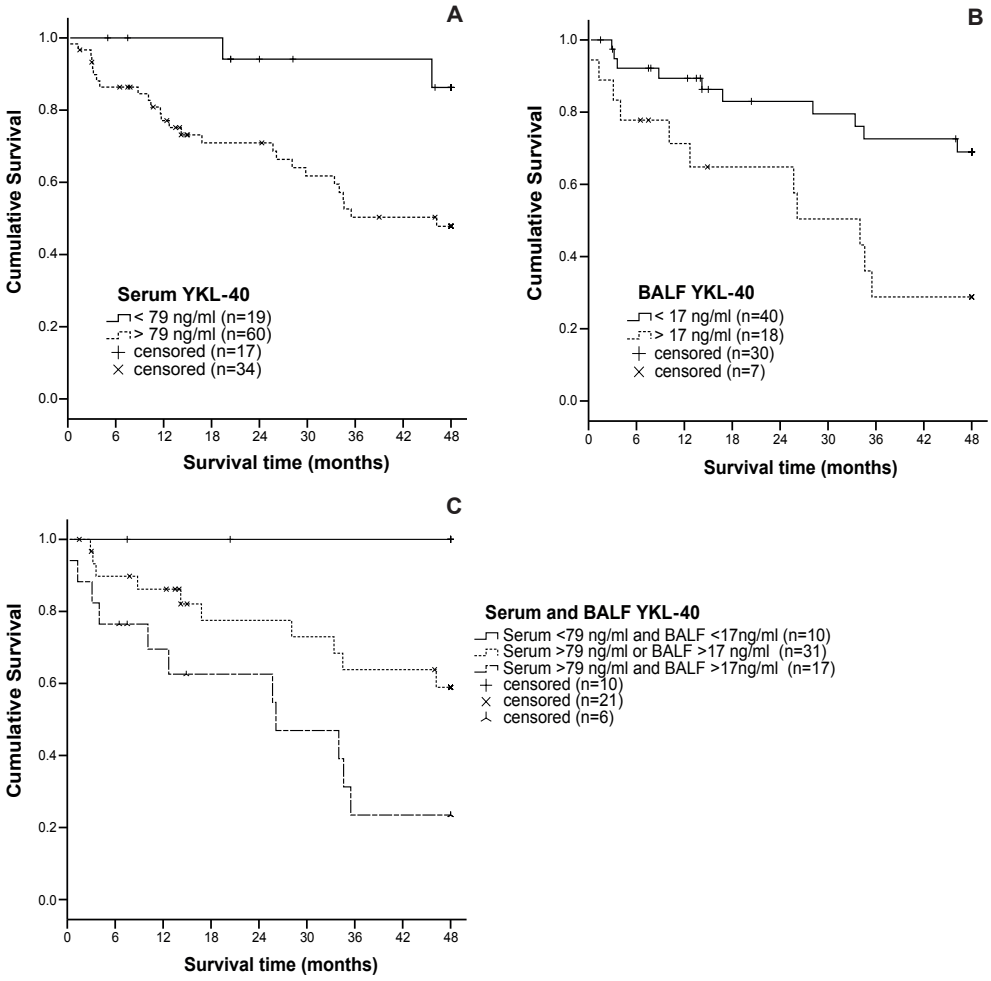

Figure 4. Kaplan-Meier survival analysis grouped by baseline YKL-40 levels. Patients that were still alive, that were transplanted or died from a cause unrelated to IPF were censored in the survival analysis. A) Patients with high serum YKL-40 had significantly worse survival estimates than patients with low serum YKL-40 ($p < 0.01$, $\chi^2 = 6.77$). B) Patients with high BALF YKL-40 had significantly worse survival estimates than patients with low BALF YKL-40 ($P < 0.01$, $\chi^2 = 7.81$). C) In the group with both low serum YKL-40 and low BALF YKL-40 there were no IPF related deaths within 48 months after diagnosis. Patients with both high serum YKL-40 and high BALF YKL-40 had significantly worse survival estimates than patients who had either high serum YKL-40 or high BALF YKL-40 ($p < 0.01$, $\chi^2 = 13.09$)

Discussion

This study investigated YKL-40 as a potential biomarker for prognosis in IPF.
In our cohort of IPF patients, high serum and BALF YKL-40 levels were significantly associated with shorter survival time (figure 4A, B). Optimum cut-off points for serum and BALF YKL-40 were determined using ROC-curve analysis and stratifying patients according to these cut-off points resulted in three groups with significantly different survival estimates. In the group with both low serum and low BALF YKL-40 levels, there were no IPF related deaths during the 4-year follow-up period (figure 4C).
Multivariate Cox regression analysis showed that there were no confounding factors in our survival analysis. In our cohort, only age, BALF neutrophil percentage and smoking were marginally associated with survival. Other IPF survival studies have shown that age, smoking, and baseline clinical parameters can be predictors for survival although findings differ and are often contradictory.[26-30] After correction for possible confounding factors, the association between serum and BALF YKL-40 levels and prognosis in IPF remained significant. However, these findings will have to be confirmed in an independent cohort.

The previously reported prognostic markers for IPF, such as SP-D and CCL18, seem to be most predictive in the first year after diagnosis.[31,32] YKL-40 remains predictive after 3 to 4 years and could thus be of use in a clinical setting. In addition, combining YKL-40 with a short-term prognostic biomarker may result in an even better estimate of survival.

This is the first study to investigate the influence of a *CHI3L1* polymorphism on BALF YKL-40 levels. In IPF patients, presence of the *CHI3L1* –329 GG genotype resulted in higher YKL-40 levels in BALF. We did not find a significant difference in genotype frequency between patients and controls and the –329 polymorphism therefore does not seem to influence IPF disease susceptibility. The –329 genotype influences BALF YKL-40 levels and thereby seems to cause an indirect effect on survival in IPF patients. However, no significant difference in survival curves was observed in our cohort. Further studies are needed to determine if an effect of *CHI3L1* genotype on survival can be found when a larger cohort is used. The –329 G-allele is almost in complete linkage disequilibrium with the –131 C-allele, as was also previously

found by Ratcke *et al.*[33] In recent studies, *CHI3L1* –131 was found to be associated with asthma and atopy,[23,34,35] liver fibrosis[22] and schizophrenia.[36] The –131 CC genotype results in higher serum YKL-40 levels and is associated with more severe liver fibrosis and a predisposition to develop asthma.[22,23] However, no effects of this polymorphism on the expression of YKL-40 in the lung have been reported. Because of the large variation in serum and BALF YKL-40 levels in our cohort of IPF patients, determining the effect of a genetic polymorphism on disease susceptibility and progression will be hampered. The study results suggest that the -329 genotype neither predisposes to IPF nor influences activity of IPF.

YKL-40, a chitinase-like glycoprotein, is expressed in many healthy tissues, and expression is higher in cells with high metabolic activity.[37] YKL-40 is also known to be upregulated in late stages of macrophage differentiation.[38] Furuhashi *et al.* showed that in lungs from IPF patients, bronchiolar epithelial cells and alveolar macrophages near areas of remodelling express YKL-40. This is the most likely source of the elevated BALF YKL-40 levels found in IPF patients. YKL-40 has been found to induce the release of profibrotic and proinflammatory cytokines by alveolar macrophages and could thus contribute to tissue remodelling in the lung.[39]

The source of serum YKL-40 is more difficult to ascertain. Blood granulocytes are the most likely source,[40] but no significant correlation between serum YKL-40 and blood granulocytes was found in our cohort. In addition, it is unlikely that protein leakage through the damaged alveolar walls is a major source of elevated serum levels, as the correlation between BALF and serum YKL-40 levels is low.

The lack of correlation between serum and BALF YKL-40 levels might reflect different sources of the protein. In the lung, alveolar macrophages and epithelial cells seem to be the main source while the source of serum YKL-40 could be specific subsets of peripheral immune cells.[16,40,41] Our results indicate that serum and BALF YKL-40 levels are independently associated with survival and could therefore also reflect different pathogenic processes. YKL-40 is known to be upregulated in inflammatory conditions and it could be a marker of peripheral immune cell activation,[41] whereas BALF YKL-40 levels may be indicative of local remodelling and macrophage activation. Activation markers of circulating immune cells have been associated with IPF progression.[42] How this is a part of IPF pathology and why peripheral YKL-40 production predicts disease progression is still unclear and deserves further

research. YKL-40 is a marker for inflammation, tissue remodelling and cancer and as such could simply reflect the pathogenic process in IPF. YKL-40 has been shown to be a connective tissue cell growth factor, to modulate collagen I fibrillogenesis, and angiogenesis, which are all involved in wound repair. [9,11,43] *In vitro* tests showed that YKL-40 is a growth factor for fibroblasts and may therefore be directly involved in the pathogenesis of fibrotic disorders.[9] Mice lacking the mouse equivalent of YKL-40 were found to have significantly less IL-13/TGF-β mediated tissue inflammation and fibrosis.[44] The elevated levels observed in IPF patients in this study, and previously in a smaller Japanese cohort,[16] could thus mediate the fibrotic process in the IPF lung.

In conclusion, understanding about the role of YKL-40 in biological pathways is growing but its exact function is not clear. It has been associated with inflammation, angiogenesis, extracellular matrix remodelling, and fibroblast growth and could therefore play a role in disease aetiology.[12] Our study showed that serum and BALF YKL-40 levels in IPF patients are upregulated in a genotype dependent manner. In addition, elevated serum and BALF YKL-40 levels are associated with shorter survival time and could be prognostic markers of survival in IPF.

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CHAPTER 6

FOLLOW-UP OF SERUM YKL-40 LEVELS IN IPF PATIENTS AND COMPARISON WITH OTHER INTERSTITIAL PNEUMONIAS

Submitted

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Abstract

Background

YKL-40 is a chitinase-like protein that is elevated in diseases with inflammation, fibrosis and tissue remodelling. Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal disease that is hallmarked by these processes. Previously, YKL-40 was shown to be elevated in the serum of IPF patients and was associated with survival. The aim of this study was to compare levels between IPF and other interstitial pneumonias at diagnosis and to investigate serial YKL-40 measurements in patients with IPF and cryptogenic organising pneumonia (COP).

Methods

Serum YKL-40 levels were measured in 124 healthy controls and a total of 315 patients, consisting of 185 patients with IPF, 25 with idiopathic NSIP, 38 with CTD-NSIP, 40 with SR-ILD and 27 with COP. Follow-up serum YKL-40 levels were available in 36 patients with IPF and 6 patients with COP.

Results

In all patient groups, serum YKL-40 levels were elevated compared to controls ($p < 0.0001$). Serum YKL-40 levels were highest in patients with idiopathic NSIP and COP. There was a significant difference between patients with idiopathic NSIP compared to CTD-NSIP ($p = 0.02$). Serum YKL-40 levels in IPF patients generally remained stable over time although levels can fluctuate within an individual. In COP patients serum YKL-40 levels decreased significantly over time (average 9.3 months, $p = 0.04$).

Conclusion

Serum YKL-40 levels are elevated in all patient groups and can not be used to differentiate between IPF and other fibrotic interstitial lung diseases. Our results suggested that serum YKL-40 may be a marker for disease activity in patients with interstitial pneumonia.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a rapidly progressing lung disease with a median survival of 2.5 to 3.5 years.[1] IPF is a chronic fibrosing form of interstitial pneumonia. Apart from lung transplantation, there is currently no therapy that has been proven to extend survival. Diagnosis and management of IPF is complex and requires distinguishing IPF from other, usually less devastating, interstitial pneumonias and identification of patients with the worst prognosis. Diagnostic and prognostic biomarkers are important for clinical practice and will facilitate timing of therapeutic intervention such as lung transplantation.

YKL-40 has been proposed as a diagnostic and prognostic biomarker for IPF.[2,3] YKL-40, a chitinase-like protein, is part of the chitinase family that also includes chitotriosidase.[4] Produced at sites of inflammation, YKL-40 is potentially involved in regulating the inflammatory tissue response.[5,6] It is a marker for diseases that are characterized by inflammation, fibrosis and tissue remodelling.[7-9] Circulating levels of YKL-40 have been found to correlate with liver fibrosis,[10] and with fibrosis in Crohn's disease.[11] YKL-40 could also be a prognostic marker for several types of cancer.[12,13]

In IPF patients, serum YKL-40 levels are elevated and were shown to be associated with survival.[2,3] However, it remains unclear whether the elevated serum levels of YKL-40 represent the activity of the underlying disease process and whether it can be used to distinguish between IPF and other interstitial pneumonias.

The aim of this study was to further evaluate the diagnostic and prognostic value of YKL-40. We compared YKL-40 levels at diagnosis between IPF patients and patients with other forms of interstitial pneumonia: non-specific interstitial pneumonia (NSIP), smoking related interstitial lung diseases (SR-ILD) (including desquamative interstitial pneumonia and respiratory bronchiolitis associated interstitial lung disease) and cryptogenic organising pneumonia (COP). Additionally, we monitored the levels of YKL-40 during follow-up of IPF and COP patients. We hypothesized that the serum YKL-40 levels in these patients might represent disease activity and would be highest in patient groups with a poor prognosis such as IPF and NSIP.

Methods

Subjects

A total of 315 patients were included in this study. Serum was available from 101 patients that were included in our study prococol between 1993 and 2007 and all of these patients gave written informed consent. In 2007 serum YKL-40 measurements became part of the clinical routine in our hospital. YKL-40 values could be retrieved from hospital records for 214 patients that were diagnosed between 2007 and September 2011.

The study included 185 patients with IPF. 81 of these patients were also included in our previous study on YKL-40 in IPF.[3] In addition, 25 idiopathic NSIP patients, 38 connective tissue disease associated (CTD-) NSIP, 40 SR-ILD patients and 27 COP patients were included.

The control group comprised 124 healthy employees of the St. Antonius Hospital, 79 males and 45 females, with a mean age of 51.6 (± 7.7, SD). The medical ethical committee in our hospital approved the study and all healthy subjects gave formal written informed consent.

Table 1. Characteristics and serum YKL-40 levels of the control and patient groups

Group (n)	Males	Females	Age	YKL-40 (ng/ml)	Significance*
Controls (124)	79	45	51.6 ± 7.7	44.7 [32.7-66.15]	
IPF (185)	153	32	63.8 ± 10.7	116.9 [78.4-207.0]	$p < 0.0001$
Idiopathic NSIP (25)	13	12	66.3 ± 9.5	159.3 [100.0-258.0]	$p < 0.0001$
CTD-NSIP (38)	20	18	57.8 ± 12.5	83.7 [54.2-164.2]	$p < 0.0001$
SR-ILD (40)	22	18	49.8 ± 9.7	96.3 [58.4-143.0]	$p < 0.0001$
COP (27)	18	9	60.8 ± 12.2	154.4 [121.2-280.9]	$p < 0.0001$

Age is reported as mean ± SD.
Serum YKL-40 level as median with IQR. Serum was available from 124 controls, 185 patients with idiopathic pulmonary fibrosis (IPF), 25 patients with idiopathic non-specific interstitial pneumonia (NSIP), 38 patients with connective tissue disease associated non-specific interstitial pneumonia (CTD-NSIP), 40 patients with smoking-related interstitial lung diseases (SR-ILD) and 27 patients with cryptogenic organising pneumonia (COP).
*P-value for difference between patients and controls (analysis of variance (ANOVA) with Bonferroni correction.).

Follow-up of serum YKL-40 was available for 36 IPF patients with informed consent and was defined as one measurement at diagnosis and at least two subsequent measurements. Average time of follow-up was 39 months (range 4 - 86) and the average number of samples per patients was 7 (range 3 - 13). From six patients with COP a second sample was available (average 9.3 months after diagnosis).

YKL-40 protein detection

YKL-40 levels were determined by YKL-40 enzyme immunoassay (Quidel Corporation, San Diego, CA, USA), which was performed in accordance with the manufacturer's instructions. Twenty microliter of serum or BALF was used; either undiluted or diluted 1:5 when exceeding the standard curve range. The lower limit of quantitation of this ELISA is 15.6 ng/ml and the lower limit of detection is 5.4 ng/ml.

Statistics

SPSS (SPSS Inc., Chicago, IL, USA) and Graphpad Prism (Graphpad software INC., San Diego, CA, USA) were used for statistical analysis. Analysis of variance (ANOVA) with Bonferroni correction or t-test was used to compare subject groups. Linear mixed modelling was used to test for changes in serum YKL-40 levels over time in IPF patients.

Results

Comparison between IPF and other interstitial pneumonia patients

Characteristics of the patients and controls that were included in this study are shown in table 1. Median serum YKL-40 levels in patients with interstitial pneumonia were at least two times higher than in healthy volunteers (figure 1). Levels were highest in patients with idiopathic NSIP and COP and lowest in patients with CTD-NSIP and SR-ILD. Serum YKL-40 levels were significantly higher in COP patients than in patients with CTD-NSIP and SR-ILD ($p = 0.016$ and $p = 0.018$ respectively). A significant difference was observed between idiopathic NSIP patients and patients with CTD-NSIP (ANOVA with Bonferroni correction resulted in $p = 0.02$ and a students t-test resulted in $p = 0.003$).

There was no significant difference between men and women in patients or controls. No difference in serum YKL-40 levels was observed between our previously described IPF cohort[3] ($n = 81$) and the new IPF cohort ($n = 104$). Evaluation in all patients with informed consent showed that the serum YKL-40 level at diagnosis in patients did not correlate with any of the lung function parameters or smoking history.

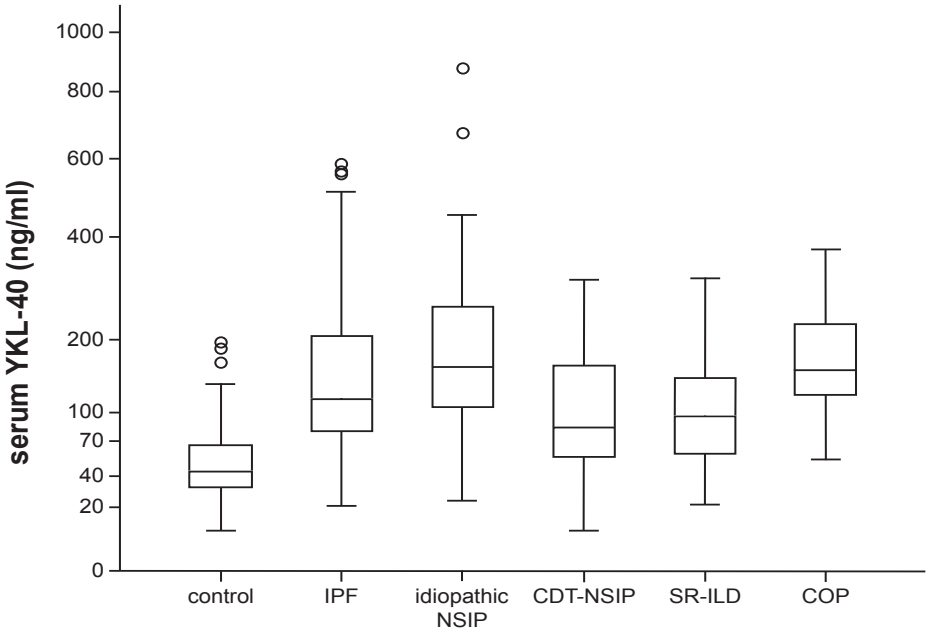

Figure 1. Serum YKL-40 levels in patients and controls. Levels are shown on a power scale (0.5). Boxplot represents median and interquartile range (IQR). Error bars represent lowest and highest (non-outlier) levels. Outliers are shown as open circles (values between 1.5*IQR and 3*IQR from the box)

Follow-up in IPF patients

Serum YKL-40 measurements remained predominantly stable over time (average 39 months, range 4 - 86 months). This was confirmed by linear mixed modelling that revealed no significant effect of time on serum YKL-40 levels in the 36 IPF patients (p = 0.4). Comparing the first measurement to the last measurement and to one sample in between that was closest to the midway time point (figure 2) also confirmed serum YKL-40 levels remain stable over time.

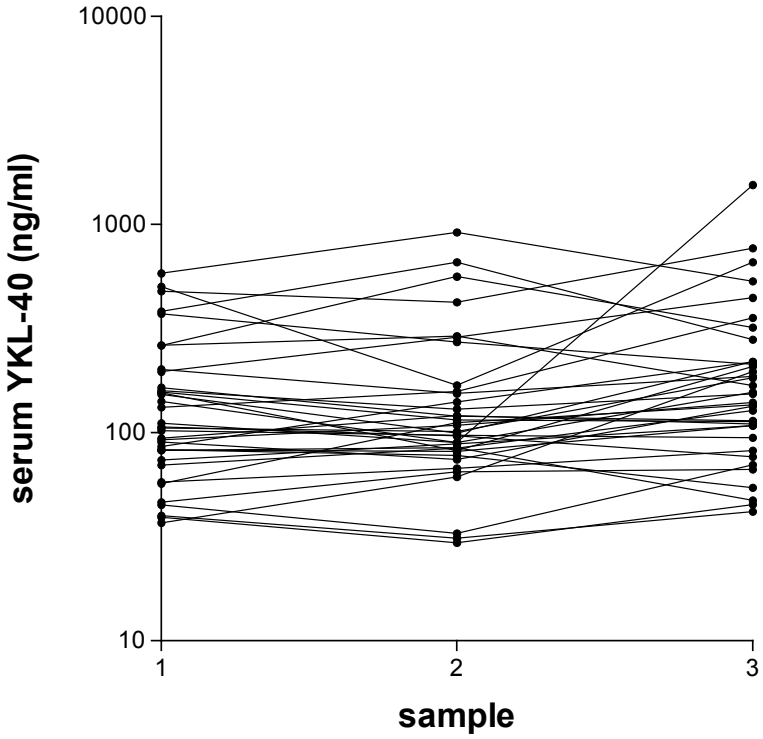

Figure 2. Follow-up of serum YKL-40 levels in IPF patients. Serum YKL-40 level is shown for 36 IPF patients. 1 = first measurement (at diagnosis), 3 = last measurement and 2 = measurement closest to the midway time point. No significant difference was observed.

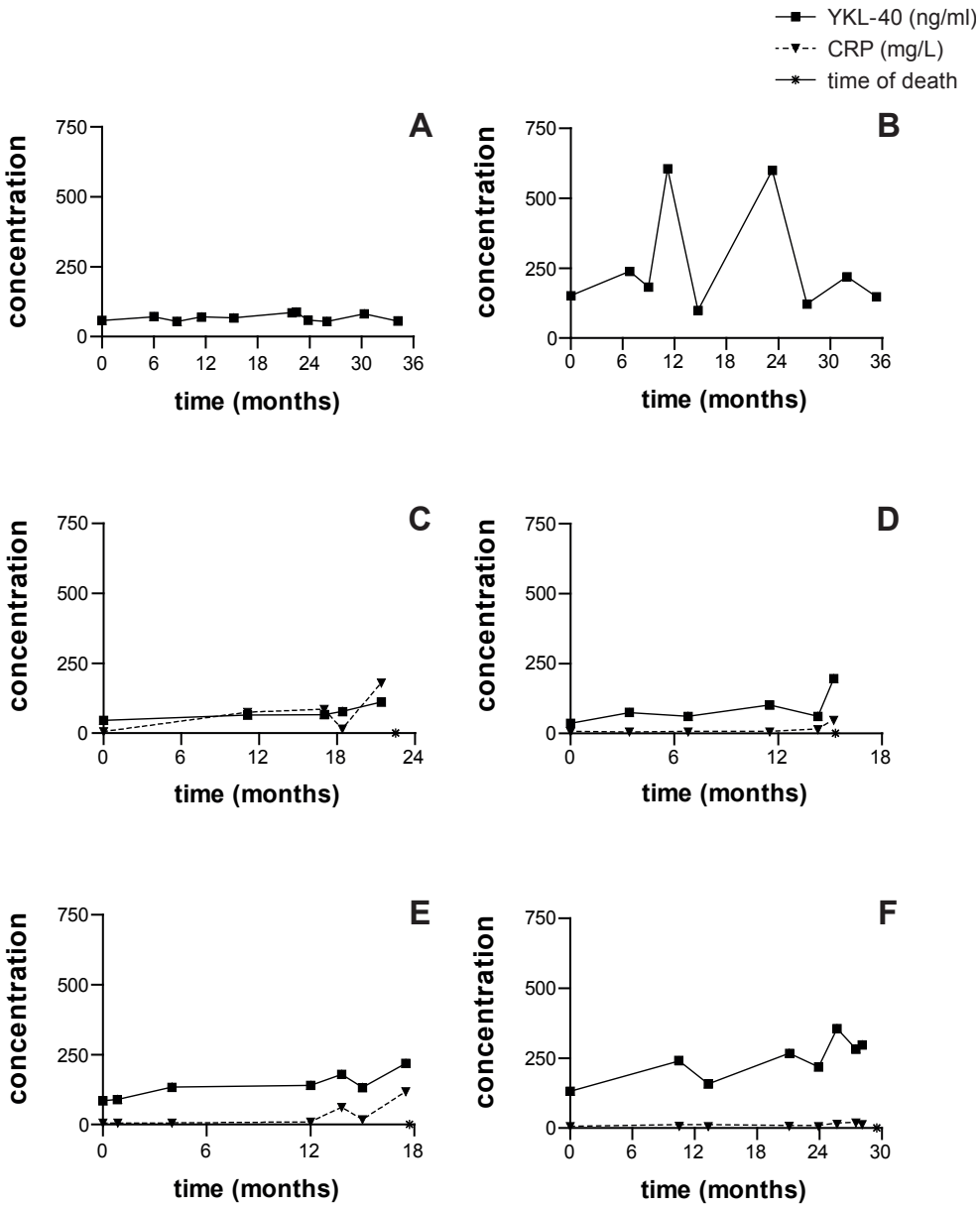

Figure 3. Follow-up of serum YKL-40 levels in IPF patients. A) Example of an IPF patient with stable serum YKL-40 levels over time. B) Example of an IPF patient with fluctuating serum YKL-40 levels over time. C-E) Examples of a sudden increase in serum YKL-40 level just before death that is accompanied by a similar increase in C-reactive protein (CRP). F) Example where the increase in serum YKL-40 level and the correlation with CRP is less obvious.

Besides stable YKL-40 levels during follow-up (figure 3A), considerable fluctuations in serum YKL-40 levels were observed in some patients (figure 3B-D). Elevated levels were found in some, but not all, patients that underwent a surgical procedure such as video assisted thoracic surgery (VATS) a few days before the measurement.
In 12 of the 36 patients samples were available until shortly before they died. A large increase (at least 50%) in YKL-40 levels was observed in 6 of these patients, 1.3 months or less before death. In 4 cases this was accompanied by an increase in the level of C-reactive protein (CRP) (figure 3C-E). A less obvious increase in serum YKL-40 level was observed in 4 cases (example is shown in figure 3F) and in two cases no increase was observed up to one week before their death.

In many patients changes in YKL-40 could not be explained by evaluation of clinical records and other routine measurements such as CRP. All patients had declining diffusion capacity for carbon monoxide (DLCO), except for two patients who already had a DLCO of around 30% of predicted at diagnosis. Survival analysis in the new cohort of IPF patients was not possible because limited follow-up time for survival was available (n = 55, median follow-up for survival 15 months).

Follow-up in COP patients

From six patients with COP the serum YKL-40 level at diagnosis and during follow-up (average 9.3 months after diagnosis) is shown in figure 4. The average serum YKL-40 level in the follow-up sample was significantly lower ($p = 0.04$, paired t-test).

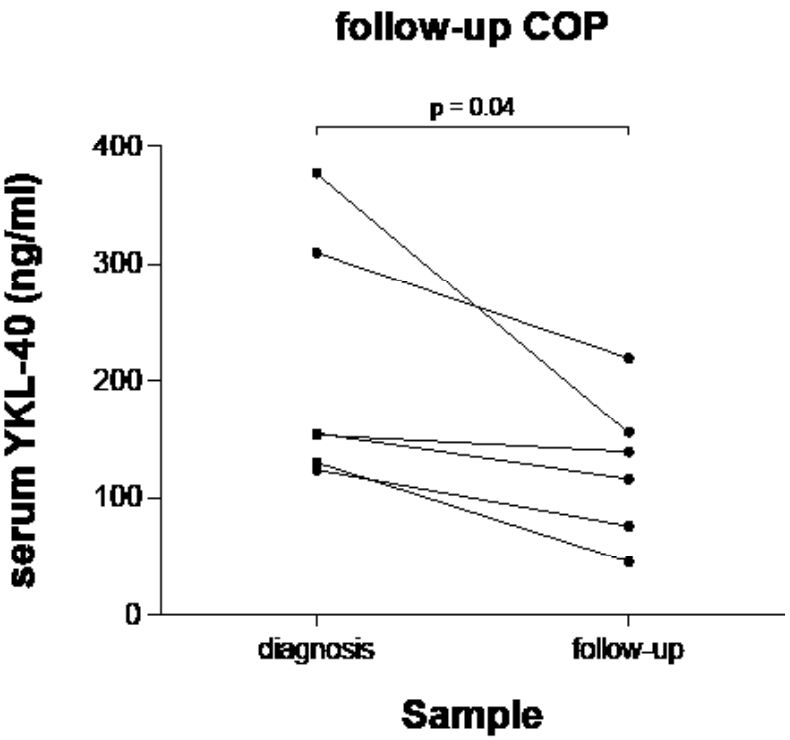

Figure 4. Serum YKL-40 levels in patients with cryptogenic organising pneumonia (COP). Comparison between serum YKL-40 level in a sample taken at diagnosis and a sample during follow-up (at least 6 months after diagnosis) p = 0.04 (paired t-test).

Discussion

Our study showed that serum YKL-40 levels were high in all interstitial pneumonias with medians at least two times higher than in controls. Levels were highest in patients with fibrotic interstitial pneumonias: IPF, idiopathic NSIP and COP. Serum YKL-40 levels were significantly lower in patients with CTD-NSIP compared to patients with idiopathic NSIP. It is known that patients with CTD-NSIP have a significantly better prognosis than patients with idiopathic NSIP.[14] This suggests that serum YKL-40 levels might be associated with prognosis in NSIP as we found earlier for IPF.[3] The better prognosis in CTD-NSIP suggests that the fibrogenesis is less active in these patients and YKL-40 levels may reflect fibrotic disease activity.

Follow-up of serum YKL-40 levels in IPF patients showed that YKL-40 levels fluctuate but generally remain stable over time. A temporary increase of serum YKL-40 levels

early in the disease course often coincided with a surgical procedure such as VATS. In addition, in at least 50% of patients a sudden increase in serum YKL-40 level was seen just prior to the patient's death. In some patients with fluctuating serum YKL-40, a correlation with CRP could be observed. Serum YKL-40 levels did not associate with lung function decline in the follow-up group. This suggests that the stable serum YKL-40 levels do not reflect the degree of disease severity in IPF patients. Instead, YKL-40 levels might be a marker for disease activity. Our previous research showed that higher YKL-40 levels were associated with diminished survival in IPF.[3] IPF is a progressive disease with untreatable, but variable degree of fibrogenesis in individual patients. The relatively high and stable levels of YKL-40 might reflect this process. This is in congruence with the observation that follow-up of serum YKL-40 levels in COP patients significantly decreased over time. Most patients with COP respond well to treatment with prednisone and the disease can show complete remission within one year. The decrease in serum YKL-40 levels in COP patients therefore suggests that serum YKL-40 levels decline when the disease is in remission. Together these findings indicate that serum YKL-40 levels may be a marker for the activity of the fibrotic process in interstitial pneumonias. Further comparison of serum YKL-40 levels with clinical data in patients is needed to assess its value as a clinical marker of disease activity.

The precise function of YKL-40 is unknown, but it may drive inflammatory and fibrotic processes by activation of macrophages.[15] Remarkably, serum YKL-40 levels were even higher in COP patients than in IPF patients. The disease process in both IPF and COP is characterized by an accumulation of fibroblasts, though in IPF the fibroblastic foci are found in the interstitium, while in COP these cells are filling the alveolar space. The ability of YKL-40 to induce fibroblast growth[16] suggests that it may play a part in the development of the fibroblast foci seen in IPF patients. However, sudden increases in serum YKL-40 levels were sometimes associated with increased levels of CRP suggesting they can reflect an inflammatory process. The association with CRP was most often observed in the months before a patient's death. In advanced stages of IPF patients become less able to fight infection, and respiratory infection often occurs in the last stage of disease.[17,18]

It has been suggested that YKL-40 is produced by macrophages. Immunohistological examination in IPF lungs by Furuhashi *et al.* has revealed that YKL-40 was mainly

produced by macrophages, but not all macrophages were positive for YKL-40.[2] Remarkably, serum YKL-40 levels were lowest in SR-ILD patients. SR-ILD is associated with extreme influx of pigmented macrophages into the lung.[19] However, these pigmented macrophages are very different from cells that are seen in IPF patients.[20] Recently, Pechkovsky *et al*. showed that the predominant type of macrophage in the lungs from IPF patients is the type 2 macrophage.[20] It seems possible that YKL-40 is produced by this specific subset of macrophages that is associated with tissue repair and remodelling.

In conclusion, we have shown that serum YKL-40 levels are elevated in all patient groups. Our results suggest that serum YKL-40 is a marker for activity of the fibrotic remodelling processes in interstitial pneumonias. Follow-up of serum YKL-40 levels showed that in IPF patients the levels stay relatively stable although large fluctuations can occur. Further follow-up studies are needed to determine whether serum YKL-40 can be used to monitor patients and can be helpful in therapeutic decision making especially with regard to lung transplantation.

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CHAPTER 7

YKL-40 PRODUCTION BY ALVEOLAR MACROPHAGES: A PILOT STUDY

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Background: Association between alveolar macrophages and YKL-40

Idiopathic pulmonary fibrosis (IPF) is a rapidly progressive lung disease characterized by interstitial remodelling and fibrosis. Median survival in IPF is approximately 3 years, but can vary between a few months and over 10 years.[1] Recently, we found that YKL-40 levels in serum and lavage fluid of patients with IPF were elevated and were correlated to survival.[2] YKL-40 is a chitinase-like protein involved in inflammation, fibrosis, and tissue remodelling. Elevated serum levels have been found in various diseases including cancer, liver cirrhosis, asthma and sarcoidosis.[3-7] The precise function of YKL-40 is unknown, but it was found to drive inflammatory and fibrotic processes by alternative activation of macrophages.[8,9] Moreover, in IPF patients it is mainly expressed by alveolar macrophages near fibrotic lesions.[10] It was recently reported that in IPF patients alveolar macrophages have shifted from the normal (M1) to the alternatively activated (M2) phenotype and that these M2 cells were involved in the pathogenesis of fibrotic interstitial lung diseases.[11] M2 macrophages are thought to influence many processes such as inflammation, fibrosis and tumorigenesis, but their main function is assumed to be stimulation of tissue repair after inflammation.[12]

M2 activation is mediated by T-helper 2 cytokines, mainly interleukin (IL)-4, IL-10, and IL-13 in contrast to the normal pro-inflammatory activation by interferon-gamma (IFN-γ) that leads to M1 macrophages. Remarkably, alternatively activated alveolar macrophages in mice produce the proteins Ym1 and Ym2 that are chitinase-like lectins belonging to the same family as YKL-40.[13] In addition, two other members of this protein family, YKL-39 and SI-CLP, were produced by macrophages stimulated with IL-4 and TGF-β but not by macrophages stimulated with IFN-γ, and therefore seem specific for M2 macrophages.[14,15]

The aim of this study was to investigate whether YKL-40 is specifically produced by alternatively activated macrophages.

Experiments and results

BAL cell isolation and culture conditions

Bronchoalveolar lavage (BAL) is a method for the recovery of cellular and non-cellular components from the lower respiratory tract by introduction and recovery of sterile saline (300 ml) through the bronchoscope. In healthy controls and patients with interstitial lung disease, the majority of cells in BAL fluid are alveolar macrophages (AM). BAL cells were obtained from individuals that underwent BAL at the university medical center in Freiburg im Breisgau, Germany, between March and June 2011. BAL cell profiles were determined using cytosmears counting at least 300 cells. When alveolar macrophage (AM) count in the BAL was below 50%, a MACS (magnetic activated cell sorting) CD-3 cell depletion was performed as previously described.[11] Cells were counted and resuspended in RPMI-1640 culture medium (Gibco with 10% foetal calf serum and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin). Cells were cultured in 24 wells plates (Nunc, Germany) at $1x10^6$ cells per well in 500 µl medium and incubated for 24 hours (h) in an incubator at 37°C and 5% CO2. To stimulate differentiation of macrophages towards an M2 phenotype the cells were cultured in the presence of IL-4 (10 ng/ml) and IL-10 (10 ng/ml). To stimulate M1 macrophage differentiation the cells were cultured in the presence of IFN-γ (10 U/ml). After 24h cells were carefully scraped from the wells using a pipette tip and resuspended in the culture medium. The culture medium was then transferred to an eppendorf vial and centrifuged for 5 minutes at 3000 rotations per minute (rpm). The supernatant was transferred to a new vial and the cell pellet was resuspended in 200 µl TRIzol reagent (Invitrogen, Germany) or washed in phosphate buffered saline (PBS). Samples were stored at -80°C.

Detection of YKL-40 production by BAL cells *in vitro*

Enzyme linked immunosorbent assay (ELISA) was used to determine YKL-40 levels in the supernatant of BAL cell cultures after 24h. The YKL-40 enzyme immunoassay (Quidel Corporation, San Diego, CA, USA), was performed in accordance with the manufacturer's instructions. After 24h incubation YKL-40 levels in culture supernatants were very low and seldom reached the minimal detection level of the ELISA (5.4 ng/ml). YKL-40 was measured in BAL cell supernatants from 11 individuals. Two of these had IPF, 6 had other ILD such as sarcoidosis, one had undergone lung

transplantation (LTX), one had rheumatoid arthritis, and one healthy person. BAL AM percentage was between 56% and 92%. The highest levels were found in the BAL cell supernatant of one IPF patient (figure 1). The AM percentage in this patient was 86%. In this case the highest YKL-40 level was found after 24h stimulation with both IL-4 and IL-10. In the patient with rheumatoid arthritis the YKL-40 level was also highest after stimulation with both IL-4 and IL-10 and reached the detection limit of the ELISA. In one sarcoidosis patient and in the LTX patient the YKL-40 level reached the detection limit only after stimulation with IFN-γ. No correlation between macrophage percentage and YKL-40 levels was observed.

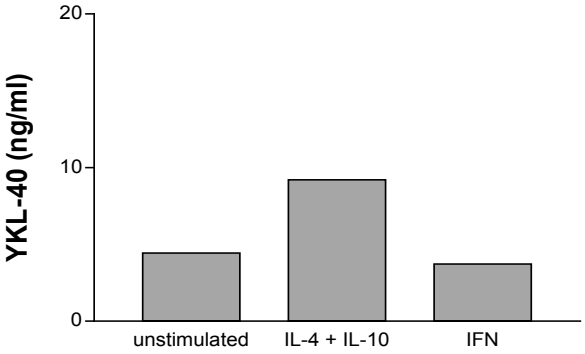

Figure 1. YKL-40 levels in BAL cell culture supernatant after 24h either unstimulated or stimulated with IL-4 and IL-10 or IFN-γ.

Determining intracellular YKL-40 protein production in BAL cells

To measure intracellular YKL-40 protein levels in BAL cells, antibodies from the ELISA kit were used in a western blot analysis. For this analysis 3 cell pellets were pooled to obtain $3x10^6$ cells. Cell pellets were lysed by incubation in 150 µl lysis buffer with 1:500 proteinase inhibitor for 30 minutes on ice. This solution was diluted 1:4 in Laemmli-buffer and loaded on a SDS-page (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) gel. The gel consisted of a 10% stacking gel and a 12% running gel. The gel was run and then blotted onto a PVDF membrane in a mini trans-blot electrophoretic transfer cell (Bio-Rad) according to standard protocols. The blot was incubated for 1-3 hours in 10% blocking solution (Roti-Block®) in tris-buffered saline (TBS) and then incubated overnight at 4°C with the primary antibody (YKL monoclonal antibody, 1:100). The first antibody was washed away and the blot was incubated for at least 1.5h with the second antibody (Donkey anti-Rabbit IRDye® 680;

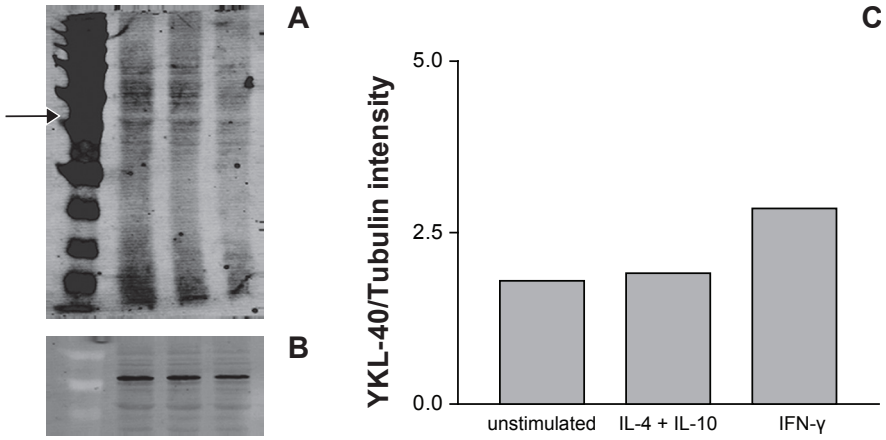

Figure 2. Western blot analysis of YKL-40 levels in BAL cells after *in vitro* stimulation with IL-4 and IL-10 or IFN-γ. A) Western blot showing YKL-40 (expected height indicated by arrow). B) Tubulin staining on the same blot. C) Relative intensity of YKL-40 compared to tubulin.

Odyssey, 1:10000). The blot was incubated again with a primary antibody against α-tubulin (secondary antibody Donkey anti-Mouse IRDye® 800CW; Odyssey, 1:10000). Fluorescence intensity of the bands on the blot was measured on the Odyssey infrared imaging system (LiCor) and quantified using imageJ software (NIMH, Bethesda, MD, USA). Western blotting with YKL-40 antibody did not yield specific bands. Only after increasing the exposure time of the blot and adjusting the contrast and brightness of the image could any bands be made visible (figure 2A). Figure 2C represents a sarcoidosis patient with YKL-40 levels in the BALF cell supernatant that were highest after stimulation with IFN-γ.

YKL-40 mRNA production in BAL cells

Finally, mRNA isolation, reverse transcription and quantitative real time PCR was used to measure YKL-40 production by BAL cells. RNA was isolated from cell pellets in TRIzol reagent (Invitrogen, Germany) according to the manufacturer's protocol. RT-PCR was performed as described previously.[16] Primers used for expression analysis of YKL-40 (*CHI3L1*) were forward 5'-TCCCAGATGCCCTTGACCGCT and reverse 5'-TCCATCCTCCGACAGACAAGAGAGT. The copy numbers were normalized by the housekeeping gene β-actin (ACTB), forward 5'-AGCCTCGCCTTTGCCGA and reverse 5'-CTGGTGCCTGGGGCG.

Quantitative PCR results for a healthy control showed that upregulation was

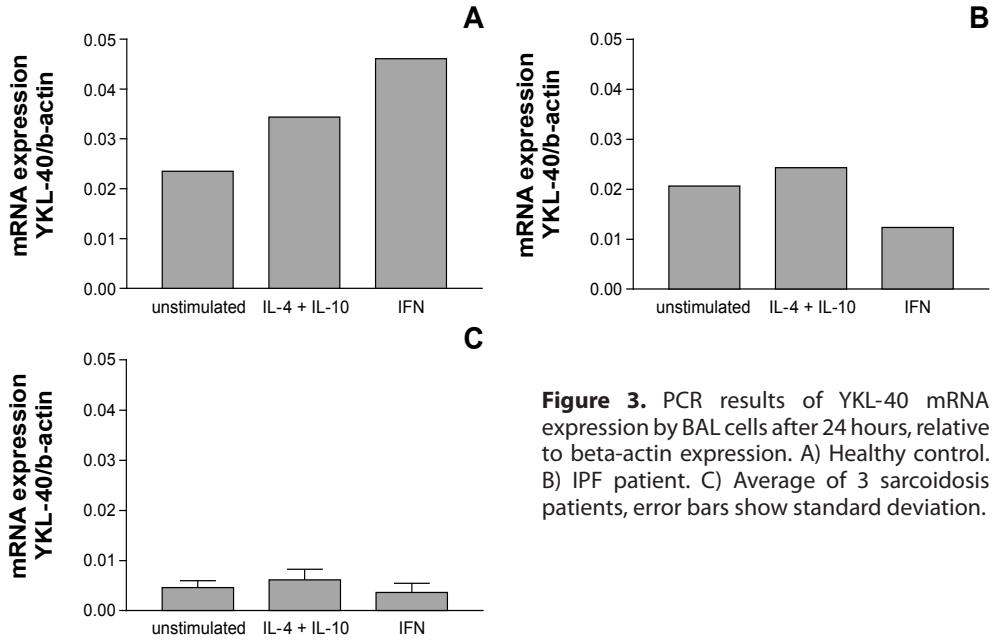

Figure 3. PCR results of YKL-40 mRNA expression by BAL cells after 24 hours, relative to beta-actin expression. A) Healthy control. B) IPF patient. C) Average of 3 sarcoidosis patients, error bars show standard deviation.

highest after 24 hours stimulation with IFN-γ (figure 3A). In patients, YKL-40 mRNA expression was upregulated after stimulation with IL-4 and IL-10 (figure 3B,C) although this difference did not reach statistical significance (n = 3, p = 0.06). Thus, the basic expression of YKL-40 can vary between individuals and a different response to stimulation was observed between patients and controls.

To investigate whether 24h is the optimal incubation time for YKL-40 expression we also performed an experiment with 2h, 24h and 48h incubation of BAL cells from a sarcoidosis patient (93% AM). The results showed much higher YKL-40 expression after 48h than after 24h (figure 4).

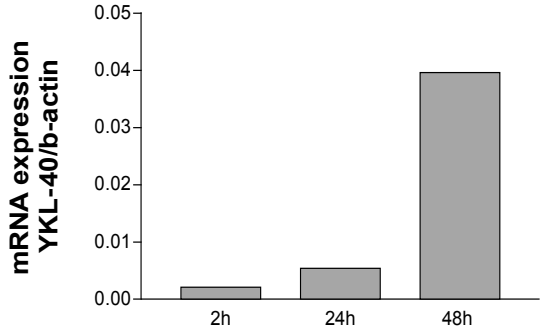

Figure 4. Relative expression of YKL-40 mRNA after 2h, 24h or 48 h incubation without stimulants.

Confirming the differentiation to M1 and M2 macrophages

To confirm that the BAL macrophages have indeed differentiated to M1 or M2 macrophages after 24h stimulation with cytokines that drive macrophage differentiation, we measured cytokines and chemokines that are associated with M1 and M2 in the culture supernatants. IL-1β, chemokine C-C motif ligand (CCL)-2, CCL17, CCL18 and CCL22 were quantified using ELISA kits (R&D Systems, UK) in accordance with the manufacturer's protocol. The influence of stimulation with IFN-γ or IL-4 and IL-10 was evaluated in 3 healthy controls and 3 sarcoidosis patients.

IL-1β and CCL2 (also known as monocyte chemoattractant protein 1, MCP-1) are thought to be produced by M1 macrophages and are induced by stimulation with IFN-γ.[17-19] However, in our experiments IL-1β upregulation after stimulation with IFN-γ was not observed except in one healthy control. Stimulation with IL-4 and IL-10 should result in M2 differentiation. Indeed, a downregulation of IL-1β and CCL2 was observed in almost all controls and patients. For CCL2, a downregulation of 20% was seen after stimulation with IL-4 and IL-10 (figure 5). Thus, after induction of M2 differentiation lower levels of M1 markers were observed.

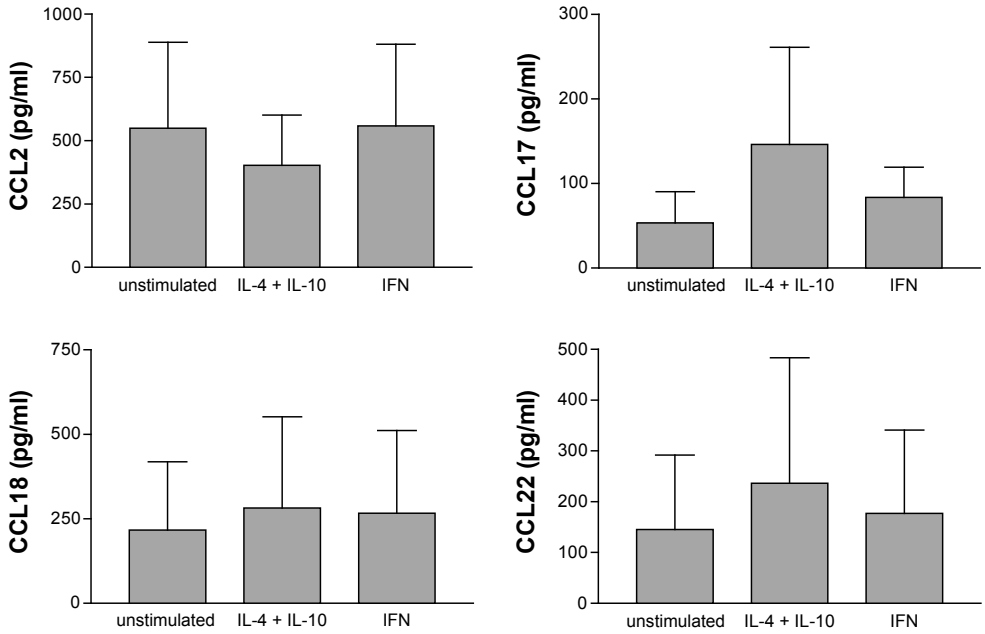

Figure 5. Levels of chemokine C-C motif ligand 2 (CCL2), CCL17, CCL18 and CCL22 in BAL cell culture supernatant after 24h either unstimulated or stimulated with IL-4 and IL-10 or IFN-γ. Mean ± SD in 3 healthy controls.

CCL17, CCL18 and CCL22 are markers for M2 macrophages. Highest upregulation after 24h stimulation with IL-4 and IL-10 was seen for CCL17 (average of 3 healthy controls). CCL17 was more 250% higher after stimulation, CCL22 increased with 60% and a 34% increase in CCL18 was observed after stimulation (figure 5). However, large differences were present between individuals; therefore, the influence of IL-4 and IL-10 stimulation did not reach significance for any of the CCL chemokines. Similar upregulation of CCL17, CCL18 and CCL22 was observed in 3 sarcoidosis patients, and in this case the upregulation of CCL22 after stimulation with IL-4 and IL-10 was significant ($p = 0.002$, paired t-test). When the healthy individuals and sarcoidosis patients were combined, the upregulation of CCL17 by IL-4 and IL-10 also became significant ($n = 6$, $p = 0.01$, paired t-test). Therefore, induction of M2 differentiation resulted in higher levels of M2 markers.

To investigate whether the M2 stimulation with IL-4 and IL-10 was optimal, we performed experiments with higher concentrations of these cytokines (100 ng/ml). This resulted in much higher levels of the M2 markers CCL17, CCL18 and CCL22 (figure 6). A similar result was obtained after stimulation with IL-13 (1 ng/ml) that should also induce M2 differentiation. Stimulation with higher levels of IFN-γ (100 U/ml) did not induce expression of M1 markers IL-1β or CCL2.

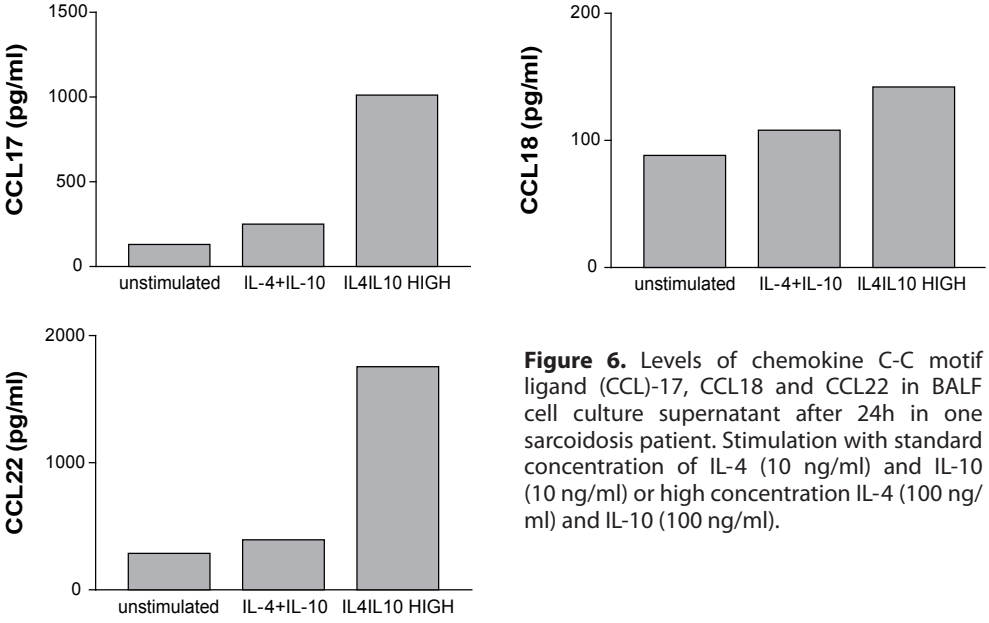

Figure 6. Levels of chemokine C-C motif ligand (CCL)-17, CCL18 and CCL22 in BALF cell culture supernatant after 24h in one sarcoidosis patient. Stimulation with standard concentration of IL-4 (10 ng/ml) and IL-10 (10 ng/ml) or high concentration IL-4 (100 ng/ml) and IL-10 (100 ng/ml).

Discussion

The aim of this study was to investigate whether YKL-40 is specifically produced by alternatively activated macrophages. We cultured alveolar macrophages in the presence of IL-4 and IL-10 to induce M2 differentiation and in the presence of IFN-γ to induce M1 macrophages.

The ELISA that was used to detect YKL-40 levels in culture supernatant was not sensitive enough and levels were often below the detection limit of this ELISA. YKL-40 levels in BAL fluid from IPF patients were previously shown to be very low.[2] This suggests that the observed levels in our experiments might still be biologically relevant. Levels in the culture supernatant differed between patients with different diagnosis. We found the highest YKL-40 levels in an IPF and rheumatoid arthritis patient after stimulation with IL-4 and IL-10, while in a sarcoidosis and LTX patient the highest levels were found after stimulation with IFN-γ. This suggests a difference in M1 and M2 induction between different diseases.

Subsequent efforts to quantify intracellular YKL-40 protein levels using western blot showed very little specific bands. Therefore, we turned to quantitative RT-PCR to measure YKL-40 mRNA levels. We found that YKL-40 expression after stimulation varied between healthy controls and patients. In patients the highest YKL-40 levels were observed after stimulation with IL-4 and IL-10, although this upregulation was not large. In one healthy control the highest upregulation was observed after stimulation with IFN-γ.

Our timeline experiment showed that 24 hours is probably not the optimum time point to observe YKL-40 mRNA expression because this expression was ten times higher after 48 hours of cell culture. This suggests that the optimum time for protein detection may be even longer.

Markers for M1 (IL-1β, CCL2) and M2 (CCL17, CCL18, CCL22) were measured in the culture supernatant after stimulation of AM towards an M1 or M2 phenotype. After induction of M2 differentiation higher levels of CCL17, CCL18, CCL22 were observed. In previous research by Pechkovsky *et al*[11] the observed upregulation of CCL18 was much higher than we observed in our study. Experiments with higher levels of IL-4 (100 ng/ml) and IL-10 (100 ng/ml) revealed that this did cause much higher levels of CCL17, CCL18 and CCL22. Additional experiments with IL-13 in two healthy controls

revealed that this also caused larger upregulation of CCL17, CCL18 and CCL22 than the combination of IL-4 and IL-10. Together this confirms that we were capable of inducing M2 differentiation and that this differentiation could be further optimized. In addition, it has previously been suggested that there are different M2 subtypes and that M2c macrophages in particular are involved in matrix deposition, tissue remodelling and tumour promotion.[20] This M2c phenotype is induced by IL-10 and characterized by the production of CCL18 while the M2a subtype is induced by IL-4 and IL-13.[20] We found that CCL17 and CCL22 were not upregulated by stimulation with only IL-10, while stimulation by IL-4 alone resulted in higher levels than the combination of IL-4 and IL-10 (results not shown). The differential induction of CCL17 and CCL22 by IL-4, and not by IL-10, suggests they represent the M2a subtype.

We found no effect of stimulation with IFN-γ on M1 markers. Stimulation with higher levels of IFN-γ also did not have an effect on expression of IL-1β or CCL2 suggesting that this expression was already at maximum and could not be further upregulated. Another possibility is that the IFN-γ solution may have been corrupted. A downregulation of IL-1β and CCL2 was observed after stimulation with IL-4 and IL-10 suggesting that the macrophage phenotype could be shifted towards an M2 phenotype.

A solution may be to use monocyte-derived macrophages. These macrophages are obtained by culturing peripheral blood mononuclear cells for several days in the presence of monocyte-colony stimulating factor (M-CSF). Previous studies suggest these macrophages can be more easily differentiated towards an M1 or M2 phenotype.[11] We have performed similar experiments and in some cases this resulted in YKL-40 levels that could be measured by ELISA, but no differences between stimulations were observed.

Recommendations for the future

Further experiments are needed to optimise the differentiation of AM to an M1 and M2 phenotype *in vitro*, before evaluating their potential to produce YKL-40. Production of YKL-40 by M2 macrophages may explain why YKL-40 is associated with survival in IPF. M2 macrophages and YKL-40 levels have independently been suggested to influence IPF disease progression. Elucidating the role of M2 macrophages and the proteins they produce in IPF may give important insights into disease aetiology and may lead to the discovery of therapeutic targets.

In conclusion, YKL-40 levels in BAL cell culture are low but mRNA experiments showed that BAL AM are capable of producing YKL-40. It remains undetermined whether YKL-40 is produced by AM with a particular phenotype. Our preliminary analysis suggests that induction of YKL-40 in AM differs between healthy controls and patients and between different diseases. Further research is needed to elucidate the relationship between macrophage differentiation and YKL-40 production and to discover whether the associations of both YKL-40 and alternatively activated macrophages with the progression of IPF is mediated by the same pathway.

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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

Summary

Idiopathic pulmonary fibrosis (IPF) is thought to be the result of tissue damage followed by aberrant wound healing and remodelling. Currently there are no molecular markers for IPF that can aid in diagnosis, prognosis and clinical decision making with regards to therapy. The aim of this thesis was to evaluate proteins and genes that play a role in the immunological response to injury and apoptosis for their potential as innovative clinical markers in IPF.

In **chapter 2**, single nucleotide polymorphisms (SNPs) in *TP53* (5 SNPs) and *CDKN1A* (4 SNPs) were determined in 77 IPF patients and 353 healthy controls. Two SNPs in *TP53*, the gene encoding tumour protein 53 (p53), were significantly associated with survival in IPF patients. Two SNPs in *CDKN1A*, the gene encoding p21, were significantly associated with an increased risk of developing IPF. Functional experiments showed that the risk allele correlated with lower p21 expression.
In damaged cells, upregulation of p53 induces apoptosis and overexpression of this protein may lead to an increased loss of cells. Induction of p21 results in cellular senescence and differentiation. Reduced levels of this protein could contribute to increased apoptosis, fibroblast growth, epithelial type II hyperplasia and cancer.

Starting with **chapter 3**, the influence of immunological processes on the pathogenesis of IPF was explored. In this chapter, BALF levels of MRP14 were determined in IPF patients, healthy controls and patients with different stages of sarcoidosis. MRP14 levels were highest in patients with IPF and patients with fibrotic sarcoidosis and higher MRP14 levels were associated with lower diffusion capacity of the lungs. This suggests that MRP14 might be a biomarker for fibrosing interstitial lung diseases. MRP14 was also correlated to BALF neutrophil percentage in IPF patients and may represent activated neutrophils. MRP14 is a protein that is highly expressed by neutrophils, macrophages and epithelial cells during chronic inflammation.

In **chapter 4**, the influence of a polymorphism in the gene encoding interleukin-1 receptor antagonist (IL-1Ra) on IPF susceptibility was evaluated. The 5 case-control studies that have investigated the association between the *IL1RN* gene and IPF were combined in a meta-analysis. This revealed that the *IL1RN* VNTR*2 haploblock is

significantly associated with susceptibility to IPF.
The influence of the VNTR polymorphism and another predisposing SNP, rs2637988, on protein expression was determined and this revealed that the risk alleles may limit the production of IL-1Ra. IL-1Ra is a cytokine that is an important inhibitor of the proinflammatory and profibrotic IL-1 cytokine. IL-1Ra is thought to be produced by alternatively activated macrophages.

In **chapter 5**, the prognostic value of serum and BALF YKL-40 levels was determined. Serum and BALF YKL-40 levels were significantly higher in IPF patients than in healthy controls and high levels were associated with poor survival. A polymorphism in *CHI3L1*, the gene encoding YKL--40, significantly influenced YKL-40 levels in serum and BALF of healthy controls, but it was not significantly associated with disease. YKL-40 is a potential marker for diseases involving inflammation, fibrosis and tissue remodelling. In IPF lungs, it is expressed by macrophages adjacent to fibroblast foci.

In **chapter 6**, the role of YKL-40 is examined further in interstitial pneumonias. Serum YKL-40 levels were found to be highest in patients with idiopathic NSIP, COP and IPF. Levels in idiopathic NSIP were significantly higher than those in NSIP associated with connective tissue disease. Idiopathic NSIP is associated with a worse prognosis, therefore serum YKL-40 levels may also be associated with prognosis in other interstitial pneumonias besides IPF. Follow-up of serum YKL-40 levels showed that levels generally remain stable over time in IPF patients while levels significantly decrease in COP patients. As COP patients often show complete remission, this suggests that serum YKL-40 levels are associated with the activity of the underlying fibrotic disease processes.

In **chapter 7**, we aimed to identify the cellular source of YKL-40 in a short pilot study. We hypothesized that YKL-40 is produced specifically by alternatively activated macrophages. We stimulated BAL cells for 24 hours with cytokines that are known to induce macrophage differentiation to the normal M1 phenotype or to the alternatively activated M2 phenotype, and determined their potential to produce YKL-40. YKL-40 levels were measured with ELISA, western blot and quantitative RT-PCR. Under these conditions YKL-40 levels were low. The results indicated that upregulation of YKL-40 production may vary between different diseases and between IPF patients,

sarcoidosis patients and healthy controls. Further experiments showed that longer incubation times may improve results and that macrophage differentiation could be optimized. Production of YKL-40 by M2 macrophages may explain why YKL-40 is associated with survival in IPF, since M2 macrophages have been suggested to influence IPF disease progression. Elucidating the role of M2 macrophages and the proteins they produce in IPF may give important insights into disease aetiology and may lead to the discovery of therapeutic targets.

General discussion

Evolving concepts of pathogenesis

The aetiology of IPF is complex and the discovery of diagnostic and prognostic biomarkers is important for clinical practise and could shed light on the cause of IPF. In the past, research aimed at unravelling IPF's cause and course had lead to the discovery of potential disease markers, and this resulted in a succession of matching hypotheses on IPF aetiology. As diagnostic criteria changed and the patient group became more accurate, these hypotheses became unsatisfying. They were subsequently revised or rejected as new concepts emerged.

IPF was first described as a new clinical and pathological entity called diffuse interstitial fibrosis by Hamman and Rich in the 1930s and 40s, although earlier case reports exist that are consistent with IPF.[1,2] At that time, tuberculosis was often proposed as the cause.[2] Hamman and Rich suggested that influenzal pneumonia, chemical irritants and hypersensitivity to various agents could be the cause.[1] Hereditary predisposition was also observed and reports on familial occurrence appear regularly since the 1950s.[2] Early reports described very short survival times of 8 to 24 weeks but they were followed by reports of more stable disease and it was soon recognized that the prognosis can be highly variable.[3]

When bronchoalveolar lavage (BAL) was introduced in the 1980s, it prompted the evaluation of the clinical usefulness of differential cell counts. Elevated percentages of neutrophil granulocytes and eosinophils were associated with a poor prognosis and it was proposed that the release of oxygen radicals by these cells damaged the lung.[4-7] Because the majority of BAL cells in IPF are alveolar macrophages, these cells were an interesting target for investigation. They were discovered to produce large

amounts of the cytokine interleukin-1.[8] Subsequently, many inflammatory cytokines were investigated and elevated levels were observed in both blood and BALF of IPF patients. The inflammation hypothesis was coined (figure 1), implicating the recruitment of inflammatory cells as the major cause of tissue injury in IPF. This has lead to extensive investigation of inflammatory markers and the assumption that treatment with anti-inflammatory corticosteroids would interrupt the inflammatory cascade and prevent fibrosis.[9,10] Some investigators proposed that the inflammatory response in IPF resembles a Th2-type immune response with increased amounts of IL-4 and IFN-γ.[11-13] However, as diagnostic criteria became more strict and other entities such as NSIP were clearly defined and excluded, it became apparent that anti-inflammatory drugs had no ameliorating effects on survival in IPF,[14] and the inflammation hypothesis was thoroughly rejected.[15-17] However, this rejection may have been somewhat premature. Recent evidence suggests that some inflammatory cytokines are profibrotic and that their effects are not influenced by anti-inflammatory drugs such as glucocorticoids.[18-20] Therefore, these immunological processes may indeed be involved in IPF disease development and progression.

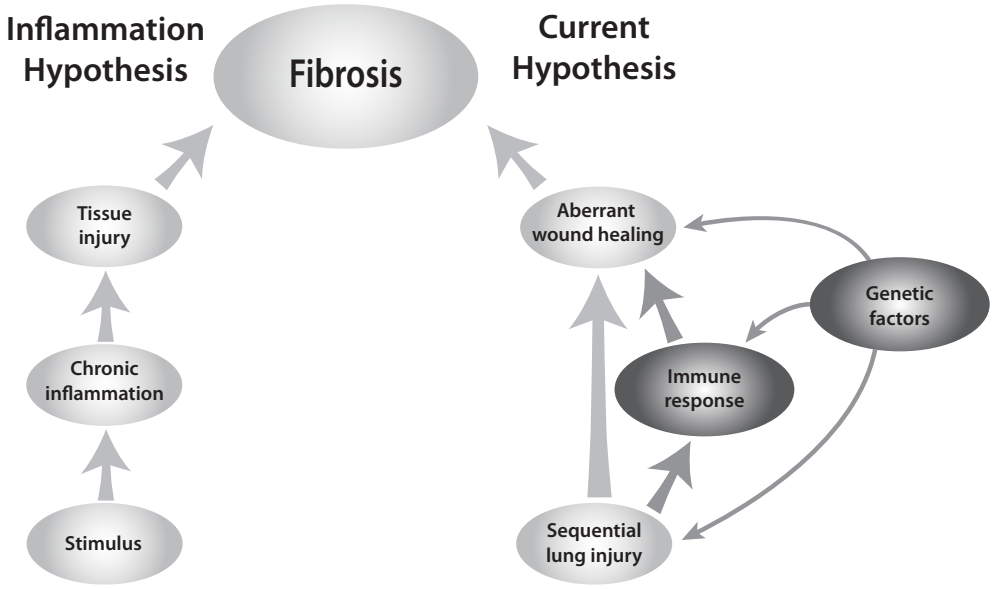

Figure 1. Schematic representation of the inflammatory hypothesis (left) and the current hypothesis on IPF pathogenesis (right) as described in this thesis.

After the rejection of the inflammatory hypothesis, the general consensus became that repeated epithelial injury is necessary to initiate the inflammatory and fibrotic response (figure 1). In the lungs of IPF patients, large amounts of apoptotic alveolar epithelial cells are seen and it is hypothesized that increased cell death contributes to the pathogenesis of IPF.[21-23] Loss of alveolar epithelial cells would lead to tissue damage that does not seem to be resolved in IPF. Instead, long rows of hyperplastic epithelial cells are seen near areas of remodelling. Because of this, we studied the influence of variations in two of the genes involved in cell death and proliferation. Variations in the gene encoding p21, which prevents cell division, were associated with an increased likelihood of developing IPF. The risk alleles also correlated with reduced levels of p21, suggesting that they would cause increased proliferation of cells. This is consistent with the rows of alveolar epithelial cells that are seen in IPF. In addition, variations in the gene encoding p53, a trigger of cell death, were associated with survival in IPF patients suggesting that increased cell death is indeed a part of IPF aetiology.

Tissue injury will allow foreign substances to enter the lung and leads to the influx of immune cells that form a protective shield by releasing antimicrobial substances. A cell type that is strongly associated with IPF is the neutrophil. Neutrophilia in the BAL has been associated with a poor prognosis, and is thought to promote fibrosis by the release of damaging oxygen radicals.[24,25] Markers that are associated with neutrophils may aid in diagnosis and prognosis especially when they represent activated neutrophils. One example of this is shown in our study of MRP14 levels in the BALF of IPF and sarcoidosis patients. We showed that it could be a marker for fibrotic interstitial lung diseases and that it may represent activated neutrophils in the lung. Activated neutrophils are associated with acute lung injury and may be overrepresented in fibrotic interstitial lung disease.[26] Thus, the phenotype of the neutrophil may be an important factor determining the development of fibrosis and it is suggested that other immune cells may also have a different phenotype in IPF patients.

The latest discovery that has inspired research in many fields of pulmonary medicine is the concept of different types of macrophages with different functions and morphologies. Remarkably, both IL-1Ra and YKL-40 are thought to be produced by a subset of macrophages that are often referred to as activated macrophages.[27-31] IL-1RA and IL-1β are thought to be markers of two distinct types of macrophages

referred to as M2 and M1, respectively.[29] M1 macrophages are recruited and participate in the antimicrobial response by releasing reactive oxygen species, IL-1 and tumour necrosis factor (TNF). M2 macrophages are thought to be important for resolving the wound repair process. They are anti-inflammatory and induce fibrosis by producing anti-inflammatory cytokines such as IL-1Ra and profibrotic cytokines such as TGF-β. Recent evidence suggests that M2 macrophages are over-induced in IPF and may predict survival.[32] Remarkably, M2 macrophages are induced by Th2 cytokines,[29] and glucocorticoids,[33,34] indicating that the inflammatory hypothesis should be reconsidered and perhaps be incorporated in the current hypothesis.

Our study on genetic variations in *IL1RN* confirmed that they increase the likelihood of developing IPF. These genetic variations were associated with lower levels of IL-1Ra and suggest that levels in IPF patients are not sufficient to prevent the initial damaging effects of IL-1. However, it is difficult to reconcile these findings with the concept that IL-1Ra producing M2 macrophages are profibrotic and influence prognosis. The explanation may be found in the events that trigger the initiation of damage in IPF. These are very different from the chronic responses that occur later in the disease and lead to extensive fibrosis. Therefore, genetic variations that increase the risk of developing IPF may differ from variations that influence response processes and disease progression. This illustrates the importance of determining genetic variations that predispose to disease and variants that determine disease progression. Predisposing variations act at a time when the patient is still healthy. These genetic variations give vital clues about the initial stages of disease and the primary pathogenic processes. When a patient reports to the clinic, extensive remodelling may already be present and factors that determine disease progression may be secondary to the initial processes. IPF is a progressive disease and although therapies that target secondary factors of disease progression may have beneficial effects, the cure for IPF will only be found when initiating disease processes can be successfully altered.

Future directions

In the current concept of IPF pathogenesis, the connection between the damaged alveolar epithelium and the induction of the profibrotic immune response that leads to aberrant wound healing needs further investigation. Little is known about the signals that are released by the damaged alveolar epithelium. Recruitment and activation of neutrophils and macrophages might be mediated by the alveolar epithelium, but the exact mechanisms remain elusive. MRP14, IL-1β and YKL-40 are also produced by the epithelium and may be important factors in neutrophil and macrophage activation.[28,35,36] The interaction between the damaged epithelium and immune cells may be the determinant in the aetiology of IPF. The immune response to epithelial injury could be responsible for the aberrant wound healing processes that lead to fibrosis in IPF.

In addition, it is becoming apparent that the classification of immune cells in the lung may be more complex than previously thought. More knowledge about the different macrophage phenotypes, and the proteins they produce, may result in a better understanding of IPF aetiology. For instance, it has been suggested that YKL-40 is produced by activated macrophages, but the exact phenotype that is associated with this YKL-40 production remains to be discovered. Finding the source of YKL-40 may provide clues to the role it plays in diseases with inflammation, fibrosis and tissue remodelling.

Genetic variations that influence epithelial injury and the subsequent immunological response can predispose to IPF and predict prognosis. Further studies of the genetics of IPF and investigation of the immune cells involved in promoting the profibrotic milieu in the lung may lead to the discovery of novel biomarkers and targets for therapy.

In conclusion, the aetiology of IPF is now thought to involve repeated epithelial injury followed by excessive wound repair. The role of inflammation as a major cause of IPF has become controversial, but a new role for immunological processes is emerging. Genes and proteins involved in apoptosis and the immunological response to injury can predict susceptibility to IPF and the progression of this devastating disease.

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NEDERLANDSE SAMENVATTING

Inleiding

Idiopathische pulmonale fibrose (IPF) is een ziekte die gekenmerkt wordt door fibrose, ofwel littekenvorming, in de longen. De fibrosevorming vindt plaats in het interstitium, het weefsel onder het oppervlak van de longblaasjes (alveoli). Normaal is dit weefsel heel dun, zodat zuurstof uit de lucht kan worden opgenomen in het bloed. Maar door de fibrose in het interstitium kan er onvoldoende zuurstof naar het bloed worden getransporteerd. Bij IPF-patiënten is de fibrosevorming chronisch en voortschrijdend. Als de fibrose eenmaal is gevormd is deze blijvend. Hierdoor heeft een IPF-patiënt een zeer slechte levensverwachting. De meeste patiënten zijn binnen vier jaar na de diagnosestelling overleden. Helaas is de ziekte moeilijk te behandelen en is er tot op heden geen werkend medicijn voorhanden. Momenteel is een longtransplantatie de enige mogelijkheid om patiënten zicht te bieden op een langere overleving.

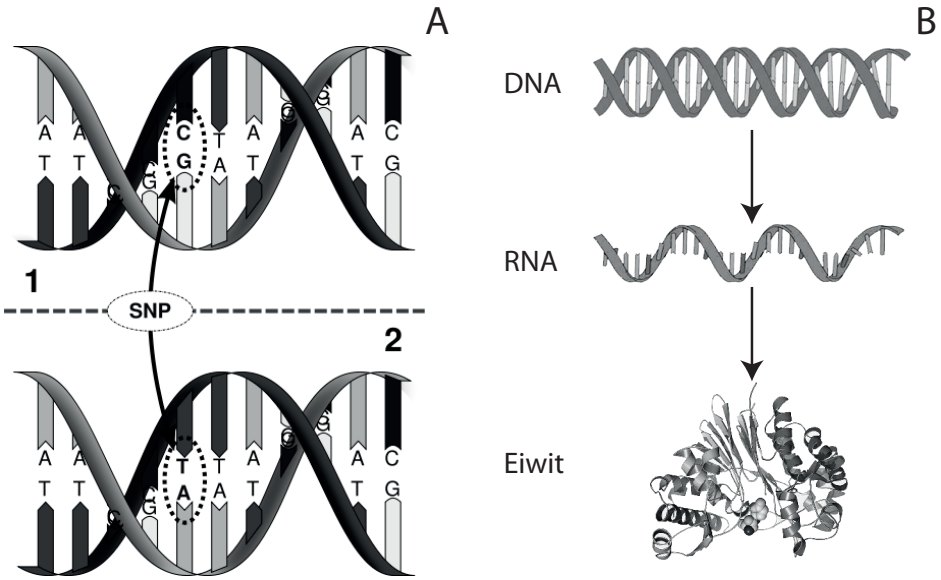
IPF komt vooral voor bij oudere mannen. Ongeveer 55-80% van de patiënten is man en de gemiddelde leeftijd bij diagnose is 60-65 jaar. In 2-19% van de gevallen komt de ziekte in de familie voor en is er dus een erfelijke component.
Er is geen eenvoudige meting voorhanden om de diagnose van IPF stellen. Daarom wordt de diagnose gebaseerd op een combinatie van bevindingen. De meeste patiënten hebben last van toenemende kortademigheid en hoesten. Longfunctieonderzoek toont een verminderde gaswisseling en een daling van het longvolume.
De belangrijkste diagnostische informatie wordt verkregen via hoge resolutie computertomografie (HRCT) scan. Dit is een vorm van zeer gedetailleerde röntgenfoto waarop de fibrose beter te zien is. Een typisch HRCT-patroon voor IPF laat met name in de periferie en basaal afwijkingen zien en wordt geclassificeerd als een usual interstitial pneumonia (UIP)-patroon. Opvallend is dat de afwijkingen heterogeen zijn: gebieden die zijn aangedaan worden afgewisseld met gebieden die niet zijn aangedaan.
Soms is aanvullende informatie nodig om de diagnose IPF te kunnen stellen. Dan kan worden overwogen om een longbiopsie uit te voeren. Dit houdt in dat operatief een klein stukje long wordt uitgenomen. De typische bevindingen in een longbiopt worden ook een UIP-patroon genoemd. Hierbij zijn ook heterogene afwijkingen

te zien die voornamelijk aan de longwanden grenzen. Omdat een longbiopsie een risicovolle operatie is wordt gezocht naar andere manieren om de diagnose IPF te stellen.
Een van de alternatieven die onderzocht worden is de longspoeling of bronchoalveolaire lavage (BAL). Hierbij wordt, tijdens een bronchoscopie, een isotone vloeistof ingebracht in de longen en weer opgezogen. De cellen en eiwitten die in deze vloeistof zitten worden bestudeerd.

De oorzaak van IPF is onbekend. Potentiële risicofactoren in het kader van IPF zijn roken, chronische aspiratie (inademing) van maaginhoud en bepaalde omgevingsfactoren, zoals contact met hout-, steen- en metaalstof. De huidige hypothese stelt dat er herhaaldelijk schade optreedt aan het oppervlak van de longblaasjes (alveolair epitheel), waarna een abnormale wondhelingsreactie leidt tot fibrose. Verschillende celtypen zouden hierbij betrokken kunnen zijn. Het is onduidelijk of en welke van deze cellen afwijkend zijn in IPF. Fibroblasten zijn cellen die direct betrokken zijn bij wondherstel en littekenvorming. Bij IPF liggen grote groepen fibroblasten, zogenaamde fibroblasten foci, onder het alveolaire epitheel. Het alveolaire epitheel is op sommige plekken verdwenen, beschadigd, of stervende terwijl op andere plekken juist cellen aan het delen zijn. Daarnaast is er een reactie van het immuunsysteem waarbij verschillende witte bloedcellen betrokken zijn.

Doel van het proefschrift

Het doel van het onderzoek in dit proefschrift is het evalueren en vinden van merkers die kunnen helpen bij het stellen van de diagnose IPF. Potentiële merkers worden onderzocht in IPF-patiënten en vergeleken met andere longziekten die moeilijk van IPF te onderscheiden zijn. Deze ziektespecifieke merkers kunnen daarnaast bijdragen aan inzichten over de oorzaak van de ziekte.
In dit proefschrift worden eiwit niveaus onderzocht die zijn gemeten in het serum en in de BAL-vloeistof van patiënten. Daarnaast zijn er genetische variaties onderzocht. Deze genetische variaties, zogenaamde single nucleotide polymorphisms (SNPs), betreffen een verandering van 1 nucleotide in een gen (figuur 1A). Deze 1-punts veranderingen kunnen invloed hebben op het eiwit waar het gen voor codeert. De volgorde van nucleotiden bepaalt namelijk hoe een eiwit eruit komt te zien en hoeveel ervan gemaakt wordt (figuur 1B).



Figuur 1. A) Schematische representatie van een single nucleotide polymorphism (SNP). Persoon 1 heeft op een bepaalde locatie in het DNA het nucleotide cytosine (C) op de ene streng en het nucleotide guanine (G) op de andere streng (omcirkeld door een stippellijn). Persoon 2 heeft op dezelfde lokatie een thymine (T) en een adenine (A). Door het verschil van nucleotiden op die ene plek verandert de genetische informatie die nodig is om het eiwit te bouwen waar dit gen voor codeert. De hoeveelheid of de functie van het eiwit dat gebouwd wordt kan hierdoor veranderen.
B) Schematische representatie van de mogelijke bronnen van moleculaire merkers en hun samenhang. In dit proefschrift zijn SNPs bepaald in het DNA, en de hoeveelheden RNA en eiwit zijn gemeten.

Hoofdstuk 2: Variaties in celcyclus genen zijn geassocieerd met IPF.

In dit hoofdstuk worden SNPs in de genen *TP53* en *CDKN1A* onderzocht in 77 IPF-patiënten en 353 gezonde controles. De genen *TP53* en *CDKN1A* coderen respectievelijk voor de eiwitten p53 en p21. Deze eiwitten zijn betrokken bij de celcyclus. Ze bepalen of een cel kan delen, in rust gaat, of sterft (apoptose). Dit zijn belangrijke processen bij groei, wondherstel en ziektes zoals kanker. Twee SNPs in *TP53* zijn significant geassocieerd met de overlevingstijd in IPF patiënten. Varianten van twee SNPs in *CDKN1A* komen vaker voor bij IPF patiënten dan bij gezonde controles, wat een verhoogd risico op IPF suggereert. Een van deze SNPs in *CDKN1A* lijkt ook een snellere achteruitgang in longfunctie te veroorzaken. Deze varianten blijken te resulteren in lagere niveaus van *CDKN1A*.

Overexpressie van p53 in beschadigde cellen kan leiden tot apoptose en dus het verlies van cellen. Een gereduceerd p21 niveau kan bijdragen aan deze apoptose maar kan ook leiden tot een toename van fibroblasten en kanker.

Hoofdstuk 3: MRP14 is verhoogd in de bronchoalveolaire lavagevloeistof van fibroserende interstitiële longziekten.

In dit hoofdstuk zijn MRP14 niveaus bepaald in de BAL vloeistof van patiënten met IPF, patiënten met sarcoïdose en van gezonde controles. De hoogste MRP14 niveaus zijn gevonden in patiënten met IPF en patiënten met fibroserende sarcoïdose. Hogere niveaus van MRP14 hangen samen met een verminderde diffussiecapaciteit van de longen. Dit suggereert dat MRP14 een merker zou kunnen zijn voor fibroserende interstitiële longziekten. In IPF-patiënten hangt MRP14 ook samen met het percentage neutrofielen in de BAL. MRP14 wordt geproduceerd door neutrofielen, macrofagen en epitheliale cellen tijdens chronische ontstekingen en zou een merker kunnen zijn voor geactiveerde neutrofielen.

Hoofdstuk 4: Associatie tussen variaties in *IL1RN* en IPF: een meta-analyse en mRNA expressiestudie.

In dit hoofdstuk is gekeken naar de invloed van variaties in *IL1RN* op de kans om IPF te ontwikkelen. De vijf studies die de associatie tussen het *IL1RN* gen en IPF hebben onderzocht zijn gecombineerd in een meta-analyse. Het resultaat toont aan dat het *IL1RN* VNTR*2 haploblok significant vaker voorkomt bij patiënten met IPF. Dragers van deze variant hebben 29% meer kans om IPF te krijgen.
Ook is in dit hoofdstuk gekeken naar de invloed van de VNTR SNP en een andere SNP, rs2637988, op de genexpressie. De risicovarianten bleken te leiden tot lagere niveaus van interleukine-1 receptor antagonist (IL-1Ra). Dit is een cytokine en een belangrijke remmer van IL-1 dat een rol speelt bij fibrose en inflammatie. IL-1Ra wordt o.a. geproduceerd door alternatief geactiveerde macrofagen.

Hoofdstuk 5: Serum en BAL YKL-40 niveaus voorspellen de overleving in IPF-patiënten.

In dit hoofdstuk wordt de prognostische waarde van YKL-40 niveaus in serum en BAL vloeistof bepaald. YKL-40 is verhoogd in serum en BAL vloeistof van IPF-patiënten. Hogere niveaus hangen samen met een slechtere prognose. Een SNP in het gen dat codeert voor YKL-40, *CHI3L1*, is significant geassocieerd met YKL-40 niveaus

in gezonde controles. Deze SNP heeft echter geen invloed op het ontstaan of het verloop van IPF. YKL-40 is verhoogd in ziektes waar inflammatie, fibrose en weefsel veranderingen een rol spelen. In IPF-patiënten wordt YKL-40 geproduceerd door macrofagen die gelegen zijn naast fibroblasten foci.

Hoofdstuk 6: Serum YKL-40 niveaus gedurende het ziekteverloop in IPF en vergelijking met andere interstitiële pneumonieën.

De rol van YKL-40 in interstitiële longziekten wordt in dit hoofdstuk verder onderzocht. Het hoogste serum YKL-40 is gevonden in patiënten met idiopathische NSIP, COP en IPF. Dit zijn de interstitiële pneumonieën die gekenmerkt worden door veel fibrose. De YKL-40 niveaus zijn hoger in patiënten met idiopathische NSIP (dus zonder bekende oorzaak) dan in patiënten waar de NSIP geassocieerd is met een systeemziekte. Omdat idiopathische NSIP een slechtere prognose heeft lijken de YKL-40 waarden in NSIP dus samen te hangen met de prognose.
Gedurende het ziekteverloop in IPF blijven de serum YKL-40 niveaus relatief stabiel terwijl in COP-patiënten de niveaus significant dalen. Opmerkelijk genoeg kan een COP-patiënt volledig herstellen waarbij ook de fibrose verdwijnt. De serum YKL-40 niveaus lijken dus samen te hangen met het onderliggende fibrotische proces.

Hoofdstuk 7: YKL-40 productie door alveolaire macrofagen: een eerste opzet.

In dit hoofdstuk wordt geprobeerd te achterhalen welk celtype in de long de bron is van YKL-40. De hypothese is dat YKL-40 in de long geproduceerd wordt door alternatief geactiveerde macrofagen. Deze witte bloedcellen zijn betrokken bij wondherstel en fibrose. Bij deze studie werden cellen uit de BAL vloeistof gedurende 24 uur blootgesteld aan stoffen die de macrofagen activeren. Er zijn twee typen geactiveerde macrofaag, de M1 en M2, die door verschillende stimulerende stoffen ontstaan. Van deze twee typen werd vervolgens gemeten of ze YKL-40 produceren. De YKL-40 productie bleek vrij laag te zijn en de beste meetmethode was het meten van RNA expressie in de cellen. Uit het onderzoek bleek dat er verschillen zijn in YKL-40 productie tussen IPF- en sarcoïdosepatiënten en gezonde controles. Verder onderzoek is nodig om deze verschillen goed in kaart te brengen. Daarbij kan een langere incubatietijd de resultaten verbeteren. Tevens moet de activatie van de macrofagen nog verder verbeterd worden.

Conclusie

In dit proefschrift werden verscheidene potentiële merkers voor IPF onderzocht. Als eerste werden genetische variaties onderzocht die betrokken zijn bij de celcyclus en apoptose. Deze bleken samen te hangen met het risico om IPF te ontwikkelen en met de prognose. Hoewel al bekend was dat de eiwitten p53 en p21 verhoogd aanwezig zijn in de longen van IPF-patiënten, waren de genetische variaties nog nooit eerder met IPF geassocieerd. Deze bevindingen kunnen bijdragen aan het achterhalen van de oorzaak van IPF.

Het is opmerkelijk dat de andere merkers die werden gevonden allemaal betrokken zijn bij specifieke immuunreacties. Zowel MRP14, IL-1Ra als YKL-40 worden geproduceerd door geactiveerde macrofagen. Er is nog niet zo veel bekend over deze macrofagen en hun functie maar ze lijken betrokken bij de immuunrespons tijdens wondherstel. Ze zouden kunnen worden geactiveerd door beschadigde epitheel cellen waarbij ze bijdragen aan het aantrekken en stimuleren van fibroblasten. Dit is een normale reactie bij een wond maar zou bij IPF-patiënten wel eens uit de hand gelopen kunnen zijn.
Als YKL-40 geproduceerd word door M2-macrofagen kan dat verklaren waarom YKL-40 niveaus samenhangen met de prognose in IPF. M2-macrofagen zijn verhoogd aanwezig in de longen van IPF patiënten en zouden invloed kunnen hebben op de prognose. Verder onderzoek naar deze M2-macrofagen is nodig om meer inzicht te krijgen hun functie en hun invloed op de ziekteprocessen bij IPF.
Meer inzicht in de immuunrespons bij IPF zou kunnen leiden tot nieuwe medicijnen.

Interessant is dat in dit proefschrift merkers worden beschreven die samenhangen met de prognose van IPF-patiënten maar ook merkers die invloed hebben op het risico om IPF te krijgen. Een van de moeilijkheden bij het achterhalen van de oorzaak van IPF is dat de meeste patiënten de ziekte al een tijd hebben voor ze er last van krijgen. Als patiënten in het ziekenhuis komen zijn er vaak al ernstige afwijkingen aanwezig in de long en is er al een flinke immuunreactie op gang gekomen. Het is dan moeilijk te achterhalen of dit een oorzaak is van de ziekte of een gevolg. Daarom is het erg nuttig om onderzoek te doen naar genetische variaties. Deze zijn namelijk ook al aanwezig als de patiënt nog gezond is en kunnen zo inzicht geven in de periode voordat de patiënt ziek wordt.

Aanbevelingen voor de toekomst

Tot voor kort werd gedacht dat ontstekingen en inflammatie geen belangrijke component zijn van de oorzaak van IPF. Er is wel inflammatie in de long van IPF-patiënten, maar dit werd gezien als aspecifiek en niet bijdragend aan het ziekteproces. De bevindingen uit dit proefschrift laten zien dat er wel degelijk een rol is voor immuunreacties in IPF. Ze hangen samen met de prognose van IPF en met het risico om IPF te ontwikkelen. Wel lijkt het te gaan om een specifieke immuunreactie van een subtype macrofaag. Verder onderzoek is nodig om meer inzicht te krijgen in de immuunreacties die betrokken zijn bij het ontstaan en het verloop van IPF. Ook de exacte rol van de macrofaag en hoe deze cel er precies uitziet, vergt nog meer onderzoek.

Daarnaast is meer inzicht nodig in de rol van het beschadigde epitheel in de long van IPF-patiënten. Beschadigde en stervende cellen geven signalen af die moeten zorgen voor het correct opruimen van de cellen. Als dit niet gebeurt, kan het immuunsysteem de stervende cellen als gevaarlijk gaan zien. Er worden dan witte bloedcellen zoals neutrofielen en macrofagen aangetrokken. Deze cellen kunnen juist nog ergere schade veroorzaken en bijdragen aan de vorming van fibrose. Hoewel er weinig bekend is over de signalen die afgegeven worden door het epitheel, zijn er aanwijzingen dat MRP14 en YKL-40 zulke signaalmoleculen zijn.

Tot slot blijft de kennis over witte bloedcellen toenemen en wordt het steeds duidelijker dat de eenvoudige classificatie niet altijd meer volstaat. De eigenschappen van witte bloedcellen kunnen veranderen onder verschillende omstandigheden en er zijn reeds een aantal verschillende subtypen geïdentificeerd. Er moet meer onderzoek gedaan worden naar de verschillende immunologische subtypen en hun potentiële rol in IPF. Dit zou inzicht kunnen geven in de oorzaak van IPF en kunnen leiden tot de ontwikkeling van medicijnen.

DANKWOORD

CURRICULUM VITAE

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Dank jullie wel,

Nikki

Curriculum vitae

Nicoline Korthagen was born on February 20th 1981 in Maarssen, the Netherlands. After graduating from College de Klop in Utrecht in 1999, she studied Science and Innovation Management at the Utrecht University for one year before switching to Biology. She quickly developed a preference for cellular and molecular biology with an emphasis on *in vitro* modelling for human tissues and diseases. After obtaining her master's degree in 2006, she started working as a researcher lung diseases in the ILD-group of the St Antonius Hospital in Nieuwegein. She investigated molecular markers for diagnosis and prognosis in Idiopathic pulmonary fibrosis (IPF).
With her research on the protein YKL-40, and its predictive value for survival in patients with IPF, she has won the 2010 Young Investigator Award from the Netherlands Respiratory Society. In 2011 she received a research fellowship from the European Respiratory Society for a three months stay at Freiburg University (Germany) to perform *in vitro* studies with lung macrophages. At the 2011 international WASOG conference, she won an award for excellence in Dutch pulmonary fibrosis research for her abstract on variations in cell cycle genes in IPF.
Nicoline lives in Utrecht and is married to André Dales (2010).