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Serum and BALF YKL-40 levels are predictors of survival in idiopathic pulmonary fibrosis

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KEYWORDS

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Summary

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.

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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.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a rapidly progressing disease that is characterized by remodelling of the lung parenchyma. Its aetiology remains unclear but tissue remodelling in IPF is thought to be caused by pneumocyte dysfunction, fibroblast proliferation, and chronic inflammation.^{1,2,3} While the median survival time is only 2.5–3.5 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 remodelling.^{10,11,12,13,14,15}

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.^{17,18,19} 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 extent 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 methods²⁴ 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 s (FEV₁), vital capacity (VC), FEV₁/VC ratio, diffusion capacity of the lungs for carbon monoxide (DLCO) and lung transfer coefficient (KCO). 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

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
Pack year	18 ± 12	NA	NA
DLCO, % predicted	48 ± 18	NA	NA
FEV ₁ , % predicted	82 ± 23	NA	106 ± 11
FVC, % predicted	78 ± 23	NA	110 ± 11
Blood monocytes, %	8 ± 3	NA	8 ± 2
Blood neutrophils, %	63 ± 13	NA	56 ± 8
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.

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

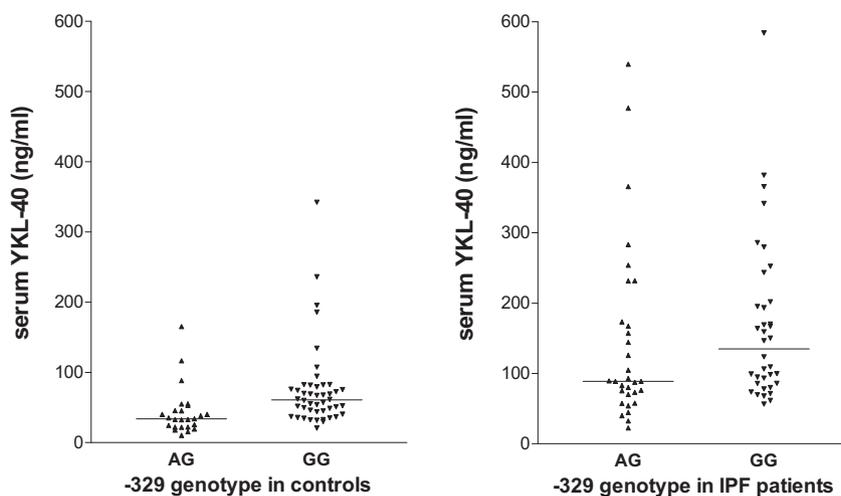


Figure 1 Serum YKL-40 levels in patients and healthy controls. In controls, serum YKL-40 levels were significantly higher ($P < 0.01$) in the GG group ($n = 46$) than in the AG group ($n = 25$). The effect of genotype on serum levels in patients was less pronounced ($P = 0.07$).

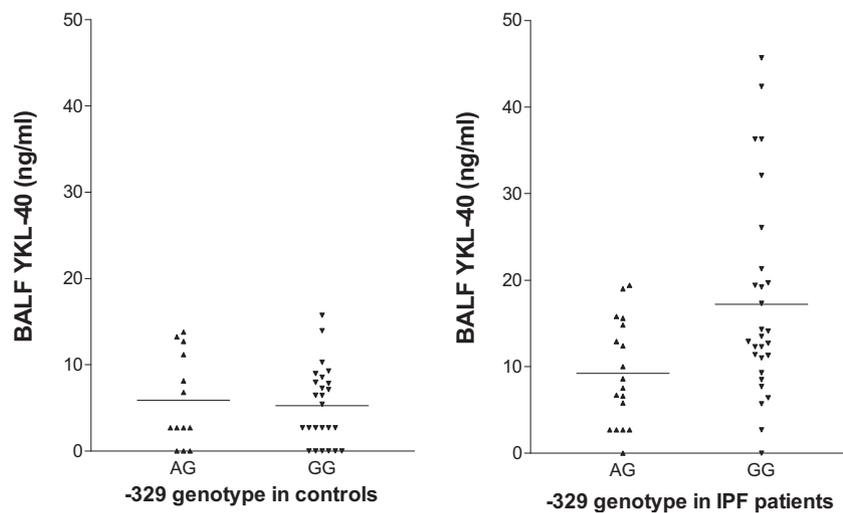


Figure 2 BALF YKL-40 levels in patients and controls. BALF YKL-40 levels were not associated with genotype in controls. In patients, BALF YKL-40 levels were significantly higher ($P = 0.01$) in the GG group ($n = 28$) than in the AG group ($n = 18$).

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 (Fig. 1). 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). In patients, BALF YKL-40 levels were genotype dependent ($P = 0.01$, Fig. 2) but no difference was found in controls.

We did not find a relevant correlation between serum and BALF YKL-40 levels (Fig. 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 ($D' = 1$, $r^2 = 0.85$).

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 ($P < 0.01$, Fig. 4 A). 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, $p < 0.01$).

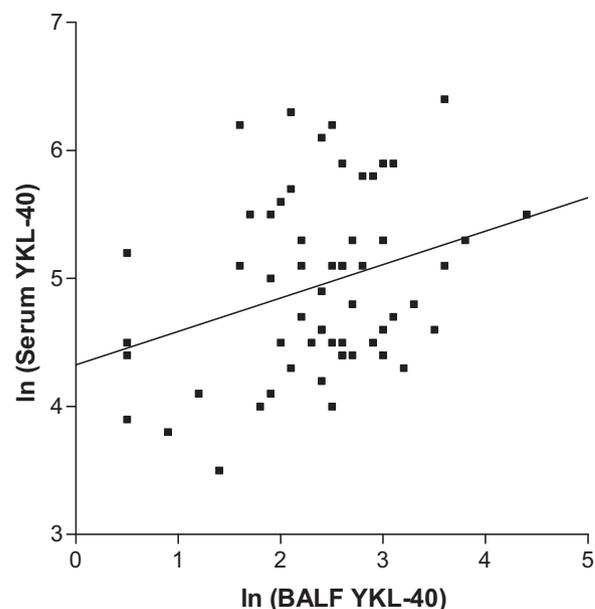


Figure 3 Correlation between Serum and BALF YKL-40 levels in IPF patients ($n = 58$). $R^2 = 0.09$ $P < 0.05$.

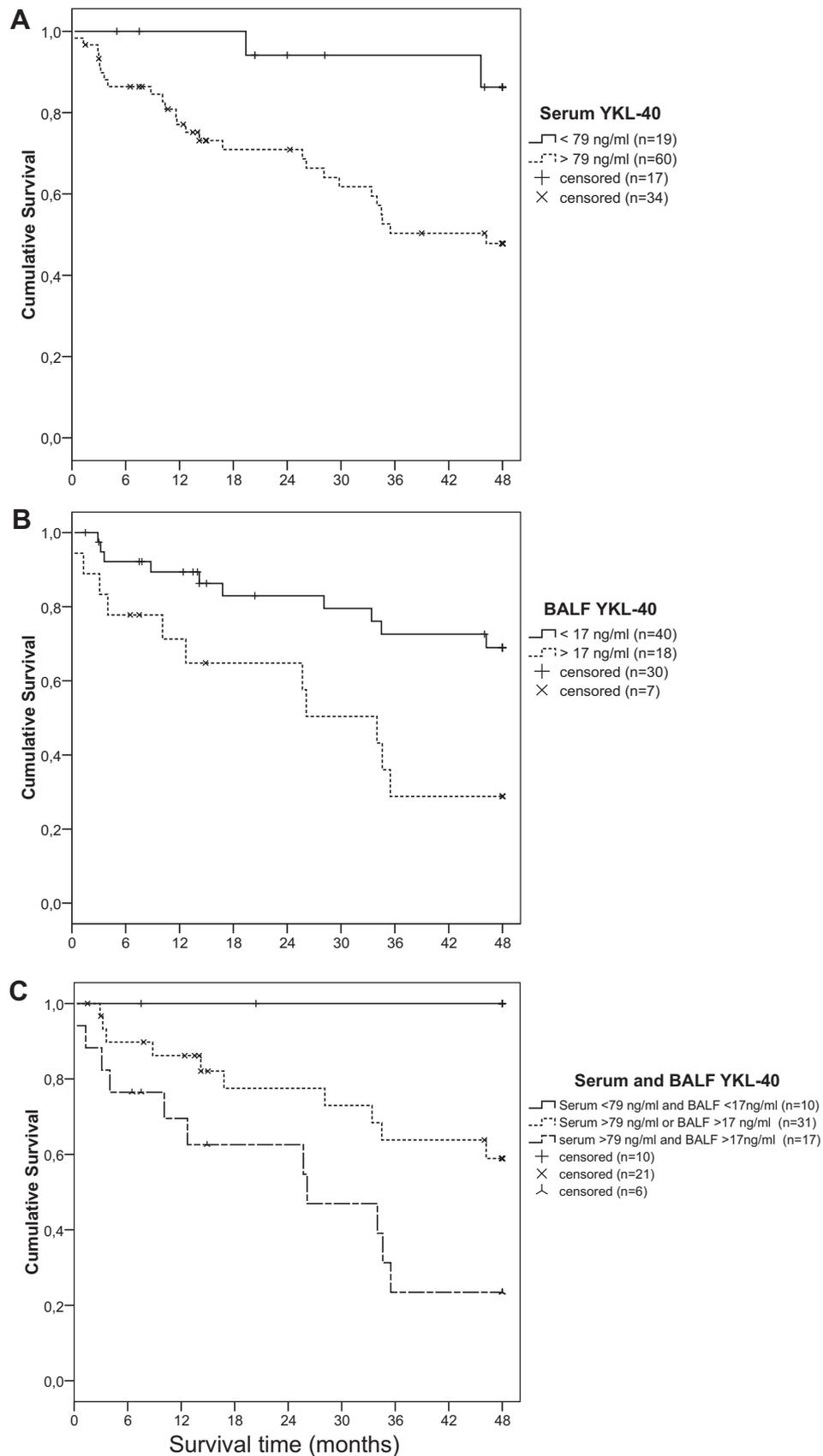


Figure 4 Kaplan–Meier survival analysis grouped by baseline YKL-40 levels. Patients that were still alive, that were transplanted or died from a cause unrelated to IPF were censored in the survival analysis. (A) Patients with high serum YKL-40 had significantly worse survival estimates than patients with low serum YKL-40 ($P < 0.01$, $\chi^2 = 6.77$). (B) Patients with high BALF YKL-40 had

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 ($P < 0.001$, Fig. 4 B). 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 ($P = 0.001$, Fig. 4 C). 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, $p < 0.01$).

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 (Fig. 4 A, 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 (Fig. 4 C).

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 3–4 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 fibrosis²² 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-40.¹⁶ 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

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 ($p < 0.01$, $\chi^2 = 13.09$).

of profibrotic and proinflammatory cytokines by alveolar macrophages and could thus contribute to tissue remodeling 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 IL13/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.

Conflict of interest statement

None of the authors of this study have any potential conflict of interest.

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