Serum YKL-40 as predictor of outcome in hypersensitivity pneumonitis

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ABSTRACT YKL-40, a chitinase-like protein mainly secreted by macrophages, neutrophils and epithelial cells, is increased in patients with idiopathic interstitial pneumonia and sarcoidosis. We aimed to investigate the role of YKL-40 as a biomarker in hypersensitivity pneumonitis (HP).

72 HP patients, 100 interstitial lung disease (ILD) controls and 60 healthy controls were studied. YKL-40 was measured by ELISA in serum and bronchoalveolar lavage fluid (BALF) at baseline and follow-up. The relationship between YKL-40 levels, clinical variables and disease outcome was evaluated.

Baseline serum YKL-40 levels were significantly higher in HP patients than in healthy controls ($p<0.001$), but lower than in patients with other ILDs. Baseline BALF YKL-40 levels in HP patients were the highest among ILD patients. In HP patients, serum YKL-40 correlated with the diffusing capacity of the lung for carbon monoxide at baseline ($p<0.01$) and over time ($p<0.001$). HP patients whose disease progressed or who died had higher baseline YKL-40 levels than those who remained stable and survived ($p<0.001$). At a cut-off of 119 $ng{\cdot}mL^{-1}$, the baseline serum YKL-40 level predicted disease progression (hazard ratio 6.567; $p<0.001$), and at a cut-off of 150 $ng{\cdot}mL^{-1}$ was associated with mortality (hazard ratio 9.989; $p<0.001$).

Serum YKL-40 may be a useful prognostic biomarker in HP patients.

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Introduction

Hypersensitivity pneumonitis (HP), also called extrinsic allergic alveolitis, is a T-cell-driven immunologic lung disease caused by repeated exposure to organic particles in susceptible subjects [1, 2]. Type III and type IV hypersensitivity reactions are involved in the pathogenesis of HP [3]. HP may progress to severe pulmonary fibrosis [1, 4]. Therefore, there is a need to identify noninvasive biomarkers that can predict disease outcome in HP patients.

YKL-40 is a chitinase-like protein [5] mainly secreted by macrophages, neutrophils and epithelial cells, which is involved in the inflammatory response to tissue injury [6, 7]. It is best known as a biomarker for diseases that are characterised by inflammation, fibrosis and tissue remodelling [6, 8–10]. For instance, circulating levels of YKL-40 have been found to be associated with liver fibrosis [9], Crohn's disease [11] and rheumatoid arthritis [12]. Recently, YKL-40 has been proposed as a diagnostic and prognostic biomarker for various forms of interstitial lung disease (ILD), especially idiopathic pulmonary fibrosis (IPF) and sarcoidosis [8, 13, 14]. Whether YKL-40 is also associated with HP is unknown.

The aim of the present study was to determine the clinical utility of YKL-40 as a biomarker in HP patients. We hypothesised that YKL-40 is elevated in HP patients and that YKL-40 level is associated with disease outcome. Some of the results of this study have been previously reported in an abstract [15].

Materials and methods

Study subjects

We retrospectively studied 72 HP patients and 100 ILD controls (45 patients with IPF, 34 with idiopathic nonspecific interstitial pneumonia (iNSIP) and 21 with cryptogenic organising pneumonitis (COP)), who were followed in our institution between January 2007 and December 2013. 60 volunteers served as healthy controls. The diagnosis of HP and ILD was based on clinical/high-resolution computed tomography (HRCT) findings, bronchoalveolar lavage fluid (BALF) characteristics and/or histopathological findings on biopsy [1, 16–19]. Acute, subacute and chronic HP were defined as previously described [1, 16]. The study was approved by the local institutional review board (approval number 15-6486-BO) and the experiments complied with current laws in Germany. Informed consent was obtained from the ILD patients and healthy controls.

Definition of disease progression in HP

Disease progression was defined as deterioration of self-reported symptoms (worsening of dyspnoea, cough, chest pain and weight loss), and/or lung function (decrease in forced vital capacity (FVC) >10% and/or diffusing capacity of the lung for carbon monoxide (D_{LCO}) ⩾15%), and/or chest imaging (increase in existing or appearance of new infiltrates compatible with HP) since the last follow-up visit [20, 21]. Otherwise, the patients were defined as stable/improved.

YKL-40 and lactate dehydrogenase laboratory assays

Serum samples were obtained from all patients at the first evaluation and in 39 HP patients also during follow-up at intervals ranging from 6 to 36 months. A total of 59 follow-up measurements were obtained. The samples were stored at −20°C or −80°C until analysis. Bronchoalveolar lavage was performed *via* a fibreoptic bronchoscope as previously described [22]; the supernatant was collected and stored at −80°C. YKL-40 in serum and BALF was measured by ELISA (Quidel, San Diego, CA, USA) as previously described [23]. Lactate dehydrogenase (LDH) in serum was routinely measured (normal value for LDH <225 $U \cdot L^{-1}$).

Pulmonary function tests and blood gas analysis

Measurements including FVC, forced expiratory volume in 1 s (FEV_1), total lung capacity (TLC), D_{LCO}, arterial oxygen tension (P_{aO_2}), arterial carbon dioxide tension (P_{aCO_2}), arterial oxygen saturation (S_{aO_2}) and alveolar–arterial oxygen tension difference (P_{A-aO_2}) were performed at the time of blood sample collection. Values were expressed as percentages of predicted normal values [24].

Statistical analysis

Continuous variables were evaluated for a normal distribution with the Kolmogorov–Smirnov test. Parametric data are presented as mean±SEM. Categorical variables are presented as either a percentage of the total or numerically, as appropriate. Multiple comparisons were performed by one-way ANOVA and least significant difference or Dunnett's *post hoc* tests. Comparison between two groups was done with t-test or Wilcoxon's rank test for continuous variables, and Chi-squared or Fisher's exact test for categorical variables. Spearman's correlation coefficient was obtained for correlations. Receiver operating characteristic (ROC) analysis was used to test the role of baseline YKL-40 in serum and BALF as a diagnostic marker of HP and a predictor of disease outcome. Univariate and multivariate Cox's proportional hazard regression models were used to analyse prognostic factors. The Kaplan–Meier method with log-rank test was used to analyse

whether baseline YKL-40 levels were associated with disease outcome. p-values of <0.05 were considered statistically significant. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of study subjects

Demographic and laboratory characteristics of the enrolled subjects are shown in table 1. The mean±SEM follow-up time of the HP patients was 38±2 months (range 1–60) from initial blood collection. Of the 72 HP patients, 11 presented with the acute/subacute form and 61 presented with the chronic form of HP. Relevant antigen exposures were fungi/moulds (4%), birds/feathers (74%), both (21%) or unknown (1%). During follow-up, 29 HP patients experienced disease progression (symptom worsening in 100%, lung function deterioration in 90%, chest imaging worsening in 38%). No patient had disease progression on the basis of symptoms alone. 16 HP patients died. The majority of patients in all groups were not treated with corticosteroids at baseline; these were those patients who were enrolled at the time of diagnosis, before treatment was started.

Baseline serum and BALF levels of YKL-40

Serum YKL-40 levels were significantly higher in HP patients (127±9 ng·mL^{-1}) than in healthy controls (39±4 ng·mL^{-1}; p<0.001), but lower than in IPF (214±20 ng·mL^{-1}; p<0.001), iNSIP (184±21 ng·mL^{-1}; p<0.05) and COP (213±33 ng·mL^{-1}; p<0.05) patients (figure 1a). Among all ILD patients, serum YKL-40 levels were higher in males than females (213±14 *versus* 163±12 ng·mL^{-1}; p<0.01) and in smokers than nonsmokers (214±13 *versus* 161±12 ng·mL^{-1}; p<0.01). Serum YKL-40 levels were higher in untreated patients than in those treated with corticosteroids (206±11 *versus* 141±13 ng·mL^{-1}; p<0.001). Among HP patients, serum YKL-40 levels were higher in patients with acute/subacute disease than in those with chronic disease (179±27 *versus* 117±9 ng·mL^{-1}; p<0.05).

TABLE 1 Demographics and characteristics of all enrolled subjects

	HP	IPF	iNSIP	COP	Controls
Subjects	72	45	34	21	60
Demographics					
Age years	57±2***	71±1***,+++	68±2***,+++	61±3***,§§,*f*	40±2
Sex male/female	28/44**	39/6**,+++	17/17§§	6/15**,§§§	37/23
Smoking status current/ex/nonsmoker	0/24/48***	3/27/15***,++	1/17/16***+	3/7/11*	10/7/33#
Steroid therapy yes/no	31/41***	8/37***,+++	9/25***	4/17**,++	0/60
Disease duration months	79±6.5§§§	31±4.5+++	50±9++	41±9++	NA
BALF cell differential					
Total cells ×10^4·mL^{-1}	29±2	17±1+++	18±3+++	18±3++	NA
Macrophages ×10^4·mL^{-1}	12±2	13±1	13±2	9±2	NA
Lymphocytes ×10^4·mL^{-1}	14±1	2±1+++	4±1+++,§	5±1+++,§	NA
Granulocytes ×10^4·mL^{-1}	4±1	2±1	2±1	3±1	NA
Neutrophils ×10^4·mL^{-1}	1±0.3	2±0.4	2±1	2±1	NA
Eosinophils ×10^4·mL^{-1}	0.3±0.1	0.3±0.1	0.4±0.1	0.5±0.2	NA
Pulmonary function¶					
FVC % pred	63±2***	72±3***,++	66±3***	83±5*,+++,§,*ff*	90±3
FEV_1 % pred	61±2***	76±3**,+++	64±3***,§§	74±5**,++,*ff*	88±9
D_{LCO} % pred	42±2***	45±2***	49±3***,+	60±4***,+++,§§,*f*	85±4
TLC % pred	70±2***	67±2***	61±2***,+	81±4+++,§§,*fff*	89±3
Blood gas analysis¶					
P_{aO_2} mmHg	69±1***	72±2***	74±2**,+	69±2***	86±4
P_{aCO_2} mmHg	38±1	37±1	38±1§	35±1++,*ff*	37±5
P_{A-aO_2} mmHg	34±1***	31±2***	28±2**,+	37±2***,*ff*	14±2
S_{aO_2} %	94±1	95±1	95±1	95±1	95±2

Data are presented as mean±SEM unless otherwise stated. HP: hypersensitivity pneumonitis; IPF: idiopathic pulmonary fibrosis; iNSIP: idiopathic nonspecific interstitial pneumonia; COP: cryptogenic organising pneumonitis; NA: not available; BALF: bronchoalveolar lavage fluid; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; D_{LCO}: diffusing capacity of the lung for carbon monoxide; TLC: total lung capacity; P_{aO_2}: arterial oxygen tension; P_{aCO_2}: arterial carbon dioxide tension; P_{A-aO_2}: alveolar–arterial oxygen tension difference; S_{aO_2}: arterial oxygen saturation. #: smoking status was available in 50 healthy controls; ¶: pulmonary function and blood gas analysis were available in 18 healthy controls. *: p<0.05,**: p<0.01,***: p<0.001, *versus* controls. +: p<0.05, ++: p<0.01, +++: p<0.001, *versus* HP. §: p<0.05, §§: p<0.01, §§§: p<0.001, *versus* IPF. *f*: p<0.05, *ff*: p<0.01, *fff*: p<0.001, *versus* iNSIP.

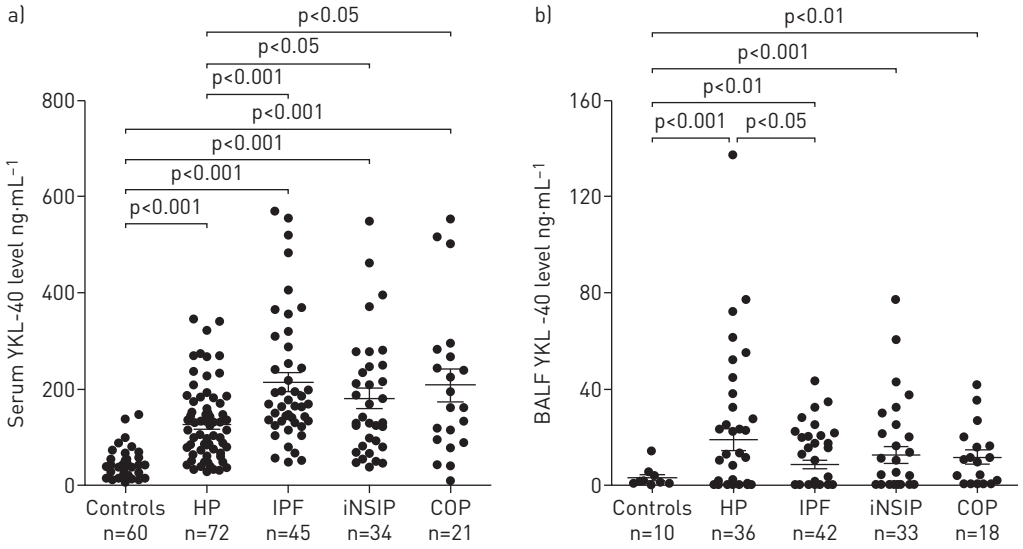


FIGURE 1 a) Serum and b) bronchoalveolar lavage fluid (BALF) YKL-40 levels in the studied subjects at baseline. Dots represent single patients. Bars represent mean±SEM values. COP: cryptogenic organising pneumonitis; HP: hypersensitivity pneumonitis; iNSIP: idiopathic nonspecific interstitial pneumonia; IPF: idiopathic pulmonary fibrosis.

BALF YKL-40 levels were significantly higher in HP patients (21±5 ng·mL^{-1}) than in healthy controls (3±1 ng·mL^{-1}; p<0.001) and in those with IPF (9±2 ng·mL^{-1}; p<0.05) (figure 1b). BALF YKL-40 levels were higher in patients with acute/subacute HP patients than in patients with chronic HP (42±10 *versus* 15±7 ng·mL^{-1}; p<0.05). No effect of sex, smoking or treatment on BALF levels was observed (data not shown).

Using ROC analysis, at a cut-off of >47 ng·mL^{-1}, serum YKL-40 levels showed the best sensitivity (88%), specificity (77%) and accuracy (88%) to discriminate HP from healthy controls (area under the curve (AUC) 0.904; p<0.001), and at a cut-off of >134 ng·mL^{-1}, serum YKL-40 levels showed the best sensitivity (76%), specificity (61%) and accuracy (71%) to discriminate HP from IPF (AUC 0.727; p<0.001). For BALF YKL-40 levels, no significantly predictive cut-off value was found.

Correlations between serum YKL-40 and other characteristics in HP

Baseline serum YKL-40 levels positively correlated with baseline serum LDH levels (r=0.554, p<0.001), BALF total cell counts (r=0.496, p<0.001) and BALF lymphocyte counts (r=0.451, p<0.001); a weak correlation was seen with age (r=0.264, p<0.05). Serum YKL-40 levels inversely correlated with *D*LCO (r=−0.310, p<0.01) (figure 2a) and FVC (r=−0.376, p<0.001) at baseline. Moreover, changes in serum

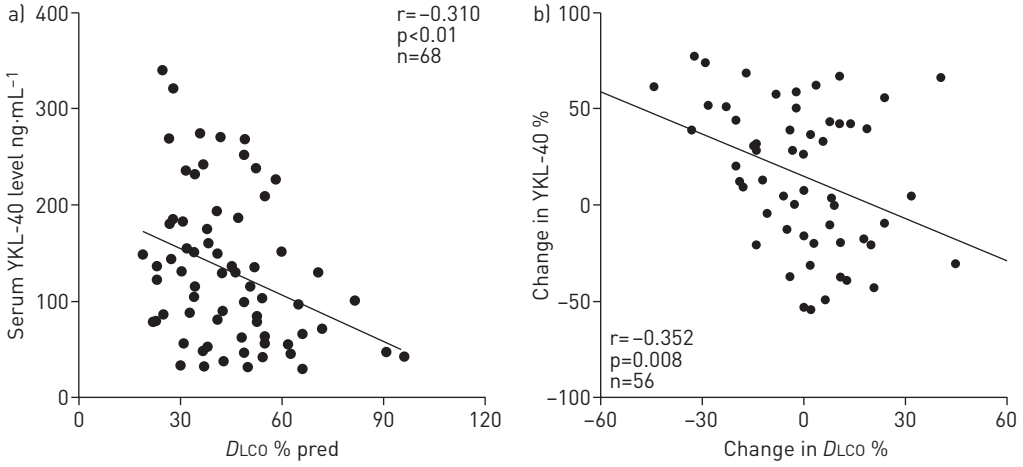


FIGURE 2 a) Correlation between baseline YKL-40 serum levels and diffusing capacity of the lung for carbon monoxide (*D*LCO) (% pred) at baseline. Dots represent single patients. b) Correlation between change in serum YKL-40 and *D*LCO over time in 39 hypersensitivity pneumonitis patients. Dots represent 56 measurements performed in 39 patients at various time intervals ranging from 6 to 36 months.

YKL-40 levels inversely correlated with changes in D_{LCO} over time (r=−0.352, p<0.001) (figure 2b), but not with changes in FVC (r=0.221, p=0.176).

Serum YKL-40 levels and disease outcome in HP

HP patients who experienced disease progression had higher baseline serum YKL-40 levels than those who remained stable during follow-up (167±12 *versus* 98±11 ng·mL^{-1}; p<0.001) (figure 3a). HP patients who died (n=16) had higher baseline serum YKL-40 levels than those who survived (n=56) (190±22 *versus* 107±9 ng·mL^{-1}; p<0.001) (figure 3b).

Predictive value of baseline serum YKL-40 levels for disease progression in HP

Using ROC analysis, at a cut-off level of >119 ng·mL^{-1}, serum YKL-40 levels showed the best sensitivity (81%), specificity (77%) and accuracy (79%) to predict disease progression (AUC 0.797; p<0.001). At a cut-off level of >303 U·L^{-1}, serum LDH showed similar results (sensitivity 78%, specificity 81%, accuracy 79%; AUC 0.811; p<0.001) (figure 4a). The combination of the two cut-offs (serum YKL-40 119 ng·mL^{-1} and serum LDH 303 U·L^{-1}) yielded better results (sensitivity 81%, specificity 91% and accuracy 80%) to predict disease progression.

According to the best cut-off obtained by ROC analysis, we also divided all HP patients into a high YKL-40 level group (n=34), with baseline concentrations >119 ng·mL^{-1}, and a low YKL-40 level group (n=38) (baseline concentrations ⩽119 ng·mL^{-1}). The characteristics of patients stratified according to this cut-off are shown in table 2.

Univariate and multivariate analyses were performed to verify whether the identified cut-off predicts disease progression. In the univariate analysis, baseline serum YKL-40 levels >119 ng·mL^{-1} were associated with an increased risk of disease progression (hazard ratio (HR) 6.567; p<0.001). In the multivariate analysis, serum YKL-40 levels >119 ng·mL^{-1} were independently associated with risk of disease progression after adjustment for age, sex, body mass index (BMI), smoking history, steroid therapy, disease type, baseline FVC, D_{LCO} and serum LDH as covariates (HR 5.208; p=0.004) (table 3).

Kaplan–Meier analysis confirmed the predictive value of serum YKL-40 for disease progression in HP (figure 5a). At 5 years, the rate of disease progression of all HP patients was 40%. The patients in the high YKL-40 group had a higher rate of disease progression (71%) than those in the low YKL-40 group (13%) (log-rank test, p<0.001).

Predictive value of baseline serum YKL-40 levels for mortality in HP

Using ROC analysis, at a cut-off level of >150 ng·mL^{-1}, serum YKL-40 levels showed the best sensitivity (78%), specificity (84%) and accuracy (83%) to predict death (AUC 0.787; p<0.001). For serum LDH, no predictive value for mortality was found (p>0.05) (figure 4b).

In the univariate analysis, baseline serum YKL-40 levels >150 ng·mL^{-1} were associated with an increased risk of death (HR 9.989; p<0.001). In the multivariate analysis, serum YKL-40 levels >150 ng·mL^{-1} were independently associated with the risk of death after adjustment for age, sex, BMI, smoking history, steroid therapy, disease type, baseline FVC, D_{LCO} and serum LDH as covariates (HR 6.413; p=0.013) (table 4).

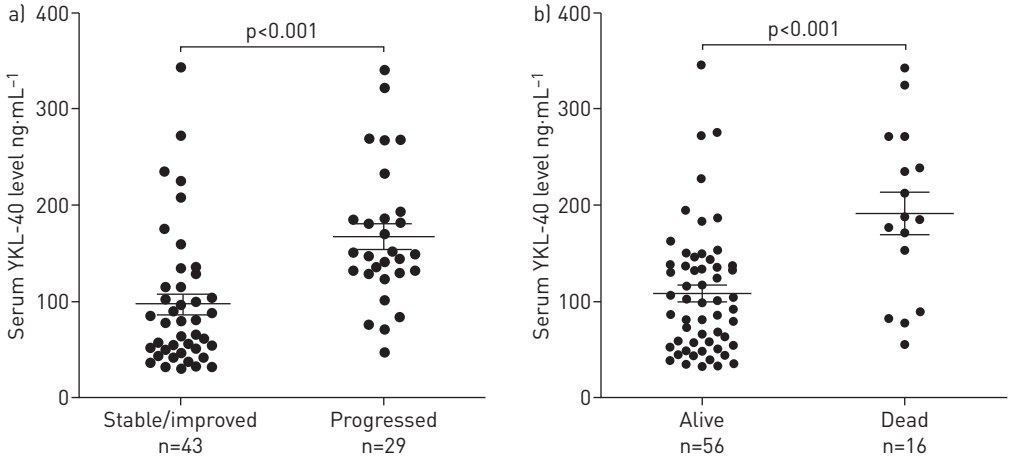


FIGURE 3 Comparisons of baseline serum YKL-40 levels in 72 hypersensitivity pneumonitis patients according to a) disease progression and b) survival. Dots represent single patients. Bars represent mean±SEM values.

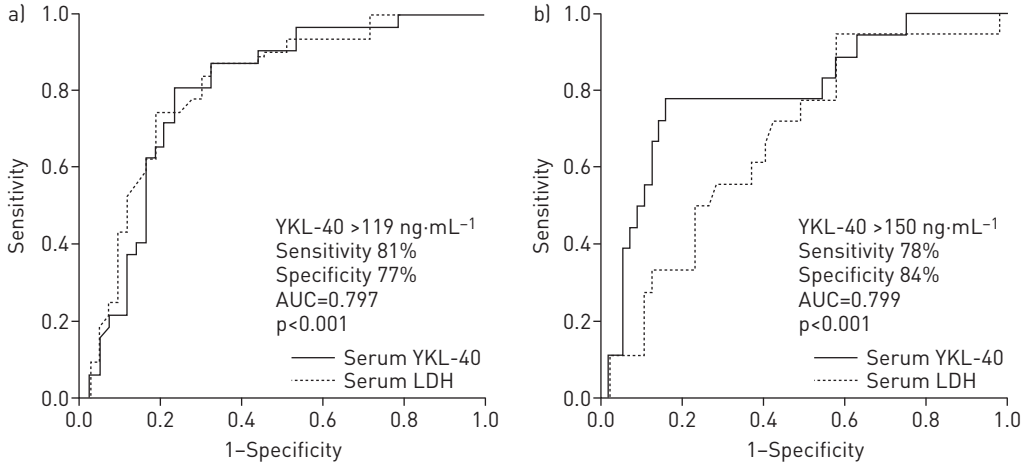


FIGURE 4 Receiver operating characteristic analysis showing the predictive value of baseline YKL-40 and lactate dehydrogenase (LDH) serum levels to predict a) disease progression and b) mortality in 72 hypersensitivity pneumonitis patients. AUC: area under the curve.

TABLE 2 Characteristics of 72 hypersensitivity pneumonitis (HP) patients stratified according to serum YKL-40 predictive cut-off level for disease progression

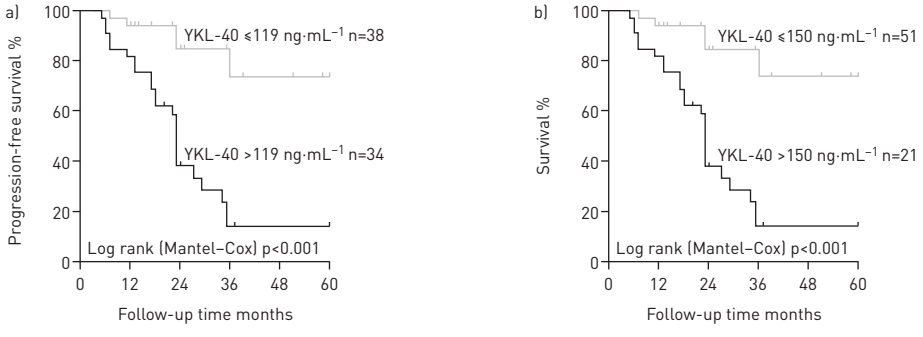
	YKL-40 ⩽119 ng·mL^{-1}	YKL-40 >119 ng·mL^{-1}	p-value
Subjects	38	34	
Demographics			
Age years	54±2	61±2	NS
Sex male/female	15/23	13/21	NS#
Smoking habits current/ex/nonsmoker	0/12/26	0/12/22	NS#
Steroid therapy yes/no	19/19	12/22	NS#
Disease type acute/subacute or chronic	2/36	9/25	0.020#
Disease outcome			
Disease progression yes/no	5/33	24/10	<0.001#
HP-related death yes/no	4/34	12/22	0.012#
BALF cell differential			
Total cells ×10^4 mL^{-1}	19±2	32±2	0.001
Macrophages ×10^4 mL^{-1}	9±2	13±2	NS
Lymphocytes ×10^4 mL^{-1}	8±1	16±1	<0.0001
Granulocytes ×10^4 mL^{-1}	2±1	4±1	NS
Neutrophils ×10^4 mL^{-1}	0.4±0.2	1.2±0.4	NS
Eosinophils ×10^4 mL^{-1}	0.1±0.04	0.3±0.08	NS
Mast cells ×10^4 mL^{-1}	0.03±0.01	0.1±0.06	NS
Pulmonary function			
FVC % pred	66±3	57±3	0.040
FEV_1 % pred	63±3	59±3	NS
D_{LCO} % pred	45±2	37±2	0.013
TLC % pred	69±3	70.5±3	NS
Blood gas analysis			
P_{aO_2} mmHg	70±2	68±2	NS
$P_{A\text{-}aO_2}$ mmHg	33±2	35±2	NS
S_{aO_2} %	95±0.4	94±0.5	NS
Biomarkers			
Serum YKL-40 ng·mL^{-1}	68±4	192±11	<0.0001
BALF YKL-40 ng·mL^{-1}	14±5	26±8	NS
Serum LDH U·L^{-1}	252±10	328±11	<0.0001

Data are presented as mean±SEM unless otherwise stated. NS: nonsignificant; BALF: bronchoalveolar lavage fluid; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; D_{LCO}: diffusing capacity of the lung for carbon monoxide; TLC: total lung capacity; P_{aO_2}: arterial oxygen tension; $P_{A\text{-}aO_2}$: alveolar–arterial oxygen tension difference; S_{aO_2}: arterial oxygen saturation; LDH: lactate dehydrogenase. #: Chi-square test or Fisher's exact test; for all other comparisons a t-test was used.

TABLE 3 Univariate and multivariate Cox proportional analysis for the risk of disease progression in 72 hypersensitivity pneumonitis patients

	Hazard ratio	95% CI	p-value
Univariate analysis			
Serum YKL-40 ng·mL^{-1} (continuous)	1.009	1.005–1.014	<0.001
Serum YKL-40 >119 ng·mL^{-1} (binary)	6.567	2.487–17.340	<0.001
Age years (continuous)	1.028	1.000–1.058	0.053
Sex (female=1)	0.991	0.466–2.106	NS
Steroid therapy (positive=1)	0.679	0.308–1.494	NS
Smoking history (positive=1)	0.997	0.974–1.021	NS
Disease type (acute/subacute=1)	0.328	0.136–0.788	0.013
Total cells ×10^4 mL^{-1} (continuous)	1.026	0.998–1.055	NS
Macrophages ×10^4 mL^{-1} (continuous)	1.013	0.981–1.047	NS
Lymphocytes ×10^4 mL^{-1} (continuous)	1.079	1.015–1.148	0.014
Granulocytes ×10^4 mL^{-1} (continuous)	1.099	0.941–1.284	NS
Neutrophils ×10^4 mL^{-1} (continuous)	1.271	1.036–1.560	0.022
Eosinophils ×10^4 mL^{-1} (continuous)	1.771	0.882–3.559	NS
Mast cells ×10^4 mL^{-1} (continuous)	6.161	1.510–25.141	0.011
FVC % pred (continuous)	0.989	0.963–1.015	NS
FEV_1 % pred (continuous)	0.997	0.947–1.021	NS
TLC % pred (continuous)	0.996	0.973–1.021	NS
D_{LCO} % pred (continuous)	0.948	0.918–0.978	0.001
P_{aO_2} mmHg (continuous)	1.003	0.974–1.034	NS
$P_{A\text{–}aO_2}$ mmHg (continuous)	0.996	0.954–1.040	NS
S_{aO_2} % (continuous)	0.977	0.855–1.118	NS
Serum LDH U·L^{-1} (continuous)	1.010	1.005–1.015	<0.001
Serum LDH >303 U·L^{-1} (binary)	4.715	2.085–10.665	<0.001
BALF YKL-40 ng·mL^{-1} (continuous)	1.011	0.999–1.024	NS
Multivariate analysis#			
Serum YKL-40 ng·mL^{-1} (continuous)	1.008	1.003–1.013	0.002
D_{LCO} % pred (continuous)	0.960	0.928–0.992	0.016
Serum YKL-40 >119 ng·mL^{-1} (binary)	5.208	1.713–15.835	0.004
Serum LDH U·L^{-1} (continuous)	1.008	1.001–1.014	0.017

Data are presented as hazard ratios, representing the relative risk of developing disease progression as a specific characteristic at baseline. Backward and forward stepwise (conditional likelihood ratio) analyses were performed. NS: nonsignificant; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; TLC: total lung capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; P_{aO_2}: arterial oxygen tension; $P_{A\text{–}aO_2}$: alveolar–arterial oxygen tension difference; S_{aO_2}: arterial oxygen saturation; LDH: lactate dehydrogenase; BALF: bronchoalveolar lavage fluid. #: after adjustment for age, sex, body mass index, smoking history, steroid therapy, disease type, baseline FVC, D_{LCO} and serum LDH.



Number at risk						
YKL-40 ⩽119 ng·mL^{-1}	38	36	34	33	33	33
YKL-40 >119 ng·mL^{-1}	34	28	17	10	10	10

Number at risk						
YKL-40 ⩽119 ng·mL^{-1}	51	51	50	48	48	47
YKL-40 >119 ng·mL^{-1}	21	17	15	12	12	9

FIGURE 5 Kaplan–Meier analysis showing a) disease progression and b) survival rate according to serum YKL-40 levels in 72 hypersensitivity pneumonitis patients. The respective cut-off levels are indicated.

TABLE 4 Univariate and multivariate Cox proportional analysis for the risk of death in 72 hypersensitivity pneumonitis patients

	Hazard ratio	95% CI	p-value
Univariate analysis			
Serum YKL-40 ng·mL^{-1} (continuous)	1.010	1.005–1.015	<0.001
Serum YKL-40 >150 ng·mL^{-1} (binary)	9.989	3.187–31.312	<0.001
Age years (continuous)	1.107	1.047–1.171	<0.001
Sex (female=1)	1.068	0.387–2.947	NS
Steroid therapy (positive=1)	1.499	0.561–4.007	NS
Smoking history (positive=1)	1.460	0.471–4.531	NS
Disease type (acute/subacute=1)	0.296	0.103–0.855	0.025
Total cells ×10^4 mL^{-1} (continuous)	1.083	1.033–1.135	0.001
Macrophages ×10^4 mL^{-1} (continuous)	1.069	1.024–1.117	0.003
Lymphocytes ×10^4·mL^{-1} (continuous)	1.044	0.958–1.317	NS
Granulocytes ×10^4·mL^{-1} (continuous)	1.087	0.933–1.266	NS
Neutrophils ×10^4 mL^{-1} (continuous)	1.399	1.107–1.768	0.005
Eosinophils ×10^4 mL^{-1} (continuous)	1.899	0.528–6.822	NS
Mast cells ×10^4 mL^{-1} (continuous)	5.540	1.286–23.860	0.022
FVC % pred (continuous)	0.949	0.907–0.992	0.021
FEV_1, % pred (continuous)	0.975	0.941–1.011	NS
TLC % pred (continuous)	0.989	0.955–1.024	NS
D_{LCO}, % pred (continuous)	0.961	0.922–1.003	0.001
P_{aO2} mmHg (continuous)	1.011	0.969–1.054	NS
P_{A-aO_2} mmHg (continuous)	0.989	0.945–1.036	NS
S_{aO_2} % (continuous)	1.013	0.839–1.223	NS
Serum LDH U·L^{-1} (continuous)	1.006	1.000–1.013	0.058
BALF YKL-40 ng·mL^{-1} (continuous)	1.002	0.980–1.024	NS
Multivariate analysis#			
Serum YKL-40 ng·mL^{-1} (continuous)	1.010	1.001–1.019	0.024
Age years (continuous)	1.132	1.054–1.215	0.001
FVC % pred (continuous)	0.945	0.901–0.990	0.018
Serum YKL-40 >150 ng·mL^{-1} (binary)	6.413	1.472–27.933	0.013
Age years (continuous)	1.137	1.055–1.226	0.001
FVC % pred (continuous)	0.950	0.907–0.995	0.029

Data are presented as hazard ratios, representing the relative risk of death as a specific characteristic at baseline. Backward and forward stepwise (conditional LR) analyses were performed. NS: nonsignificant; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; TLC: total lung capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; P_{aO_2}: arterial oxygen tension; P_{A-aO_2}: alveolar–arterial oxygen tension difference; S_{aO_2}: arterial oxygen saturation; LDH: lactate dehydrogenase; BALF: bronchoalveolar lavage fluid. #: after adjustment for age, sex, body mass index, smoking history, steroid therapy, disease type, baseline FVC, D_{LCO} and serum LDH.

Kaplan–Meier analysis showed that higher serum YKL-40 was associated with mortality in HP (figure 5b). The mortality rate of all HP patients after 5 years was 22%. The patients in the high YKL-40 group had a higher mortality rate (57%) than those in the low YKL-40 group (8%) (log-rank test, p<0.001).

Discussion

In this study, we show that serum and BALF YKL-40 levels are elevated in HP patients and other ILD patients compared with healthy controls, and correlate with pulmonary function tests (PFTs) and BALF cell counts. Moreover, serum YKL-40 was found to be an independent predictor of disease progression and mortality in HP. To the best of our knowledge, this is the first investigation of YKL-40 levels in serum and BALF and its potential role as a biomarker in HP.

HP is caused by repeated inhalation of organic particles; thus, antigen presentation and hypersensitivity reactions are involved in the development of this disorder [1, 3]. As previously reported, antigen-presenting cells produce YKL-40 in response to specific stimulation [25, 26]. Thus, it can be speculated that activated antigen-presenting cells in the lung of HP patients are responsible for the elevated levels of YKL-40 in BALF of these patients. In support of this hypothesis, we found a positive correlation between serum YKL-40 levels and BALF lymphocyte counts in HP patients. Although lymphocytes cannot directly produce YKL-40, they can induce the production of interleukin-13, which can

stimulate YKL-40 production in macrophages, dendritic cells and epithelial cells [7]. On the other hand, YKL-40 levels were higher in serum than BALF of patients with HP and other ILDs, suggesting a possible spill-over of YKL-40 from the bloodstream to the alveolar space. This has also been reported in a previous study on YKL-40 in IPF [23]. As postulated for asthma [27], the elevated concentration of YKL-40 in serum of HP patients may reflect the activity of antigen-presenting cells and the systemic immune response. In line with this hypothesis, serum and BALF levels of YKL-40 were significantly higher in acute/subacute HP patients than in those with chronic HP.

Serum YKL-40 was inversely correlated with PFTs at baseline; this finding has also been observed in IPF [13] and sarcoidosis [28]. Interestingly, change in YKL-40 levels in serum also correlated with change in *D*LCO over time. Moreover, the serum level of YKL-40 correlated well with LDH, a nonspecific marker of tissue injury widely used to monitor the course of acute lung disease and chronic fibrotic disease [29].

We found that HP patients with progressive disease had higher levels of YKL-40 than those who remained stable or improved. Serum YKL-40 levels >119 ng·mL^{-1} predicted disease progression with a sensitivity of 81% and specificity of 77%, similar to serum LDH (cut-off 303 U·L^{-1}, sensitivity 78%, specificity 81%). Moreover, the combined assessment of serum YKL-40 and LDH provided better results (sensitivity 81%, specificity 91%) than either single marker alone. By using the identified cut-off to stratify the patients, those with serum YKL-40 levels >119 ng·mL^{-1} had worse PFTs and a higher rate of disease progression (71%) than those with serum YKL-40 ⩽119 ng·mL^{-1} (13%). The multivariate analysis confirmed that both a serum YKL-40 level >119 ng·mL^{-1} and YKL-40 as a continuous variable were independently associated with disease progression, even after adjusting for covariates. The same was not observed for serum LDH.

Moreover, HP patients with serum YKL-40 levels >150 ng·mL^{-1} had worse PFTs and a worse survival (57% mortality rate) compared with those with ⩽150 ng·mL^{-1} (8% mortality rate). The multivariate analysis confirmed that a serum YKL-40 level >150 ng·mL^{-1} was associated with mortality, even after adjusting for covariates. This predictive cut-off for survival in our cohort is approximately two-fold higher than that reported in IPF patients (79 ng·mL^{-1}) [23]. This suggests that production of YKL-40 level is enhanced in HP patients with active disease, and correlates with outcome.

The thresholds for serum YKL-40 described in this study might be different in other cohorts. However, this does not impact our main finding that HP patients with high serum YKL-40 levels at baseline may have a higher rate of disease progression (or death) than those with lower levels. In recent years, there has been increasing interest in defining accurate predictors of outcome in HP: a higher fibrosis score on HRCT [30], increasing severity of traction bronchiectasis [31], lower lymphocyte levels in BALF [32] and a usual interstitial pneumonia-like pattern on histology [31, 32] have been found to correlate with disease outcome. Whereas Myazaki *et al.* [32] previously identified a low lymphocyte level in BALF as a potential risk factor for acute exacerbation of HP, in our cohort a higher BALF lymphocyte count was seen in the HP group with high YKL-40 levels, driven by the higher number of patients with acute/subacute disease in this group. However, univariate and multivariate analyses demonstrated that lymphocyte count was not an independent predictive factor for disease progression or survival.

Besides clinical characteristics, two biomarkers have been studied in the past to evaluate outcome in HP patients: KL-6 [33, 34] and CCL17 [35]. Serum KL-6 has been shown to predict disease progression and survival in HP patients [33, 34], whereas serum CCL17 was shown to be a predictor of disease progression but not of survival [35].

With regard to the factors responsible for YKL-40 variability, we found, in agreement with others [36, 37], that YKL-40 serum levels correlated with age in our entire cohort, but the correlation was weak (data not shown). As reported previously [36, 38], we also found that former and current smokers had higher serum YKL-40 levels than never-smokers. Cigarette smoke increases the permeability of the air–blood membrane [39] and could increase both production and spill-over of YKL-40; it has been shown that YKL-40 expression is increased in alveolar macrophages and epithelial cells in lung tissue of cigarette smokers with chronic obstructive pulmonary disease and cigarette smoke-exposed mice [37]. We believe that smoking habits should be taken into consideration when interpreting YKL-40 serum levels. The relevance of this aspect for HP patients is low, since the majority of HP patients are nonsmokers. Moreover, we observed that corticosteroid-treated patients had significantly lower levels of serum YKL-40 levels than untreated patients, suggesting that corticosteroids may suppress YKL-40 release [40, 41]. This observation has not been reported in other studies of ILD patients and could be of clinical utility in assessing the clinical response to steroid treatment in HP patients.

Despite the novel findings of this study, it has several limitations. First, the healthy controls were younger than the ILD patients, and this difference could affect the respective serum levels of YKL-40. Secondly, only limited data on serum YKL-40 levels during follow-up were available, and these data did not allow us

to assess the clinical value of serial YKL-40 measurements. Thirdly, baseline single nucleotide polymorphisms in the chitinase 3-like 1 (CHI3L1) encoding gene, known to influence YKL-40 levels in serum, were not determined [23]. Finally, immunohistochemistry to demonstrate YKL-40 in lung tissue obtained by biopsy was not performed, and therefore we can only speculate on the source and localisation of this protein in HP and other ILDs.

In conclusion, this study indicates that serum YKL-40 can be a prognostic biomarker in HP, suggesting a clinical role in predicting disease progression and survival, but its serum levels should be carefully interpreted in light of the confounding factors. Although the data are promising, a multicentre validation study is necessary to determine whether serum YKL-40 measurements should be routinely used in HP.

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