Internal Medicine
The Japanese Society of Internal Medicine
doi: 10.2169/internalmedicine.8716-16
Intern Med 56: 3009-3018, 2017
http://internmed.jp

【 ORIGINAL ARTICLE 】

The Serum Level of KL-6 Is Associated with the Risk of Insulin Resistance and New-onset Diabetes Mellitus: The Tanno-Sobetsu Study

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Abstract:

Objective Inflammatory cytokines generated in visceral fat have been shown to contribute to the development of insulin resistance. The involvement of pulmonary inflammation in insulin resistance remains unclear, but smoking is known to be a risk factor for diabetes as well as chronic obstructive pulmonary disease. We herein examined the hypothesis that increased serum levels of lung interstitial injury biomarkers [surfactant protein (SP)-A, SP-D and Krebs von den Lungen (KL)-6] are associated with the risk of diabetes development.

Methods For cross-sectional and longitudinal analyses, we enrolled 750 apparently healthy non-diabetic subjects who received annual examinations in 2011 or 2012 in the Tanno-Sobetsu cohort.

Results A cross-sectional analysis showed that distinct clinical parameters were associated with SP-A, SP-D and KL-6. In a multiple regression analysis, independent explanatory variables were Brinkman index and brain natriuretic peptide (BNP) for SP-A, sex (women), BNP and body mass index (BMI) for SP-D, and age and BMI for KL-6. A longitudinal analysis of 415 subjects who received annual examinations in both 2011 and 2014 showed that 13 (3.1%) of the patients developed type 2 diabetes during the 3-year follow-up. A multiple logistic regression analysis showed the KL-6 levels, systolic blood pressure and homeostasis model assessment of insulin resistance (HOMA-IR) in 2011 to be independently associated with new-onset diabetes. In a multiple regression analysis for HOMA-IR in 2014, the KL-6 level and BMI in 2011 were selected as explanatory variables.

Conclusion A modest elevation of the serum KL-6 level is therefore considered to be associated with the risk for insulin resistance development and new-onset diabetes mellitus in a general population.

Key words: diabetes mellitus, insulin resistance, SP-A, SP-D, KL-6

(Intern Med 56: 3009-3018, 2017)
(DOI: 10.2169/internalmedicine.8716-16)

Introduction

The number of patients with diabetes mellitus is rapidly increasing in all areas of the world (1), and the World Health Organization (WHO) has undertaken an initiative for the prevention of and early intervention for diabetes mellitus (http://www.who.int/diabetes/en/). In addition to general

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Received: December 14, 2016; Accepted: March 23, 2017; Advance Publication by J-STAGE: September 25, 2017
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public education, the selection of individuals at high risk for new-onset diabetes for intensive education and guidance is a reasonable approach in light of limited social resources. A number of cohort and clinical studies have revealed factors that are closely associated with new-onset diabetes (2-7). Besides a sedentary life style (2), the established risk factors of new-onset type 2 diabetes are demographic and metabolic parameters such as obesity, family history of diabetes, fasting plasma glucose (PG) level, and the indices of insulin resistance (3-7). Recently, inflammation has received attention as a factor playing a role in the pathogenesis of diabetes (8-15). The plasma levels of pro-inflammatory cytokines, such as tumor necrotic factor-alpha (TNF-alpha) and interleukin-6 (IL-6) are elevated and, conversely, the level of anti-inflammatory cytokine adiponectin is reduced in diabetic patients (9-12). A causal role of the cytokines in diabetes is supported by the findings that pharmacological agents with anti-inflammatory actions were demonstrated to improve glycemic control in an animal model of diabetes (13) and in patients with type 2 diabetes (14, 15).

Visceral adipose tissue has been suggested to be a major source of pro-inflammatory cytokines in type 2 diabetes (8). However, theoretically, inflammation in other tissues may also contribute to the development of insulin resistance. In fact, the incidence of diabetes mellitus has been reported to be higher in patients with rheumatoid arthritis and those with psoriasis (16, 17). The lungs are exposed each day to more than 7,000 L of air containing particles that can potentially increase inflammatory reactions. Active smoking increases the risk of diabetes development by 1.6-fold (18), and its mechanism possibly includes smoking-induced inflammation, though the activation of sympathetic nerves by smoking has been shown to acutely impair glucose tolerance (19). Interestingly, a few earlier studies (20, 21) indicated associations between insulin resistance and biomarkers of interstitial lung injury. Fernández-Real et al. (20) showed that the serum level of surfactant protein-A (SP-A) was inversely correlated with an index of insulin sensitivity in men, and Takahashi et al. (21) reported that the serum level of Krebs von den Lungen-6 (KL-6) was significantly higher in patients with type 2 diabetes. However, besides lung diseases, the factors that influence serum levels of SP-A, KL-6 and surfactant protein-D (SP-D) have not yet been characterized, and the relationships between the three biomarkers and the risk of diabetes development have not so far been systematically examined.

The aim of the present study was to determine whether the commonly used biomarkers of interstitial lung injury, SP-A, SP-D and KL-6 (22, 23), are associated with insulin resistance and/or risk of new-onset diabetes mellitus in the general population. To this end, we retrieved data from the Tanno-Sobetsu study, a prospective cohort study (4, 24-26). First, a cross-sectional analysis was conducted using the data collected in 2011-2012 to clarify the distribution of SP-A, SP-D and KL-6 data in 750 apparently healthy subjects and also to examine whether any of the demographic and clinical parameters are associated with variations in each of the three biomarkers. Second, a longitudinal analysis was performed using data collected in 2011 and 2014 to determine the factors that are significantly associated with new-onset diabetes during a 3-year follow-up. The results of the cross-sectional and longitudinal analyses suggest that KL-6, but not SP-A or SP-D, is independently associated with the risk of developing insulin resistance and it is therefore a risk factor for new-onset diabetes in Japanese.

Materials and Methods

The present study was approved by the Ethics Committee of Sapporo Medical University and was conducted in strict accordance with the principles expressed in the Declaration of Helsinki. The Tanno-Sobetsu study (4, 24-28) is a prospective cohort study in which residents of two towns, Tanno and Sobetsu, were recruited for annual or biannual medical examinations. Written informed consent was obtained from all subjects who participated in this study. The present study was performed as one of the SPRUCE (Sapporo Medical University Affiliates for Common Disease and Education) projects in Sapporo Medical University.

Study subjects in Study 1, a cross-sectional analysis

We screened the subjects who participated in the Tanno-Sobetsu study and enrolled subjects who met the following criteria: 1) attended annual examination in Sobetsu in 2011 or 2012, 2) were not on regular medications at the time of the examination and 3) were free from diseases that require strict medical follow-up and/or treatments at the time of the examination. Information regarding past and concurrent diseases and treatments was obtained from the study participants by public health nurses at the time of annual examinations.

Study subjects in Study 2, a longitudinal analysis

The subjects included in the longitudinal analysis met the following criteria: 1) attended annual examinations in both 2011 and 2014, 2) were not on regular medications at the examination in 2011, and 3) were free from diseases, including diabetes, that require strict medical follow-up and/or treatments at the time of the examination in 2011. At the examination in 2011 and 2014, the subjects were diagnosed to have diabetes if they meet one of the following criteria: fasting PG of ≥126 mg/dL and hemoglobin A1c (HbA1c) of ≥6.5% (29); history of new-onset diabetes diagnosed after the examination in 2011; or concurrent medical treatments for diabetes.

Measurements

The study subjects received medical examinations in the early morning after an overnight fast. Systolic blood pressure (BP) and diastolic BP were measured twice by medical doctors after a 5-min rest on a seat and the values were averaged. Peripheral venous blood was drawn to determine the

Table 1. Baseline Characteristics of the Study Subjects in Study 1.

	Total (n=750)	Male (n=314)	Female (n=436)
Age (years)	65.7±13.8	66.0±13.4	65.5±14.1
BMI (kg/m^2)	23.3±3.6	23.9±3.4	22.9±3.7**
Systolic BP (mmHg)	137.9±22.4	139.5±19.4	136.7±24.3
Diastolic BP (mmHg)	76.7±11.7	78.4±11.0	75.4±12.0**
Brinkman index	618.0±468.6	705.4±475.4	395.3±369.3**
Current smoker, n (%)	213 (28.4%)	153 (48.7%)	60 (13.8%)**
Triglyceride (mg/dL)	87 (28-1,482)	97.5 (28-1,482)	83 (30-438)**
HDL-cholesterol (mg/dL)	65.6±17.8	59.9±15.8	69.8±18.0**
LDL-cholesterol (mg/dL)	120.7±29.7	116.8±30.4	123.6±28.9**
Fasting PG (mg/dL)	99.7±23.6	104.0±26.2	96.5±21.0**
HbA1c (%)	5.6±0.7	5.7±0.8	5.5±0.6**
Uric acid (mg/dL)	5.2±1.3	5.9±1.2	4.7±1.2**
eGFR (mL/min/1.73 m^2)	61.8±15.7	54.0±13.4	67.4±14.8**
HOMA-IR	1.66±2.08	1.82±2.21	1.54±1.98
hsCRP (mg/dL)	0.032 (0.004-0.50)	0.036 (0.004-0.50)	0.027 (0.004-0.50)
BNP (pg/mL)	20.3 (4.0-610.4)	33.9 (4.0-610.4)	22.5 (4.0-529.5)
SP-A (ng/mL)	33.8 (10.2-171.0)	32.6 (10.2-171.0)	34.3 (10.5-132.0)
SP-D (ng/mL)	51.4 (17.2-688.0)	55.7 (17.2-688.0)	47.4 (17.2-360.0)**
KL-6 (U/mL)	266.0 (112.0-2,070)	280.0 (121.0-1,590)	262.0 (112.0-2,070)*

Data are presented as mean±SD, n (%), or median (range). *p<0.05, **p<0.01 vs. Male.
BMI: body mass index, BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, PG: plasma glucose, eGFR: estimated glomerular filtration ratio, HOMA-IR: homeostasis model assessment-insulin resistance, hsCRP: highly sensitive C-reacting protein, BNP: B-type natriuretic polypeptide, SP: surfactant protein, KL: Krebs von den Lungen

PG and serum biochemical parameters: high-density lipoprotein cholesterol (HDL-cholesterol), total cholesterol (TCHO), triglycerides, serum creatinine, high-sensitivity C-reactive protein (hs-CRP) and brain natriuretic peptide (BNP). The Friedewald formula (TCHO - HDL-cholesterol-triglycerides/5) was used to calculate the low-density lipoprotein cholesterol (LDL-cholesterol). The estimated glomerular filtration rate (eGFR) was calculated from the data for serum creatinine, age and sex by use of equations for Japanese (30). HbA1c was determined using the latex coagulation method and expressed according to the National Glycohemoglobin Standardization Program (NGSP) scale. The PG level was measured by the hexokinase method. Homeostasis model assessment of insulin resistance (HOMA-IR), an index of insulin resistance, was calculated as previously reported (31). The serum levels of SP-A and KL-6 were measured by a chemiluminescent enzyme immunoassay (HISCL SP-A kit, Sysmex, Kobe, Japan; Picolumi KL-6, Eisai, Tokyo, Japan) and the SP-D level was measured by an enzyme immunoassay (SP-D Yamasa-EIA II, Kyowa-Medex, Tokyo, Japan).

Statistical analysis

Numeric variables are expressed as the means±SD for normally distributed variables and as median (ranges) for variables without a normal distribution. The normality of data distribution was evaluated using the Shapiro-Wilk W test. Student's t-test was used for testing differences between the group means of data that showed a normal distribution. Because the triglycerides, BNP, hs-CRP, SP-A, SP-D, KL-6 and HOMA-IR data were not normally distributed, inter-group differences in the means of these parameters were tested by the Mann-Whitney U test. Data for the variables that were not normally distributed were logarithmically transformed in regression analyses. The relationships between parameters were examined using simple and multiple linear regression analyses. In a multiple linear regression analysis, we selected the dependent variables using the stepwise method based on Akaike's Information Criterion (AIC). A multiple logistic regression analysis was used to closely examine the parameters associated with new-onset diabetes mellitus. A receiver operating characteristic (ROC) curve analysis was performed to calculate the cutoff values to predict new-onset diabetes. Statistical analyses were carried out using JMP Pro (version 12.2.0, SAS Institute, Cary, USA). Inter-group differences in the rate of smokers were examined by the chi-square test. A difference was considered to be statistically significant if p was less than 0.05.

Results

1. Study 1: A cross-sectional analysis

Demographic and clinical characteristics of the study subjects

Demographic parameters and metabolic profiles of the subjects are summarized in Table 1. In comparison to men, women had a smaller BMI, lower diastolic BP, smaller Brinkman index, a lower rate of smokers, lower levels of tri-

Table 2. Correlation Coefficients among SP-A, SP-D, KL-6 and Clinical Parameters.

	Log SP-A		Log SP-D		Log KL-6	
	r	p	r	p	r	p
Age (years)	0.260	<0.001	0.090	0.014	0.250	<0.001
BMI (kg/m^2)	-0.021	0.560	-0.077	0.034	0.220	<0.001
Systolic BP (mmHg)	0.200	<0.001	0.034	0.350	0.180	<0.001
Diastolic BP (mmHg)	0.015	0.680	-0.045	0.220	0.130	0.001
Brinkman index	0.190	0.005	0.170	0.015	0.240	<0.001
Log Triglyceride (mg/dL)	0.140	<0.001	-0.076	0.038	0.180	<0.001
HDL-cholesterol (mg/dL)	-0.058	0.110	0.001	0.970	-0.150	<0.001
LDL-cholesterol (mg/dL)	0.089	0.015	0.012	0.740	0.085	0.019
Fasting PG (mg/dL)	0.045	0.220	0.000	0.990	0.130	<0.001
HbA1c (%)	0.081	0.027	0.051	0.160	0.180	<0.001
Uric acid (mg/dL)	0.036	0.330	0.069	0.057	0.120	<0.001
eGFR (mL/min/1.73 m^2)	-0.110	0.004	-0.110	0.002	-0.220	<0.001
Log HOMA-IR	-0.034	0.360	-0.011	0.770	0.170	<0.001
Log hsCRP (mg/dL)	0.087	0.033	0.066	0.110	0.220	<0.001
Log BNP (pg/mL)	0.300	<0.001	0.180	<0.001	0.093	0.012
Log SP-A (ng/mL)	-	-	0.280	<0.0001	0.240	<0.001
Log SP-D (ng/mL)	-	-	-	-	0.270	<0.001

BMI: body mass index, BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, PG: plasma glucose, eGFR: estimated glomerular filtration ratio, HOMA-IR: homeostasis model assessment-insulin resistance, hsCRP: highly sensitive C-reacting protein, BNP: B-type natriuretic polypeptide, SP: surfactant protein, KL: Krebs von den Lungen

Table 3. Multiple Linear Regression Analysis for Log SP-A.

	Estimate	SE	β	p
Age (years)	0.00035	0.003	0.011	0.900
Sex (women)	-0.021	0.037	-0.040	0.570
Brinkman index	0.00017	0.0001	0.170	0.022
Log BNP (pg/mL)	0.14	0.039	0.290	<0.001
BMI (kg/m^2)	-0.0041	0.009	-0.030	0.640

R^2=0.13

Table 4. Multiple Linear Regression Analysis for Log SP-D.

	Estimate	SE	β	p
Age (years)	0.0021	0.002	0.044	0.37
Sex (women)	-0.067	0.028	-0.100	0.015
eGFR (mL/min/1.73 m^2)	-0.0025	0.002	-0.06	0.200
Log BNP (pg/mL)	0.14	0.031	0.200	<0.001
BMI (kg/m^2)	-0.019	0.007	-0.100	0.007
HbA1c (%)	0.075	0.038	0.076	0.048

R^2=0.060

glycerides, fasting PG, HbA1c and SP-D, and higher levels of HDL-cholesterol, LDL-cholesterol and eGFR. These profiles of the study subjects are consistent with those in previous reports of the Tanno-Sobetsu study (24-28).

Simple regression analyses for SP-A, SP-D and KL-6

To determine the factors that are potentially associated with the serum levels of SP-A, SP-D and KL-6, we first performed a simple linear regression analysis between each of the lung injury biomarkers and demographic and clinical parameters. Because the data for SP-A, SP-D and KL-6 were not normally distributed, these parameters were transformed to logarithmic variables. As shown in Table 2, the serum levels of all three biomarkers correlated with age, the Brinkman index, Log Triglyceride and Log BNP and inversely correlated with eGFR. In addition, the SP-A level correlated with the systolic BP, LDL-cholesterol, HbA1c and the hs-CRP, SP-D level correlated with the BMI, and the KL-6 level correlated with the BMI, systolic and diastolic BPs, HDL-cholesterol, LDL-cholesterol, fasting PG, HbA1c, uric acid, HOMA-IR, and hs-CRP. SP-A, SP-D and KL-6 significantly correlated with each other, though the correlation coefficients were not large (i.e., 0.24-0.28).

Multiple regression analyses for SP-A, SP-D and KL-6

By using demographic and metabolic parameters as independent variables, multiple regression analyses for SP-A, SP-D and KL-6 were performed. For Log SP-A, the Brinkman index and Log BNP were selected as significant independent variables (Table 3). For Log SP-D, significant independent explanatory variables were sex (women), Log BNP, BMI and HbA1c (Table 4). This result for Log SP-D might appear to be inconsistent with the lack of any significant correlation between Log SP-D and HbA1c (Table 2), but the apparent inconsistency is explained by interactions between the explanatory variables. Log SP-D negatively correlated with the BMI (Table 2), the BMI positively correlated HbA1c (r=0.2, p<0.001), and a multiple regression analysis for Log SP-D using a BNP-HbA1c interaction term (i.e., Log BNP - HbA1c) indicated a significant interaction of the

Table 5. Multiple Linear Regression Analysis for Log KL-6.

	Estimate	SE	β	p
Age (years)	0.0053	0.003	0.170	0.042
Sex (women)	-0.010	0.036	-0.021	0.770
Brinkman index	0.00013	0.0001	0.140	0.058
eGFR (mL/min/1.73 m^2)	-0.0028	0.002	-0.110	0.220
BMI (kg/m^2)	0.028	0.008	0.220	<0.001

R^2=0.16

two parameters (Supplementary material Table 1). For Log KL-6, significant explanatory variables were age and the BMI (Table 5).

2. Study 2: Longitudinal analysis

Demographic and clinical characteristics of the study subjects

The baseline characteristics of study subjects in 2011 are presented in Table 6. The data for demographic and metabolic parameters and their differences between men and women were similar to those in the cross-sectional analyses (Table 1) except for the fact that the percentage of current smokers was lower (15.0% vs. 28.4%).

New-onset diabetes mellitus

Of the 415 study subjects enrolled in 2011, 13 subjects (3.1%) were found to have developed diabetes mellitus in 2014. As shown in Table 7, there were differences in the baseline clinical characteristics between the subjects who developed diabetes and the subjects who remained non-diabetic; namely, the age was higher, the BMI was larger, the levels of systolic BP, fasting PG, HbA1c, HOMA-IR and KL-6 were higher and the serum HDL-cholesterol level was lower in the subjects who developed diabetes during the following three years. In contrast to the KL-6 level, the SP-D and SP-A levels in 2011 were similar in the subjects with and without diabetes in 2014. In the subjects who later developed diabetes, the mean values of fasting PG and HbA1c in 2011 were slightly above the upper limits of normal values of the parameters, suggesting that "prediabetic" subjects at baseline were included in this longitudinal analysis.

A multiple logistic regression analysis for new-onset diabetes mellitus

As the number of subjects with new-onset diabetes was relatively small, we selected four variables in addition to sex and age to prepare a model by a step-wise method. Since fasting PG, HbA1c and HOMA-IR are closely correlated with each other, we prepared three models in which one of the three metabolic parameters was incorporated together with Log KL-6 (models 1-3, Table 8). As in earlier studies (3-7), the fasting PG (model 1), HbA1c (model 2) and Log HOMA-IR (model 3) were significantly associated with new-onset diabetes. Log KL-6 was not selected as an independent explanatory variable for new-onset diabetes in models 1 and 2, but Log KL-6 and systolic BP were significantly associated with new-onset diabetes in the model in which Log HOMA-IR was incorporated as an independent variable (model 3). Neither SP-A nor SP-D was selected as a significant explanatory variable for new-onset diabetes.

As a *post-hoc* analysis, we selected patients with both fasting PG and HbA1c being within normal ranges (i.e., fasting PG <110 mg/dl, HbA1c <6.2%) in 2011 (n=352) and repeated a multiple logistic regression analysis for new-onset diabetes mellitus. However, since the number of subjects with new-onset diabetes in 2014 was only 4 (Supplementary material Table 2), no significant association was detected between new-onset diabetes and any of clinical parameters presumably due to a lack of sufficient statistical power (Supplementary material Table 3).

A multiple regression analysis for HOMA-IR

Consistent with the results of a cross-sectional analysis for the relationships of SP-A, SP-D, KL-6 and HOMA-IR, none of the lung injury biomarkers in 2011 was selected as an explanatory variable of Log HOMA-IR in 2011 (data not shown). However, the BMI and Log KL-6 in 2011 were found to be independent variables of Log HOMA-IR in 2014 (Table 9).

A receiver operating characteristic (ROC) curve analysis

Since KL-6 was found to be a significant independent variable for new-onset diabetes in the present cohort, the predictive value of this parameter was examined by a ROC curve analysis. The area under the curve (AUC) for KL-6 was 0.63 [95% confidence interval (CI): 0.53-0.73] (Figure). The optimal cut-off point of KL-6 was 376.0 U/mL with a sensitivity of 44% and specificity of 84%.

Discussion

The present study showed differences between SP-A, SP-D and KL-6 in their association with clinical parameters in a general population not receiving any medical treatment (Table 3-5). BNP was an independent explanatory variable for the serum levels of SP-A and SP-D, but not for the level of KL-6. Interestingly, the KL-6 level correlated with the BMI and other indices of pre-diabetes individuals (i.e., HOMA-IR, fasting PG, triglycerides), but age and BMI were factors that independently explained the variation in the serum KL-6 level (Table 2, 5). Furthermore, the serum KL-6 level was one of the significant explanatory parameters for new-onset diabetes three years later (Table 8) and HOMA-IR three years later (Table 9). These findings indicate that distinct parameters are involved in the variations of the SP-A, SP-D and KL-6 levels in the serum and suggest that KL-6 is a predictor of the risk for insulin resistance and new-onset diabetes in the general population.

SP-A and SP-D are surfactant lipoproteins produced in airway Clara cells and alveolar epithelial cells, and KL-6 is a mucin-like glycoprotein synthesized in alveolar type II cells and bronchiolar epithelial cells (23). Different patterns of elevation of these three biomarkers are observed depending on the type of lung injury, and they are currently util-

Table 6. Baseline Characteristics in Study 2: Men vs. Women.

	Total (n=415)	Men (n=182)	Women (n=233)
Age (years)	65.0±12.6	64.5±12.9	65.3±12.4
BMI (kg/m^2)	23.3±3.6	24.1±3.6	22.7±3.5**
Systolic BP (mmHg)	137.8±22.2	138.5±19.6	137.2±24.1
Diastolic BP (mmHg)	76.6±11.5	78.5±10.9	75.1±11.7**
Brinkman index	616.1±478.8	688.0±488.4	396.6±375.4**
Current smoker, n (%)	62 (15.0%)	46 (25.4%)	16 (6.9%)**
Triglyceride (mg/dL)	87.0 (30.0-1,070)	91.5 (33.0-1,070)	84.0 (30.0-344)**
HDL-cholesterol (mg/dL)	68.0±17.8	62.3±17.4	72.5±16.7**
LDL-cholesterol (mg/dL)	122.2±28.2	117.3±29.5	126.0±26.7**
Fasting PG (mg/dL)	98.6±23.1	102.5±25.8	95.5±20.2**
HbA1c (%)	5.6±0.8	5.7±0.9	5.5±0.6*
Uric acid (mg/dL)	5.0±1.3	5.8±1.4	4.5±1.0**
eGFR (mL/min/1.73m^2)	70.8±13.7	71.9±13.4	70.0±13.8
HOMA-IR	1.57±2.55	1.83±3.60	1.36±1.16
hsCRP (mg/dL)	0.036 (0.004-0.50)	0.041 (0.005-0.50)	0.032 (0.004-0.50)*
BNP (pg/mL)	17.6 (4.0-476.4)	14.8 (4.0-461.7)	21.8 (4.0-476.4)
SP-A (ng/mL)	35.7 (11.3-163.0)	33.8 (11.3-163.0)	36.8 (13.6-150.0)
SP-D (ng/mL)	46.9 (17.2-291.0)	50.7 (17.2-291.0)	45.1 (17.2-221.0)*
KL-6 (U/mL)	261.5 (107.0-1,190)	278.0 (132.0-1,190)	253.0 (107.0-1,120)

Data are presented as mean±SD, n (%) or median (range). *p<0.05, **p<0.01 vs. Male.

BMI: body mass index, BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, PG: plasma glucose, eGFR: estimated glomerular filtration ratio, HOMA-IR: homeostasis model assessment-insulin resistance, hsCRP: highly sensitive C-reacting protein, BNP: B-type natriuretic polypeptide, SP: surfactant protein, KL: Krebs von den Lungen

ized for the differential diagnosis of lung diseases (22, 23). However, variations in the serum levels of SP-A, SP-D and KL-6 in a general population and the factors that influence such variations have not yet been characterized. The results of a multiple regression analysis indicate that distinct variables are associated with each of the three biomarkers of lung injury (Table 3-5). Small R^2 values (i.e., 0.06-0.16) of the statistical models for SP-A, SP-D and KL-6 appear to reflect good specificity of the molecules as biomarkers of lung injury. However, the results of the present study suggest that the BMI, heart failure and age need to be taken into account when interpreting the marginal changes in the serum levels of SP-A, SP-D and KL-6.

Fasting PG, HbA1c and HOMA-IR are major predictors of diabetes development (3-7). Significant associations of the three parameters with the risk of new-onset diabetes were confirmed in the present cohort (Table 8). An association of KL-6 with new-onset diabetes was indicated in a multiple regression model in which HOMA-IR was included as an independent variable (model 3, Table 8), but not in a model that included fasting PG or HbA1c as a metabolic parameter. The follow-up period was relatively short (i.e., three years), and fasting PG and HbA1c were included in the diagnostic criteria of diabetes. In contrast, the impact of inflammation on PG is mediated by indirect mechanisms, such as a reduction in insulin sensitivity (8, 9), and a period of the pre-diabetic metabolic state precedes elevation of the PG level to the diabetic level. Thus, a plausible explanation for the different contributions of KL-6 in the three models of predictors of new-onset diabetes (Table 8) is the involvement of inflammation detected by a KL-6 elevation in the development of diabetes as a mechanism upstream of the elevation of the PG level. Although a ROC analysis (Figure) indicated that the KL-6 level is not a sensitive predictor of diabetes development as is HOMA-IR, the specificity of KL-6> 376 U/mL was 84%, thus suggesting some practical value.

Earlier studies (20, 32, 33) showed an association of insulin resistance with an elevation of SP-A or a reduction of SP-D within "its normal range". Fernández-Real et al. (20) reported that serum SP-A level was higher in non-smoking men with glucose intolerance and that there was an inverse correlation between the serum SP-A level and insulin sensitivity. In contrast, the serum SP-D level was positively associated with insulin sensitivity (32), though SP-D gene polymorphism may contribute to insulin resistance independently of the serum SP-D level (33). We attempted to confirm the associations of SP-A and SP-D with the change in insulin sensitivity in the present cohort. Although the BMI was a negative explanatory variable of SP-D (Table 3), HOMA-IR was not correlated with either SP-A or SP-D (Table 2), and HOMA-IR or fasting PG was not selected as an independent variable of SP-A and SP-D (Table 3, 4). Furthermore, neither of these surfactant proteins was selected as an independent variable contributing to the development of diabetes (Table 8) or to the increase in HOMA-IR (Table 9) during a three-year follow-up. The reason for these discrepant results is unclear, but differences in the clinical characteristics of the study subjects and differences in the methods of insulin

Table 7. Baseline Characteristics in Study 2: Subjects who Later Developed Diabetes (Diabetes) vs. Subjects who Remained Non-diabetic (Non-Diabetes).

	Diabetes (n=13)	Non-Diabetes (n=402)
Male (n, %)	6 (46.2%)	177 (44.3%)
Age (years)	68.9±9.1*	64.5±12.9
BMI (kg/m^2)	25.4±4.4**	23.1±3.4
Systolic BP (mmHg)	151.2±18.7**	136.2±22.1
Diastolic BP (mmHg)	79.3±13.9	76.2±11.2
Brinkman index	698.1±465.9	606.1±481.1
Current smoker, n (%)	2 (15.4%)	60 (15.0%)
Triglyceride (mg/dL)	107.0 (37.0-296)	85.5 (30.0-1,070)
HDL-cholesterol (mg/dL)	61.2±18.7**	68.8±17.5
LDL-cholesterol (mg/dL)	124.3±29.8	121.9±28.1
Fasting PG (mg/dL)	113.3±20.2*	98.1±23.0
HbA1c (%)	6.3±0.6*	5.6±0.8
Uric acid (mg/dL)	5.3±1.3	5.0±1.3
eGFR (mL/min/1.73 m^2)	69.3±14.4	71.0±13.6
HOMA-IR	3.29±6.9**	1.37±1.2
hsCRP (mg/dL)	0.052 (0.010-0.50)	0.034 (0.0040-0.50)
BNP (pg/mL)	16.1 (4.0-476.4)	17.6 (4.0-461.7)
SP-A (ng/mL)	39.9 (14.3-150.0)	35.1 (11.3-163.0)
SP-D (ng/mL)	48.1 (17.2-254.0)	46.8 (17.2-291.0)
KL-6 (U/mL)	306.0 (163.0-1,170)**	258.0 (107.0-1,190)

Data are presented as n (%), mean±SD or median (range). *p<0.05, **p<0.01 vs. Non-Diabetes.

BMI: body mass index, BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, PG: plasma glucose, eGFR: estimated glomerular filtration ratio, HOMA-IR: homeostasis model assessment-insulin resistance, hsCRP: highly sensitive C-reacting protein, BNP: B-type natriuretic polypeptide, SP: surfactant protein, KL: Krebs von den Lungen

Table 8. Multiple Logistic Regression Analysis for the New-onset Type 2 Diabetes.

Model 1	Estimate	SE	χ^2	p
Sex (women)	0.0104	0.228	0.002	0.960
Age (years)	0.0305	0.024	1.60	0.210
BMI (2011) (kg/m^2)	0.143	0.062	5.31	0.021
Systolic BP (2011) (mmHg)	0.0283	0.011	6.62	0.010
Fasting PG (2011) (mg/dL)	0.0948	0.014	45.0	<0.001
Log KL-6 (2011)	0.716	0.521	1.89	0.170
Model 2	Estimate	SE	χ^2	p
Sex (women)	0.145	0.209	0.479	0.490
Age (years)	0.0213	0.023	0.865	0.350
BMI (2011) (kg/m^2)	0.0871	0.060	2.11	0.150
Systolic BP (2011) (mmHg)	0.0206	0.010	3.91	0.048
HbA1c (2011) (%)	2.28	0.357	40.7	<0.001
Log KL-6 (2011)	0.544	0.498	1.19	0.270
Model 3	Estimate	SE	χ^2	p
Sex (women)	0.277	0.183	2.31	0.130
Age (years)	0.0173	0.017	1.00	0.320
BMI (2011) (kg/m^2)	0.0332	0.053	0.393	0.530
Systolic BP (2011) (mmHg)	0.0246	0.009	7.38	0.007
Log HOMA-IR (2011)	0.946	0.287	10.8	0.001
Log KL-6 (2011)	1.02	0.412	6.15	0.013

Model 1: R^2=0.455, Akaike's information criterion (AIC)=162.1, Model 2: R^2=0.373, AIC=184.4, Model 3: R^2=0.175, AIC=238.1.

Table 9. Multiple Linear Regression Analysis for Log HOMA-IR (2014).

	Estimate	SE	β	p
Sex (women)	0.0299	0.027	0.048	0.270
Age (years)	-0.00190	0.002	-0.039	0.430
BMI (2011) (kg/m^2)	0.0812	0.008	0.472	<0.001
Systolic BP (2011) (mmHg)	-0.000138	0.001	-0.005	0.920
Log KL-6 (2011)	0.162	0.068	0.108	0.018

R^2=0.25

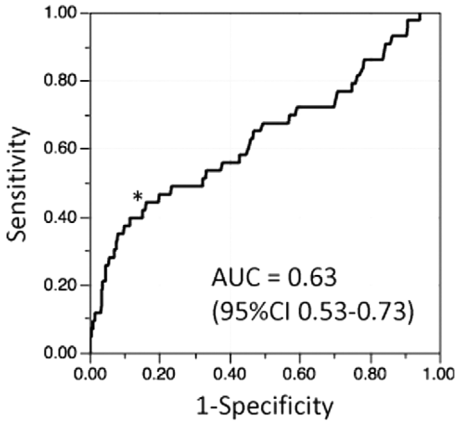

Figure. A ROC curve of the use of KL-6 as a predictor of diabetes mellitus developmentduring a 3-year period. A ROC analysis for KL-6 (2011) to predict new-onset diabetes is shown. The optimal cut-off point (*) was 376.0 with a sensitivity of 0.44 and specificity of 0.84.

resistance assessment may be involved. The age of the study subjects was higher and subjects with diabetes were excluded at the time of enrollment in the present study. An intravenous glucose tolerance test with glucose and insulin injection was used to assess insulin sensitivity in earlier studies (20, 31, 33), while we used HOMA-IR. Thus, the present findings do not exclude the possibility of an association of SP-A or SP-D with insulin resistance, but suggest that the change in insulin sensitivity associated with changes in the serum SP-A and SP-D levels was not large enough to be detected by HOMA-IR or by the incidence of new-onset diabetes during the three-year follow-up of this study.

To our knowledge, the changes in the serum KL-6 level in diabetic patients without lung disease has only so far been examined in one study by Takahashi et al. (21), and it was shown that the KL-6 level was significantly higher in diabetic patients than in healthy controls (643.0±32.7, n=176 vs. 206.5±6.1 U/mL, n=237). Interestingly, although the serum KL-6 level was higher in diabetic patients on insulin or oral hypoglycemic agents than in those on diet and exercise therapy only, the KL-6 level in diabetic patients did not correlate with the level of glycemic control (21). These findings suggest that the severity of diabetic complications, but not hyperglycemia *per se*, determines an elevation of serum KL-6 level in patients with diabetes. The present study showed for the first time that an elevation of the serum KL-6 level in non-diabetic subjects is associated with a risk of diabetes development (Table 7-9) and that the level of KL-6 in pre-diabetic subjects was lower (400.5±240.5 U/mL) than the level in diabetic patients reported by Takahashi et al. (21).

The mechanism underlying the association of the serum KL-6 level with the risk of diabetes development is unclear. However, there are a few possible explanations for the association between KL-6 and diabetic risk. First, inflammatory cytokines produced together with KL-6 in the lungs may induce insulin resistance, in the same manner as that for visceral fat-derived TNF-alpha and IL-6. In fact, the KL-6 level predicted an increase in HOMA-IR three years later (Table 9). Although direct evidence of such cytokines is lacking, this explanation is supported by the result of a case-control study showing that the incidence of diabetes was 2.7-fold higher in patients with idiopathic pulmonary fibrosis on no steroid therapy than in control subjects (34). However, it is notable that both KL-6 and HOMA-IR were selected as explanatory variables of new-onset diabetes (Table 8), indicating that insulin resistance associated with a KL-6 elevation is not the only mechanism of diabetes development in subjects with elevated KL-6 levels. Second, an elevation of KL-6 may be associated with novel risk factors of diabetes. Recent studies using a metabolomic analysis of blood samples revealed that a few small peptides and amino acid profile predict new-onset diabetes (35, 36). KL-6 is expressed not only in the cells in the respiratory system, but also in renal tubular cells (22). The relationship between the KL-6 expression in those cells and the blood metabolomes associated with risk of diabetes development is therefore an interesting issue to be examined in the future.

There are some limitations associated with the present study. First, because chest X-rays were not included in the annual examinations in the Tanno-Sobetsu study, we cannot rule out the possibility that there were mild cases of interstitial lung diseases in the study subjects. The mean values of SP-A, SP-D and KL-6 were similar to those reported previously for healthy controls (20-22, 32, 33), but in a few subjects, one of the three biomarkers was high despite the absence of any respiratory symptoms. Second, the follow-up period in the longitudinal analysis was relatively short. Thus, risk of diabetes associated with KL-6 elevation might have been underestimated, and we cannot exclude the possibility that the follow-up period was too short to detect any associations of the SP-A and/or SP-D levels with the risk of

new onset diabetes. Third, since we did not use the 75 g-oral glucose tolerance test for the diagnosis of diabetes mellitus, we cannot completely rule out the possibility that some undiagnosed diabetic patients (i.e., those with fasting PG<126 mg/dL or HbA1c<6.5%) at baseline may have been included in the longitudinal analysis. Fourth, we did not collect information regarding passive smoking from the study subjects. A cohort study in which full-time employees in 12 large companies were enrolled indicated that passive smoking raised the risk of diabetes (37), and thus the possible contribution of passive smoking to variations in the SP-A, SP-D or KL-6 levels and to the new-onset diabetes could not be examined. Finally, because of the lack of any interventions for KL-6, whether the association of the serum KL-6 level with the risk of diabetes mellitus is causative or not remains unclear.

In conclusion, a modest elevation of the serum KL-6 level was found to be associated with the risk of insulin resistance development and new-onset of diabetes mellitus in a cohort of the Japanese general population. However, no such association with the risk of diabetes was observed for SP-A or SP-D during a three-year follow-up.

Author's disclosure of potential Conflicts of Interest (COI).
Tetsuji Miura: Honoraria, Daiichi-Sankyo, Behringer-Ingelheim Japan and MSD.

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