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Hospital plaza Building 1F
9-15 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan
TEL: +81-3-3265-0079
FAX: +81-3-3265-0083
E-mail: info@ningen-dock.jp
URL : http://www.ningen-dock.jp/

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"AminoIndex Technology" for Lifestyle-related Disease Risk Screening

Minoru Yamakado

Abstract

Recently, both overnutrition and undernutrition have become social issues. Overnutrition has led to an increase in the number of individuals with metabolic syndrome and lifestyle-related diseases, while undernutrition due to loss of appetite in the elderly or severe weight-loss diets in young women may lead to loss of skeletal muscle. Early detection of these risks followed by appropriate interventions is critical for preventing the development of serious diseases such as cardiovascular and cerebrovascular diseases and locomotive syndrome.

Recent studies have reported that future risks for developing lifestyle-related diseases include changes in plasma amino acid profiles and that malnutrition changes plasma amino acid profiles in a different manner from overnutrition. Based on these findings, other studies have developed and evaluated the performance of multivariable models based on plasma amino acid concentrations for use as screening tests. We reviewed one of these screening tests (AminoIndex$_{TM}$ LifeStyle diseases) regarding the development of diabetes within four years as a result of overnutrition as well a status of undernutrition.

Keywords plasma amino acid, diabetes, multivariable analysis, biomarker

In recent years, the nutritional issues that people in contemporary society face have become increasingly complex. Lack of exercise and excessive nutritional intake increase the risk for obesity and metabolic syndrome, which may lead to lifestyle-related diseases such as diabetes[1]. In contrast, loss of appetite resulting from physical changes in elderly people and changes in their living environment as their age may cause undernutrition[2]. The latter has also been seen in young women who suffer nutritional deficiency as a result of excessive weight loss diets[3]. Thus, malnutrition in the form of the simultaneous presence of overnutrition and undernutrition is a double burden for modern-day Japanese society that requires immediate measures.

Both overnutrition and undernutrition lead to serious diseases. Overnutrition increases the risk for diabetes and the resulting cerebrovascular accidents and cardiovascular disease. Undernutrition, a particular feature of modern society, is characterized by a substantially insufficient intake of protein, resulting in a lack of amino acids and other plasma constituents, and muscle volume. This increases the risks for anemia and sarcopenia, and people may eventually develop locomotive syndrome or become bed-ridden. The onset of these diseases and symptoms is not sudden and it is thought that the risk for developing them gradually increases as a result of poor everyday lifestyle habits. Therefore, we believe it is important to properly screen for these risks at an early stage so that daily preventive behavior can be practiced by patients[4].

Fluctuations in plasma levels of amino acids and relationships between them and lifestyle-related diseases such as diabetes[5,6], hyperglycemia[7,8], insulin resistance[9–17], nonalcoholic fatty liver disease (NAFLD)[18,19], and cardiovascular disease[20–22], as well as related findings have been reported. Studies have also assessed fluctuations in plasma levels of amino acids under states of undernutrition[23,24] or low protein intake[25]. In other research, the health status and risk for developing cancer[26–29], liver disease[30], and inflammatory bowel disease (IBD)[31] have been evaluated by creating index formulas consisting of multiple amino acid levels and applying multivariate analysis. In recent years, a number of studies have developed index formulas for nutritional status and lifestyle-related diseases[32–34].

Among screening tests using plasma levels of amino acids examined in recent studies, this review article focused on AminoIndex$_{TM}$ LifeStyle diseases (AILS),

Faculty of Nursing, Ashikaga Institute of Technology; Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital
Contact : Minoru Yamakado, Faculty of Nursing, Ashikaga Institute of Technology; 268–1 Omaecho, Ashikaga, Tochigi, 326-8558, Japan.
Tel : +81–284–62–0605 ; Fax : +81–284–21–1270 ; E-mail : yamakado.minoru@v90.ashitech.ac.jp

which evaluates the risk for lifestyle-related diseases based on amino acid levels. AILS comprises two tests, namely AILS (risk for diabetes), which evaluates the risk for developing diabetes within four years, and AILS (amino acid level), which evaluates decreases in plasma concentrations of essential and semi-essential amino acids. In the following, we review the features of AILS and the findings regarding diabetes, as well as the risks due to decreased levels of essential and semi-essential amino acids, which are important to the body.

Evaluation of risk for diabetes using "AminoIndex Technology"

Insulin resistance due to visceral fat accumulation is a major cause of diabetes and insulin resistance also causes the balance of plasma amino acid levels to fluctuate[35]. In order to examine fluctuations in the balance of plasma amino acid levels in the early stages of diabetes rather than before its onset, data from 8,070 subjects who visited the Center for Multiphasic Health Testing and Services at Mitsui Memorial Hospital were analyzed. Ability to discriminate between subjects that developed diabetes within four years from AILS testing (215 subjects) and those that had already developed diabetes at the time of testing (367 subjects) were assessed relative to subjects that had not developed diabetes within four years (7,488 subjects). The results for the area under the receiver operating characteristic (ROC) curves for each amino acid are shown in **Fig. 1**. The amino acid balance of subjects that developed diabetes within four years was similar to that of subjects with diabetes, suggesting that changes in amino acid metabolism may occur prior to the onset of diabetes. Therefore, the development of an AILS (risk for diabetes) formula using plasma amino acid levels to assess the risk for diabetes was discussed. As shown in **Fig. 2**, this formula was developed as a multivariate formula using plasma amino acid levels, which correlate to visceral fat area, as an indicator of pre-diabetic visceral fat accumulation. Data from 650 individuals who visited the Center for Multiphasic Health Testing and Services of Mitsui Memorial Hospital for health screening were used for the derivation of candidate formulas and data from the 215 subjects were used for verification. Asparagine, glycine, alanine, valine, tyrosine, and tryptophan are included in the AILS (risk for diabetes) formula derived in this study. Alanine, valine, tyrosine, and tryptophan levels were significantly higher ($p < 0.05$) in subjects that developed diabetes within four years compared to the levels in those who did not develop it within this period, while glycine levels were significantly lower ($p < 0.05$). The possibility of predicting the development of diabetes within four years using this formula was evaluated using the data from 7,703 people undergoing health screening at this hospital who had not developed diabetes at the time of examination.

AILS (risk for diabetes) value and risk for developing diabetes within four years

The AILS (risk for diabetes) value is an index that indicates the risk for developing diabetes. Based on the AILS (risk for diabetes) formula, when the specificity for developing diabetes within four years is 40%, the value is 5.0, and a specificity of 80% corresponds to a value of 8.0. The minimum and maximum values are 0.0 and 10.0, respectively. In addition, AILS (risk for diabetes)

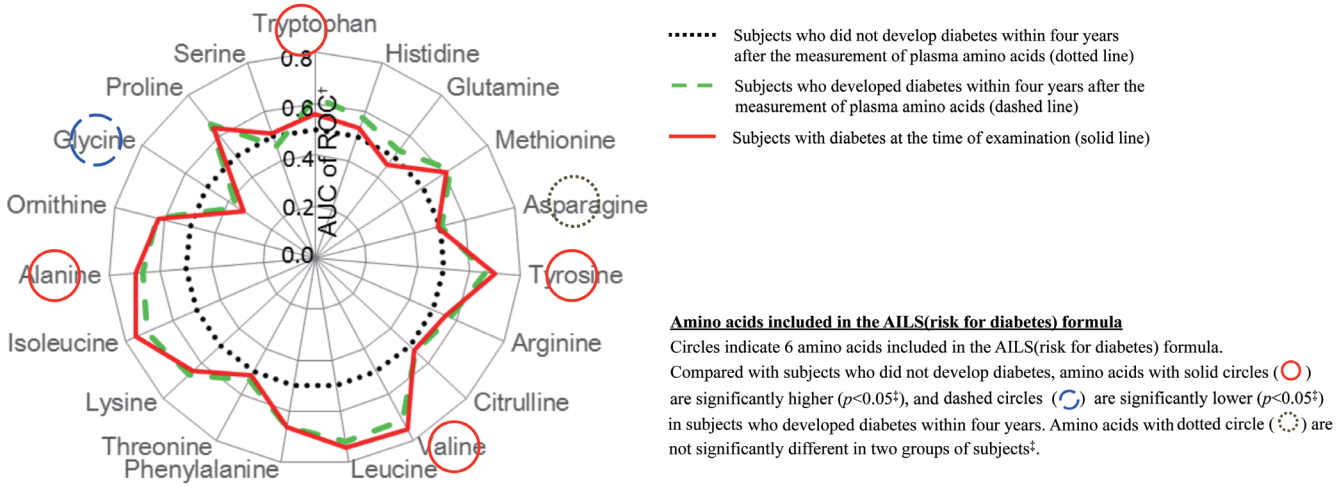


Fig. 1. Balance of Plasma Amino Acid Concentrations in Diabetic Subjects and Subjects who Developed Diabetes within Four Years

† The values on this axis represent the area under the ROC curve, indicates ability to discriminate between group of subjects who did not develop diabetes and the other groups. ‡ Mann-whitney U test.

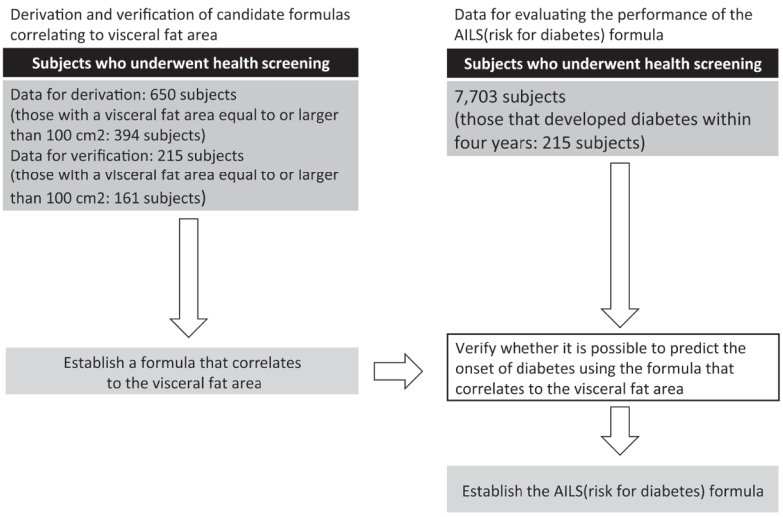


Fig.2. Flow of Derivation of AILS (risk for diabetes) Formula

Table 1. Specificity Sensitivity and Positive Predictive Value of AILS (risk for diabetes) for Onset of Diabetes within Four years

A. Analysis of all subjects who underwent health screening

Rank B or Rank C			Rank C		
Specificity	Sensitivity	Positive predictive value	Specificity	Sensitivity	Positive predictive value
40% (2996/7488)	94% (203/215)	4% (203/4695)	80% (5991/7488)	50% (107/215)	7% (107/1604)

B. Analysis by sex

Sex	Rank B or Rank C			Rank C		
	Specificity	Sensitivity	Positive predictive value	Specificity	Sensitivity	Positive predictive value
Male	21% (936/4416)	97% (179/185)	5% (179/3659)	70% (3094/4416)	52% (97/185)	7% (97/1419)
Female	67% (2060/3072)	80% (24/30)	2% (24/1036)	94% (2897/3072)	33% (10/30)	5% (10/185)

A total of 7,703 subjects that had not developed diabetes at the time of examination and whose data were available for the following four years were included in the analysis.

values are classified as Rank A, for values lower than 5.0, Rank B, for values from 5.0 to less than 8.0, and Rank C for values of 8.0 or higher. The higher the AILS (risk for diabetes) value, the greater the risk for developing diabetes within four years. Among those who underwent health screening, 8,164 (4,988 men and 3,176 women, including those who were diabetic or had unknown diabetes status at the time of examination) were followed up for four years and the distributions of AILS (risk for diabetes) ranks were analyzed according to sex and age. In this population, 81% of the men and 34% of the women were categorized as Rank B or Rank C. This indicates that men tended to be more likely to come under Rank B or Rank C, which is similar to the sex difference in those affected by diabetes[36]. In addition, as AILS (risk for diabetes) is intended to be applied to subjects who are not diabetic at the time of examination, the rank distribution of those who were diabetic at the time of examination, and therefore out of the scope of evaluation, was used as a reference. Among the subjects with diabetes, however, 94% (96% of the men and 82% of the women) were categorized as Rank B or Rank C, indicating that the AILS was accurate in detecting those with diabetes at the time of examination.

We next examined the predictive accuracy of AILS (risk for diabetes) for onset of diabetes within four years. **Table 1** shows the specificity, sensitivity, and positive predictive value for onset of diabetes within four years. The sensitivity for Rank B or Rank C, that is, the accuracy of correctly predicting the onset of diabetes within four years was 94%, indicating that AILS (risk for diabetes) is an effective formula for predicting the risk for developing diabetes within this period. In order to assess whether a person is diabetic at the present moment, diabetes associated data from 8,089 subjects at the time of the initial examination and the ensuing four years were examined. The specificity, sensitivity, and positive predictive value for those categorized as Rank B or Rank C were 39% (3,010/7,722 subjects), 94% (344/367 subjects), and 7% (344/5,056 sub-

jects), respectively. The results for Rank C were 79% (6,111/7,722 subjects), 65% (239/367 subjects), and 13% (239/1,850 subjects), respectively. This demonstrates that AILS (risk for diabetes) is an accurate test for assessing whether a person is diabetic at the present moment. In addition, the AILS (risk for diabetes) rank distributions of those who did and those who did not develop diabetes within four years of AILS testing were also analyzed. Twenty percent of those that did not develop diabetes within four years were categorized as Rank C and 40% were Rank B, while 6% of those that developed diabetes within four years were Rank A. **Fig. 3 A** shows the risk for developing diabetes within four years by AILS (risk for diabetes) rank. When the risk for developing diabetes within four years for Rank A subjects was expressed as 1.0, the odds ratios of developing diabetes for Rank B and Rank C subjects were 8.0 (relative risk: 7.8) and 17.8 (relative: risk 16.7). Thus the risk was significantly higher for both ranks compared to that for Rank A. **Fig. 3 B** shows the results of similar analyses for men and women separately. They show that the risk was still significantly higher for those categorized as Rank B and Rank C as compared to Rank A, regardless of sex. These results indicate the feasibility of AILS (risk for diabetes) values for assessing the risk for developing diabetes within four years.

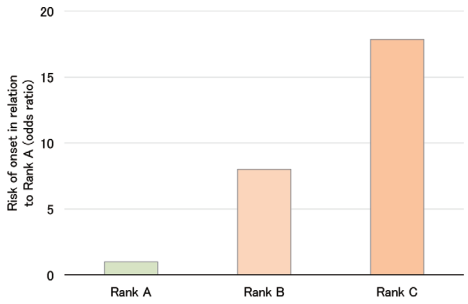
Characteristics of AILS (risk for diabetes) values

The potential of using AILS (risk for diabetes) to determine the risk for developing diabetes within four years was also assessed in subjects not affected by diabetes, hyperlipidemia, or hypertension, that is, subjects belonging to a subgroup with low probablility of developing lifestyle-related diseases. Relative to Rank A, the risks of onset (odds ratios) were 6.2 and 8.9 for Rank B and Rank C, respectively, indicating a significantly higher risk for both categories. In order to examine whether the risk for developing diabetes within four years was higher in Rank B and Rank C as compared to Rank A in subjects whose fasting blood glucose, glycated hemoglobin (HbA1c), glycated albumin, and homeostatic model assessment for insulin resistance (HOMA-IR) values were within normal or standard ranges, we conducted a subgroup analysis according to different ranges

A. Overall

B. Risk for developing diabetes within four years

AILS (risk for diabetes)	Percentage developing diabetes within four years	Odds ratio relative to Rank A		Relative risk relative to Rank A*
		Odds ratio	Confidence interval	
Rank A	0.4% (12/3,008 subjects)	1.0		1.0
Rank B	3.1% (96/3,091 subjects)	8.0	4.4 – 14.6	7.8
Rank C	6.7% (107/1,604 subjects)	17.8	9.8 – 32.5	16.7



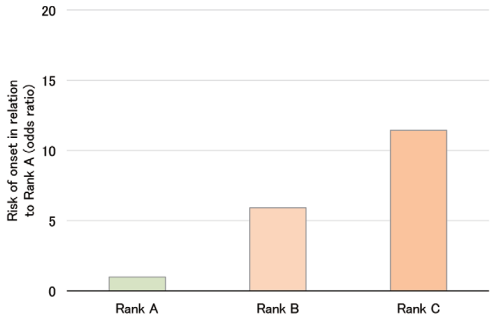
B. By sex

Risk for developing diabetes within four years (males)

AILS (risk for diabetes)	Percentage developing diabetes within four years	Odds ratio relative to Rank A		Relative risk relative to Rank A
		Odds ratio	Confidence interval	
Rank A	0.6% (6 /942 subjects)	1.0		1.0
Rank B	3.7% (82/2,240 subjects)	5.9	2.6 – 13.6	5.7
Rank C	6.8% (97/1,419 subjects)	11.4	5.0 – 26.2	10.7

Risk for developing diabetes within four years (females)

AILS (risk for diabetes)	Percentage developing diabetes within four years	Odds ratio relative to Rank A		Relative risk with respect to Rank A
		Odds ratio	Confidence interval	
Rank A	0.3% (6/2,066 subjects)	1.0		1.0
Rank B	1.6% (14/851 subjects)	5.7	2.2 – 15.0	5.7
Rank C	5.4% (10/185 subjects)	19.6	7.0 – 54.6	18.6



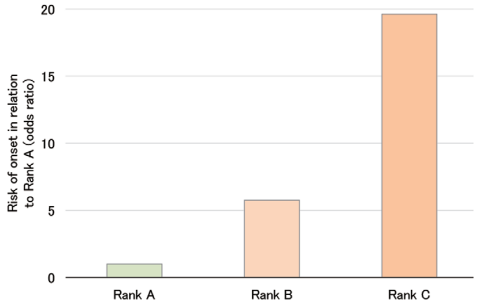


Fig. 3. Risk for Developing Diabetes within Four Years According to AILS (risk for diabetes) Classification

for each of these indexes. Among subjects with a fasting blood glucose level lower than 100 mg/dL or between 100 and 109 mg/dL, the risk of onset (odds ratio) was significantly higher for subjects in Rank B and Rank C compared to subjects in Rank A. The risks of onset (odds ratio) for these ranges were 10.1 (95% confidence interval [CI] 1.3–80.7) and 4.7 (95%CI 1.1–20.1) for Rank B and 16.3 (95%CI 1.9–139.3) and 5.2 (95%CI 1.2–22.9) for Rank C, respectively. Among subjects with an HbA1c level below 5.6% or from 5.6–5.9%, none of the subjects in Rank A developed diabetes within four years; however, subjects in Rank B and Rank C did develop diabetes within this period. Among subjects with an HbA1c level of 6.0–6.4%, the risk of onset (odds ratio) was significantly higher for those categorized as Rank B and Rank C compared to Rank A. The risks of onset (odds ratio) were 7.5 (95%CI 2.7–21.1) and 13.6 (95%CI 4.9–37.8) for Rank B and Rank C, respectively. In addition, among subjects with glycated albumin levels of 16% or less, the odds ratios of developing diabetes within four years relative to Rank A were 10.8 (95%CI 1.4–82.2) and 21.7 (95%CI 2.8–165.9) for Rank B and Rank C, respectively. Furthermore, among those with HOMA-IR values less than 2.5, the odds ratios of developing diabetes within four years relative to Rank A were 7.8 (95%CI 4.2–14.8) and 11.3 (95%CI 5.8–22.2) for Rank B and Rank C, respectively. Therefore, the risk of onset (odds ratio) significantly increases as the AILS (risk for diabetes) rank increases, even when the above indices are within the standard ranges[37]. **Fig. 4** shows an analysis of 215 subjects that developed diabetes within four years regarding a relationship between AILS (risk for diabetes) ranks and fasting blood glucose and HbA1c levels at the time of the initial examination. The results showed that the incidence of onset was higher for Rank B and Rank C than in Rank A regardless of the fasting blood glucose or HbA1c level at the time of examination. This finding suggests that evaluation by AILS (risk for diabetes) would be significant for those with low values in clinical tests used for assessment of diabetes, such as 109 mg/dL or lower for fasting blood glucose or 5.9% or lower for HbA1c.

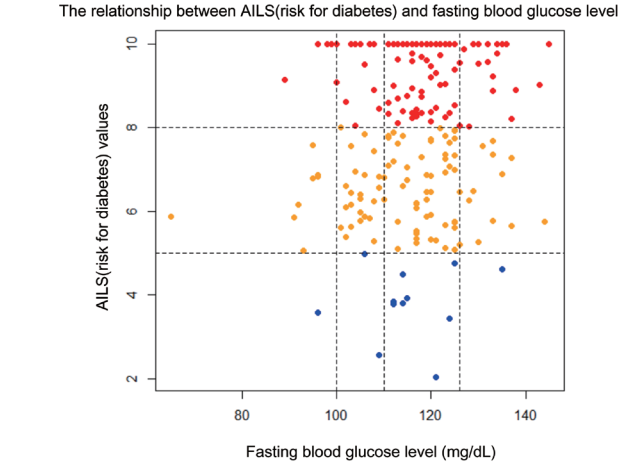
Japanese (Asians) accumulate fat differently from Westerners and among the former, there are a substantial number with hidden obesity; that is, those who have visceral obesity with a BMI lower than 25 kg/m^2 [38]. This means that Japanese may be at risk for developing diabetes even they are not considered obese according to their BMI or do not have metabolic syndrome. We therefore examined whether the onset of diabetes within four years was predictable with AILS (risk for diabetes) in people who were not obese or did not have metabolic syndrome. Among those with a BMI lower than

Relationship with fasting blood glucose level at time of examination (those that developed diabetes within four years)

AILS (risk for diabetes)	Fasting blood glucose (mg/dL)			
	100>	100–109	110–125	126≤
Rank A	0.5% (1/215 subjects)	0.9% (2/215 subjects)	3.7% (8/215 subjects)	0.5% (1/215 subjects)
Rank B	3.7% (8/215 subjects)	10.7% (23/215 subjects)	24.2% (52/215 subjects)	6.0% (13/215 subjects)
Rank C	2.3% (5/215 subjects)	7.9% (17/215 subjects)	30.7% (66/215 subjects)	8.8% (19/215 subjects)

Relationship with HbA1c level at time of initial examination (those that developed diabetes within four years)

AILS (risk for diabetes)	HbA1c (%)			
	5.6>	5.6–5.9	6.0–6.4	6.5≤
Rank A	0.0% (0/215 subjects)	0.0% (0/215 subjects)	1.9% (4/215 subjects)	3.7% (8/215 subjects)
Rank B	0.9% (2/215 subjects)	4.2% (9/215 subjects)	25.1% (54/215 subjects)	14.4% (31/215 subjects)
Rank C	0.0% (0/215 subjects)	5.1% (11/215 subjects)	31.2% (67/215 subjects)	13.5% (29/215 subjects)



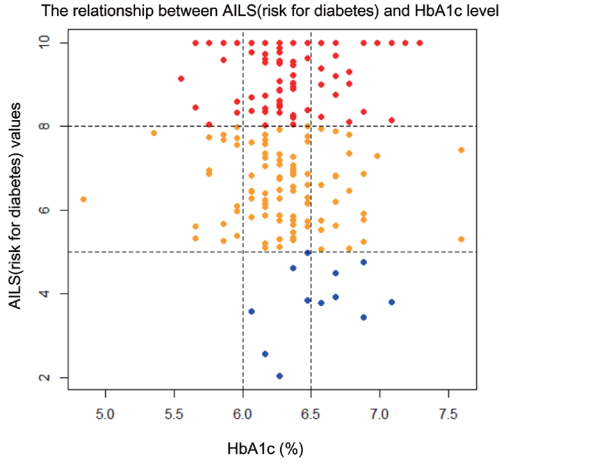


Fig. 4. Relationship between AILS (risk for diabetes) Value of Subjects who Developed Diabetes within Four Years and Their Fasting Blood Glucose and HbA1c Levels at Time of Initial Examination

Blue dot: AILS(risk for diabetes) rank A, Yellow dot: rank B, Red dot: rank C. The dotted lines represent the borders of AILS (risk for diabetes) ranks. The ranges for fasting blood glucose and the HbA1c are taken from the 2016–2017 diabetes treatment guide and Ningen Dock classifications, respectively.

25 kg/m^2, the risk of onset (odds ratio) relative to Rank A was significantly higher for those categorized as Rank B and Rank C. The risks of onset (odds ratio) were 7.3 (95% confidence interval 3.8–13.8) and 12.3 (95% confidence interval 6.3–24.2) for Rank B and Rank C, respectively. Similarly, in those without metabolic syndrome, the risk of onset (odds ratio) was also significantly higher relative to Rank A, at 6.5 (95% confidence interval 3.4–12.3) and 10.6 (95% confidence interval 5.5–20.6), respectively. These results suggest that AILS (risk for diabetes) could be used to assess the risk for developing diabetes when it cannot be predicted from the presence of other lifestyle-related diseases associated with diabetes or indices related to diabetes. In addition, individuals at risk for diabetes could be discovered through a more comprehensive assessment in consideration of the risk of developing diabetes within four years based on AILS (risk for diabetes) and existing risk factors for diabetes.

As AILS (risk for diabetes) was created as a test for assessing the risk for developing diabetes within four years, the time elapsed before disease onset was also analyzed for each rank. **Fig.5** shows cumulative percentages of subjects who developed diabetes in each year after the initial examination for each rank. The results showed that a higher rank was related to a higher risk of onset, starting from the first year. In addition, intergroup comparisons using log-rank tests were performed on the number of years that had elapsed between the initial examination and the onset of diabetes, with $p < 0.001$ for Rank A vs. Rank B and Rank A vs. Rank C. The time elapsed before the onset of diabetes was shorter for Rank B and Rank C compared to Rank A.

So far, we have focused on AILS (risk for diabetes) ranks and the onset of diabetes within four years. A relationship between the AILS (risk for diabetes) ranks and various lifestyle-related disease findings was also assessed. In the assessment, 99% of subjects with postprandial hyperglycemia, one of the diagnostic criteria of diabetes, fell under Rank B or Rank C. This shows that AILS (risk for diabetes) can be used to detect not only the risk for developing diabetes within four years but also the current presence of postprandial hyperglycemia, and metabolic changes that arise in association with diabetes may also be able to be detected. Also, 79%, 76%, 97%, 85%, 94% and 86% of those with hypertension, dyslipidemia, metabolic syndrome, visceral fat obesity, fatty liver and liver dysfunction, respectively, were categorized as Rank B or Rank C.

In the foregoing, we have examined the characteristics of AILS (risk for diabetes). A previous study examined the changes in the AILS (risk for diabetes) value as a result of interventions such as lifestyle guidance[39]. The study included 85 subjects (53 men, 32 women) with a BMI of 30 kg/m^2 or higher and an abdominal girth measurement of 85 cm or higher in men or 90 cm or higher in women; or a BMI≥25 kg/m^2 and one of the following conditions: hyperglycemia (fasting blood glucose level of 110 mg/dL or higher), dyslipidemia (triglyceride level of 150 mg/dL or higher, or high-density lipoprotein [HDL] cholesterol concentration of 40 mg/dL or lower), or hypertension (systolic blood pressure of 130 mmHg or higher or diastolic blood pressure of 85 mmHg or higher). The subjects received advice on nu-

Year of onset by AILS (risk for diabetes) rank

AILS (risk for diabetes)	Number of subjects	Onset after one year	Onset after two years	Onset after three years	Onset after four years
Rank A	3,008	3 (0.1%)	5 (0.2%)	1 (0.0%)	3 (0.1%)
Rank B	3,091	28 (0.9%)	27 (0.9%)	23 (0.7%)	18 (0.6%)
Rank C	1,604	41 (2.6%)	26 (1.6%)	23 (1.4%)	17 (1.1%)

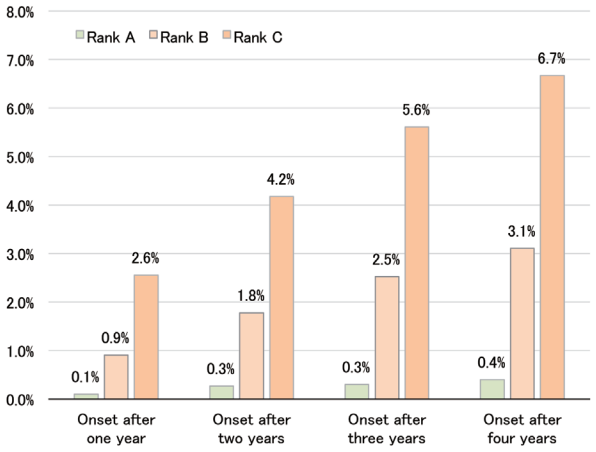


Fig.5. Cumulative Incidence of Diabetes by AILS (risk for diabetes) Rank

trition and exercise advice[40] for three months based on obesity treatment guidelines that recommend reduced calorie intake and daily exercise. Changes in the AILS (risk for diabetes) values were examined in 50 subjects whose body weight and abdominal girth decreased. Comparisons between before and after receiving advice using Wilcoxon matched-pairs signed rank tests revealed that the AILS (risk for diabetes) value in this group was significantly decreased ($p < 0.001$). Therefore, the effects of changes in lifestyle habits could be confirmed by AILS (risk for diabetes).

Next, we look at AILS (amino acid level), the other test included in AILS.

Evaluation of decreased essential and semi-essential amino acid levels using AminoIndex Technology

The AILS (amino acid level) formula was devised to evaluate plasma levels of essential and semi-essential amino acids because of their importance to the body. As shown in **Fig.6**, from among 7,685 individuals that underwent health screening at Mitsui Memorial Hospital, 1,890 persons (901 men, 989 women) were selected as standard subjects on the basis of satisfying conditions to be considered standard[41–43] based on the guidelines of the Japan Society of Ningen Dock. The AILS (amino acid level) formula was developed in three steps: setting reference intervals for plasma amino acid levels, converting the amino acid levels into standard scores, and deriving an AILS (amino acid level) formula. Also, in the selection of the standard subjects those who were receiving regular drug treatment for a chronic disease or those with abnormal test values, anemia, or inflammation had been excluded. In addition, for each amino acid, individuals with a plasma amino acid concentration above the average plus four standard deviations or below the average minus four standard deviations were excluded from the set of standard subjects before compiling data used to establish the evaluation formula. Standard values were set separately for men and women because levels of essential and semi-essential amino acid levels are significantly higher in men than those in women. Also, when amino acid levels were not normally distributed, they were converted into a normal distribution[44] using the Box-Cox transformation, a generally accepted statistical method, before the standard score for each amino acid was calculated. The relationship between the resulting AILS (amino acid level) formula and each clinical test item and its effectiveness were examined using data from 10,102 individuals undergoing health screening at Mitsui Memorial Hospital.

AILS (amino acid level) value and ratio

The AILS (amino acid level) value represents the lowest standard value of the plasma amino acid levels of ten amino acids. They include nine essential amino acids (threonine, valine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine, and methionine) and one semi-essential amino acid (arginine). When the standard values of all amino acids except methionine is 30.0 and the standard value of methionine is 28.8, the AILS (amino acid level) value is 28.8. In other words, a low AILS (amino acid level) value indicates that the level of one of the amino acids is low. An AILS (amino acid level) value of 30.0 or higher is defined as "normal", while an AILS (amino acid level) value of less than 30.0 is defined as "low". The distributions of AILS (amino acid level) values were examined using data from

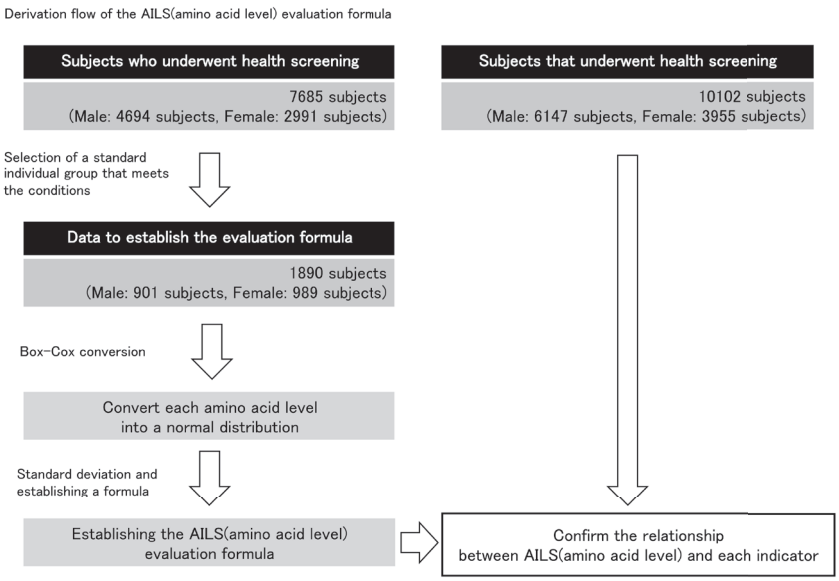


Fig.6. Flow of Derivation of AILS (amino acid level) Evaluation Formula

Table 2. Distributions of AILS (amino acid level) Results by Sex

A. Overall

AILS (amino acid level)	Percentage	Number of subjects
Normal	83%	(8426/10102)
Low	17%	(1676/10102)

B. Males

AILS (amino acid level)	Percentage	Number of subjects
Normal	82%	(5060/6147)
Low	18%	(1087/6147)

C. Females

AILS (amino acid level)	Percentage	Number of subjects
Normal	85%	(3366/3955)
Low	15%	(589/3955)

	Plasma albumin (g/dL)		Total
	4.0 ≤	4.0 >	
Normal	82% (8,300 subjects)	1% (126 subjects)	83% (8,426 subjects)
Low	16% (1,603 subjects)	1% (73 subjects)	17% (1,676 subjects)
Total	98% (9,903 subjects)	2% (199 subjects)	100% (10,102 subjects)

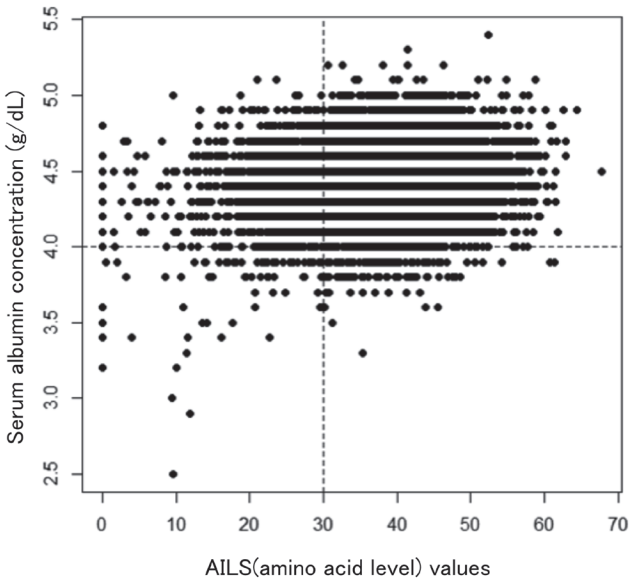


Fig. 7. Relationship between AILS (amino acid level) Value and Serum Albumin Levels

10,102 individuals (6,147 men and 3,955 women) that underwent health screening at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. As shown in **Table 2**, 83% (82% of the men and 85% of the women) of the individuals fell under the normal category and 17% (18% of the men and 15% of the women) had low values [45,46]. In addition, the results of an analysis performed on the AILS (amino acid level) rank distributions compiled after stratifying by age and sex revealed that the percentage of men with low values was higher among those over the age of 70 years (27%) compared to the percentage for all age groups (18%), while the percentage of women with low values was higher among those in their 40s (21%) or younger compared to those over the age of 70 (19%) and all age groups (15%).

Characteristics of AILS (amino acid level)

Serum albumin level is generally used as a marker for protein-energy malnutrition; however, it does not decrease under conditions of mild undernourishment and changes little in short-term protein-energy malnutrition. Hence, there are limitations on tests that use serum albumin as a marker to assess nutritional status[47–50]. Therefore, a relationship between serum albumin level and AILS (amino acid level) value was analyzed. The results in **Fig. 7** show that serum albumin levels of 199 individuals (2%) were below the reference

value (less than 4.0 g/dL) for "C: Requires follow-up and lifestyle improvement" in the classification[50] of the Japan Society of Ningen Dock, while 1,676 individuals (17%) were categorized as low using AILS (amino acid level). These results suggest that more individuals with protein-energy malnutrition can be detected using AILS (amino acid level). However, the possibility remains that the number of individuals with low AILS (amino acid level) values was high due its threshold settings. Therefore, it was examined whether there was a difference in the clinical profile of those with normal and low AILS (amino acid level) values. **Fig.8** shows the odds ratios for individuals with low AILS (amino acid level) values having values below or above the reference values for each clinical test item relative to subjects with normal AILS (amino acid level) values. With regard to nutrition, anemia, and immunological/inflammation indicators, subjects with low AILS (amino acid level) values were more likely to have measurements outside the reference ranges of these test items. Therefore, the AILS (amino acid level) value could be a comprehensive indicator of nutritional status.

In this regard, 5.1% (333/6,493 subjects) of those with normal AILS (amino acid level) values developed anemia within four years, whereas 8.7% (103/1,178 subjects) with low values developed anemia in the same time period, with an odds ratio of 1.8 (95%CI 1.4–2.2). Therefore, the risk for developing anemia within four years was significantly higher. These results indicate the importance of evaluating the plasma levels of essential and semi-essential amino acid levels based on AILS (amino acid level). Similar to AILS (risk for diabetes), changes in AILS (amino acid level) values as a result of nutritional guidance and interventions including supplements were examined. Changes in AILS (amino acid level) values were examined in 10 individuals (6 men and 4 women) with low AILS (risk for diabetes) values after an eight-week intervention including lifestyle guidance focusing on energy intake and amino acids based on plasma amino acid profile evaluation and types of lifestyle-related disease risk, and two packs of essential amino acid supplements (essential amino acid "Amino L40" with 40% leucine) per day. Wilcoxon matched-pairs signed rank tests were used to

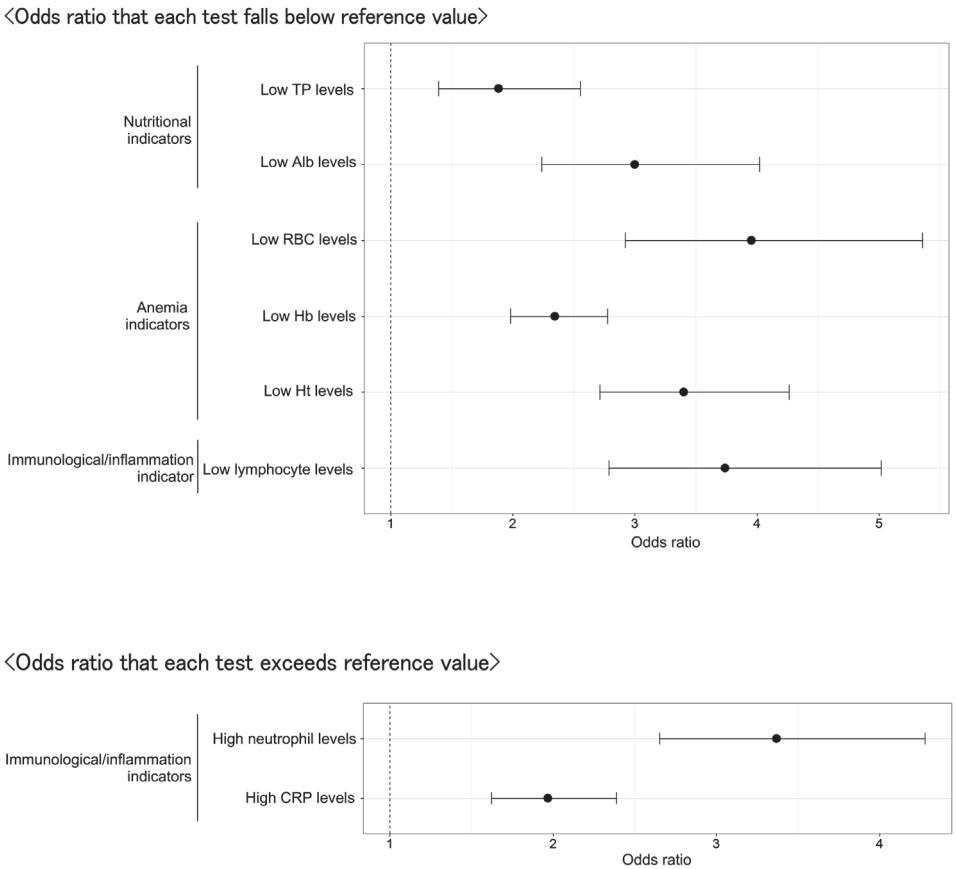


Fig.8. Clinical Test Items for which Odds Ratio was Significantly Higher in Subjects with Low AILS (amino acid level) Values

* The odds ratio was based on the group with values at normal levels (●: odds ratio, line: 95% confidence interval). Low clinical test values were those that were lower than the reference values, while high values were those that exceeded the reference values. Japan Society of Ningen Dock classifications (revised April 1, 2017) were used for total protein level, serum albumin level, erythrocyte count, hemoglobin level, hematocrit, and CRP level; the standards described in Medical Examination (2015) were used for lymphocyte and neutrophil counts.

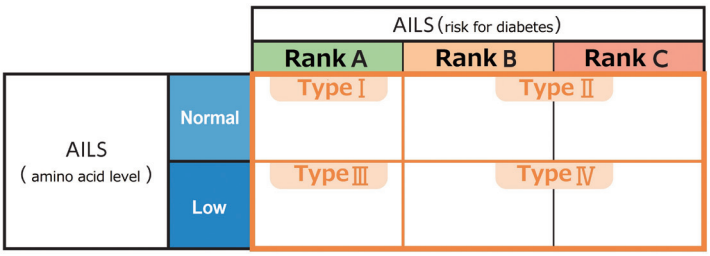


Fig. 9. Types Based on Classifications and Test Results for AILS (risk for diabetes) and AILS (amino acid level)

compare AILS (amino acid level) values before and after guidance and revealed a significant increase in AILS (amino acid level) values ($p<0.01$). In other words, the AILS (amino acid level) value reflected the improvement in these subjects following the intervention.

Use of AILS

The features of AILS include its ability to be used as a single test to evaluate the risk for developing diabetes within four years due to overnutrition and decreased plasma levels of essential and semi-essential amino acids, which is a form of undernutrition. Thus, AILS is a screening test that bridges the prediction and prevention of disease and can be performed during health screening and health check-ups.

There are various indicators for diabetes, which include fasting blood glucose, HbA1c, and oral glucose tolerance test (OGTT) values, as well as fasting insulin levels. They aid in the early detection of the onset of diabetes. In addition, fasting blood glucose and HbA1c levels can be used to identify subjects with borderline diabetes who are at high risk of developing diabetes. Not only can AILS (risk for diabetes) be used to evaluate the risk for developing diabetes within four years in those that do not fall under the borderline category, it can also be used in combination with fasting blood glucose or HbA1c levels to identify individuals at even higher risk. Therefore, using AILS together with these tests can provide valuable information. In addition, although the OGTT is effective for detecting postprandial hyperglycemia and postprandial hyperinsulinemia, it requires an extended period of time in hospital and imposes a great burden on medical institutions. AILS could be a substitute for this test.

While serum albumin level is the standard test for undernutrition, it is difficult to assess short-term nutritional status using it[51]. The plasma amino acid profile may be a more sensitive indicator of changes in nutritional status than albumin and may therefore, be used as a new indicator for nutritional evaluation.

In addition, by combining the 2 AILS tests the nutritional status of an individual can be evaluated in terms of the risks of overnutrition and amino acid deficiency in the body. By doing this, nutritional status risks can be classified into four types, as shown in **Fig. 9**, based on risks determined by AILS (risk for diabetes) and AILS (amino acid level). Conventional measures are weighted toward either overnutrition or undernutrition, and we believe that the combined use of AILS (risk for diabetes) and AILS (amino acid level) would be more specific for those undergoing health screening. In recent years, "sarcopenic obesity"[52], has been attracting increased attention. In this condition, obesity progresses due to aging and excessive weight loss as well as a simultaneous decrease in basal metabolism. We believe that those falling under type IV (Rank B or Rank C with AILS (risk for diabetes) and low with AILS (amino acid level) would be at high risk for developing sarcopenic obesity, and therefore, AILS could be used to detect it. Regarding actual measures to be taken for each of the different types, those categorized as types II and IV, with high AILS (risk for diabetes) values and a BMI of 25 kg/m^2 or higher, would be recommended to adjust their energy intake, increase their intake of dietary fiber (by adjusting consumption of grains, etc.), and exercise (aerobic exercise, etc.) in accordance with the Treatment Guide for Diabetes 2016[53]. Also, regarding AILS (amino acid level), consumption of high-quality proteins and resistance exercise in accordance with the "Standards for dietary intake for Japanese (2015)"[54] would be recommended.

As described above, AILS is an effective screening method for determining lifestyle-related risks. It is also a method that bridges preventive and preemptive medicine. In addition, healthy life expectancy would be expected to increase as a result of improving lifestyle habits by following early stage guidance based on AILS test results.

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Conflict of Interest

There is nothing to declare with regard to the content of this review article.

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Original Article

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Relationships of Skeletal Muscle Mass and Visceral Fat with Atherosclerosis Risk Factors in Middle-aged Japanese People: An Assessment Using Accurate, Simple Bioelectrical Impedance Methods

Tetsuya Sato[1,3], Kenji Kanaji[2], Hiroyuki Nishikawa[2], Takashi Miyawaki[1,2]

Abstract

Objective: Recently, a decrease in skeletal muscle mass was found to be related to atherosclerosis risk. We investigated the relationship between skeletal muscle mass and atherosclerosis risk factors according to visceral fat accumulation status, using accurate, simple bioelectrical impedance methods.

Methods: The study included 1,161 middle-aged Japanese people (998 men and 163 women). The skeletal muscle was assessed using a bioelectrical impedance method between the upper and lower limbs, and visceral fat area (VFA) was measured using a dual bioelectrical impedance method. The subjects were divided into two groups according to VFA (VFA $\geq$100 cm^2 and VFA $<$100 cm^2) by sex and the relationship between percentage of skeletal muscle of body weight (PSM) and the number of atherosclerosis risk factors in each group was investigated. Multiple linear regression analysis was performed to determine the relative contribution of explanatory variables (age, VFA, subcutaneous fat area, and PSM) to the response variable (number of risk factors).

Results: The mean PSM significantly decreased as the number of risk factors increased in both men and women. In men with VFA $<$100 cm^2, the number of risk factors was significantly correlated with PSM ($B = -0.164$, $p < 0.001$), whereas in men with VFA $\geq$100 cm^2, there was no correlation. In women, the number of risk factors was not correlated with PSM irrespective of VFA.

Conclusions: Skeletal muscle mass assessed using the present accurate, simple bioelectrical impedance methods may be a new index for assessing the possibility of atherosclerosis risk factors in men.

Keywords skeletal muscle mass, bioelectrical impedance, atherosclerosis risk, visceral fat

Sarcopenia refers to age-related loss of skeletal muscle mass and muscle strength[1], and is a marker of frailty and poor prognosis among the elderly. In addition, it is known that sarcopenia is related to metabolic disorders. Insulin resistance has a stronger association with sarcopenic obesity, which is the combination of sarcopenia and obesity, than with sarcopenia or obesity alone in elderly people[2,3]. Additionally, a decrease in muscle mass may be a risk factor for atherosclerosis in middle-aged people[4] and young adults[5].

It is well known that metabolic syndrome (MS) is closely associated with atherosclerosis risk. MS is a cluster of atherosclerosis risk factors including visceral fat accumulation, hypertension, dyslipidemia, and diabetes[6]. Visceral fat accumulation increases the risk of atherosclerosis, and previous studies have shown that visceral fat accumulation is related to insulin resistance[7,8]. In addition, the coexistence of a decrease in muscle mass and MS increases the risk of cardiovascular diseases[9]. Thus, measurements of both visceral fat and muscle mass may be important for assessing the possibility of atherosclerosis risk factors.

The gold standard methods for the assessment of visceral fat and skeletal muscle are computed tomography (CT)[10] and magnetic resonance imaging (MRI)[1], respectively. In Japan, visceral fat accumulation is defined as visceral fat area (VFA) of $\geq$100 cm^2 using CT and MRI, both in men and women[6]. However, CT involves radiation exposure, and both of these methods are complex, costly, and time-consuming. Additionally, they are not suitable for body composition screening. Although dual energy X-ray absorptiometry (DXA) is an accu-

[1] Graduate School of Home Economics, Kyoto Women's University ; [2] Health Administration Center, Rakuwakai Toji-Minami Hospital ; [3] Omron Healthcare Co., Ltd.

Contact : Tetsuya Sato, Graduate School of Home Economics, Kyoto Women's University, 35, Kitahiyoshi-cho, Imakumano, Higashiyama-ku, Kyoto 605–8501, Japan. Tel : +81–75–925–2224 ; Fax : +81–75–925–2044 ; E-mail : k5420351@kyoto-wu.ac.jp

rate method for measuring body composition, it also involves radiation exposure and is complex, and the assessment is influenced by body thickness[11].

Recently, two simple, highly accurate bioelectrical impedance methods for measuring VFA, subcutaneous fat area (SFA), and percentage of skeletal muscle of body weight (PSM) have been developed[12,13,14]. In this study, we investigated the relationship between skeletal muscle mass and atherosclerosis risk factors according to visceral fat accumulation status, using accurate, simple bioelectrical impedance methods, in middle-aged Japanese people.

Subjects and Methods

Subjects

We initially enrolled 1,441 adults (1,250 men and 191 women; mean age, 50.3±7.2 (SD) and 49.3 ± 8.8 (SD) years, respectively) who underwent an annual medical check-up at a health check-up center in the Kinki area of Japan between February 2012 and April 2015. We excluded 252 men and 28 women who were taking antihypertensive agents, antidiabetic agents, and lipid-lowering agents, and/or had dehydrative or edematous diseases. The study finally included 1,161 adults (998 men and 163 women; mean age, 49.4 ± 7.4 (SD) and 48.2 ± 8.9 (SD) years, respectively).

Anthropometric and atherosclerosis risk factor assessments

In the morning, the body weight of each subject was measured to the nearest 0.1 kg, with the subject in light clothes, and the height was measured to the nearest 0.1 cm. The waist circumference was measured to the nearest 0.5 cm at the umbilical level in the late exhalation phase while standing, by a well-trained examiner. Blood pressure measurements were obtained in the morning while the subjects were resting in a seated position. Blood samples were collected after an overnight fast for assessment of fasting plasma glucose, triglyceride, and high-density lipoprotein (HDL)-cholesterol levels.

Diagnosis of atherosclerosis risk factors

The diagnosis of atherosclerosis risk factors was in accordance with the definitions of the Examination Committee of Criteria for the Metabolic Syndrome in Japan[7,15]. Hypertension was defined as systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, diabetes was defined as a fasting plasma glucose level ≥ 110 mg/dL, and dyslipidemia was defined as a triglyceride level ≥ 150 mg/dL and/or HDL-cholesterol level ≤ 40 mg/dL.

Body composition (PSM, VFA, and SFA) measurements

Measurement of PSM

Skeletal muscle mass was measured with the HBF-354 prototype (Omron Healthcare Co. Ltd., Kyoto, Japan), which used bioelectrical impedance between the upper and lower limbs[14].

Bioelectrical impedance was determined by measuring the voltage induced by applying a current to electrodes fixed on bilateral palms and soles while shorting each of the current and voltage electrodes fixed on each palm and sole, in the standing position with bilateral upper limbs extended forward. A constant current of 500 μA at 50 kHz was applied. The impedance measured using this method was whole-body impedance (Zw). Skeletal muscle mass was calculated using the BI index (height^2/Zw), the body surface area (BSA), sex, and age, using the following formula:

Skeletal muscle mass (kg) = (0.147 × BI index) + (1.625 × BSA) + (− 0.056 × age) + (− 2.098 × sex) − 2.282

The reference skeletal muscle mass was obtained using MRI performed in the supine position, every 20 mm from the hand to the foot. There was a good correlation between the skeletal muscle mass determined using the HBF-354 prototype and that measured using MRI ($r = 0.959$, $p < 0.001$).

Measurement of VFA and SFA

VFA and SFA were measured with HDS-2000 (DUALSCAN®, Omron Healthcare Co. Ltd., Kyoto, Japan), which used a dual bioelectrical impedance method[12,13]. Bioelectrical impedance was measured by applying a constant current of 500 μA at 50 kHz between the upper and lower limbs and the surface of the abdominal area in the supine position.

DUALSCAN (DS) calculates the cross-sectional area of visceral fat at the umbilical level based on a dual bioelectrical impedance method. DS uses two components (Zt: fat free area, Zs: subcutaneous fat area) to measure two kinds of bioelectrical impedance, and the width (A) and height (B) of a cross-sectional area of the abdomen. Using DS, the VFA was calculated according to the following formula:

$VFA = a_1 A + a_2 B^2 - a_3 (A^2 + B^2)^{1/2} Zs - a_4 / Zt + a_5$

a_1, a_2, a_3, a_4, and a_5 are constants

Reference VFA and SFA values were obtained using CT. There were good correlations between VFA determined using DS and that measured using CT ($r = 0.888$, $p < 0.001$)[13], and between SFA determined using DS and that measured using CT ($r = 0.887$, $p < 0.001$).

With the bioelectrical impedance method, as there is intra-day variability in body composition measurements[14], skeletal muscle mass, VFA, and SFA were measured after an overnight fast.

Statistical analysis

We divided the subjects into two groups according to visceral fat accumulation, and analyzed the relationship between PSM and the number of risk factors. Visceral fat accumulation was defined as VFA ≥ 100 cm^2 according to the Japanese guidelines for obesity treatment of the Japan Society for the Study of Obesity[6].

We used PSM, the percentage of skeletal muscle mass of body weight, for the evaluation of skeletal muscle.

Data are presented as mean ± standard deviation. We used the Mann-Whitney U test to assess significant differences between the groups, and Spearman's correlation coefficient to assess the relationship between each atherosclerosis risk factor and PSM. We determined the relationship between the number of risk factors and PSM, using the Jonckheere-Terpstra trend test. Multicollinearity analysis was performed on BMI, age, VFA, SFA, and PSM. Explanatory variables with a low contribution rate were excluded and multiple linear regression analysis was performed to determine the relative contribution of explanatory variables to the response variable (number of risk factors), grouped by presence or absence of visceral fat accumulation. All statistical analyses were performed using SPSS Statistics version 21 for Windows (IBM Corp., Armonk, NY). Statistical significance was set at $p < 0.05$.

Ethical statement

Informed consent was obtained from each study subject, and this study was approved by the Research Ethics Committee of Kyoto Women's University (Approval number 25–26).

Results

The subjects' characteristics are presented in **Table 1**. The mean height, body weight, BMI, waist circumference, VFA, skeletal muscle mass, and PSM were significantly higher in men than in women. There were no significant differences in the mean age or SFA between men and women.

The mean PSM significantly decreased with aging in both men and women (p for trend < 0.001). PSM significantly decreased as VFA increased in both men (r = -0.59, $p < 0.001$) and women (r = -0.58, $p < 0.001$).

Table 1. Clinical Characteristics of Study Subjects

		Men	Women	*p* value
Number		998	163	
Age (years)		49.4 ± 7.4	48.2 ± 8.9	0.216
Height (cm)		171.4 ± 5.8	158.7 ± 5.2	<0.001
Weight (kg)		68.9 ± 9.1	57.3 ± 8.7	<0.001
BMI (kg/m^2)		23.4 ± 2.7	22.7 ± 3.3	<0.001
Waist circumference (cm)		84.0 ± 7.5	79.7 ± 8.9	<0.001
VFA (cm^2)		72.8 ± 30.9	48.9 ± 23.1	<0.001
SFA (cm^2)		148.2 ± 49.5	158.1 ± 72.5	0.541
Skeletal muscle mass (kg)		21.9 ± 2.8	14.6 ± 1.9	<0.001
PSM (%)		31.8 ± 1.9	25.6 ± 2.1	<0.001
SBP (mmHg)		122.9 ± 15.6	112.6 ± 17.0	<0.001
DBP (mmHg)		78.3 ± 10.0	70.9 ± 11.0	<0.001
FPG (mg/dL)		102.9 ± 10.8	99.7 ± 10.0	<0.001
TG (mg/dL)		125.6 ± 102.1	85.7 ± 54.6	<0.001
HDL-C (mg/dL)		60.2 ± 14.6	67.8 ± 14.5	<0.001
number of risk factors	0	431	118	
	1	373	33	
	2	160	9	
	3	34	3	
				mean ± SD

VFA: visceral fat area, SFA: subcutaneous fat area, PSM: percentage of skeletal muscle mass of body weight, SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, TG: triglyceride, HDL-C: HDL-cholesterol

Table 2. Relationship between Risk Factors and PSM

	Men		Women	
	r	*p* value	r	*p* value
SBP (mmHg)	-0.319	<0.001	-0.440	<0.001
DBP (mmHg)	-0.354	<0.001	-0.425	<0.001
FPG (mg/dL)	-0.255	<0.001	-0.322	<0.001
TG (mg/dL)	-0.366	<0.001	-0.336	<0.001
HDL-C (mg/dL)	0.211	<0.001	0.229	0.003

PSM: percentage of skeletal muscle mass of body weight, SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, TG: triglyceride, HDL-C: HDL-cholesterol

Table 2 shows the relationship between atherosclerosis risk factors and PSM in both men and women. Systolic blood pressure, diastolic blood pressure, fasting plasma glucose level, and triglyceride level had significant negative correlations with PSM in both men ($p < 0.001$) and women ($p < 0.001$), while the HDL-cholesterol level had a significant positive correlation with PSM in both men ($p < 0.001$) and women ($p = 0.003$).

Fig. 1 shows the relationship between the number of atherosclerosis risk factors and PSM. The mean PSM significantly decreased as the number of risk factors increased in both men and women (p for trend < 0.01).

Table 3 shows the results of multiple linear regression analysis on the relationship between the number of atherosclerosis risk factors and age, VFA, SFA, and PSM. There was multicollinearity between BMI and SFA, and the contribution rate of BMI was lower than that of SFA and therefore, BMI was excluded. In men with VFA <100 cm^2, the number of risk factors was significantly correlated with PSM (B = -0.164, $p < 0.001$), whereas in men with VFA ≥ 100 cm^2, the number of risk factors was significantly correlated with VFA (B = 0.182, $p = 0.031$). In women with VFA <100 cm^2, the number of risk factors was significantly correlated with VFA (B = 0.259, $p = 0.006$); however, in women with VFA ≥ 100 cm^2, there was no significant correlation between the number of risk factors and any of the variables.

Discussion

The number of risk factors was negatively correlated with PSM only in men with VFA<100cm^2 (B=-0.164, $p<0.001$), and positively correlated with VFA both in men (VFA<100cm^2 B=-0.189, $p<0.001$, VFA >100cm^2 B=0.182, $p=0.031$) and women with VFA<100cm^2 (B=0.259, $p=0.006$). No such relationships were seen between SFA and number of risk factors. To our knowledge, this is the first study to investigate the relationship between skeletal muscle mass and cardiovascular risk factors according to visceral fat accumulation using accurate, simple bioelectrical impedance methods.

Skeletal muscle, which accounts for the largest amount of tissue in the human body, plays important roles in energy metabolism, uptake of glucose, and physical activity. Approximately 15% of the circulating blood volume is supplied to skeletal muscle at rest,

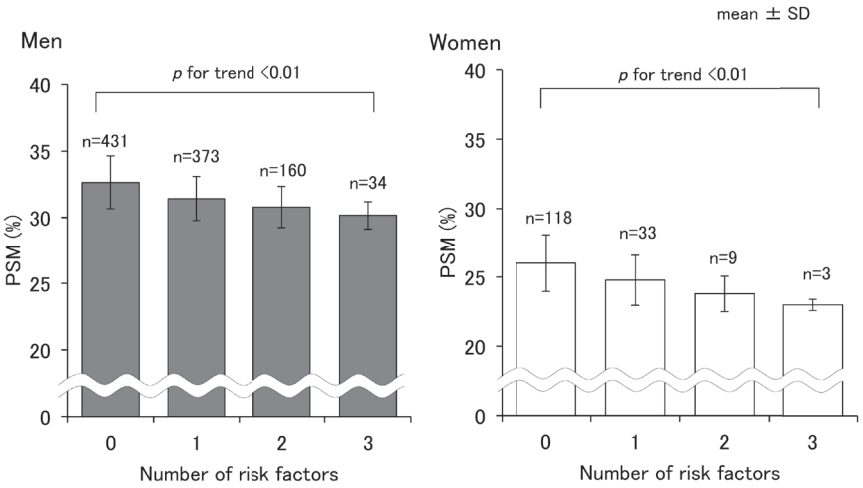

Fig. 1. Relationship between Number of Risk Factors and PSM
PSM: percentage of skeletal muscle mass of body weight

Table 3. Results of Multiple Linear Regression Analysis on Relationship between Number of Risk Factors and Age, VFA, SFA, and PSM

	Men				Women			
	VFA < 100cm^2 (n=810)		VFA ≥ 100cm^2 (n=188)		VFA < 100cm^2 (n=156)		VFA ≥ 100cm^2 (n=7)	
	B	*p* value	B	*p* value	B	*p* value	B	*p* value
Age	0.158	<0.001	0.129	0.113	0.212	0.015	0.803	0.349
VFA	0.189	<0.001	0.182	0.031	0.259	0.006	0.098	0.906
SFA	0.058	0.234	0.171	0.071	-0.011	0.922	-0.322	0.679
PSM	-0.164	<0.001	0.103	0.233	-0.104	0.408	-0.376	0.672

Objective variable: number of risk factors, Explanatory variables: age, VFA, SFA, and PSM, B: standardized partial regression coefficient, VFA: visceral fat area, SFA: subcutaneous fat area, PSM: percentage of skeletal muscle mass of body weight

and approximately 20% of the oxygen used in the body is consumed by skeletal muscle. Glucose in the blood is carried to skeletal muscle, and metabolized with oxygen[16]. Sarcopenia has been shown to aggravate impaired glucose tolerance[4]. A previous study showed that among older adults, muscle mass and muscle strength were lower in those with type 2 diabetes than in those without diabetes[17] and another study found that in Japanese patients with type 2 diabetes, the muscle mass of the lower limbs was significantly decreased[18].

The exact mechanism by which atherosclerosis risk factors develop with a decrease in muscle mass is not yet fully understood. It is considered that insulin resistance due to a decrease in skeletal muscle mass leads to atherosclerosis risk factors such as diabetes, dyslipidemia, and hypertension[3]. Skeletal muscle is responsible for a major part of insulin-stimulated whole-body glucose disposal and therefore plays an important role in the pathogenesis of insulin resistance. Previous studies revealed that a decrease in skeletal muscle mass caused a decline in and hypofunction of mitochondria in skeletal muscle[19,20]. Skeletal muscle mitochondrial dysfunction is involved in the accumulation of intra-myocellular lipid metabolites and therefore, the fatty acid in skeletal muscle is not metabolized, and becomes a factor of insulin resistance[19–21].

An exercise-induced transient increase in interleukin-6 (IL-6), a myokine derived from skeletal muscle contraction, stimulates glucose uptake and fatty-acid oxidation[22]. Although exercise levels were not examined in this study, a relative decrease in the contraction of skeletal muscle in subjects with low PSM might be associated with insulin resistance. Further studies are required to explore the molecular mechanisms associated with the development of atherosclerosis risk factors with decreasing skeletal muscle mass.

The results of multiple regression analysis suggested that VFA had the strongest association with atherosclerosis risk factors in men without visceral fat accumulation, and PSM was the second largest contributing factor next to VFA, but not to SFA or age, while in women, the number of risk factors was not significantly correlated with PSM. One of the reasons for the gender difference might be that the volume of muscle mass in women is relatively smaller than that in men, and therefore the contribution of muscle mass is lower in women than in men. Another might be the influence of sex hormones. The male hormone testosterone has been shown to induce skeletal muscle protein anabolism and have an influence on muscle size and muscle strength[23]. On the other hand, estrogen, a female hormone, is probably associated with a protective effect against atherosclerosis risks and an increasing effect with respect to subcutaneous fat[24]. Taken together, in men, a measurement not only of VFA, but also of the volume of muscle, an organ related to energy metabolism in humans, might be required for an assessment of the possibility of atherosclerosis risk factors.

In the present study, we used simple, highly accurate bioelectrical impedance methods to measure PSM, VFA, and SFA. Skeletal muscle mass, VFA, and SFA can be measured with the two bioelectrical impedance methods used in this study. One of them involves bioelectrical impedance between the upper and lower limbs, and has been shown to be highly accurate for measurement of skeletal muscle mass when compared with MRI in two previous studies ($r = 0.92$, $p < 0.001$ and $r = 0.85$, $p < 0.001$, respectively)[25,26]. The other method involves dual bioelectrical impedance, and has been shown to be accurate for measurement of VFA when compared with CT ($r = 0.821$, $p < 0.0001$)[13,27]. Results for both methods in clinical application are available[27–35].

Various indices are used for the assessment of skeletal muscle, such as skeletal muscle mass, appendicular skeletal muscle mass (ASM), and the skeletal muscle index (SMI), which involves dividing appendicular muscle mass by the square of height[1,36]. In the present study, we used PSM to assess skeletal muscle because it allows a relative assessment of body composition to be made, and the percentage of body fat and muscle mass (fat free mass) do not increase linearly with an increase in weight, although findings have suggested that the increase in fat mass is almost linear[37]. Additionally, there may be a good correlation between PSM and insulin resistance[38], which may be related to arteriosclerosis risk with a decrease in muscle mass.

The present study has several limitations. First, we did not measure each segment of skeletal muscle in the lower or upper limbs. In a previous study, the rate of decrease in skeletal muscle mass and the percentage of skeletal muscle mass were higher in the lower limbs than in the upper limbs[23,39]. Additionally, metabolic parameters were more closely associated with sarcopenia defined as ASM/Weight than with sarcopenia defined as ASM/square height[3]. Kim, *et al.*[5] reported that a lower muscle mass estimated using DXA was significantly associated with MS and its components only in men and women who were not obese. Therefore, further studies on the relationship of each segment of skeletal muscle assessed using bioelectrical impedance and atherosclerosis risk should be performed. Second, we could not evaluate functional aspects of skeletal muscle, such as muscle strength. It has been reported that training to increase muscle strength reduced the risk of MS[40]. Finally, we did not evaluate humoral factors such as insulin, myokines, and sex hormones, for which findings have suggested a relationship with the mechanisms of

arteriosclerosis risk with a decrease in muscle mass.

In conclusion, the present study showed that a decrease in PSM is related to an increase in the number of atherosclerosis risk factors in middle-aged Japanese men without visceral fat accumulation. Measurement of muscle mass with the accurate, simple bioelectrical impedance methods used in this study may be useful for assessing the possibility of atherosclerosis risk factors.

Conflict of Interest Statement

Tetsuya Sato is an employee of OMRON HEALTHCARE Co., Ltd. The other authors declare no conflict of interest.

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Original Article

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Chronological Reduction in Hepatitis B Virus Antibody Titer After Vaccination in Adult Humans

Hideki Hayashi[1,3], Taduko Shimada[1], Mikiko Teraya[1], Yuki Azuma[1], Yukiko Fujii[1], Emi Goto[1], Kaoru Nakayama[2], Junko Sugimoto[2], Katsuya Miyanaga[3]

Abstract

Objective: Blood exposure and subsequent blood-borne infections are major risk factors for acquired occupational diseases. Hepatitis B (HB) viral infection is among them, and it is well known that it causes cirrhosis and liver cancer in two or three decades. It can be prevented with HB vaccination, which is recommended for medical staff. However, the antibody titer for acquired immunization generally decreases with time. The authors investigated the time-dependent decrease in HB virus antibody titers after vaccination.

Methods: Three hundred and seventy-five (70 males and 305 females) hospital employees were immunized with HB vaccine at their workplace and thereafter when needed. HB virus surface antibody titers were measured in 2004, 2007, 2010 and 2015, using a semi-quantitative photohemagglutination assay. Measured titers were defined as negative for 8 times or less and positive for 16 times or more.

Results: Out of the total of 375 persons who had been injected with HB vaccine, there had been significant titer loss in 132 (35%) in five years. There was no significant statistical difference between two subgroups, one of them consisting of persons who had continued to have positive results and the other of persons who had newly obtained immunity. Conversion from a positive to a negative result was more likely in males than in females (50% vs 32%, $p < 0.01$).

Conclusions: The titers of approximately one-third of the subjects converted from positive to negative in five years. After HB vaccination, periodical antibody titer measurements and additional vaccination may be recommended for persons with negative results.

Keywords hepatitis B antibody titer, titer reduction, hepatitis B vaccination, occupational vaccination

Blood exposure and subsequent blood-borne infections are among major risks for acquired occupational diseases, and are most likely to occur in medical institutions and ambulances. Hepatitis B (HB) virus is typically transmitted via the blood from human to human and results in HB viral infection[1]. It is well known that persistent infection causes liver cirrhosis and cancer in two or three decades[2,3]. HB viral infection can probably be prevented with HB vaccination, which is highly recommended for medical staff[4].

After three injections of HB vaccine, immunity is thought to continue for decades. However, the efficacy or antibody titer of acquired immunity generally decreases with time, and the duration of efficacy differs among individuals[5]. A preliminary study revealed that HB virus antibody titers after vaccination decreased more rapidly than expected. Therefore, periodical measurement of HB virus antibody titers in high-risk persons may be important. The authors investigated the time-dependent decrease in HB virus surface antibody titers after vaccination in employees of a general hospital in the present study.

Methods

Study Population

Out of 408 persons who were employed at a designated hospital in January 2016, 375 were evaluated in this study. Thirty-three were excluded for the following reasons: 1) non-responder[5], 2) refused vaccination, 3) HB virus surface antibody positive and 4) person who had not completed the full vaccination course in January 2016.

[1] Section of Health Assessment and Preventive Medicine, Hayashi General Hospital ; [2] Section of Personnel, Hayashi General Hospital,
[3] Department of Surgery, Hayashi General Hospital
Contact : Hideki Hayashi, Section of Health Assessment and Preventive Medicine, Hayashi General Hospital, 1–5–7 Fuchu, Echizen, Fukui Prefecture 915–8511, Japan. Tel : +81–778–22–0336 ; Fax : +81–778–23–4014 ; E-mail : hp-hayashi@ma.interbroad.or.jp

All persons enrolled were Japanese and had physical performance status I. Their laboratory data were not considered in this study, but they had not been restricted in their work by doctors. The subjects enrolled in this study consisted of 70 males and 305 females. Their age ranged from 18 to 68 years, and employment periods ranged from one to 49 years.

Vaccination

Each person was subcutaneously vaccinated with 0.5 mL of recombinant sedation HB vaccine (General Incorporated Foundation, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), Kumamoto, Japan) three times – 1) first shot, 2) second shot (1 month later) and 3) third shot (4–6 months after first shot), as recommended[6]. Each employee was vaccinated against HB virus unless they refused, and was further vaccinated when the measured titer was negative unless the person was a non-responder.

Outcome Measures

Peripheral blood was withdrawn and HB virus surface antibody titers were measured using a semi-quantitative photohemagglutination assay. Measured titers were classified as negative for 8 times or less and positive for 16 times or more.

Study Design

HB virus surface antibody titers were measured in 2004, 2007, 2010 and 2015. Persons whose titers were negative in 2004, 2007 and 2010 received further vaccine injections. The titers were again measured in 2015.

Statistical Analysis

Statistical significance was evaluated by the chi-squared test with Yates modification and considered to be $p < 0.05$.

Results

The distribution of antibody titers measured in 2015 is shown in **Table 1**.

Ninety-five persons received vaccine shots in 2010 or later. They included those who became negative in 2010 and those who were employed in 2010 or later. Out of the 95 persons, 31 (33%) became negative in 2015. Out of 280 persons who continued to have a positive response in 2010, 101 (36%) became negative in 2015. There was no statistical difference between these two subgroups (**Table 2**). There was also no significant difference in gender ratio, age distribution or employment years between the two groups. Out of the total of 375 (280 + 95), 132 (31+101) (35%) became negative in five years.

Out of the 70 males, 35 (50%) became negative in 2015 and out of the 305 females 97 (32%) became negative in the same period of time. The gender difference for the positive-to-negative conversion rate was statistically significant ($p<0.01$) (**Table 3**).

Discussion

The number of asymptomatic HB virus carriers in Japan has decreased since the national pre-blood transfusion HB virus screening program started in 1972[7], and was estimated to have dropped to less than 1% of general population by 2002[8]. Seventy to eighty percent of patients with HB viral infection are asymptomatic[2],

Table 1. Distribution of Measured HB Virus Antibody Titers and Numbers of Persons in 2015

Titer	Male	Female	Total
8 or less	35	97	132
16	9	42	51
32	6	33	39
64	6	41	47
128	4	30	34
256	6	26	32
512	2	15	17
1024	1	9	10
2048	1	8	9
4096 or more	0	4	4

Table 2. No Significant Difference in Negative Conversion Rates for HB Virus Antibody Titers between Persons with Positive Conversion in 2010 or Later and Persons who Continued to be Positive in 2010

	Total	persons		% negative conversion	
		positive in 2015	negative in 2015		
Persons with positive conversion in 2010 or later	95	64	31	33%	NS
Persons continuing to be positive in 2010	280	179	101	36%	

NS: notstatistically significant

Table 3. Gender Difference in Negative Conversion Ratio

Immunity	Male	Female
Negative	35	97
Positive	35	208

Out of 70 men, there was negative conversion in 35, and out of 305 women, there was negative conversion in 97, in five years

and most of them are not aware of their infection unless blood tests are carried out. Persons at risk of HB viral infection should be provided with immunity against it. This study revealed that acquired antibody titers against HB virus diminished much more rapidly than thought. HB vaccination over a single period of time may not be sufficient for persons who work for decades in an environment with possible exposure to the virus. In the absence of a new antigenic stimulus, antibody levels generally diminish in the long term. The status of persons who have low positive titers for HB virus antibodies, such as 16 times or 32 times, tended to be converted from positive to negative status in this study. Persons who had high positive titers, such as 64 times or more, tended to continue to be positive in five years (data not shown).

A scientist at the vaccine supplier stated in a personal communication that acquired HB viral antibody titers after vaccination decreased by approximately half in three years. This was consistent with the above observations. Therefore, continuous monitoring and additional vaccination may be crucial for persons at risk of HB viral infection.

There are a few limitations in the present study. It was a retrospective study, and was based on analyses of data under occupational health care regulations. Thus, the vaccination and observation periods varied among the persons enrolled. In consideration of cost, the titers were measured using a semi-quantitative assay, not a quantitative one. There is also the issue of variation in the stereo structure of the HB virus antigen epitope used in manufacturing the vaccine among companies, which may affect efficacy[9]. This is given as a limitation because there are 2 commercial suppliers of HB vaccine in Japan and many more worldwide. Prospective studies are required in the future to confirm our observations and determine the optimal schedule for titer monitoring and additional vaccination.

Several studies[10,11] have reported that persons who received HB vaccination were at low risk of acute or chronic hepatitis over the long term even if their HB virus antibody titers subsequently decreased. There is some skepticism regarding the requirement for booster vaccination to protect against acute and chronic hepatitis[12], and it is controversial in terms of preventing liver cirrhosis or cancer.

The World Health Organization (WHO) has recommended universal vaccination. The recommendation for HB vaccination is three injections, including the first injection within 24 hours after birth[13]. The Japanese Government has started an HB vaccination program following the WHO recommendation.

Approximately 10% of persons were reported to be non-responders[14], which is consistent with this study. The ability to acquire a significant antibody titer is reported to be higher in young people than in those who are older[8]. The possibility of age and gender influencing titer decreases and negative conversion rates is under investigation.

The implementation of the WHO's universal vaccination policy will probably increase the number of persons who have effective immunity against HB viral infection for decades. The content of this study will be subject to further consideration one generation later.

Conclusion

The HB virus surface antibody titers acquired through vaccination gradually decreased, and converted from positive to negative in approximately one-third of the subjects in five years. Titer monitoring after HB vaccination may be important for protecting those who are at risk of HB viral infection and subsequent onset of liver cirrhosis and cancer. Periodical titer measurements and additional vaccination may be recommended for persons with negative results.

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Conflicts of Interest

The authors disclose no conflicts of interest. This study was approved by the Institutional Committee of Ethics of Hayashi General Hospital.

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Original Article

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Examination of Lifestyle Habits at Age 35 that Influence Lipid Data at Age 40 in Men

Emi Moriya[1], Shigeki Muto[2], Mutsuko Tobayama[3]

Abstract

Objective: We investigated whether the lifestyle habits of males at the age of 35 influenced their blood lipids at the age of 40.

Methods: From among 2,583 males who underwent health check-ups at 35 and 40 years old in the period from 2008 to 2015, 1,655 without medication for dyslipidemia were selected. We retrospectively examined whether lifestyle habits at 35 years old influenced the occurrence of blood lipid disorders at 40 years old or not.

Results: We assigned 1,503 subjects with normal TG levels at 35 and 40 years old to a TG maintenance group. Also, we assigned 152 subjects with normal TG levels at 35 years old but levels outside the standard range at 40 years old to a TG aggravation group. In addition, 1,363 subjects were assigned to a LDL-C maintenance group and 292 to a LDL-C aggravation group, 1,622 to a HDL-C maintenance group and 33 to a HDL-C aggravation group, in a similar manner to that for TG data. Regarding lifestyle habits at 35 years old, skipping breakfast, smoking, and risky drinking were associated with TG aggravation and risky drinking was associated with HDL-C aggravation.

Conclusions: When there are poor lifestyle habits at 35 years old, we should determine risk factors for increases in lipid data to levels beyond the standard ranges at 40 years old even then the levels at the age of 35 are considered to be normal. Such data are no problem in youth but we still need to pay attention to them.

Keywords lipid metabolism, young men, lifestyle, longitudinal study

Hypertension, dyslipidemia, diabetes mellitus and obesity are well known risk factors for atherosclerosis, and dyslipidemia is an especially important risk factor for it. Although the prevalence of metabolic syndrome and pre-metabolic syndrome increases more in the forties than thirties[1], the lifestyle-related risk factors in the forties develop well before the forties. Changes in total cholesterol concentrations and triglyceride concentrations are remarkable in age groups below the forties[2] and increases in BMI or body weight after the twenties may influence healthcare costs[3,4]. Therefore, lifestyle management in the twenties and thirties is important for preventing lifestyle diseases. To our knowledge, no previous longitudinal studies regarding a relationship between dyslipidemia and lifestyle habits in the thirties have been conducted in Japan.

This study investigated whether lifestyle habits at the age of 35 influenced blood lipid levels at the age of 40.

Methods

Subjects

A total of 2,583 males underwent health check-ups at 35 and 40 years old between 2008 and 2015 at Seirei Social Community Healthcare Division in Shizuoka, Japan. From among them, 1,655 (64.1%) males with normal TG, LDL-C and HDL-C levels but no medication for dyslipidemia were selected.

This study was approved by the Health Ethics Committee of Seirei Welfare Community Healthcare Division.

Study design

This longitudinal study retrospectively examined whether lifestyle habits at the age of 35 influenced the

[1] Seirei Healthcare Support Center Shizuoka ; [2] Seirei Center for Health Promotion and Preventive medicine ; [3] Seirei Social Welfare Community Health Division

Contact : Shigeki Muto, Seirei Center for Health Promotion and Preventive medicine, 2-35-8 Sumiyoshi, Naka-ku, Hamamatsu, Shizuoka 430-0906, Japan. Tel : +81–53–471–2247 ; Fax : +81–53–474–2505 ; E-mail : smuto@sis.seirei.or.jp

occurrence of blood lipid disorders at the age of 40 or not.

Data collection

The data of subjects at 35 and 40 years old was extracted from our database.

It included Triglyceride (TG), LDL-cholesterol (LDL-C) and HDL-Cholesterol (HDL-C) and the cut off levels were 150 mg/dL or greater for TG, 140 mg/dL or greater for LDL-C and less than 40 mg/dL for HDL-C. We assigned subjects with normal TG levels at 35 and 40 years old to a TG maintenance group. In the same way, we assigned subjects to a LDL-C maintenance group and a HDL-C maintenance group. Also, we assigned subjects with normal TG concentrations at 35 years old and concentrations outside the standard range at 40 years old to a TG aggravation group. Subjects were assigned to a LDL-C aggravation group and HDL-C aggravation group in the same manner.

We collected lifestyle habit data using the standard questionnaire for the Specific Health Check-up and other questions regarding eating habits and lifestyle. There were 2 possible responses for speed of eating - "fast or moderate" and "slow". In investigating drinking habit, frequency and daily amount of alcohol intake, we regarded an ethanol intake of 40 grams or greater a day every day or a few days a week as risky drinking[5]. We considered current smokers as having a smoking habit (**Table 1**).

We could not know whether subjects had received health guidance when they were 35 years old but they probably had not received it because their blood lipid concentration data were normal. They also might not have received Specific Health guidance because almost all of it was received by those 40 years or older.

Statistical analysis

We analyzed differences in the means of blood lipid data between 35 years old and 40 years old. We used Student's *t*-test to compare mean lipid levels in the maintenance groups with those in the aggravation groups. To compare before and after levels in each group, we used the paired *t*-test. To investigate whether lifestyle habits of the subjects at 35 years old were associated with presence of blood lipid disorders at 40 years old, we performed multiple logistic regression analysis and calculated odds ratios. The dependent variable was presence of blood lipid disorder at 40 years old, the independent variables were each lifestyle habit at 35 years old, and the adjustment variable was lipid concentrations at 35 years old. We excluded subjects with missing data in each analysis. We used SPSS version 20.0 (IBM Japan, Tokyo) and considered that p values less than 0.05 indicated statistical significance.

Results

Change in blood lipid concentrations from 35 years old to 40 years old

Mean values (SD) of blood lipid concentrations and BMI at 35 years old and 40 years old and differences between 35 years old and 40 years old are shown in **Table 2**.

Mean values (SD) for TG, LDL-C and HDL-C at 35 years old were 76.9 (28.2) mg/dL, 104.5 (19.8) mg/dL and 60.4 (14.1) mg/dL, respectively. Those at 40 years old were 89.7 (47.9) mg/dL, 115.3 (26.0) mg/dL and 62.9 (15.0) mg/dL, respectively, so all blood lipid concentrations had increased.

The rates of aggravation from normal lipid at 35 years old to lipid disorder at 40 years old were 9.2% for TG, 17.6% for LDL-C and 2.0% for HDL-C. Mean values

Table 1. Questions Regarding Lifestyle Habits at the Age of 35

Skipping breakfast more than three times a week (skipping breakfast)
Eating within 2 hours before going to bed more than three times a week (eating before going to bed)
No moderation in eating (not moderate in eating)
Not getting enough vegetables (not getting enough vegetables)
Eating out or taking out bought prepared foods more than three times a week (eating out or taking out)
Eating midnight snacks more than three times a week (eating midnight snacks)
Not taking moderate amount of salt (not moderate in amount of salt)
Not avoiding fatty foods one time a day (not avoiding fatty foods)
Consuming sweetened drinks more than three times a week (consumption of sweetened drinks)
Eating more quickly than other persons (eating quickly)
Not exercising more than 30 minutes a time and more than two days a week (no exercise habit)
Not engaging in physical activities more than one hour a day (insufficient physical activities)
Not walking more quickly than similar aged persons (not walking quickly)
Not getting enough rest through sleep (insufficient rest through sleep)
Smoking habit (smoking)
Drinking habit (drinking)
Alcohol consumption of more than 40g/day every day or some days (risky drinking)

(SD) for blood lipids in the aggravation groups significantly changed; from 102.2 (27.5) mg/dL for TG, 122.9 (12.4) mg/dL for LDL-C and 45.7 (1.8) mg/dL for HDL-C at 35 years old to 198.4 (62.5) mg/dL, 154.2 (13.1) mg/dL and 37.3 (5.2) mg/dL at 40 years old, respectively. In the maintenance groups, however, though mean blood lipid values also significantly changed, the changes were slight, and HDL-C concentrations increased.

The mean (SD) value of BMI for all subjects slightly increased, from 22.1 (2.9) to 22.5 (3.0). Similarly, BMIs for both the maintenance groups and the aggravation groups slightly increased (**Table 2**).

Associations with lifestyle habits

Table 3 shows the results of multiple logistic regression analysis.

Regarding associations of lifestyle habits at the age of 35 and TG aggravation at the age of 40, the highest odds ratio (95% Confidence Interval) was 1.943 (1.332–2.834) for risky drinking, followed by 1.524

Table 2. Means (SD) of Each Lipid Parameter and BMI in Maintenance Groups and Aggravation Groups

		n(%)	mean (SD) at 35 y/o	p^{*1}	mean (SD) at 40 y/o	mean (SD) of difference (40 y/o–35 y/o)	p^{*2}	p^{*3}
TG	subjects	1655 (100.0)	76.9 (28.2)		89.7 (47.9)	12.8 (43.2)		<0.001
	maintenance	1503 (90.8)	74.3 (27.0)	<0.001	78.7 (28.5)	4.4 (28.6)	<0.001	<0.001
	aggravation	152 (9.2)	102.2 (27.5)		198.4 (62.5)	96.2 (67.7)		<0.001
LDL-C	subjects	1655 (100.0)	104.5 (19.8)		115.3 (26.0)	10.8 (18.9)		<0.001
	maintenance	1363 (82.4)	100.6 (18.9)	<0.001	107.0 (19.7)	6.4 (16.2)	<0.001	<0.001
	aggravation	292 (17.6)	122.9 (12.4)		154.2 (13.1)	31.3 (16.8)		<0.001
HDL-C	subjects	1655 (100)	60.4 (14.1)		62.9 (15.0)	2.5 (9.2)		<0.001
	maintenance	1622 (98.0)	60.7 (14.0)	<0.001	63.4 (14.7)	2.7 (9.1)	<0.001	<0.001
	aggravation	33 (2.0)	45.7 (1.8)		37.3 (5.2)	−8.4 (5.5)		<0.001
BMI	subjects	1655 (100)	22.1 (2.9)		22.5 (3.0)	0.5 (1.4)		<0.001
	TG maintenance	1503 (90.8)	22.0 (2.9)	<0.001	22.3 (3.0)	0.4 (1.3)	<0.001	<0.001
	TG aggravation	152 (9.2)	23.1 (2.8)		24.2 (3.0)	1.1 (1.3)		<0.001
	LDL-C maintenance	1363 (82.4)	22.0 (2.9)	<0.001	22.3 (3.0)	0.3 (1.3)	<0.001	<0.001
	LDL-C aggravation	292 (17.6)	22.5 (2.9)		23.5 (3.1)	1.0 (1.3)		<0.001
	HDL-C maintenance	1622 (98.0)	22.0 (2.9)	<0.001	22.5 (3.1)	0.4 (1.3)	<0.001	<0.001
	HDL-C aggravation	33 (2.0)	23.2 (2.0)		24.5 (1.9)	1.2 (1.6)		<0.001

*1 comparison of means between maintenance group and aggravation group (Student's *t*-test)
*2 comparison of means of difference (40 y/o - 35 y/o) between maintenance groups and aggravation groups (Student's *t*-test)
*3 before-after comparison between 35 y/o and 40 y/o for the same item (paired *t*-test)

Table 3. Odds Ratios of Aggravation for Each Lipid Paramater by Lifestyle Habit

lifestyle	TG				LDL-C				HDL-C			
	odds ratio	95% CI		*p*	odds ratio	95% CI		*p*	odds ratio	95% CI		*p*
skipping breakfast	1.524	1.031	2.253	0.035*	0.923	0.619	1.376	0.694	1.208	0.526	2.774	0.656
eating before going to bed	1.196	0.834	1.714	0.331	1.144	0.809	1.617	0.446	1.875	0.918	3.829	0.085
no moderation in eating	0.742	1.061	0.746	1.508	0.924	0.681	1.254	0.613	1.079	0.530	2.196	0.834
not getting enough vegetables	1.224	0.859	1.744	0.264	0.962	0.719	1.287	0.794	1.359	0.665	2.777	0.400
eating out or taking out	1.363	0.914	2.033	0.129	1.172	0.874	1.573	0.289	1.608	0.734	3.524	0.236
eating midnight snacks	0.683	0.419	1.114	0.127	1.290	0.914	1.821	0.147	0.815	0.325	2.044	0.663
not moderate in amount of salt	1.204	0.837	1.731	0.317	0.959	0.667	1.378	0.820	1.310	0.618	2.778	0.481
not avoiding fatty foods	1.339	0.842	2.128	0.218	1.059	0.790	1.421	0.701	0.964	0.420	2.210	0.931
consumption of sweetened drinks	1.015	0.714	1.444	0.932	1.269	0.881	1.829	0.201	1.036	0.509	2.110	0.922
eating quickly	1.213	0.852	1.726	0.284	1.086	0.813	1.450	0.576	1.398	0.687	2.847	0.356
no exercise habit	0.995	0.614	1.614	0.985	0.895	0.667	1.202	0.462	1.568	0.533	4.610	0.414
insufficient physical activities	1.139	0.772	1.680	0.513	0.960	0.664	1.389	0.828	1.367	0.599	3.116	0.458
not walking quickly	1.197	0.836	1.712	0.326	1.235	0.905	1.683	0.183	0.981	0.478	2.013	0.958
insufficient rest through sleep	0.825	0.576	1.183	0.295	1.284	0.958	1.719	0.094	0.955	0.462	1.974	0.900
smoking	1.801	1.264	2.565	0.001*	1.104	0.824	1.480	0.508	1.318	0.645	2.695	0.449
drinking	1.444	0.983	2.119	0.061	1.226	0.916	1.642	0.171	1.233	0.599	2.539	0.569
risky drinking	1.943	1.332	2.834	0.001*	1.073	0.753	1.528	0.698	2.575	1.190	5.571	0.016*

* $p<0.05$

(1.031–2.253) for skipping breakfast.

Regarding associations of lifestyle habits at the age of 35 and HDL-C aggravation at the age of 40, at 2.575 (1.190–5.571), only the odds ratio for risky drinking was significantly higher.

No lifestyle habits at the age of 35 were associated with LDL-C aggravation at the age of 40.

Discussion

This study demonstrated that lifestyle habits at the age of 35 influenced blood lipid data at the age of 40. To our knowledge, almost all studies regarding associations between lifestyle habits and lipid disorders have been cross-sectional. Although it is retrospective in design, our longitudinal study suggests that lifestyle habits at the age of 35, are important for future health even in the absence of lipid disorders at this age.

The change in TG data from 35 years old to 40 years old was +4.4mg/dL in the maintenance group and +96.2mg/dL in the aggravation group. The changes in LDL-C were +6.4mg/dL and +31.3mg/dL, respectively, and those in HDL-C were +2.7mg/dL and −8.4mg/dL, respectively. All changes in lipid data in the aggravation groups were greater than those in the maintenance groups. This was not only because data at age 35 were close to normal limits in the aggravation groups; the changes from 35 to 45 years in these groups were also remarkable. We consider that this suggests that subjects in the aggravation groups already had problems regarding lifestyle habits at the age of 35.

For each lipid parameter, BMI at the age of 35 in the aggravation groups was higher than in the maintenance groups. Also, while BMI increased slightly in both the maintenance groups and the aggravation groups from 35 years old to 40 years old, the rate of increase was more remarkable in the aggravation groups. Therefore, we consider that aggravation of blood lipid data may have been influenced by an increase in BMI, namely an increase in body weight. As previous studies showed that blood lipid data were higher when BMI was greater[6], and body weight reduction was effective for improving blood lipid data[7], it is important that health guidance leads to maintaining BMI or bodyweight.

Our results suggest that skipping breakfast at the age of 35 resulted in TG aggravation at the age of 40 and a previous study showed that people with higher TG levels had a higher rate of skipping breakfast[8]. It has also been found that a high frequency of skipping breakfast raises energy absorption per meal and significantly increases body fat percentage[9-12] and that energy consumption for people skipping breakfast is lower than that for people who eat breakfast[13]. Thus, we consider that skipping breakfast may decrease metabolism, and as a result, TG may rise. Since people in Japan in their twenties or thirties sometimes skip breakfast[14], this may be an important point in health guidance for younger people.

Almost all previous studies have shown that smoking raises TG and LDL-C levels and reduces HDL-C levels[15,16]. The present study suggests that risk of TG aggravation at the age of 40 in subjects with a smoking habit at the age of 35 is 1.8. On the other hand, smoking did not aggravate LDL-C and HDL-C. According to a previous longitudinal study, TG levels in males in their forties with a smoking habit was higher than in those without a smoking habit, which is consistent with our results[17]. Smoking in itself is a cardiovascular risk and as it also, increases TG levels, the risk of cardiovascular disease will be even greater. It is therefore, necessary to include smoking cessation in health guidance. Though many studies have shown that smoking can raise LDL-C levels, Kuzuya *et al.* found that they did not differ in smokers and non-smokers. This result is also consistent with that in our study[17]. On the other hand, while many studies have demonstrated that HDL-C in smokers is lower than in non-smokers, this is not consistent with our finding[15–17]. Kuzuya *et al.* found that HDL-C in smokers was already lower than in non-smokers in the early thirties[17], and this situation continued until the sixties. The subjects in the present study had normal HDL-C levels at the age of 35, and as those with lower HDL-C levels at the age of 35 may already have been excluded, smoking habit may not be additional risk for HDL-C aggravation. Therefore, it may be necessary to recommend smoking cessation before the age of 35.

In this study, risky drinking was associated with TG and HDL-C aggravation. It is well known that moderate alcohol consumption will increase HDL-C levels[18,19]while excessive alcohol consumption, of 30 mg/day and over will increase TG levels and decrease HDL-C levels[8]. In our study, risky drinking meant alcohol consumption of 40 mg/day and over, and therefore, the results of the previous study support our finding that excessive alcohol consumption increases TG concentrations and decreases HDL-C concentrations[18]. The present study retrospectively evaluated the effect of lifestyle habits from 35 years old to 40 years old, so we should consider the long-term effect of alcohol consumption. It has been found that long-term alcohol consumption will increase TG levels, which is consistent with our result[20]. Therefore, health guidance should also be given in consideration of the quantity and period of alcohol consumption.

In our study, the lipid that had the highest rate of aggravation from 35 years old to 40 years old was LDL-C. It is known that the causes of increases in LDL-C concentrations are excessive consumption of exogenous cholesterol, disturbance of reverse transfer due to lower-

ing of LDL receptor activity caused by excessive intake of saturated fatty acids and trans unsaturated fatty acids, acceleration of cholesterol synthesis in the liver and decreased excretion[21]. The Japan Atherosclerosis Society Guidelines for Prevention of Atherosclerotic Cardiovascular Diseases recommend restricting consumption of fatty meals and eggs and increasing consumption of water soluble dietary fiber and plant-derived cholesterol[22]. In the present study, affirmative answers to none of the questions (including those regarding lack of vegetable consumption and fatty food consumption) were associated with LDL-C aggravation. Although seaweed, kelp, jelly and fruit have a high soluble dietary fiber content and would be included in the category of vegetables, the questions in this study did not ask about the kind or quantity of vegetables, making us unable to explain the association between LDL-C aggravation and vegetable consumption. Similarly, because the question about fatty food does not distinguish different kinds of fat, such as saturated fatty acids, unsaturated fatty acids and trans fatty acids, the fatty food consumption lifestyle habit evaluated in this study may not be associated with LDL-C aggravation. To clarify associations between lifestyle habits and LDL-C aggravation, questions concerning water-soluble dietary fiber and kind and quantities of vegetable foods may be needed.

Lifestyle habits were associated with lipid aggravation in this study so, needless to say, people have to lead a better lifestyle. Even if lipid values are normal at the age of 35, they may be aggravated at the age of 40 and therefore, we have to carefully examine people with the lifestyle habits that aggravated lipid levels in this study.

The present study has some limitations. First, we investigated lifestyle habits at the age of 35, and could not make a lifestyle habit evaluation for before and after 35 years old. Unfavorable lifestyle habits may have been present before the age of 35. To improve this aspect, we may need to conduct a study on younger people. Secondly, although there is the possibility of lifestyle being improved by health guidance, we did not consider whether the subjects had health guidance or not. However, as mentioned in the Methods section, lipid concentrations were normal at the age of 35, so it was unlikely that the subjects had received health guidance. Finally, the generalizability of the results in this study is limited because almost all of the subjects lived in Shizuoka Prefecture, Japan.

Conclusion

Our findings suggest that skipping breakfast and smoking at the age of 35 may aggravate TG levels at the age of 40, risky drinking may aggravate TG levels and HDL-C levels and that increase in BMI is associated with lipid aggravation. Regardless of lipid data at the age of 35, we should pay attention to lifestyle habits in order to prevent future lipid disorders.

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There are no conflicts of interest to declare.

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Original Article

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Hyperuricemia Augments Incident Hypertension in a Healthy Japanese Male Population with No Influence from Proteinuria or Creatinine Clearance: Results from Nagoya Health Check Study (NHC Study)

Hiroka Suzuki[1,2], Akari Mizuno[1,2], Ruri Hoshino[1,2], Yasuyo Shirata[1,2], Izumi Takeichi[1,2], Junji Yoshino[1,2], Kengo Maeda[1,2]

Abstract

Objective: Hyperuricemia is an established risk factor of incident hypertension. However, the impact of renal function on the relationship between hyperuricemia and incident hypertension has not been fully elucidated. We investigated the relationship between hyperuricemia and incident hypertension stratified by estimated creatinine clearance (Ccr) or proteinuria using health check-up data.

Methods: We conducted a retrospective cohort study of 11,043 adult males who underwent health check-ups at our institutions and had no history of either chronic renal failure or cerebrocardiovascular diseases. Participants were divided into quartiles according to serum urate levels, and the fourth quartile was defined as the hyperuricemia group (serum urate level ≥ 6.9 mg/dL). We followed up the participants for five years using annual health check-up data.

Results: During 33,672 person-years of follow-up, 1,614 participants developed hypertension. The cumulative incidence of hypertension was significantly different among the quartiles (Log-rank test, $p < 0.001$). The multivariate Cox proportional hazard model revealed that hyperuricemia was significantly associated with incident hypertension (hazard ratio: 1.346, $p < 0.001$). In the analyses stratified by proteinuria or Ccr, hyperuricemia was associated with incident hypertension in the proteinuria-negative and proteinuria-positive groups, as well as in the Ccr-high and Ccr-low groups (divided by median of Ccr). Neither proteinuria nor Ccr had a significant interaction with the relationship between hyperuricemia and incident hypertension (likelihood ratio test: interaction $p = 0.8747, 0.5638$, respectively).

Conclusions: Hyperuricemia was significantly associated with incident hypertension in a healthy male population with no influence from proteinuria or creatinine clearance.

Keywords hypertension, hyperuricemia, renal function

Hypertension is one of the most common diseases found in health check-ups. Previous studies have revealed that the risk of cardiovascular diseases increases with increasing blood pressure past the optimum level[1]. Thus, the prevention of incident hypertension is a crucial issue in Japan as well as worldwide. Although approximately 90% of hypertension cases are essential hypertension, substantial efforts have been made to optimize lifestyles to prevent incident hypertension.

Previous studies have shown that hyperuricemia is a risk factor of incident hypertension, at least in male populations[2-4]. Serum urate levels are positively correlated with age, body weight, and alcohol intake, and negatively correlated with renal function. Hyperuricemia can cause renal dysfunction, which results in retention of sodium and water. Excessive sodium and water can augment incident hypertension. Thus, hyperuricemia may be more closely associated with incident hypertension in those with renal injury than in those without such injury. A previous study reported that the effect of hyperuricemia on incident hypertension was stronger in older males (≥ 40 years old) than in younger males (< 40 years old)[5]. However, few data on factors that influence the association between hyperuricemia and incident hypertension are available.

[1] Kokusai Central Clinic ; [2] Dai-Nagoya-Building Central Clinic

Contact : Kengo Maeda, Kokusai Central Clinic, 1-47-1 Nagono, Nakamura-ku, Nagoya, Aichi, 450-0001, Japan. Tel : +81–52–561–0633 ; Fax : +81–52–561–0631 ; E-mail : maedaken@central-cl.or.jp

We therefore conducted a retrospective cohort study using the data from health check-ups to investigate the relationship between incident hypertension and hyperuricemia. We investigated the cumulative incidence of hypertension in healthy adult male Japanese subjects, and performed a subgroup analysis stratified by proteinuria or estimated creatinine clearance (Ccr) to elucidate whether or not renal function affected the relationship between hypertension and hyperuricemia in a healthy male population.

Methods

Study Population

We conducted a retrospective and prospective observational study of health check-up examinees, named the Nagoya Health Check Study (NHC Study). The NHC Study was registered with UMIN-CTR, number UMIN000028954, and designed to investigate the risk factors associated with lifestyle-related disease, malignancy, cardiovascular disease, and arteriosclerosis. The participants in the present study were recruited from the NHC Study. Briefly, the Japanese male population (20–79 years old) who visited our clinic for annual health check-ups from April 2010 to March 2011 was recruited for the present study (n = 20,233). Prospective participants with hypertension, a history of cerebrocardiovascular disease, or chronic renal failure, and those taking urate-lowering or anti-hypertensive medicine were excluded (n = 4,576). Those who did not visit our institutions from April 2011 to March 2012 were also excluded (n = 4,614). The final analytic sample included 11,043 participants. The serum creatinine levels of participants ranged from 0.4 to 1.9 mg/dL (interquartile ranges: 0.8–0.9 mg/dL), and Ccr ranged from 36.7 to 265.6 mL/min (interquartile range: 91–121 mL/min). Annual health check-ups were conducted every year for five years. The observation was censored when a participant started urate-lowering medications. Written informed consent was obtained from all participants. The protocol of the NHC Study was approved by the Ethics Committee of Japan Medical Association (Reference no. 29-5).

Definitions

Hypertension was defined as a detected blood pressure ≥ 140/90 mmHg and/or the use of antihypertensive medications. The incidence of hypertension was defined as a newly detected blood pressure ≥ 140/90 mmHg and/or the initiation of antihypertensive medications. Diabetes mellitus was defined as a fasting blood sugar > 125 mg/dL and/or the use of antidiabetic medications. Dyslipidemia was defined as low-density lipoprotein cholesterol (LDL-C) ≥ 140 mg/dL, triglyceride (TG) ≥ 150 mg/dL, high-density lipoprotein cholesterol (HDL) < 40 mg/dL, and/or taking lipid-lowering medications. The Ccr was estimated using the formula of Cockcroft and Gault: Ccr = (140 - age) × body weight (kg) / (72 × serum creatinine [mg/dL]). Participants were divided into quartiles according to serum urate levels, and the highest quartile was defined as the hyperuricemia group (serum uric acid level ≥ 6.9 mg/dL).

Measurements

Blood pressure was measured with an automated sphygmomanometer. Participants rested in a seated position before the initial blood pressure reading was obtained. If the systolic blood pressure was ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg, an additional measurement was performed, and the average of the 2 measurements was used for the analysis. Urine and blood samples were obtained after overnight fasting. Urinalysis was conducted with dipsticks and measurements made by an automated urine analyzer. Proteinuria positivity was defined as a dipstick test trace of (±) or more. Blood analysis was conducted at a single center (Handa- Ishikai laboratory center). All participants were individually interviewed with a structured questionnaire. The smoking status, alcohol intake, medical history, and medications taken were recorded. The smoking status was classified as current smoker or non-current smoker, and the alcohol intake was classified as habitual or non-habitual.

Statistical analyses

A Kaplan-Meier survival analysis was used to compare the cumulative incidence of hypertension between the control and hyperuricemia groups. Differences between the groups were analyzed using the log-rank test. The Cox proportional hazard model was used to estimate the hazard ratios (HRs) and 95 % confidential intervals (CI) for incident hypertension. In the subgroup analyses, we stratified Ccr (divided by the median of Ccr; Ccr ≥ 105 mL/min, and < 105 mL/min) and proteinuria (proteinuria-negative: [–] by dipstick, and proteinuria-positive: [±] or more). We also tested the interactions using the likelihood ratio test. All p values were two-tailed, and $p < 0.05$ was considered statistically significant. The analyses were performed using the STATA/MP software program version 13 (StataCorp LP, Texas, USA).

Results

Baseline characteristics

The clinical characteristics and laboratory data of the participants are summarized in **Table 1**. There were statistically significant trends in all parameters among the quartiles. The participants in the higher quartiles tended to have a significantly higher body mass index (BMI), baseline blood pressure, and fasting blood sugar than those in the first quartile. The lipid profile was less favorable and the prevalence of alcohol drinkers sig-

Table 1. Baseline characteristics

	1st quartile (*n* = 2708)	2nd quartile (*n* = 2941)	3rd quartile (*n* = 2384)	4th quartile (*n* = 3010)	*p* value
Age	44 (38–53)	43 (37–52)	43 (37–51)	43 (38–51)	0.009
BMI	22.0 (20.3–23.9)	22.4 (20.9–24.3)	23.1 (21.5–25.0)	24 (22.1–26.2)	<0.001
Systolic BP (mmHg)	117 (109–126)	118 (110–126)	119 (111–127)	122 (114–130)	<0.001
Diastolic BP (mmHg)	67 (61–75)	68 (61–75)	69 (63–76)	72 (65–78)	<0.001
LDL–C (mg/dL)	112 (94–132)	116 (97–136)	120 (101–139)	125 (105–146)	<0.001
HDL –C (mg/dL)	56 (48–67)	55 (47–64)	54 (47–63)	52 (45–61)	<0.001
Triglyceride (mg/dL)	86 (62–122)	94 (68–133)	105 (75–148)	126 (87–182)	<0.001
Urate (mg/dL)	4.8 (4.3–5.1)	5.8 (5.6–6.0)	6.5 (6.3–6.6)	7.5 (7.1–8.0)	<0.001
Cr (mg/dL)	0.8 (0.7–0.9)	0.8 (0.8–0.9)	0.9 (0.8–0.9)	0.9 (0.8–1.0)	<0.001
Ccr (mL/min)	104 (90–119)	105 (91–120)	105 (91–121)	106 (92–123)	0.003
FBG (mg/dL)	92 (87–98)	92 (87–98)	92 (88–98)	94 (88–100)	<0.001
Current smoking (%)	41.2%	42.0%	39.9%	34.6%	<0.001
Alcohol intake (%)	65.9%	71.1%	74.2%	78.0%	<0.001

BMI, body mass index; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Cr, creatinine, Ccr, estimated creatinine clearance; FBG, fasting blood glucose. Data are presented as medians (interquartile ranges) for continuous variables, and percentages for categorical variables. For continuous variables, differences between groups were evaluated using Kruskal-Wallis test.
For categorical variables, trends across groups were tested with an extension of the Wilcoxon rank-sum test.

Table 2. Complications and Medications of Participants at Baseline

	1st quartile (*n* = 2708)	2nd quartile (*n* = 2941)	3rd quartile (*n* = 2384)	4th quartile (*n* = 3010)	*p* value
Complications					
Diabetes mellitus	2.95%	1.94%	1.55%	1.20%	<0.001
Dyslipidemia	31.1%	36.6%	43.1%	57.3%	<0.001
Proteinuria-positive	8.20%	8.02%	8.47%	8.01%	0.933
Medications					
Diabetes mellitus	2.95%	1.94%	1.55%	1.20%	<0.001
Dyslipidemia	3.32%	2.41%	3.27%	3.09%	0.892

Data are presented as percentages.
Trends across groups were tested with an extension of the Wilcoxon rank-sum test.

nificantly higher in the higher quartiles than in the first quartile. The Ccr was significantly but modestly higher in the higher quartiles than in the first quartile. **Table 2** shows the complications and medications of the participants at baseline. The prevalence of dyslipidemia was significantly higher in the higher quartiles, while the prevalence of diabetes mellitus was higher in the lower quartiles. The prevalence of proteinuria was not significantly different among the quartiles.

Relationship between hyperuricemia and incident hypertension

During 33,672 person-years of follow up, 1,614 participants developed hypertension. The cumulative incidence of hypertension was 17.0% in the first quartile, 19.3% in the second quartile, 20.0% in the third quartile, and 28.8% in the fourth quartile (hyperuricemia group) (log-rank test, $p < 0.0001$; **Fig. 1**). The multivariate Cox proportional hazard model revealed that the fourth quartile was significantly associated with incident hypertension (HR, 1.346, 95% CI, 1.161–1.560, $p < 0.001$; **Table 3**). Other variables significantly associated with incident hypertension in the multivariate analysis were age, BMI, systolic blood pressure, diastolic blood pressure, LDL-C, fasting blood sugar, and smoking habit.

Subgroup analyses

Fig. 2 shows a stratified analysis divided by the median of Ccr. The adjusted HRs were calculated as a reference for subjects with Ccr ≥ 105 mL/min and serum urate level < 6.9 mg/dL, as the first - third quartiles were not significantly associated with incident hypertension in the multivariate Cox model (**Table 3**). The variables with $p < 0.05$ in the univariate model were used for the adjustment (age, BMI, systolic blood pressure, diastolic blood pressure, fasting blood sugar, triglyceride, LDL-C, smoking habit, alcohol intake, baseline Ccr, and proteinuria). Hyperuricemia augmented incident hypertension in both the high-Ccr group (divided by the median of Ccr; Ccr ≥ 105 mL/min) and low-Ccr group (Ccr < 105 mL/min). The likelihood ratio test revealed that the Ccr groups had no significant interaction with the association of hyperuricemia and

Table 3. Hazard Ratios for Incident Hypertension

	Univariate analysis			Multivariate analysis*		
Factor	HR	95% CI	*p* value	HR	95% CI	*p* value
Serum urate levels						
1st quartile		Reference			Reference	
2nd quartile	1.144	0.984–1.331	0.081	1.111	0.955–1.294	0.173
3rd quartile	1.266	1.084–1.478	0.003	1.109	0.947–1.298	0.198
4th quartile	1.874	1.634–2.151	<0.001	1.346	1.161–1.560	<0.001
Age	1.047	1.042–1.052	<0.001	1.043	1.036–1.051	<0.001
BMI	1.143	1.128–1.159	<0.001	1.063	1.041–1.086	<0.001
Systolic BP (mmHg)	1.116	1.109–1.122	<0.001	1.068	1.059–1.076	<0.001
Diastolic BP (mmHg)	1.127	1.120–1.134	<0.001	1.062	1.054–1.071	<0.001
LDL-C (mg/dL)	1.005	1.003–1.006	<0.001	0.998	0.997–1.000	0.044
HDL-C(mg/dL)	1.000	0.996–1.003	0.926			
Triglyceride (mg/dL)	1.002	1.001–1.002	<0.001	1.000	0.999–1.000	0.911
FBG (mg/dL)	1.010	1.008–1.012	<0.001	1.002	1.000–1.004	0.032
Ccr (mL/min)	1.003	1.000–1.005	0.005	1.002	0.999–1.006	0.116
Proteinuria–positive	1.307	1.113–1.534	0.001	1.134	0.965–1.333	0.127
Current smoking	0.904	0.817–0.999	0.049	1.114	1.004–1.237	0.043
Alcohol intake	1.296	1.155–1.454	<0.001	1.000	0.889–1.125	0.997

HR, hazard ratio; CI, confidential interval; BMI, body mass index; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; Ccr, estimated creatinine clearance.
*Variables with *p* value < 0.1 in univariate analysis were used in the multivariate model.

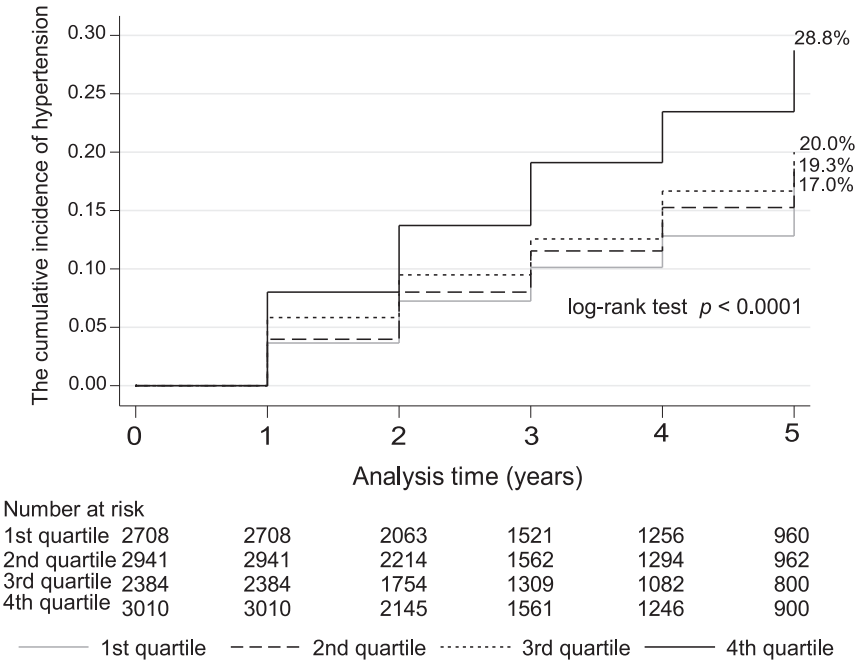
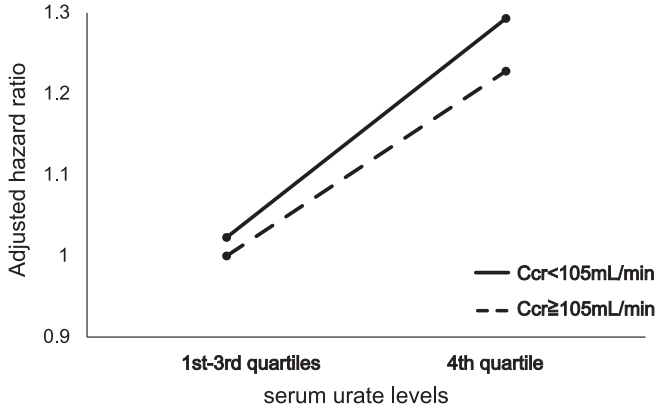


Fig. 1. Cumulative Incidence of Hypertension in Quartiles According to Serum Urate Levels Estimated Using Kaplan-Meier Method

incident hypertension (interaction $p = 0.8747$).

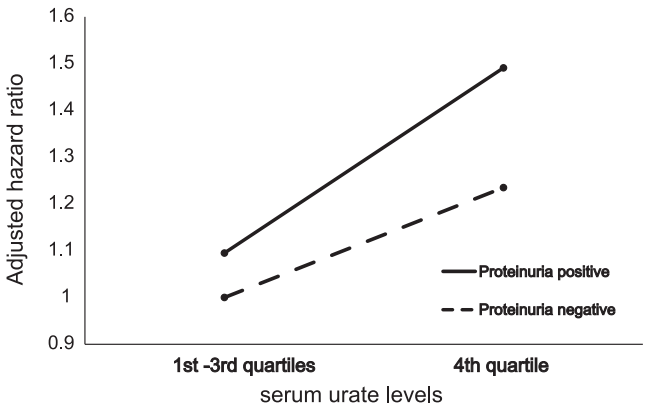
We also conducted an analysis stratified by proteinuria (**Fig. 3**). Adjusted HRs were calculated as a reference for subjects who were proteinuria-negative and had a serum urate level < 6.9 mg/dL. The variables used in **Fig. 2**, except for proteinuria, were used for the adjustment. The likelihood ratio test revealed that proteinuria also had no significant interaction with the relationship of hyperuricemia and incident hypertension (interaction $p = 0.5638$), although the HRs were higher in the proteinuria-positive group than in the proteinuria-negative group, regardless of hyperuricemia. Taken together, these results suggest that neither decreased Ccr nor proteinuria had an effect on the association between hyperuricemia and incident hypertension in a healthy male population.



	*Adjusted Hazard ratio (95%CI)	
	Ccr ≧105 mL/min (*n*=5520)	Ccr < 105 mL/min (*n*=5523)
1st - 3rd quartiles	1 (reference)	1.023 (0.866-1.209)
4th quartile	1.228 (1.056-1.428)	1.293 (1.076-1.556)

Fig. 2. Stratified Analysis Divided by Median of Ccr

*Hazard ratios were adjusted for age, BMI, systolic blood pressure, diastolic blood pressure, FBG, triglyceride, LDL-C, smoking habit, alcohol intake, baseline Ccr, and proteinuria.



	*Adjusted Hazard ratio (95% CI)	
	Proteinuria negative (*n*=10142)	Proteinuria positive (*n*=901)
1st - 3rd quartiles	1 (reference)	1.095 (0.892-1.345)
4th quartile	1.235 (1.102-1.384)	1.491 (1.152-1.930)

Fig. 3. Analyses Stratified by Proteinuria

*Hazard ratios were adjusted for age, BMI, systolic blood pressure, diastolic blood pressure, FBG, triglyceride, LDL-C, smoking habit, alcohol intake, and baseline Ccr.

Discussion

The present study revealed that hyperuricemia was a risk factor for incident hypertension in a healthy Japanese male population, even after adjustments for age, baseline blood pressure, BMI, fasting blood sugar, and smoking habit. Furthermore, neither the estimated Ccr nor proteinuria significantly influenced the effect of hyperuricemia on incident hypertension in the stratified analyses. These findings suggest that difference in renal function is not involved in the mechanisms underlying hyperuricemia-mediated hypertension, at least in healthy Japanese males. A recent cohort study conducted in a general Japanese population also suggested that hyperuricemia was an independent risk factor of incident hypertension[6]. The results of our study were consistent with those of this study.

The prevalence rate of hyperuricemia (UA ≥ 7.0 mg/dL) is estimated to be about 30%, which seems to be in accordance with the present findings[7]. Thus, our results seem to represent the real-world setting in Japan well. The recent meta-analysis conducted by Wang *et al.* showed that the overall adjusted relative risk of incident hypertension in hyperuricemia was 1.48 (95% CI, 1.33–1.65)[8]. However, the adjusted HR in our study was 1.346 (95% CI, 1.161–1.560), which was lower than that observed by Wang *et al.* This discrepancy might be attributable to the differences in our study settings, as our study population included only Japanese males. Although the influence of ethnicity on the association of hyperuricemia and hypertension remains unclear, a previous study suggested that the unfavorable effect of hyperuricemia on blood pressure might be more profound in females than males[9]. In addition, the participants of the present study were relatively young (median age: 43 years old, interquartile range: 38–52 years old), as the majority of them were office workers in Nagoya City. Furthermore, the observation period of the present study was shorter (up to 5 years, mean follow-up period: 3.05 years) than in previous studies. Although these factors may have influenced our results, the relationship between hyperuricemia and incident hypertension was reproducible in the present study, suggesting that hyperuricemia was a crucial risk factor for incident hypertension.

The present study revealed that the association between hyperuricemia and incident hypertension was not affected by renal function (either decreased Ccr or proteinuria), at least in healthy Japanese males. At present, the mechanisms underlying the association between hyperuricemia and incident hypertension are largely unknown. However, some experimental studies have provided valuable clues for resolving this issue. Mazzali *et al.* proved the causal relationship of hyperuricemia and incident hypertension using the hyperuricemia rat model[10]. They induced mild hyperuricemia in rats by feeding the animals an uricase inhibitor

without inducing any acute histological changes in the kidney. They found that mild hyperuricemia rats developed hypertension resistant to salt-restriction. They also observed interstitial fibrosis, decreased endothelial nitrogen oxide synthase (eNOS) expression, and an increased population of renin-positive cells in the kidneys of the rats, which indicated excessive microvascular constriction. Watanabe *et al.* reported salt sensitivity and microvascular disease in their hyperuricemia rat model[11]. They found that urate was able to augment the platelet-derived growth factor (PDGF)-mitogen activated protein kinase (MAPK) signaling system, which might lead to the proliferation of vascular smooth muscle cells and subsequent development of microvascular disease. Other studies have described oxidative stress, inflammation, and endothelial dysfunction in hyperuricemia animal models[12–14]. Taken together, the present and previous findings suggest that systemic and/or renal vascular disorders, but not sodium or water retention caused by renal dysfunction, play a vital role in urate-mediated hypertension, at least in a healthy population. Further studies will be needed to obtain a comprehensive picture of the mechanisms underlying urate-mediated hypertension.

Several limitations associated with the present study warrant mention. First, we were unable to obtain detailed information on alcohol intake. Seki *et al.* reported that blood pressure and serum urate levels were significantly correlated in regular alcohol drinkers but not in non-drinkers[15]. Those who drank > 25 g ethanol/day were categorized as the drinkers in their study. Their findings suggested that the alcohol intake might be involved in urate-mediated tissue injury in addition to increasing serum urate levels, although the precise mechanisms remained to be elucidated. In addition, we were also unable to obtain detailed information on smoking or dietary habits. Second, the present study did not include any patients with severe renal disease. We therefore cannot rule out the possibility that hyperuricemia might increase blood pressure through exacerbation of renal function in patients with evident renal failure. Third, hemoglobin A1c data were unfortunately missing.

Mild hyperuricemia is one of the most frequent diseases observed during health check-ups. At present, preventive antihypertensive management using urate-lowering drugs is not generally accepted. However, given the results of the present study, early interventions, such as lifestyle optimization and reducing alcohol intake, may be needed in males with hyperuricemia but no renal dysfunction.

Conflicts of Interest

None.

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Case Report

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A Case of Pulmonary Pleomorphic Carcinoma Discovered During Optional Low-dose Computed Tomography Screening and Resected at the Early Stage

Nobuya Mino[1], Naoko Yoshii[2], Hirofumi Okabe[1], Seishiro Takahashi[3], Hiroshi Ogawa[3], Masayuki Tanahashi[2], Hiroshi Niwa[2], Shigeki Muto[1]

Abstract

Pulmonary pleomorphic carcinoma (PPC) is a subgroup of non-small cell lung cancer that contains both epithelial and mesenchymal (sarcomatoid) components. Because of its rarity and aggressiveness, there are few reports describing the early stage of PPC. In this report, we describe a case of PPC detected as a small shadow during optional low-dose computed tomography (CT) screening that was completely resected at the early stage after follow-up.

During a health check-up for a 64-year-old man at our institution, optional low-dose chest CT screening revealed a small ground-glass opacity-like shadow in the peripheral area of the upper lobe of the right lung. Afterwards, the lesion was monitored using standard-dose CT. Although no remarkable changes had been observed up to 12 months, it had become enlarged by the 18-month follow-up. Further examinations did not provide a definitive diagnosis, so a surgical operation was performed for both diagnosis and treatment purposes. As the intraoperative rapid pathologic diagnosis revealed malignancy, a typical right upper lobectomy and mediastinal lymph node dissection were performed. Microscopic images of the tumor showed both epithelial and sarcomatoid components. The pathological diagnosis obtained using permanent sections was PPC resected completely at stage IA.

Keywords Pulmonary pleomorphic carcinoma, lung cancer, low-dose computed tomography

Pulmonary pleomorphic carcinoma (PPC) is a subgroup of non-small cell lung cancer (NSCLC) that contains both epithelial and mesenchymal (sarcomatoid) components. Owing to its rarity and aggressiveness, there have been few reports describing the early stage of PPC. This report describes a case of PPC detected as a small shadow during optional low-dose computed tomography (CT) screening that was completely resected at the early stage after follow-up.

This report was approved by the Ethics Committee of Seirei Social Welfare Community, and general informed consent was obtained from the patient.

Case Report

A 64-year-old man with diabetes, hyperlipidemia, and prostatomegaly underwent a health check-up during a visit to our institution. He had history of smoking 45 packs of cigarettes a year, but was symptom free.

Optional low-dose chest computed tomography (CT) screening revealed a ground-glass opacity (GGO)-like shadow with a diameter of 7–8 mm in the peripheral area of the upper lobe of the right lung (**Fig. 1**). It had been difficult to detect the shadow on a chest radiograph.

The lesion was monitored using standard-dose helical CT after 3, 6, 12, and 18 months. Although the shadow was GGO-like on low-dose CT and the 5-mm slice of standard-dose CT, it was solid on the 2.5-mm slice of standard-dose CT (**Fig. 1**). Up to 12 months, there had been no remarkable change in the shadow but after 18 months, its diameter had increased to approximately 1 cm.

Further examinations were performed, but a definitive diagnosis could not be obtained. The results of the QuantiFERON-TB test (Japan BCG Laboratory, Tokyo), cryptococcal antigen test, aspergillus antigen test, and serum β-D-glucan level were normal. Serum levels of common tumor markers of lung cancer - carcinoembry-

[1]Seirei Center for Health Promotion and Preventive Medicine ; [2]Division of Thoracic Surgery, Respiratory Disease Center, Seirei Mikatahara General Hospital ; [3]Department of Pathology, Seirei Mikatahara General Hospital

Contact : Nobuya Mino, Seirei Center for Health Promotion and Preventive Medicine, Sumiyoshi 2–35–8, Naka-ku, Hamamatsu, Shizuoka, 430–0906, Japan. Tel : +81–53–473–5506 ; Fax : +81–53–474–2505 ; E-mail : 028nononon@gmail.com

onic antigen (CEA), Cytokeratin-19 fragments (CYFRA), and pro-gastrin-releasing peptide (pro-GRP) - were all within normal limits (**Table 1**). The SCC antigen level was slightly high, but this is common for smokers. In bronchoscopy that was performed, there were no findings that could lead to a definitive diagnosis. In fluorodeoxyglucose positron emission tomography (FGD-PET), the SUVmax (value = 3.1) indicated weak uptake by the lesion. A whole body search using FGD-PET and magnetic resonance imaging (MRI) of the brain did not reveal any additional lesions.

Surgery for both diagnosis and treatment purposes was performed. Initially, only the tumor and surrounding lung parenchyma were resected for intraoperative rapid pathologic diagnosis (**Fig.2a**, **2b**). As the resected tumor tissue showed malignancy, a typical right upper lobec-

Table 1. Tumor Markers

CEA (carcinoembryonic antigen)	2.1	ng/mL (≤5.0)
SCC (squamous cell carcinoma antigen)	2.0 ↑	ng/mL (<1.5)
CYFRA (cytokeratin 19 fragment)	1.0	ng/mL (<3.5)
ProGRP (pro-gastrin-releasing peptide)	33.5	ng/mL (<81.0)

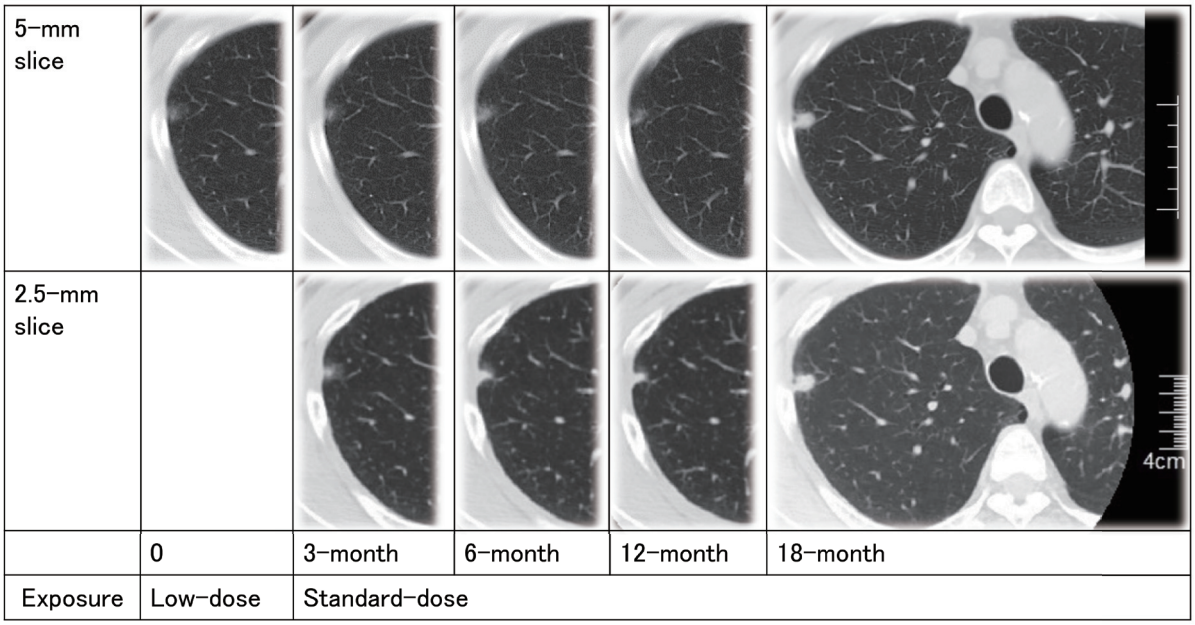

Fig.1. Computed Tomography (CT) Findings

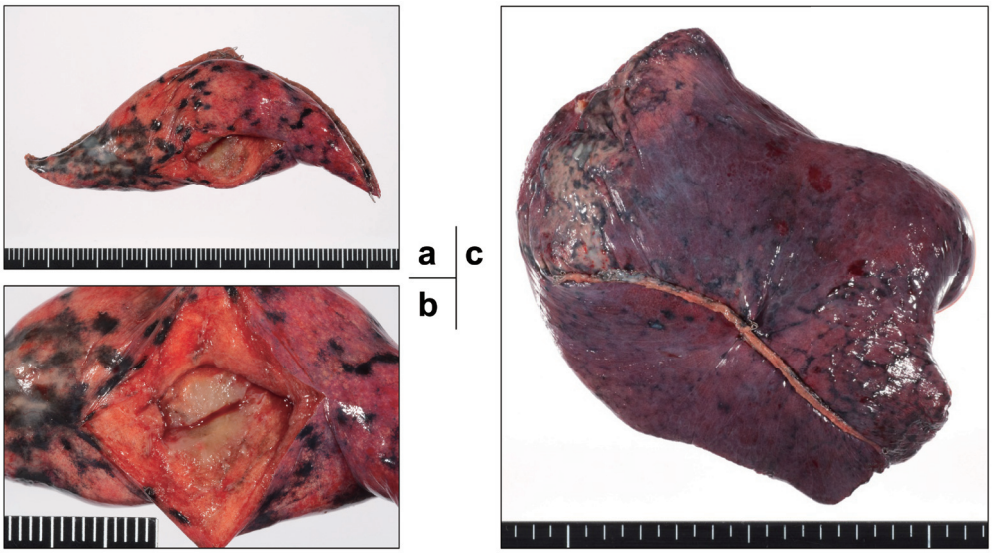

Fig.2. Resected Specimens
(a) Specimen from partially resected lung. (b) Cut surface of tumor. (c) Right upper lobe resected after partial resection.

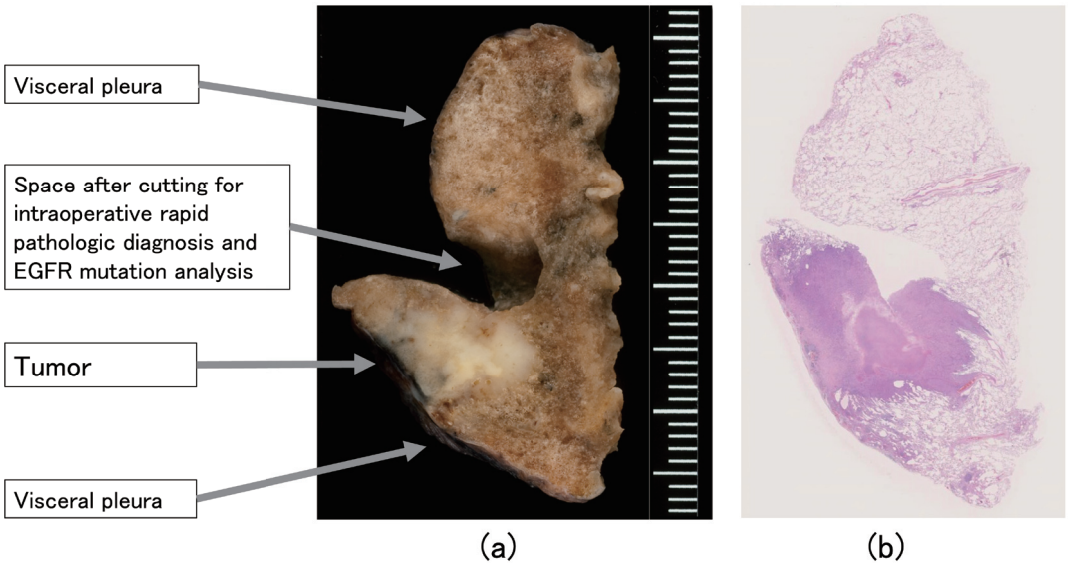

Fig. 3. Macroscopic Findings
(a) Formalin fixed. (b) Hematoxylin and eosin (HE) staining

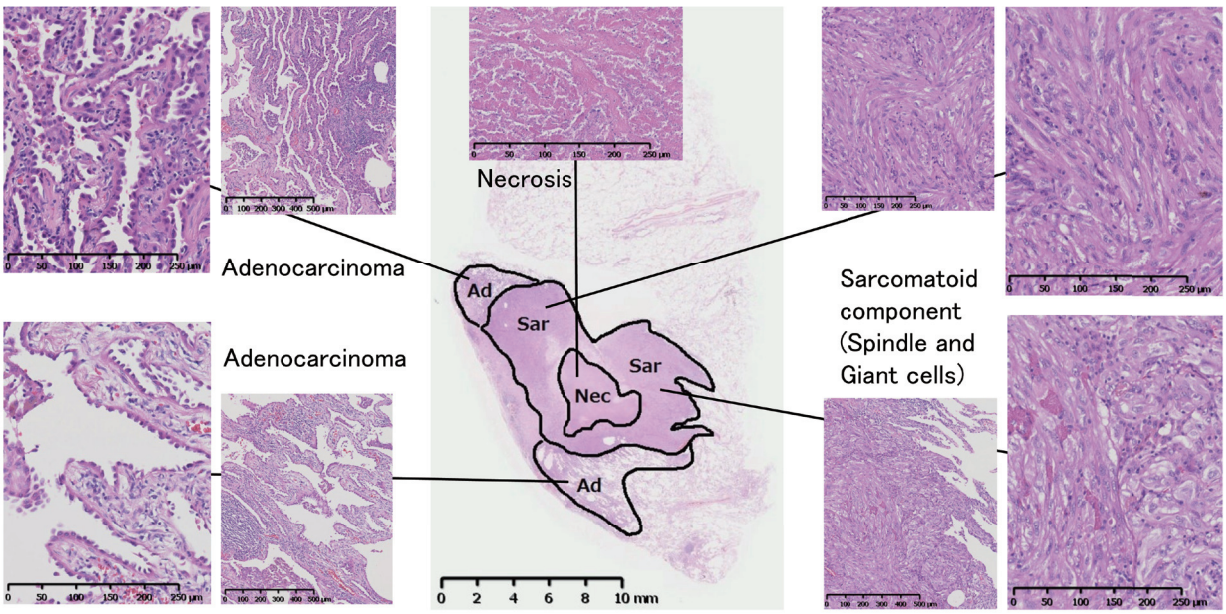

Fig. 4. Microscopic Features (HE staining)
Nec: Necrosis, Sar: Sarcomatoid component, Ad: Adenocarcinoma.

tomy and mediastinal lymph node dissection were performed sequentially (**Fig. 2c**). The operation concluded without any major problems.

The gross features of formalin-fixed tissue are shown in **Fig. 3a**. The tumor was solid, yellowish-white, without cavitation, relatively well-circumscribed, and approximately 1.6 x 1 x 1 cm in size.

Microscopic images showed a three-layered structure (**Fig. 3b**, **Fig. 4**). The central area revealed necrosis, the middle layer consisted of a sarcomatoid component (spindle cells and giant cells), and the outer area contained an adenocarcinoma (**Fig. 4**). The central necrosis consisted of non-specific necrotic tissue and neither caseous necrosis nor cavity formation were observed. Most of the sarcomatoid component was occupied by spindle cells, and giant cells had a scattered distribution. The sarcomatoid component accounted for approximately 80% of the whole area of the tumor. The adenocarcinoma was well to moderately differentiated. Inflammatory fibrous tissue was observed between it and the visceral pleura. These pathological findings gave the impression that the sarcomatoid component was infiltrating the adenocarci-

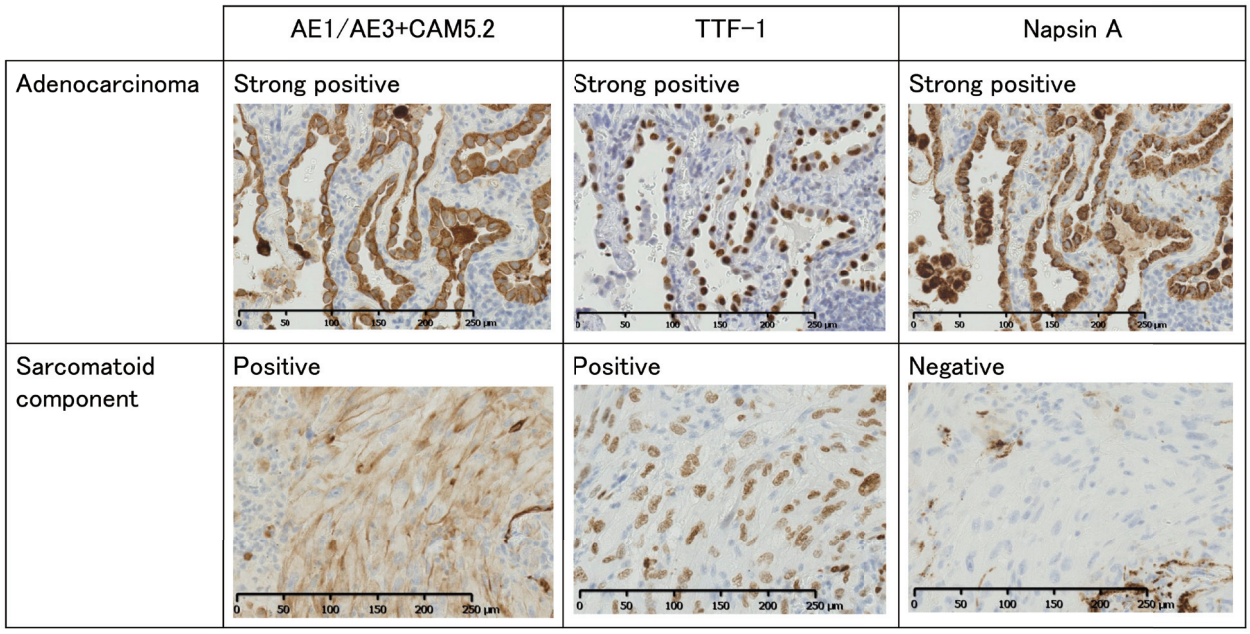

Fig.5. Immunohistochemical Study

noma towards the outside.

Immunostaining showed that the adenocarcinoma was strongly positive for all pan-epithelial markers (AE1/AE3 + CAM5.2), thyroid transcription factor-1 (TTF-1), and Napsin A (**Fig.5**). This staining pattern is commonly found in lung adenocarcinomas. The spindle cells were positive for pan-epithelial markers and TTF-1, but negative for Napsin A, indicating that the spindle cells were partially epithelial in character. If the spindle cells had been completely mesenchymal, the reactions for all these antibodies would have been weak or negative.

From these histopathological findings, a final diagnosis of "pleomorphic carcinoma (80%) with adenocarcinoma (20%)" was made. The TNM classification at the time of the diagnosis was pT1aN0M0, stage IA (according to the current TNM classification 8th edition, pT1bN0M0, stage IA2)[1,2]. The tumor was negative for pleural invasion, pleural dissemination, pulmonary metastasis, and lymph duct invasion, but positive for invasion into the vein. The EGFR gene had a wild-type phenotype.

The patient remains recurrence-free at 4 years after the surgery.

Discussion

PPC is a subgroup of NSCLC that contains both epithelial and mesenchymal (sarcomatoid) components[3-5]. PPC is defined as "a poorly differentiated non-small cell carcinoma namely a squamous cell carcinoma, adenocarcinoma, or undifferentiated non-small cell carcinoma that contains at least 10% spindle and/or giant cells or a carcinoma consisting only of spindle and giant cells"[3].

PPC was first defined in the World Health Organization classification of 1999. In the revised third edition of 2004, PPC was classified under "Sarcomatoid carcinoma"[4]. Currently, in the fourth edition of 2015, pleomorphic carcinoma and the other four types that had been classified under "Sarcomatoid carcinoma" are listed independently[3].

PPC tends to occur in elderly men with a history of smoking and resides in a peripheral location in the upper lobe of the lung[5-15]. In this case report, the patient was a 64-year-old male smoker, and the lesion was located in the periphery of the right upper lobe. All the features of this case were similar to those generally characteristic of PPC.

According to the literature, PPC is rare, accounting for 0.4–3.9% of resected lung cancers[6-10] and therefore, there are few reports of PPC. In addition, due to its aggressiveness, there are even fewer reports describing PPC at the early stage and its behavior[3-5]. In the present case, the lesion was discovered at an early stage in optional low-dose CT screening, and the early stage and behavior were monitored during the follow-up period. There were no remarkable changes in the lesion up to 12 months but it had become enlarged by the 18-month follow-up. So, why was its progression like this?

Currently, the major hypothesis for the histogenesis of PPC is the "divergent hypothesis", i.e., both the epithelial and sarcomatoid components originate from a single clone[3-5]. In this regard, the phenomenon of an epithelial cell converting to a mesenchymal cell in a malignant tumor has been described as "epithelial-to-mesenchymal transition (EMT)"[16]. Also, when EMT occurs, the epithelial cell becomes spindle-shaped, and intracellular

junctions are decreased[16], which raises the question: does "divergence" in PPC indicate EMT?

Applying the divergent hypothesis to the current case, the lesion might have been pure adenocarcinoma during the period when the CT shadow was stable. Then, part of the adenocarcinoma could have converted to sarcomatoid tissue, and the sarcomatoid component might have proliferated rapidly, which could be the reason that the CT shadow became enlarged later on.

The microscopic features support the above assumptions. The three-layered structure, i.e. central necrosis, middle sarcomatoid component and outer epithelial component, seems to indicate the process of the sarcomatoid component infiltrating the initial epithelial component towards the outside. The central necrosis probably occurred with the rapid increase of the sarcomatoid component.

In the immunohistochemical examination, spindle cells were positive for pan-epithelial markers and TTF-1, but negative for Napsin A, which indicates that the spindle cells were partly epithelial in character and supports the possibility that they were derived from adenocarcinoma.

In the present case, the tumor consisted of a combination of epithelial and sarcomatoid components with the adenocarcinoma as the epithelial component and spindle and giant cells as the sarcomatoid component. To date, various combinations of epithelial and sarcomatoid components have been reported, among them adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and other types as epithelial components; and spindle cells only, giant cells only, or both spindle and giant cells as sarcomatoid components. According to the literature, the rate of adenocarcinoma as the epithelial component of PPC was 24–73%, and that of both spindle and giant cells as the sarcomatoid component was 16–57%[6–13].

If the surgery in our case had been performed later, how might the lesion have progressed? The sarcomatoid component might have infiltrated deeper into the adenocarcinoma, and finally, the whole tumor might have consisted of the sarcomatoid component only. The last part of the definition of PPC mentioned above has the statement "or a carcinoma consisting of only of spindle and giant cells" so lung cancer consisting of only a sarcomatoid component is clearly PPC. Apart from a few studies, the rate of this type among all PPCs has been reported to be 0–22%[6–14]. PPC consisting only of a sarcomatoid component may be formed when the progress of this component is faster and completely infiltrates the epithelial component.

The prognosis of PPC is generally poorer compared with that of other NSCLC because metastasis or recurrence is more frequent and most chemotherapies and radiotherapies are ineffective[4,5,7,8]. According to the literature, the 5-year survival rate of patients with PPC was 20–48%[7,8,10,12,13,15], and the median survival was 8–19 months[6–8,11]. Yuki *et al.* reported that subtype according to epithelial or sarcomatoid components did not affect prognosis[12]. As EMT plays an important role in the metastatic mechanism of a malignant tumor[16] and influences its sensitivity to chemoradiotherapy[17], if "divergence" in PPC does indicate EMT, it would be natural for PPC to be more aggressive, highly resistant to chemoradiotherapy, and have a poorer prognosis.

Similar to the other types of NSCLC, disease stage and lymph node metastasis have been reported as prognostic factors of PPC, considering tumor progression[6,10,11,13]. Therefore, the treatment strategy in the present case was early detection and resection.

Conflict of Interest

The authors have no conflict of interest to declare.

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Case Report

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Two Cases of Pulmonary Adenocarcinoma *in Situ* Resected After 9 and 12 Years' Follow-up

Shigeaki Moriura[1], Satoko Ono[1], Shinichi Ito[1], Masashi Yosihara[2], Toshio Kato[3], Tetsuya Mitsudomi[4]

Abstract

Two cases of the long-term observation of pulmonary adenocarcinoma *in situ* (AIS) are reported. In case 1, the patient presented with an area of ground glass opacity (GGO) measuring 20 mm in diameter on chest computed tomography (CT) during a health check-up conducted at the age of 54 years. The GGO nodule was followed up with yearly CT scans and grew to 29 mm in diameter over 9 years. Thoracoscopic middle lobectomy was performed, and histopathological examination revealed AIS. In case 2, an area of GGO 7.7 mm in diameter was found when the patient was 49 years old. It was monitored via periodic CT examinations over 12 years. During this time, it grew to 19 mm in diameter. The surgical specimen exhibited AIS. In both cases, the tumor grew at a regular speed without showing any signs of invasion. The tumor doubling times for cases 1 and 2 were 1,878 and 1,071 days, respectively. However, cases in which GGO nodules grew rapidly and then developed into advanced adenocarcinoma have also been reported, so careful follow-up is mandatory for GGO lesions that are suggestive of AIS.

Keywords lung neoplasms, adenocarcinoma *in situ*, long-term observation, CT, PET

Pulmonary adenocarcinoma *in situ* (AIS)[1] involves the replacement of the alveolar lining cells without invasion, and surgical resection does not seem to be urgently required if the nature of the tumor remains unchanged. However, its natural history is unclear as only a few cases of the long-term periodic follow-up of pulmonary AIS have been reported[2,3]. We describe the growth of AIS during follow-up periods of 9 and 12 years.

Case Reports

Case 1

A 54-year-old man visited us for a health check-up in 2006. Chest computed tomography (CT) revealed an area of ground glass opacity (GGO) measuring 20 mm in diameter in the middle lobe of the right lung. He had been receiving medical treatment for diabetes mellitus, which was fairly well controlled. He had undergone surgery for volvulus soon after birth. The patient had a history of smoking (20 cigarettes a day from 25 to 32 years old and 60 cigarettes a day from 33 to 45 years old). He was examined by chest CT and positron emission tomography (PET) every year in almost the same month after detection of the lesion in almost the same month since the detection of the lesion. The GGO nodule slowly increased in size (**Fig. 1**), but was not positive for radiotracer uptake on PET. In 2013, we referred him to the surgical department with suspected lung cancer because of the size and gradual growth of the lesion. However, it was decided that the GGO nodule should continue to be observed because of its slow growth and the patient's wishes. Two years later, the GGO nodule had grown to 29 mm in diameter, and a sagittal preoperative CT image showed pleural indentation.

We referred the patient to the surgical department again. Finally, thoracoscopic middle lobectomy was performed. Histopathological examination of the specimen revealed AIS (**Fig. 2**). No findings were indicative of invasive adenocarcinoma. The pleural indentation had been caused by fibrous and granulomatous changes along the peripheral border adjacent to the pleura.

Case 2

A region of GGO measuring 7.7 mm in diameter was detected in segment 3 of the right lung in a 49-year-old male. He had smoked between the ages of 19 and 23 years (maximum: 20 cigarettes a day). The area of GGO was followed up on CT twice a year at a cancer hospital and our center. PET examinations were also performed

[1]Check-up Center, Daiyukai Daiichi Hospital ; [2]Department of Surgery , Daiyukai General Hospital; [3]Department of Pathology, Daiyukai General Hospital ; [4]Department of Surgery, Kindai University Hospital

Contact : Shigeaki Moriura, Check-up Center, Daiyukai Daiichi Hospital, 1-6-12 Hagoromo Ichinomiya, Aichi, 491-0025, Japan.
Tel : +81–586–26–2008 ; Fax : +81–586–23–3388 ; E-mail : moriura@daiyukai.or.jp

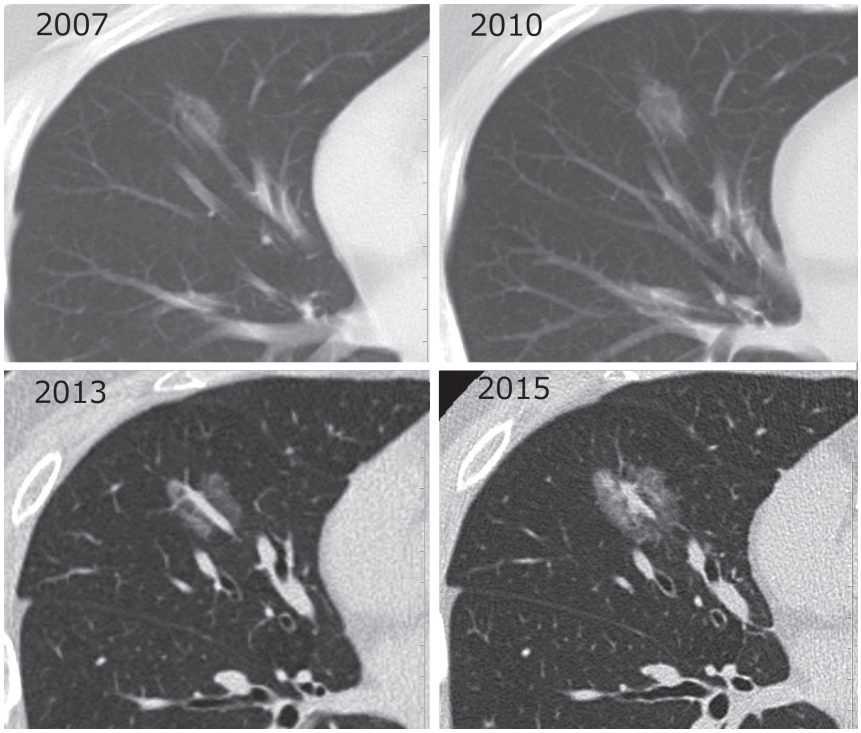

Fig. 1. Annual Changes in CT Findings in Case 1

The region of GGO in the middle lobe grew from 20 mm to 29 mm in diameter over 9 years. The CT images for 2006 were not available. The radiologist's report for 2006 stated that the maximum diameter of the tumor was 20 mm. Surgical resection was first suggested in 2013.

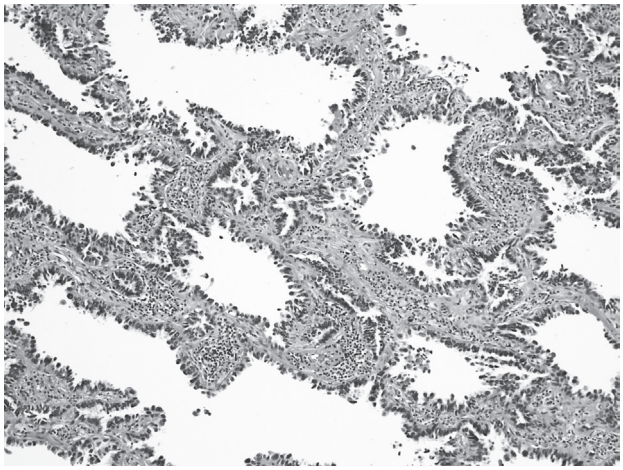

Fig. 2. Microscopic View of the Resected Specimen in Case 1

A single layer of atypical cells was arranged on the alveolar surface. Fibrous interstitial thickening was noted (HE, × 100).

every year, but the tumor was not determined to be positive for radiotracer uptake. The GGO nodule grew slowly but steadily, and in 2013 surgical resection was suggested at our check-up center because of a gradual increase in the diameter of the tumor. In August 2014, a surgeon explained that definitive treatment would probably be necessary, and he recommended surgical resection 6 months later because of a further increase in the tumor's diameter seen on CT. Finally, as the diameter of the tumor had reached 19 mm, the patient accepted the need to undergo surgery. Segmental resection of the right lung was performed in October 2015 (at 12 years and 2 months after the initial detection of the lesion) (**Fig. 3**). The pathological diagnosis was AIS, and no findings were suggestive of invasion (**Fig. 4**).

Growth of the tumors in the 2 cases

The annual changes in the maximum diameter of the tumor in cases 1 and 2 were almost constant over time

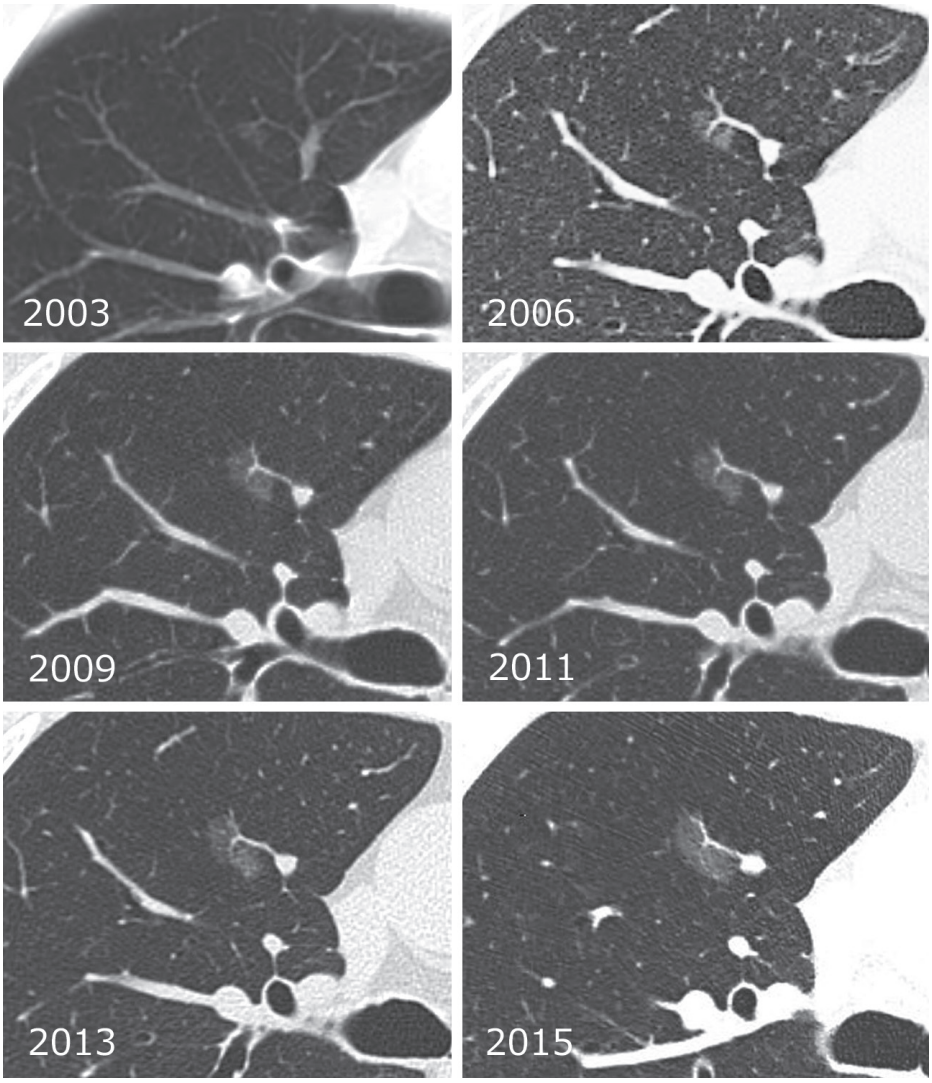

Fig. 3. Annual Changes in CT Findings in Case 2

The area of GGO in the upper lobe of the right lung grew from 7.7 mm to 19 mm in diameter over 12 years. Surgical resection was first suggested in 2013.

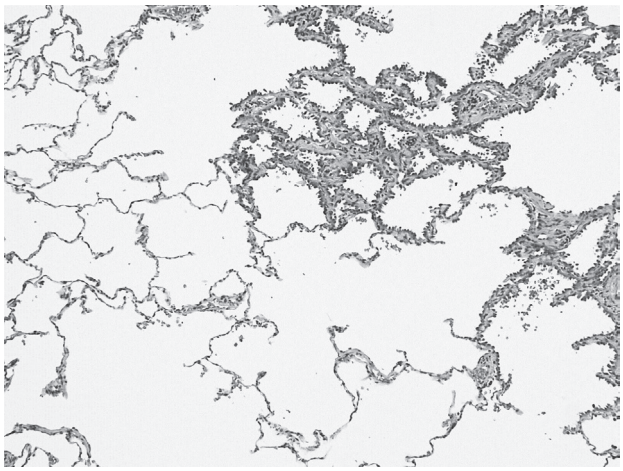

Fig. 4. Microscopic View of the Resected Specimen in Case 2 (HE, × 40)

The border of the tumor and the normal lung are shown. The upper right part of the photograph shows the AIS. A single layer of atypical cells was arranged on the alveolar surface, but fewer interstitial fibrous changes were seen than in case 1. The lower left part of the image shows normal pulmonary tissue.

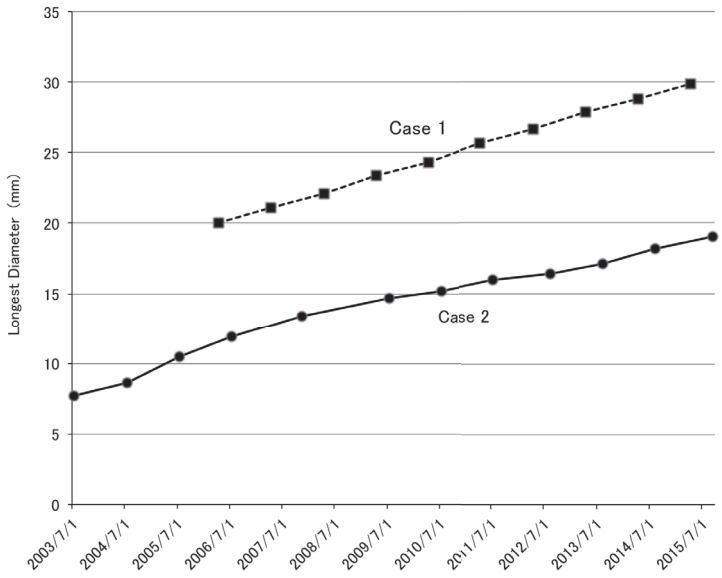

Fig. 5. Annual Changes in Maximum Diameter of the Tumor in Cases 1 and 2

(**Fig. 5**). The calculated tumor volume also exhibited linear growth. The tumor doubling time was 1,878 and 1,140 days in cases 1 and 2, respectively.

Discussion

Pulmonary AIS corresponds to Noguchi's Type A[4], localized bronchioloalveolar carcinoma. It involves the replacement of alveolar lining cells while the alveolar spaces remain intact[1,4]. Consequently, AIS presents as a GGO nodule on CT. Such lesions are sometimes described as subsolid nodules in the literature[5]. Improvements in CT have led to an increase in the detection frequency of pulmonary GGO nodules[3].

Lee *et al.*[3] reported that 45 of 175 GGO nodules that persisted for more than 2 years displayed significant increases in size (>2 mm). Of the 45 GGO nodules, 29 were surgically resected, of which 25 exhibited pathological findings of AIS or invasive adenocarcinoma. Though clear details of the number of nodules that decreased in size or disappeared were not given, their study suggests that most GGO nodules are benign. It is suspected that GGO nodules that disappear spontaneously are caused by unidentified interstitial inflammation, although the exact histopathology of such lesions is unknown. On the other hand, lymphoproliferative disorders and interstitial fibrosis were reported as histological findings of resected GGO that grew[2].

If GGO nodules do not disappear quickly, they can be distressing for patients because of their long and indolent course. It is a great help that guidelines for the management of GGO nodules have been published in both English[5] and Japanese[6]. According to the recommendations in them, surgical resection should have been performed earlier in the cases described in this study based on the size and growth of the tumors. The follow-up CT scans should have been compared with those from the earliest available study as was recommended in a statement from the Fleischner society[5].

The tumor doubling time has been reported to be a significant independent prognostic factor for lung cancer patients[7]. The mean tumor doubling time of adenocarcinoma was found to be 223.1 ± 209.4 days in 86 patients. However, this result seems to be based on cases of invasive adenocarcinoma because the study was published before the description of bronchioloalveolar carcinoma, which was later renamed AIS, as a separate entity[4]. In the present report, tumor doubling times of 1,878 and 1,140 days were observed during follow-up periods of 9 and 12 years in cases 1 and 2, respectively.

The tumors in the present cases grew very slowly, and the tumor growth curves based on tumor diameter and tumor volume were both linear. In elderly patients, a conservative approach to AIS seems acceptable, despite the current recommendations, if the tumor exhibits linear growth without signs of invasion. However, a change in the speed of tumor growth was reported in a case of bronchioloalveolar carcinoma that was followed up for 9 years[8], in which the tumor doubling time was 718 days during the first 7 years and 273 days in the subsequent 2 years. In another case[9], a GGO nodule developed into advanced cancer with metastasis to the mediastinal lymph nodes after 3 years' follow-up. Thus, very careful periodic observation is mandatory for GGO nodules that are suspected to be AIS. The appearance of solid transformation or an increasing focal density is suggestive of microinvasion, and surgery should be performed in such cases[5].

The authors have no conflicts of interest related to this manuscript.

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The Regulations of the International Society of Ningen Dock

Article 1
Name
The name of the association shall be the International Society of Ningen Dock.

Article 2
Office
The Society has its principal office in Japan Society of Ningen Dock.

Article 3
Aims
The Society, an organization of Japan Society of Ningen Dock for international operations, aims to contribute to global health promotion by enhancing the development of ningen dock as a medical check-up system.

Article 4
Tasks
The Society conducts the following tasks to achieve the aims described in the preceding section.
1. Holds congress (World Congress on Ningen Dock), board meetings, lectures, and committee meetings
2. Publishes journals and news magazines
3. Communicates and cooperates with related academic societies both in Japan and overseas
4. Promotes research activities in ningen dock and related fields
5. Does whatever is necessary to achieve the aims of the Society

Article 5
Membership
1. The Society consists of the following members
   1) Regular member
      A regular member shall be a member of the International Society of Ningen Dock who agrees to the aims of the Society, and has expert knowledge, techniques, or experience in the areas associated with the Society.
   2) Supporting member
      A supporting member shall be a person, a corporation, or a group that agrees to the aims of the Society, and supports its programs.
   3) Honorary member
      An honorary member shall be recommended, from those who have significantly contributed to the areas associated with the Society, by the executive board.
2. Those who want to apply for regular or supporting membership of the Society shall submit the prescribed application form with the membership fee.
3. The board meeting will process applications mentioned in the preceding section, and promptly notify the applicants of its decision.

Article 6
Officials
1. The Society shall appoint the following honorary advisors and officials.

Honorary advisor: Number not decided
Congress president: 1
President: 1
Vice president: 3 (from Japan : 2, overseas: 1)
Board members: up to 25 (from Japan : 15 or less, overseas : 10 or less)
Auditor: 2

Article 7
Honorary advisor
1. An honorary advisor shall be appointed by the president from those who have contributed to the development of the Society for a long period, and approved by the executive board.
2. Honorary advisors shall be eligible to attend the board meeting, and to express opinions; honorary advisors will not have voting rights.

Article 8
Congress president
1. The congress president shall be recommended by the executive board and appointed by the president.
2. The congress president shall represent the Society and host the World Congress on Ningen Dock as a scientific meeting.

Article 9
President
1. The president shall be selected by and from among board members and delegated by the president of Japan Society of Ningen Dock.
2. The president shall preside the Society.

Article 10
Vice president
1. The vice president shall be appointed, from among board members, by the president.
2. The vice president shall assist the president. In the case of accident, one of the vice presidents will be appointed by the president and will temporarily take over the duties.

Article 11
Board members
1. Board members from Japan shall be selected among candidates from regular members at Japan Society of Ningen Dock.
2. Overseas board members shall be selected at the recommendation of the executive board.
3. Board members execute duties for the Society under the orders from the president.
4. Board members, together with the president and the vice president, comprise the executive board.

Article 12
Board meeting
1. The president will call a board meeting on an as-needed basis, and serves as the chairman of the meeting.
2. The board meeting will pass resolutions on important matters of the Society.
3. The board meeting shall have the right to start proceedings if the majority of all the board members (including a letter of proxy) attend the meeting.
4. The board meeting shall pass resolutions with the majority votes of attendances.

Article 13
Auditor
Auditors shall audit accounts of the Society, and report to the board meeting.

Article 14
Commissioner
For the aims of successful programs of the Society, the president will set up committees and divisions through the resolutions of the executive board, and delegate the commissioners to regular members or other members of the Society.

Article 15
Accounting
1. The fiscal year for the Society starts on April 1 every year and ends on March 31 the following year.
2. Expenses required for the Society shall be covered by the following revenues.
   1) Membership fees
   2) Grants
   3) Donations
   4) Others

Article 16
Modification of rules
The rules of the Society can be amended by the resolution of the executive board.

Article 17
Miscellaneous provisions
Detailed regulations necessary for the enforcement of the rules of the Society are defined elsewhere by the president with the approval of the executive board.

Article 18
Additional clause
The Regulations of the International Society of Ningen Dock will come into effect on September 15, 2006.

Detailed Regulations of the International Society of Ningen Dock

Detailed regulations of the International Society of Ningen Dock are defined as follows:

(Detailed regulations on members)

Article 1

1. Members shall pay the following annual membership fee; honorary members will be exempt from membership fee.
   1) Regular member : 2,000 yen
   2) Supporting member : from one unit (unit: 20,000 yen)
2. Annual membership fee paid shall not be refunded for any reason.
3. Members with foreign citizenship shall pay a 3-year membership fee of 50 dollars.

Article 2

Members will be given priority in the following events :

1) Participation in scientific meetings hosted by the Society;
2) Contributions of articles to and receipt of the journal of the Society.

Article 3

Members shall lose their memberships in the event of the following:

1) Withdrawal from membership;
2) Adjudication of incompetence or quasi-incompetence;
3) Death or adjudication of disappearance, or dissolution of the group in the case of a member of a supporting group;
4) Delinquency in payment of membership fee for over three year.

Article 4

Those intending to withdraw from the Society must submit the notice of withdrawal in the prescribed form to be approved by the executive board.

Article 5

The Society can expel a member to whom either of the following would apply, with a resolution of the executive board:

1) Those who violate their duty as members of the Society;
2) Those who damage the honor of members of the Society or act against the aims of the Society.

Article 6

Those who satisfy Sections 1 and 2 of Article 5 of the Regulations of the International Society of Ningen Dock will be accepted as members of the Society.

(Detailed regulations on officials)

Article 7

1. The president will be selected from the board members of Japan Society of Ningen Dock.
2. In principle, the majority of board members from Japan will be selected from among the board members of Japan Society of Ningen Dock.

3. Overseas board members will essentially be selected from Asia, Pacific Rim, North America, or Europe.

Article 8

1. The term of the congress president will be from the end of the congress of which he/she is in charge to the next congress.
2. The term of board members will be six years (two terms of three years).

(Detailed regulations on congress and board meeting)

Article 9

Congress and board meeting will be held as follows :

1) The title of the congress will be World Congress on Ningen Dock.
2) In principle, the congress and the board meeting will be held once every three years; with the resolution of the executive board, however, the congress and the board meeting will be held as needed.
3) The congress and the board meeting will be held at the same time.
4) The name of the congress president and the location of the next congress will be announced.

Article 10

1. Those who want to take part in the congress shall pay the participation fee, which is defined separately.
2. Participation fee for the congress will be defined accordingly by the congress president.
3. Only regular members shall be allowed to present the results of their studies, except those who have been approved by the congress president.

(Enforcement of the detailed regulations)

Article 11

1. The detailed regulations will come into effect on September 15, 2006.

INSTRUCTIONS TO AUTHORS

Ningen Dock International
Official Journal of Japan Society of Ningen Dock

Ningen Dock International is the official journal of Japan Society of Ningen Dock, in which original articles, case reports, and review articles in both Japanese and English are published. Ningen Dock accepts only manuscripts that are original work in the field of ningen dock and related areas not previously published or being considered for publication elsewhere, except as abstracts. The manuscripts published in Ningen Dock will appear on the website of our society.

If the manuscript concerns a clinical study, it must be in accordance with the Declaration of Helsinki of 1964 (subsequent revisions included). Therefore, for a manuscript whose content is epidemiological or clinical research, the approval of the facility's Institutional Review Board (IRB) or the Ethics Committee of Japanese Society of Ningen Dock must have been obtained for the study described. Also, in the text, it should be indicated that informed consent has been obtained from subjects. Additionally, for case reports, it should be stated that adequate care has been taken to ensure the privacy of the subject concerned.

Online submission system

Ningen Dock International uses an online submission system called ScholoarOne Manuscripts.
Please access http://mc.manuscriptcentral.com/ningendock
This site is only in Japanese at this time.

Preparation of manuscript

All manuscripts must be written in English with MS-Word, Excel, PowerPoint and/or a common graphic format. Authors who are not fluent in English must seek the assistance of a colleague who is a native English speaker and is familiar with the field of the manuscript.

The title, abstract, text, acknowledgments, references, tables, and figure legends should begin on separate sheets, with pages numbered, and be typed double-spaced using the 12-point font size in MS-Word.
Files for submission should be prepared in English in a Microsoft Word or other file format that may be uploaded to the online system.

Available formats for files to be uploaded: doc (docx), xls (xlsx) ppt (pptx), jpg, tiff, gif, ai, eps, psd File names must consist of alphanumeric characters and an extension.

Example file names: Manuscript.doc, Fig 1 .jpg, Table 1 .xls, etc.

Please indicate the version of Microsoft Office used in a cover letter accompanying the uploaded files.

All measurements should be expressed in SI units. Less common abbreviations should be spelled out at first usage and the abbreviated form used thereafter.

Title page

Titles should be concise and informative. Include the full names of authors, names and addresses of affiliations, and name and address of a corresponding author to whom proofs are to be sent, including a fax number, telephone number and e-mail address.

Abstract

The abstract should not exceed 250 words, and should be arranged under the following subheadings: Objective, Methods, Results, Conclusions, and have up to 4 keywords.

Types of articles

Original articles: An original article should not exceed 3,000 words, and should be arranged as follows: Abstract, Objective, Methods, Results, Discussion, (Conclusion), (Acknowledgments), and References.

Case reports: A case report should not exceed 2,000 words, and be arranged as follows: Abstract (which should be a brief summary of the content without headings), Introduction, Case report, Discussion, and References.

Review articles: Review articles should not exceed 4,000 words. Review articles are usually by invitation. However, articles submitted without an invitation may also be considered by the Editorial Board.

References

References should be numbered consecutively in order of appearance in the text and cited in the text using superscript numbers. For example, according to the study by Sasamori[1)]. For journals, the names and initials of the first three authors, followed by "et al" if there are other coauthors, the complete title, abbreviated journal name according to Index Medicus, volume, beginning and end pages, and year should be included. For books, the names and initials of the first three authors, followed by "et al" if there are other coauthors, the complete title, book name, edition number, beginning and end pages, name and city of publisher, and year should be included. Examples of references are given below.

Journal: Ishizaka N, Ishizaka Y, Nagai R, et al: Association between white cell count and carotid arteriosclerosis in Japanese smokers. Atherosclerosis 2004; 175: 95-100.

Book: Kaplan NM: Measurement of blood pressure. In: Kaplan NM(ed), Kaplan's Clinical Hypertension. 7th ed., Lippincott William & Wilkins, Philadelphia, 2002, 25-55.

Tables

Tables should be cited in the text, and numbered sequentially with Arabic numerals. Each table should be given a number and a brief informative title, and should appear on a separate page. Explain in footnotes all abbreviations used.

Figures

Figures should be cited in the text, and numbered sequentially with Arabic numerals. A brief descriptive legend should be provided for each figure. Legends are part of the text, and should be appended to it on a separate page. Color figures can be reproduces if necessary, but the authors will be expected to contribute towards the cost of publication.

Conflict of Interest (COI)

All authors are required to disclose any conflict of interest (COI) on the form designated by the Japan Society of Ningen Dock.

If no author has any COI, this should be indicated in the manuscript.

Page proofs

The corresponding author will receive PDF proofs, the author should correct only typesetting errors. After correcting, page proofs must be returned promptly.

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Thirty reprints of each paper are free, and additional reprints are available at charge in lots of 10, but for a minimum order of 50. Reprints should be ordered on submission of the manuscript as follows: For example, "I order 100 reprints: 30 (free) + 70."

The Editorial Board considers only manuscripts prepared according to the Instructions to Authors, and makes decisions regarding the acceptance of manuscripts as well as the order of printing them. All published manuscripts become the permanent property of Japan Society of Ningen Dock, and may not be published elsewhere without written permission from the Society.

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Official Journal of Japan Society of Ningen Dock

Categories of manuscript:

- ☐ Original article (not more than 3,000 words)
- ☐ Case report (not more than 2,000 words)
- ☐ Review article (not more than 4,000 words)

Typing:

- ☐ Manuscript on A4 paper with wide margins
- ☐ Type double space using 12-point

Title page:

- ☐ Title of paper
- ☐ Full names of authors and affiliations without title of MD, PhD, etc
- ☐ Full name and address of a corresponding author including fax number, telephone number and e-mail address.
- ☐ Running title not more than 50 characters.

Abstract:

- ☐ Not more than 250 words.
- ☐ Arranged in the order of Background, Methods, Results, and Conclusion.
- ☐ Up to four key words.

Text of paper:

- ☐ Manuscript is arranged in the order of Objective, Methods, Results, Discussion, (Conclusion), (Acknowledgments), and References.
- ☐ Measurements are expressed in SI units.
- ☐ Abbreviations are spelled out at first usage.

References:

- ☐ References are numbered consecutively in order of appearance in the text and cited in the text using superscript numbers.
- ☐ Format is consistent with examples in Instructions for Authors.

Tables:

☐ Each table is given a number and a brief informative title, and appears on separate page.

☐ All abbreviations used are explained in footnotes.

Figures:

☐ Figure legends are appended to the text on a separate page.

☐ The top of the figure, the first author's name, and the figure number are indicated lightly in soft pencil on the back of the four figures.

Submission:

☐ Check list, agreement, cover letter, manuscript (title page, abstract, text, acknowledgments, and references), figure legends, tables, figures and/or photos prepared in due form.

☐ One set of the original manuscript and three sets of the copies (with original photos, if any) are submitted.

☐ All pages are numbered.

Date: _____

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Abbreviations

1	**1,5-AG**	1,5-anhydroglucitol	61	**hCG**	human chorionic gonadotropin
2	**17-OHCS**	17α-hydroxycorticosteroid	62	**HCV**	hepatitis C virus
3	**95% CI**	95% confidence interval	63	**HDL-C**	high-density lipoprotein cholesterol
4	**α-GI**	α-glucosidase inhibitor	64	**HLA**	histocompatibility [leucocyte] antigen
5	**β_2-MG**	β_2-microglobulin	65	**HPLC**	high-performance liquid chromatography
6	**γ-GTP**	γ-glutamyl transpeptidase	66	**Ht**	hematocrit
7	**A/G ratio**	albumin-globulin ratio	67	**ICD**	International Classification of Disease
8	**ABI**	ankle-brachial index	68	**ICU**	intensive care unit
9	**ACTH**	adrenocorticotropic hormone	69	**IFG**	impaired fasting glucose
10	**ADL**	activities of daily living	70	**IGT**	impaired glucose tolerance
11	**AFP**	α-fetoprotein	71	**IMT**	intima-media thickness
12	**ALP**	alkaline phosphatase	72	**LAP**	leucine aminopeptidase
13	**ALT**	alanine aminotransferase	73	**LDH**	lactate dehydrogenase
14	**Apo (a)**	apolipoprotein (a)	74	**LDL-C**	low-density lipoprotein cholesterol
15	**APTT**	activated partial thromboplastin time	75	**Lp(a)**	lipoprotein (a)
16	**AST**	aspartate aminotransferase	76	**LPL**	lipoprotein lipase
17	**BMI**	body-mass index	77	**MCH**	mean corpuscular hemoglobin
18	**CA125**	carbohydrate antigen 125	78	**MCHC**	mean corpuscular hemoglobin concentration
19	**CA19-9**	carbohydrate antigen 19-9	79	**MCV**	mean corpuscular volume
20	**cAMP**	cyclic adenosine 3', 5'-monophosphate	80	**METs**	meatbolic equivalent
21	**CAPD**	continuous ambulatory peritoneal dialysis	81	**MetS**	metabolic syndrome
22	**CBC**	complete blood cell count	82	**MMG**	mammography
23	**Ccr**	creatinine clearance	83	**MRA**	magnetic resonance angiography
24	**cDNA**	complementary deoxyribonucleic acid	84	**MRI**	magnetic resonance imaging
25	**CEA**	carcinoembryonic antigen	85	**mRNA**	messenger RNA
26	**cGMP**	cyclic guanosine 3', 5'-monophosphate	86	**MRSA**	methicillin-resistant *Staphylococcus aureus*
27	**ChE**	cholinesterase	87	**MSW**	medical social worker
28	**CKD**	chronic kidney disease	88	**NMR**	nuclear magnetic resonance
29	**COI**	conflict of interest	89	**PET**	positron emission tomography
30	**COPD**	chronic obstructive pulmonary disease	90	**PSA**	prostate-specific antigen
31	**CK**	creatinine kinase	91	**PTH**	parathyroid hormone
32	**CRP**	c-reactive protein	92	**PWV**	pulse wave velocity
33	**CT**	computed tomography	93	**QOL**	quality of life
34	**CVA**	cerebrovascular accident	94	**RBC**	red blood cell
35	**D-Bil**	direct bilirubin	95	**RF**	rheumatoid factor
36	**DBP**	diastolic blood pressure	96	**RI**	radioactive isotope
37	**DNA**	deoxyribonucleic acid	97	**RIA**	radioimmunoassay
38	**DRG**	diagnosis-related group	98	**RNA**	ribonucleic acid
39	**dsDNA**	double stranded deoxyribonucleic acid	99	**SBP**	systolic blood pressure
40	**EBM**	evidence-based medicine	100	**SD**	standard deviation
41	**ECG**	electrocardiogram	101	**SEM**	standard error of the mean
42	**eGFR**	estimated glomerular filtration rate	102	**STD**	sexually transmitted disease
43	**EIA**	enzyme immunoassay	103	**T-Bil**	total bilirubin
44	**ELISA**	enzyme-linked immunosorbent assay	104	**T_3**	triiodothyronine
45	**EPO**	erythropoietin	105	**T_4**	thyroxine
46	**ESR**	erythrocyte sedimentation rate	106	**TC**	total cholesterol
47	**FBG**	fasting blood glucose	107	**TG**	triglyceride
48	**FDA**	Food and Drug Administration	108	**TIA**	transient (cerebral) ischemic attack
49	**FEV**	forced expiratory volume	109	**TIBC**	total iron binding capacity
50	**FEV_1**	forced expiratory volume in one second	110	**tPA**	tissue plasminogen activator
51	**FEV_1%**	forced expiratory volume % in one second	111	**TPHA**	*Treponema pallidum* hemagglutination assay
52	**FPG**	fasting plasma glucose	112	**TSH**	thyroid stimulating hormone
53	**FSH**	follicle stimulating hormone	113	**TTT**	thymol turbidity test
54	**FT3**	free triiodothyronine	114	**UCG**	ultrasonic echocardiography
55	**FT4**	free thyroxine	115	**UIBC**	unsaturated iron binding capacity
56	**FVC**	forced vital capacity	116	**UN**	urea nitrogen
57	**GFR**	glomerular filtration rate	117	**VLDL**	very-low-density lipoprotein
58	**GH**	growth hormone	118	**WBC**	white blood cell
59	**Hb**	hemoglobin	119	**WHO**	World Health Organization
60	**HbA1c**	hemoglobin A1c	120	**ZTT**	zinc sulfate (turbidity) test

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Yukito Shinohara
President
Japan Society of Ningen Dock

FAX: +81-3-3265-0083

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Affiliation

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E-mail Address

2. Specialty (Circle one)

Doctors (internal medicine, primary care, surgery, gynecology, ophthalmology, pediatrics, radiology, orthopedics, pharmacology, epidemiology, other:　)

Nurse, Public Health Nurse, Dietician, Clinical Technologist,

Clinical Radiological Technologist, Pharmacist, Other:

3. Annual Dues

☐Regular Member

Annual dues in Japanese yen ……2,000

☐Supporting Member

Annual dues in Japanese yen …… 20,000

☐Regular Member -International

3-year dues in US$ ……50.00

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