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doi: 10.1016/j.diabres.2020.108002(https://doi.org/10.1016/j.diabres.2020.108002)

Title Page

Liver fat accumulation assessed by computed tomography is an independent risk factor for diabetes mellitus in a population-based study: SESSA (Shiga Epidemiological Study of Subclinical Atherosclerosis)

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Declaration of interest: none

Word count: 4131 words excluding supplements

Structured Abstract: 199 words

Number of tables and figures: 3

Prior Presentation:

Parts of this study were presented in abstract form at the 78th Scientific Sessions of the American Diabetes Association, Orlando, FL, 22–26 June 2018 and the 61st Annual Meeting of the Japan Diabetes Society, Tokyo, 24–26 May 2018.

Structured Abstract

Aims: Ectopic fat accumulation is related to insulin resistance and diabetes mellitus (DM). However, the effect of fatty liver on DM in non-obese individuals has not been clarified. We investigated whether liver fat accumulation assessed by computed tomography (CT) is associated with the incidence of DM.

Methods: In a prospective population-based study, 640 Japanese men were followed up for 5 years. The liver to spleen (L/S) ratio of the CT attenuation value was used as the liver fat accumulation index. We calculated the odds ratio (OR) and 95% confidence interval (CI) for the DM incidence of per 1 standard deviation (SD) lower L/S and those of L/S <1.0 compared with L/S ≥1.0, using logistic regression models.

Results: Both per 1 SD lower L/S and L/S <1.0 were significantly associated with a risk for DM incidence (1 SD lower L/S: OR=1.57, 95%CI=1.14–2.16; L/S <1.0: OR=2.27, 95%CI=1.00–5.14). The relationship between L/S and incidence of DM was consistent in the obese and non-obese groups, with thresholds of BMI 25 kg/m^2, waist circumference 85 cm, or visceral adipose tissue 100 cm^2.

Conclusions: Liver fat accumulation assessed by CT was associated with the incidence of DM.

Keywords: Fatty liver; CT; Diabetes mellitus

1. Introduction

Ectopic fat accumulation is defined as the presence of lipid droplets within non-adipose tissues that do not normally contain much lipid. Many studies reported that ectopic fat accumulation leads to insulin resistance and causes diabetes mellitus [1-3]. Fatty liver, including non-alcoholic fatty liver disease (NAFLD) and alcohol-induced fatty liver, is a form of ectopic fat accumulation and is increasing globally [3].

The Asian population is known to be more susceptible to both fatty liver and diabetes mellitus even with a normal body mass index (BMI) compared with the Western population [4, 5]. However, the clinical importance of fatty liver in non-obese individuals appears to be less recognized than that in the obese population, because population that develop diabetes mellitus tend to be obese.

Epidemiologic studies demonstrated a relationship between fatty liver and the incidence of diabetes mellitus based on ultrasonography images [6-8]. Although ultrasonography is a well-established imaging technique for the screening of fatty liver, this method is restricted to qualitatively detecting the presence or absence of fatty liver. By contrast, computed tomography (CT) and magnetic resonance imaging can quantitatively measure mild to severe liver fat accumulation. The liver to spleen ratio (L/S ratio) using CT is correlated with the hepatic fat content and is used as an index for the assessment of fatty liver [9, 10]. However, to date, only a few prospective studies on liver fat accumulation assessed by CT and the incidence of diabetes mellitus have been reported [11, 12]. Currently, the relationship between the L/S ratio and the incidence of diabetes mellitus has not been clarified.

Therefore, our study aimed to evaluate the association between liver fat accumulation as determined quantitatively using the L/S ratio and the risk for diabetes

mellitus in a prospective cohort of general Japanese men in both an obese and a non-obese group.

2. Subjects, materials, and methods

2.1 Study designs and participants

A prospective population-based longitudinal study of Japanese men in Kusatsu City, Shiga, Japan was performed as the Shiga Epidemiological Study of Subclinical Atherosclerosis (SESSA) [13, 14]. Study participants were randomly selected from the Kusatsu City Basic Residents' Register, aged 40–79 years at the baseline. In total, 1094 men (participation rate 46.0%) agreed to participate in the SESSA. We performed a baseline survey from 2006 to 2008 and a follow-up survey from 2010 to 2014. Among the 853 men who participated in both the baseline and follow-up studies, we excluded those with diabetes at baseline (n=189), a lack of diabetes information at baseline (n=1), and missing variables (n=23; diabetes information at follow-up n=4, CT information n=3, adjustment variables for pack-year n=5, ethanol intake n=1, waist circumferences n=3, visceral adipose tissue (VAT) n=5, daily step count n=2). Finally, 640 participants were analyzed in the present study. This study followed the Declaration of Helsinki and was approved by the Institutional Review Board of Shiga University of Medical Science. Every participant provided written informed consent.

2.2 Measurements

We performed anthropometries, questionnaires, examinations, including blood sampling, CT scan, and further examinations, both at the baseline and follow-up surveys.

2.2.1 Anthropometries and questionnaires

We measured body weight and height. The BMI was calculated as weight (kg) divided by height squared (m^2). The waist circumference was measured twice at the umbilicus level in a standing position at the end of the exhalation phase, and mean values were calculated. The office blood pressure was obtained from the mean of two consecutive measurements on a participant's right arm in a seated position after a 5-minute rest using an automated sphygmomanometer (BP-880; Omron Health Care Co. Ltd, Tokyo, Japan). Step counts for 8 days consecutively were obtained using a pedometer (DIGI-WALKER DW-200; Yamasa Tokei Keiki Co. Ltd, Tokyo, Japan). Because the step counts of the first day were recorded for only a half day, we calculated the mean of seven consecutive days from the second to the eighth day. We excluded any extreme counts; <100 or ≥50000 steps/day.

Self-administered questionnaires were used to obtain demographic information, family history of diabetes mellitus, smoking status, alcohol drinking status, medication, medical history, and other lifestyle factors. Once the participants had completed the questionnaires, trained nurses confirmed the provided data. A family history of diabetes mellitus was defined as its diagnosis in the participant's father and/or mother. Alcohol consumption was calculated as the daily ethanol intake (g/day) from self-reported

questionnaires. The pack-year was calculated by multiplying the mean number of cigarette packs smoked daily by the number of years smoking.

2.2.2 Blood sampling

Blood samples were obtained after overnight fasting for a minimum of 12 hours. Plasma glucose levels were determined from NaF-treated plasma using a hexokinase glucose-6 phosphate-dehydrogenase enzymatic assay, hemoglobin A1c (HbA1c) and insulin levels were determined using a latex agglutination immunoassay and an enzyme-linked immunosorbent assay, respectively (Kyowa Medix, Tokyo, Japan). Lipid measurements were standardized according to the protocol of the Centers for Disease Control and Prevention/Cholesterol Reference Method Laboratory Network [15]. Total cholesterol and triglycerides were measured using enzymatic assays, and high-density lipoprotein cholesterol was determined using a direct method. Aminotransferase and gamma-glutamyl transferase were measured using the modified Japan Society of Clinical Chemistry reference method [16].

2.2.3 Assessment of diabetes mellitus

The HbA1c level was converted from the method of the Japan Diabetes Society (JDS) to the method of the National Glycohemoglobin Standardization Program (NGSP) using the following formula: HbA1c (NGSP) (%) = 1.02 * HbA1c (JDS) (%) + 0.25 [17]. In the present study, for both the baseline and follow-up surveys, we diagnosed diabetes as

having an HbA1c (NGSP) level of ≥6.5% and/or a fasting plasma glucose level of ≥7 mmol/L, and/or any diabetes medication use.

2.2.4 Assessment of liver fat accumulation and VAT by non-contrast CT

We used the L/S ratio in non-contrast CT as the index of liver fat accumulation as previously described [18]. The hepatic and splenic Hounsfield unit (HU) attenuation values were measured at the T12–L1 disc space and at three regions of interest (ROI) for both the liver and the spleen. Each ROI was approximately 120 mm^2. The L/S ratio was calculated by taking mean HU measurement of liver ROIs and dividing it by the mean HU measurements of spleen ROIs. The ROI for the liver was placed manually to avoid major vessels. We defined an L/S ratio <1.0 as prevalent fatty liver and an L/S ratio ≥1.0 as non-fatty liver individuals. This definition was applied because an L/S ratio <1.0, which is equivalent to liver attenuation <51 HU was reported as an indicator for any form (mild-moderate-sever) of fatty liver [19-22]. To estimate the VAT and subcutaneous adipose tissue areas, a single CT image of the L4–L5 vertebral space was selected [14, 18]. The inner and outer aspects of the abdominal walls were manually tracked, and the respective areas were calculated using image analysis software (SliceOmatic; Tomovision, Montreal, Canada).

CT imaging was performed as previously described [4, 14]. Two types CT scanner were used during the examination period: a GE-Imatron C150 Electron Beam Tomography system (EBCT; GE Medical Systems, South San Francisco, CA; slice thickness, 6 mm) from May 2006 through to August 2007 and thereafter a 16-row multi detector row CT system (MDCT, Aquilion-16™; Toshiba Medical Systems, Tochigi,

Japan; slice thickness, 7 mm). All CT images were analyzed at Shiga University of Medical Science by trained researchers who were blinded to the participants' characteristics.

2.3 Statistical analysis

Characteristics of the participants are presented as the mean ± standard deviation (SD), median (interquartile range), or percentage. For the differences between the L/S ratio <1.0 and the L/S ratio ≥1.0, a t-test or Man-Whitney test was applied for continuous variables and a χ^2 test was used for categorical variables. To calculate the odds ratio (OR) and 95% confidence interval (CI) for the incidence of diabetes mellitus in 5 years, logistic regression was used. We estimated the OR and 95% CI of the L/S ratio <1.0 compared to the L/S ratio ≥1.0 and those of the per 1 SD lower in the L/S ratio whether the change in continuous variable would also have the effect on the incidence of diabetes mellitus. Adjusting covariates were as follows: Model 1, age (years) and CT type; Model 2, family history of diabetes, smoking (pack-year), ethanol intake (g/day), and exercise (step count/day) in addition to Model 1; Model 3, BMI (kg/m^2) in addition to Model 2; Model 4, waist circumference (cm) in addition to Model 2; Model 5, VAT (cm^2) in addition to Model 2. We investigated whether the impact of fatty liver on the incidence of diabetes mellitus was similar both in the obese and non-obese groups using stratified analysis. We assigned the data according to the obesity indices, whereby non-obese was defined as BMI <25 kg/m^2, waist circumference <85 cm, or VAT <100 cm^2 and obese as BMI ≥25 kg/m^2, waist circumference ≥85 cm, or VAT ≥100 cm^2 [23]. P-values for the interaction between obesity category and L/S ratio were also determined.

Statistical significance was defined as a two-tailed P-value <0.05. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC, USA).

3. Results

Baseline characteristics of the participants are presented in Table 1. The mean age was 63.5 years, BMI 23.4 kg/m^2, and waist circumference 85.1 cm. The mean (SD) value of the L/S ratio among all participants were 1.12 (0.18). The prevalence of an L/S ratio <1.0 was 18.9%. Among the 640 participants, 36 (5.6%) developed diabetes mellitus in 4.93 ± 1.35 years follow-up. The L/S ratio <1.0 group compared with the L/S ratio ≥1.0 group was significantly younger, had significantly higher fasting plasma glucose, HbA1c, triglycerides, liver enzymes, C-reactive protein (CRP), homeostasis model assessment of insulin resistance (HOMA-IR), and homeostasis model assessment of β, and had significantly lower high-density lipoprotein cholesterol.

Table 2 presents the ORs and 95% CIs of diabetes mellitus incidence. An L/S ratio <1.0 was significantly associated with a higher risk for diabetes mellitus incidence (OR [95% CI]: Model 2; 2.78 [1.30–5.95]). This association remained even after taking obesity indices into consideration, although the relationship was slightly attenuated (OR [95% CI]: Model 5; 2.27 [1.00–5.14]). Similarly, per 1 SD (0.18) lower in L/S ratio was significantly associated with a higher risk for diabetes mellitus incidence even when adjusted for obesity parameters (OR [95% CI]: Model 2; 1.67 [1.24–2.25], Model 5; 1.57 [1.14–2.16]).

We performed a stratified analysis according to the obesity indices (Table 3). The relationship between the L/S ratio and incidence of diabetes mellitus was consistent

across the subgroups of both the non-obese and obese groups. Per 1 SD lower in L/S ratio was partly significant with the risk for diabetes mellitus incidence in some stratified analysis, group of waist circumference ≥85 cm (OR [95% CI]: 1.50 [1.04–2.16]) and group of VAT ≥100 cm^2 (OR [95% CI]: 1.57 [1.11–2.21]). Although the ORs were higher in the obese group (BMI ≥25 kg/m^2, waist circumference ≥85 cm, or VAT ≥100 cm^2) compared with non-obese group (BMI <25 kg/m^2, waist circumference <85 cm, or VAT <100cm^2), the ORs in the non-obese group were >1.0 and all the p-values for the interaction were not significant ($p \geq 0.05$) between the obese and non-obese groups.

4. Discussion

The present study indicated that the L/S ratio assessed by CT was associated with a higher risk for diabetes mellitus incidence during the 5 year follow-up. This association was attenuated in the obesity index added models, but a statistically significant association was still observed. Additionally, the association between the L/S ratio and diabetes mellitus were consistent in both the non-obese and obese groups. To the best of our knowledge, our study is the first to demonstrate a long-term association of the L/S ratio with diabetes mellitus.

In the recent NAFLD/Nonalcoholic Steatohepatitis guideline in Europe and Japan, the L/S ratio is an established estimate for the assessment of liver fat accumulation [24-26]. A longitudinal relationship between liver fat accumulation assessed by CT and an increased risk for type 2 diabetes mellitus (T2DM) was reported from the Multi-Ethnic Study of Atherosclerosis, using the other variable from the L/S

ratio [11]. The Framingham Heart Study also reported a longitudinal association between liver fat accumulation and increased risk for T2DM using the CT liver-phantom ratio [12]. From Asia, one cohort study showed liver fat accumulation associated with T2DM incidence in addition to pancreatic fat accumulation [27].

Our data showed that the L/S ratio was continuously associated with the incidence of diabetes mellitus. From our stratified analysis, the ORs among the obese group were slightly higher than those of the non-obese group (Table 3). All p-values for the interaction between each obesity category and the L/S ratio were not statistically significant. Our data suggested that a lower L/S ratio has an impact on the incidence of diabetes in not only the obese but also the non-obese group.

The potential mechanism of fatty liver in T2DM development may originate from expanded and inflamed adipose tissue. Insulin resistance strongly associates with a fatty liver and plays a key role in its development. The accumulation of ectopic fat in the liver impairs the ability of insulin to suppress glucose production and of very low-density lipoproteins, resulting in hyperglycemia and hypertriglyceridemia, respectively. Ectopic fat accumulation increases the generation of reactive oxygen species, fluxes of free fatty acid, inflammatory hepato-cytokines, including CRP, interleukin-6, and tumor necrosis factor-alpha, and coagulation-fibrinolytic factors, which increase both hepatic and systemic insulin resistance [28-30]. In our study, CRP in the L/S ratio <1.0 group was higher than in the L/S ratio ≥1.0 group, suggesting an interaction between ectopic fat accumulation and systemic inflammation at baseline (Table 1). Furthermore, the HOMA-IR in the L/S <1.0 group was also significantly higher than in the L/S ≥1.0 group, suggesting hepatic insulin resistance in the L/S <1.0 group (Table 1).

Hepatokines, including fetuin-A, fibroblast growth factor-21, and retinol binding protein-4, may contribute to insulin resistance, although we did not measure these [31].

Fatty liver occurs not only in the obese, but also frequently in the non-obese population [2, 32, 33]. In Asian countries, 8–25% of all NAFLD patients were reported to be non-obese or lean subjects, with a BMI <25 kg/m² [34]. In an international comparison study, Japanese in Japan were reported to have a higher liver fat content and a significantly lower L/S ratio than non-Hispanic whites in the United States despite a lower mean BMI [18]. One possible explanation for the discrepancy between obese and NAFLD individuals in Asia is a genetic difference. Single nucleotide polymorphism of the patatin-like phospholipase domain-containing protein 3 (PNPLA3), PNPLA3 rs738409, displays a strong association with hepatic steatosis [35-37]. PNPLA3 rs738409 polymorphism is more frequent in Asians than Caucasians or Africans [38, 39]. It was suggested that this polymorphism causes apolipoprotein B deficiency and decreases the liver lipid excretion, leading to the risk for fatty liver disease. In addition, PNPLA3 rs738409 polymorphism was also associated with the incidence of T2DM in Japanese in a genome-wide association study [40]. Therefore, PNPLA3 polymorphism might be one of the key pathophysiologic factors why the Asian population tends to display a greater incidence of both fatty liver and diabetes mellitus with a lower BMI. Assessment of the presence of a fatty liver appears to be important for the prediction of future diabetes mellitus, particularly in the Asian population.

Our data suggested that the L/S ratio assessed by a CT scan may provide more precise information about the potential risk for developing diabetes mellitus. Compared with ultrasonography, a CT scan is able to evaluate the severity of fatty liver quantitatively, however, radiation exposure is detrimental for patients. Therefore, in

clinical settings, for those who display risk factors for fatty liver, ultrasonographic measurement might be important to detect its impact on diabetes mellitus.

Our study has some strengths. The study participants were randomly selected from the general Japanese population, including both non-alcohol drinkers and regular alcohol drinkers, whereas many previous clinical studies focused only on the prognosis of NAFLD [41]. Assessment methods of our population survey were standardized and carefully controlled. The current study also has several limitations. First, this was a prospective cohort study conducted in only Japanese men. Therefore, this study cannot be generalized to the other ethnicities or females. Additionally, we diagnosed the incidence of diabetes mellitus using one-point glucose and HbA1c assessment and self-reported medication use. And the effect of self-selection bias might remain. Finally, we did not consider the medication class effect which relate to insulin secretion or sensitivity although some of the antihypertensive and lipid-lowering agents were reported to be related with the new onset of diabetes [42] or reduce the risk of it [43-45].

In conclusion, the L/S ratio, indicating liver fat accumulation as assessed by CT, was associated with a higher risk for diabetes mellitus during a 5-year follow-up in a Japanese community-based study. Our data implied that the assessment of liver fat accumulation is an important predictor for diabetes mellitus regardless of whether or not a subject is obese. Prevention and early detection of fatty liver might be important to reduce diabetes mellitus incidence in both obese and non-obese populations.

Acknowledgements

We thank the SESSA investigators, staff, and study participants for their commitment and outstanding dedication. A full listing of the SESSA investigators can be found in Supplemental List 1.

Funding

The SESSA was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP13307016, JP17209023, JP21249043, JP23249036, JP25253046, JP15H02528, JP18H04074, JP26293140, JP24790616, JP21790579, JP23590790 and JP23590791 from the Ministry of Education, Culture, Sports, Science and Technology Japan, by grant R01HL068200 from the NIH, and from Glaxo-Smith Kline GB. The present study was initiated and the data analyzed by the authors. The funding sources listed above played no role in the study design nor in the collection, analyses, or interpretation of the results.

Authors' contributions:

KF, AK, KK, HM, K Miura, and HU conceived and designed the study. KF, AK, and KK performed the statistical analysis, and KF, AK, KK, and K Morino drafted the manuscript. All authors critically revised the manuscript for important intellectual content and contributed to and approved the final version.

Conflict of interest:

The authors do not have any conflicts of interest to disclose.

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Table 1 – Baseline characteristics of the participants (n=640; men aged 40–79 years, 2006–2008, SESSA, Japan).

	Total (n=640)	L/S ≥1.0 (n=519)	L/S <1.0 (n=121)	P-value *
Age (year)	63.5±9.9	64.0±9.9	61.4±9.5	0.009
BMI (kg/m²)	23.4±2.8	23.1±2.6	25.0±2.9	<0.001
Waist circumference (cm)	85.1±7.7	84.1±7.4	89.2±7.7	<0.001
Visceral adipose tissue (cm²)	116.0±53.6	107.8±48.9	151.2±58.5	<0.001
Systolic blood pressure (mmHg)	134.5±18.4	134.5±18.6	134.2±17.8	0.877
Diastolic blood pressure (mmHg)	79.5±11.0	79.2±10.8	80.8±11.6	0.151
Fasting plasma glucose (mmol/L)	5.3±0.5	5.3±0.5	5.5±0.6	<0.001
HbA1c (%)	5.7±0.3	5.7±0.3	5.8±0.3	<0.001
HbA1c (mmol/mol)	39±4	39±4	40±4	<0.001
Total cholesterol (mmol/L)	5.4±0.8	5.4±0.8	5.5±0.9	0.065
HDL cholesterol (mmol/L)	1.6±0.5	1.6±0.5	1.4±0.4	<0.001
Triglycerides (mmol/L)	1.1 (0.9-1.6)	1.1 (0.8-1.5)	1.6 (1.1-2.1)	<0.001
GOT (AST) (IU/L)	24.0 (20.0-30.0)	23.0 (20.0-28.0)	29.0 (23.0-39.0)	<0.001

GPT (ALT) (IU/L)	21.0 (16.0-29.0)	20.0 (15.0-25.0)	32.0 (22.0-50.0)	<0.001
γ-GTP (IU/L)	36.0 (24.0-55.0)	32.0 (22.0-51.0)	50.0 (37.0-87.0)	<0.001
High sensitivity CRP (mg/mL)	0.4 (0.2-0.8)	0.37 (0.2-0.8)	0.54 (0.4-0.9)	<0.001
HOMA-IR	0.9 (0.7-1.4)	0.9 (0.6-1.2)	1.5 (1.0-2.0)	<0.001
HOMA-β (%)	47.1 (33.8-64.7)	43.6 (31.9-59.0)	61.3 (44.8-90.0)	<0.001
Step count (/day)	8508±3715	8632±3799	7979±3293	0.081
Pack-year	22.8 (5.0-41.0)	22.0 (4.5-40.1)	26.0 (9.5-44.0)	0.153
Ethanol intake (g/day)	14.0 (1.0-37.0)	14.0 (1.0-37.0)	14.0 (1.4-43.0)	0.806
EBCT, n (%)	462 (72.2)	365 (70.3)	97 (80.2)	0.032

Results are the mean ± standard deviation, n (%) or median (interquartile range).

*: Comparison according to L/S using t test, Mann-Whitney U test, or χ^2 test appropriately.

n, number; BMI, body mass index; HDL, high-density lipoprotein; CRP, C-reactive protein; HOMA-IR, homeostasis model assessment of insulin resistance; SESSA, Shiga Epidemiological Study of Subclinical Atherosclerosis; EBCT, electron beam computed tomography.

Table 2 – Odds ratio for diabetes mellitus incidence according to the L/S ratio <1.0 and the per 1 SD lower in L/S ratio in the SESSA (2006–2008).

	Odds ratio (95% confidence interval)		
	L/S ratio ≥1.0	L/S ratio <1.0	Per 1 SD lower in L/S ratio
N events/total	23/519	13/121	36/640
Model 1	Ref	2.79 (1.34-5.82)	1.67 (1.26-2.21)
Model 2	Ref	2.78 (1.30-5.95)	1.67 (1.24-2.25)
Model 3	Ref	2.08 (0.94-4.63)	1.47 (1.07-2.03)
Model 4	Ref	2.18 (0.99-4.83)	1.53 (1.11-2.11)
Model 5	Ref	2.27 (1.00-5.14)	1.57 (1.14-2.16)

1 SD difference for L/S was 0.18.

N events/total = Incidence diabetes mellitus/Population at risk.

Model 1: age + CT type

Model 2: Model 1 + parent DM history, smoking (pack-year), ethanol intake (g/day), and exercise (steps/day)

Model 3: Model 2 + BMI (kg/m^2)

Model 4: Model 2 + Waist (cm)

Model 5: Model 2 +VAT (cm^2)

SD, standard deviation; SESSA, Shiga Epidemiological Study of Subclinical Atherosclerosis; CT, computed tomography; DM, diabetes mellitus, BMI, body mass index; Waist, waist circumference; VAT, visceral adipose tissue.

Table 3 – Odds ratio for diabetes mellitus incidence according to the L/S ratio <1.0 and the per 1 SD lower in L/S ratio stratified by obesity indices in the SESSA (2006–2008).

	N events/ total	Odds ratio (95% confidence interval)			P for interaction	
		L/S ratio ≥1.0	L/S ratio <1.0	Per 1 SD lower in L/S ratio	* P	† P
BMI <25	15/465	Ref	1.83 (0.45-7.39)	1.40 (0.65-3.01)	0.42	0.49
BMI ≥25	21/175	Ref	2.16 (0.76-6.10)	1.45 (0.99-2.13)		
Waist <85	11/314	Ref	1.54 (0.26-9.08)	1.12 (0.42-3.00)	0.85	0.34
Waist ≥85	25/326	Ref	2.18 (0.84-5.68)	1.50 (1.04-2.16)		
VAT <100	10/261	Ref	5.04 (0.68-37.35)	1.02 (0.30-3.43)	0.65	0.36
VAT ≥100	26/379	Ref	2.01 (0.78-5.17)	1.57 (1.11-2.21)		

1SD difference for L/S was 0.18.

N events/total = Incidence diabetes mellitus/Population at risk.

Adjusted for: age, CT type, parent diabetes mellitus history, smoking (pack-year), ethanol intake (g/day), exercise (steps/day), and obesity indices BMI (kg/m^2), Waist (cm), or VAT (cm^2).

* P for interaction on obesity indices (BMI, waist circumference, VAT) and L/S ratio <1.0.

† P for interaction on obesity indices (BMI, waist circumference, VAT) and per 1 SD lower in L/S ratio.

SD, standard deviation; SESSA, Shiga Epidemiological Study of Subclinical Atherosclerosis; CT, computed tomography; BMI, body mass index; Waist, waist circumference; VAT, visceral adipose tissue.

Supplemental List 1

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