The independent association between salivary alpha-amylase activity and arterial stiffness in Japanese men and women: the Toon Health Study

Tomokazu Tajima, MSc[1], Ai Ikeda, PhD[1,2], Andrew Steptoe, DPhil[3], Kaho Takahashi[1], Koutatsu Maruyama, PhD[4], Kiyohide Tomooka, PhD[1], Isao Saito, MD[5], Takeshi Tanigawa, MD[1]

[1] Department of Public Health, Juntendo University Graduate School of Medicine, Tokyo, Japan

[2] Faculty of International Liberal Arts, Juntendo University, Tokyo, Japan

[3] Department of Epidemiology and Public Health, Faculty of Population Health Sciences, University College London, London, UK

[4] Laboratory of Community Health and Nutrition, Special Course of Food and Health Science, Department of Bioscience, Graduate School of Agriculture, Ehime University, Matsuyama, Japan

[5] Department of Public Health and Epidemiology, Faculty of Medicine, Oita University, Yufu, Japan

Address for correspondence:

Takeshi Tanigawa, MD, PhD

Department of Public Health, Juntendo University Graduate School of Medicine,

2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

Phone: +81 (3) 5802-1048 Fax: +81 (3) 3814-0305 Email: tataniga@juntendo.ac.jp

ABSTRACT

Aims: Psychological stress is considered to be a potential contributor in the development of arterial stiffness. However, an independent association between arterial stiffness and biological markers of stress has not yet been established. We examined the independent association between salivary alpha-amylase (sAA) activity and arterial stiffness, not mediated by cardiometabolic disease associated with arterial stiffness, in a sample of healthy Japanese men and women.

Methods: Participants (839 in total, 237 men and 602 women aged 30–79 years) had neither previous cardiovascular events or stroke, nor coexisting hypertension, diabetes, or dyslipidemia. Arterial stiffness was measured by the cardio-ankle vascular index (CAVI), and increased CAVI was defined as a CAVI value of 9 or higher. A saliva sample was collected in the morning and sAA was measured with a commercial assay kit.

Results: Higher sAA activity was positively associated with greater arterial stiffness particularly among women (β=0.081; 95% CI=0.022–0.140; *p*=0.01), and not across all participants (β=0.050; 95% CI=-0.001–0.101; *p*=0.05) and in men (β =-0.004; 95% CI=-0.108–0.100; *p*=0.94). The association was strongest in the group of women aged 60 years and older (β=0.137; 95% CI=0.023–0.252; *p*=0.02). Although the association between sAA and increased CAVI (CAVI≥9) was not significant in all and sex subgroups, odds ratios (OR) for CAVI≥7 were significantly high in all participants (OR=1.36; 95% CI=1.10-1.69) and women (OR=1.73; 95% CI=1.19-1.96).

Conclusions: Elevation of sAA was associated with an increase in arterial stiffness, particularly for women aged 60 years or older.

Key Words: salivary alpha-amylase activity, arterial stiffness, stress

Running title: Salivary alpha-amylase and arterial stiffness

Introduction

Ischemic heart disease, stroke, and chronic obstructive pulmonary disease were the top three leading causes of death worldwide in 2019 [1]. In Japan, heart disease and cerebrovascular disease are the second and fourth leading causes of death, respectively, following malignant neoplasm in both men and women, particularly those aged 30 years or older [2]. Previous epidemiological studies have confirmed that these life-threating illnesses are associated with arterial stiffness and atherosclerosis [3]. In recent years, the cardio-ankle vascular index (CAVI) was introduced, and this scale has been used to assess arterial stiffness and to diagnose atherosclerosis [4].

Psychological stress is a potential contributor in atherosclerosis development [5-7]. From a pathophysiological perspective, stress is considered to activate autonomic nervous system and neuroendocrine system, resulting in dysfunction of coagulation, metabolic, and inflammation pathways that contribute to atherosclerosis [8]. In a previous small experimental study with 17 young men (mean age=20.1 years), CAVI scores were significantly elevated at 5, 15, and 30 minutes after experiencing acute mental stress [9]. Increased CAVI scores were reported in both healthy people and cardiovascular patients just after the Great East Japan Earthquake in 2011, with emotional stress as the suspected mechanism [10].

Psychological stress leads to an increase in autonomic nervous system activation through combined sympathetic and parasympathetic innervation of the salivary glands, leading to the release of biomarkers including cortisol, alpha-amylase (sAA), and chromogranin A (CgA) [11-12]. Salivary cortisol has been recognized as a reliable biological maker for the hypothalamic-pituitary-adrenal (HPA) axis stress response [13], whereas sAA and CgA have been used as surrogate markers of the sympathetic-adrenal-medullary (SAM) axis [14]. In contrast to Cortisol and CgA, sAA levels often continue to increase even after

prolonged exposure [15], which suggests that sAA could be a useful biomarker for chronic psychological stress.

Our previous study reported an association between sAA and cardiometabolic abnormalities, particularly in women. sAA was associated with systolic and diastolic blood pressure, fasting and 2-hour post load glucose levels, and homeostasis model assessment index for insulin resistance among women [16]. Association between arterial stiffness measured by CAVI and cardiometabolic diseases such as coronary artery disease, cerebral infarction, hypertension, diabetes, and dyslipidemia has been well recognized [17]. Therefore, history or presence of these cardiometabolic diseases are considered to mask impact of psychological stress on arterial stiffness. As far as we know, no study has investigated an independent association between sAA and arterials stiffness, not mediated via these cardiometabolic diseases, to assess independent association between psychological stress response and arterial stiffness. The aim of this study was therefore to examine the association between sAA and arterial stiffness in a large sample of Japanese men and women without a history of cardiovascular diseases, stroke, or coexisting cardiometabolic abnormalities.

Methods

Study population

The Toon Health Study (THS) has been described in detail elsewhere [16, 18]. In brief, the THS is a longitudinal study established from 2009–2012 to investigate new risk factors for diabetes and cardiovascular disease, and includes 2,032 community-dwelling men and women from Toon City, Ehime Prefecture, Japan who were aged between 30 and 79 years old at the time of entry. Toon City is in a rural area located in the southern part of Japan with a population of approximately 22,000. Participants were voluntarily recruited through newspaper advertisements, posters, or invitations. Participants were asked to return for on-site physical examinations and questionnaires every five years. A total of 1,777 individuals (n=1,396 enrolled between 2009 and 2012; n=381 newly enrolled) participated in the five-year follow-up study between 2014–2017; our study population was therefore slightly different from the original THS population. Each participant's medical history (cardiovascular diseases and/or stroke) and coexisting diseases including hypertension, dyslipidemia, and diabetes was obtained by physicians. In the present study, individuals aged 80 years or older were excluded; atherosclerosis is strongly associated with age, and no relationship between atherosclerosis and cardiometabolic markers in older adult populations (≥80 years old) has been reported [19]. Besides the individuals who were 80 years or older (n=79), we also excluded those who had been diagnosed with coronary heart disease or stroke (n=58); those who had hypertension (defined as systolic blood pressure [SBP]≥140 mmHg, diastolic blood pressure [DBP]≥90 mmHg, or if they were taking antihypertensive drugs), diabetes (defined as taking diabetes medication including insulin therapy) or dyslipidemia (defined as taking antilipidemic drugs) under treatment (n=789); those who did not have sAA level or saliva weight measured (n=10); those who did not have CAVI measured (n=1); and those who had an unknown menopausal status (n=1). Ultimately, 839 individuals (237 men

and 602 women) between the ages of 30 and 79 were included in the analysis. The study protocol was approved by the ethics committees of Juntendo University and Institutional Review Board of Ehime University Hospital. Informed consent was obtained from each study participant.

Measurements

Collection of chewing-gum-stimulated saliva and assay for salivary alpha-amylase activity

All participants were required to fast for at least 10 h before the study's health examination. Saliva was collected from each participant in the morning during stimulation by chewing gum. The participants chewed 1 g of bland and flavorless Salivar Gum (Tokyo Shizaisha, Tokyo, Japan) for 5 min. While they were chewing, their saliva was collected in plastic tubes. Collected saliva was weighed, and the salivary flow rate was calculated in g/min. The saliva collecting tubes were centrifuged at 1,960 × g for 15 min at 4 °C, and saliva samples were then stored at −80 °C until they were assayed. All samples were tested in the same series to avoid any variations between tests. A kinetic reaction assay kit (Salimetrics LLC, State College, PA, USA) was used for sAA activity measurements (unit: U/mL). A plate reader (Vmax PowerWave XS, Bio-Tech Instruments, Tokyo, Japan) was used for salivary determination with 405 nm filters for sAA. The intra-assay coefficient of variation (CV) and inter-assay reproducibility for sAA activity were 5.47% ± 1.49% and 4.7% ± 0.15%, respectively.

Measurement of CAVI

The CAVI reflects the degree of arterial stiffness from the heart to the ankles. As atherosclerosis progresses, the CAVI increases. The CAVI was measured according to a

standardized method using a Vasera VS-100 (Fukuda Denshi Co., Ltd., Tokyo, Japan). All CAVI measurements were obtained during morning hours using cuffs applied to the bilateral upper arms and ankles, with the participant lying supine and their head held in the midline position. The examinations were performed after the participant had rested for 5 min. The CAVI was calculated based on the stiffness parameter β, which represents the natural vascular stiffness independent of blood pressure, as measured using carotid echography.

The PWV between the heart and ankle was obtained according to the L/T ratio, where L is the distance from the aortic valve to the ankle and T is the time during which the PWV propagates from the aortic valve to the ankle. Scale conversion from PWV to CAVI was performed using the following formula:

$$CAVI = a\ \{(2\rho/DP)\ \times\ \ln (Ps/Pd)\ PWV^2\} +\ b$$

where Ps and Pd are the SBP and DBP values, respectively, PWV is the pulse wave velocity between the heart and ankle, a and b are constants, DP is Ps minus Pd and ρ is blood density. This equation was derived from Bramwell-Hill's equation [20] and the stiffness parameter β [21]. The scale conversion constants were determined to match the CAVI with the PWV according to Hasegawa's method [22]. After automatically obtaining the measurements, the right and left CAVI values were calculated and analyzed using the VSS-10 software program (Fukuda Denshi). The higher value, from either the right or left CAVI measurement, was used for the subsequent analysis.

The reproducibility of the CAVI measurements was demonstrated by a 3.8% CV; this value is within a satisfactory range, as a CV of 5% is generally accepted as within the limit for clinical laboratory testing [23]. In line with the manufacturer's recommendation, a CAVI less than 8 was considered to be normal, a CAVI between 8 and 9 was considered borderline, and a CAVI of 9 or above was considered abnormal [24]. Further theoretical details of the CAVI method are described elsewhere [23].

Blood measures

Overnight fasting blood samples were drawn from the antecubital vein into vacuum tubes containing a serum separator gel. The serum tube was centrifuged immediately at 3,000 × g for 15 min and the separated serum was sent to the laboratory for analysis. Blood pressure was measured twice in the sitting position after a rest of at least 5 min using an automatic sphygmomanometer (BP-103iII; OMRON Colin Co., Tokyo, Japan). The mean of the two measurements was used for analysis.

Covariates

A self-administrated questionnaire was used to assess smoking habits, alcohol consumption, sleep hours, menopausal status, educational attainment level, marital status, and employment status. Physical activity levels were assessed using a validated questionnaire consisting of 14 questions on occupation, locomotion, housework, sleep time, and leisure time physical activities [25]. Responses for each physical activity category were converted to metabolic equivalents (METs), according to the Compendium developed by Ainsworth *et al.* (2000) and expressed as METs·h/day. Body mass index (BMI) was calculated as weight divided by height squared. Overweight status was defined as having a BMI greater than or equal to 25 kg/m^2 [26].

Statistical analysis

We analyzed the activity of sAA divided by the volume of saliva collected during the timed sampling period. For the entire study group including both sexes and the sex-specific subgroups, linear regression or logistic regression models were used to estimate associations between sAA activity and each demographic, behavioral, or biomedical factor including age,

BMI, current smoking status, current alcohol intake, physical activity, sleep duration, LDL cholesterol, HDL cholesterol, triglyceride, blood glucose, and menopause status (for women). To understand the distribution and correlation coefficients between sAA (not log-transformed) and CAVI, as well as log-transformed sAA and CAVI, we drew scatterplots for all participants, separated by sex and age (<60 vs. ≥60 years old). For the entire study group, including both sexes and the sex-specific subgroups, a multivariate linear regression model was used to estimate the association between sAA activity (not log-transformed) and CAVI, as well as between log-transformed sAA and CAVI. For the entire study group, including both sexes and the sex-specific subgroups, a multivariate logistic regression model was used to estimate the association between sAA activity and increased CAVI (CAVI≥9). To seek other suitable CAVI cut-off values, we drew the receiver operating characteristics (ROC) curves for CAVI cut-off of 6, 7, 8, 9,10 and 11, and the same logistic regression analysis was conducted for these other CAVI cut-off values. In these multivariate regression and logistic models, age (years), education attainment level (pre-college education or college-level or higher education), marital status (married or non-married), employment status (unemployed, full-time, part-time, or self-employed), BMI (kg/m^2), smoking status (non-smoker or current smoker), alcohol consumption status (non-drinker or current drinker), physical activity (METs), sleep duration (<7 hours, or ≥7 hours), LDL cholesterol (mg/dL), HDL cholesterol (mg/dL), triglyceride (mg/dL), blood glucose (mg/dL), and menopause (yes or no) were used for adjustments. Missing data were addressed with specific dummy variables in the multivariate analyses. Further stratification by age group (< 60 years old or ≥ 60 years old) was done in order to assess the effect modification on the association between sAA and arterial stiffness [8]. Statistical significance was assumed at $p < 0.05$. All statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

The characteristics of the study sample and the sex-specific mean differences in sAA activity in relation to descriptive characteristics are shown in Table 1. When assessed across all participants, mean log-transformed sAA was higher in older individuals (3.30±1.00 for 70-79 y.o, 3.12±0.85 for 60-69 y.o, 2.92±0.86 for 50-59 y.o, 2.75±0.88 for 40-49 y.o, and 2.72±0.98 for 30-39 y.o, $p < 0.001$), those with a lower education level (3.06±0.90 vs. 2.72 ±0.92, $p < 0.001$), in non-smokers (2.99±0.91 vs. 2.65±0.97, $p = 0.01$), in non-drinkers (3.09±0.91 vs. 2.86±0.91, $p < 0.01$), people with higher total cholesterol (2.97±0.87 for >220, 2.90±0.95 for 200-219, 2.84±0.93 for 150-199, and 2.80±0.77 for <150, $p < 0.01$), with higher LDL cholesterol (3.02±0.84 vs. 2.95±0.94, $p = 0.01$), and those who were unemployed or part-time workers (3.14±0.91 for unemployed, 3.04±0.90 for part-time, 2.86±0.84 for self-employed, and 2.50±0.87, $p = 0.03$). sAA was higher in non-married men (3.01±0.90 vs. 2.59±0.90, $p = 0.02$), in men with longer sleep (2.74±0.90 vs. 2.49± 0.90, $p = 0.04$). It was also higher in women with lower blood glucose (3.10±0.89 vs. 2.84± 1.10, $p < 0.01$) and in menopausal women (3.21±0.86 vs. 2.92±0.90, $p < 0.01$). Median sAA without log transformation was high in women than in men (22.0 vs. 12.8 U/mL, not shown in Table).

The scatterplots of all participants, separated by sex and age (< 60 vs. ≥ 60 years old), shown in Appendix 2. For all participants, the CAVI correlation coefficient (r) was higher for log-transformed sAA than for sAA without log-transformation ($r = 0.159$ vs. $r = 0.142$). Log-transformed sAA showed a relatively strong correlation with CAVI compared with sAA value without log tranformation in all age and sex groups except for young women (young men: $r = 0.090$ vs. $r = 0.042$; old men: $r = -0.028$ vs. -0.015; young women: $r = 0.081$ vs. $r = 0.123$; old women: $r = 0.200$ vs. $r = 0.150$).

The multivariate adjusted linear associations between log-transformed sAA per standard deviation (1-SD) and arterial stiffness as CAVI values are shown in Table 2. There were significant positive associations between sAA and arterial stiffness in all participants (β=0.155; 95% confidence interval [CI]=0.0089–0.220) as well as male and female subgroups (men: β=0.148, 95% CI=0.015–0.280; women: β=0.233; 95% CI=0.157–0.308). These associations remained significant after adjusting for demographic, behavioral, and biomedical factors in the female subgroup (β=0.081; 95% CI=0.022–0.140), but not in the all participants (β=0.050; 95% CI=−0.001–0.101) and in the male subgroup (β=−0.004; 95% CI=−0.108–0.100). The multivariate adjusted linear associations between sAA (not log-transformed value) and CAVI values showed the similar results, but association was relatively weak (Appendix 1). The analysis stratified by age group (<60 vs. ≥60 years old) is shown in Table 3. The association between sAA and arterial stiffness was more evident in older (≥60 years old) compared with younger (<60 years old) female participants although the interaction by age group was not statistically significant (older group: β=0.137; 95% CI=0.023–0.252; younger group: β=0.031; 95% CI=−0.033–0.095, *p* for interaction=0.18).

ROC curves with AUC were drawn for CAVI cut-off values ranging from 6 to 11 for each sex and age group (Appendix 3). A CAVI score of 7 or 9 were the two best cut-off values with respect to predicting high CAVI and log-transformed sAA. The associations between log-transformed sAA per SD and CAVI ≥7 or ≥ 9 are shown in Table 4. There were significant positive associations between sAA and CAVI ≥7 in all participants (Odds ratio [OR]=1.42; 95% CI=1.21–1.67) as well as the female subgroup (OR = 1.69; 95% CI = 1.38–2.06), but not for the male subgroup. These associations remained significant in all participants (OR = 1.36; 95% CI = 1.10–1.69) and in the female subgroup (OR = 1.53; 95% CI = 1.19–1.96) after adjusting for demographic, behavioral, and biomedical factors. There were also significant positive associations between sAA and CAVI≥9 in all participants (OR

= 1.47; 95% CI = 1.20–1.80) as well as both male and female subgroups (men: OR = 1.50; 95% CI = 1.09–2.08; women: OR = 1.90; 95% CI = 1.42–2.53). However, these associations became attenuated after multivariate adjustment.

Discussion

In this cross-sectional analysis of a healthy Japanese population sample, we found a independent association between sAA activity and arterial stiffness, which was not mediated via cardiometabolic-related diseases (e.g., coronary heart disease, stroke, hypertension, diabetes, and dyslipidemia). This relationship was more evident in women than men, particularly for older participants. Although we found an association in men, the association was attenuated and no longer significant after adjusting for age, which indicates that the relationship is strongly influenced by age, particularly among men. The association between sAA activity and increased CAVI (CAVI≥9) was not significant, but the association between sAA and CAVI≥7 was significant overall and particularly in women.

Our study suggests that higher sAA levels are independently associated with increasing arterial stiffness. The statistically significant coefficient values between log-transformed sAA and CAVI were around 0.052–0.233, which accounts for the ΔCAVI of 1.05–1.26 associated with one-unit sAA (U/mL) changes. Considering that patients with cardiovascular disease occurrence within a 2.9±1.0 years follow-up period were reported to have higher CAVI scores than disease-free patients (10.55±0.83 vs. 9.89±0.87, $p < 0.001$) at 6th month in the study [27], a ΔCAVI 1.05–1.26 has clinical significance. Common CAVI cut-off scores are 8.0 and 9.0 (< 8 for normal, ≥ 8 and < 9 for borderline, ≥ 9 for abnormal) [24]. Patients with cardiovascular diseases and other risk factor diseases were excluded from this study, therefore, many with ≥ 9 CAVI scores were excluded, which may explain why we could not see a statistically significant association between CAVI ≥ 9 scores and sAA. Even below the cut-off values, carotid atherosclerosis can develop as CAVI increases above 7.41, and a CAVI ≥ 7 is associated with conventional risk factors for cardiovascular diseases [24]. CAVI values between 7 and 8 are associated with very early stage cardiovascular disease development. Therefore, in our study a CAVI ≥ 7 is significantly associated with sAA, and

can be considered a risk factor for potential patients.sAA is secreted in salivary glands upon β-adrenergic receptor activation by norepinephrine (NE) as a result of sympathetic-adrenal-medullary (SAM) axis activation by psychological stress [11, 13, 28]. sAA can therefore be considered a surrogate marker for SAM function in response to stress [29]. Given that sAA secretion induced by a given stressor was suppressed with β-adrenergic receptor blocker (BB) intake in healthy volunteers [28], β-adrenergic activation in the salivary gland is assumed to play major role in sAA secretion. Higher arterial stiffness indexes have been reported in cohort studies examining experimentally induced stress or heightened psychological stress [9, 30-31]. Blood pressure increases as psychological stress affects the sympathetic nervous system, which affects CAVI score and other arterial stiffness indexes due to vessels damages from physical or chemical stress, such as from reactive oxygen species [32]. An animal model study has reported that free radical production was inhibited by the antioxidative properties of BB agents [33]. Although the direct biological mechanism between psychological stress and arterial stiffness is not fully understood, one possible mechanism for the association between high sAA levels and arterial stiffness may be an increase in β-adrenergic activation in the sympathetic nervous system under psychological stress.

In this study, median sAA level was higher in women than in men (22.0 vs. 12.8 U/mL), and this trend was consistent across all age groups, particularly in their 60's and 70's. Considering that other reports showed no significant difference in sAA level between healthy men and women [34-35], we have interpreted the sex difference observed in this study as a difference in perceived psychological stress. Higher stress in middle-aged and older women could be explained by a high sensitivity to chronic stress, which is associated with traditional gender roles [36]. Women also experience greater subjective sadness and fear than men [37]. Our study has found that being an woman, particularly one aged 60 years or older, has a significant effect on sAA level and CAVI score, even after adjusting for various

demographic, behavioral, and biomedical factors. However, a similar association was not found in men. One possible explanation for the age-dependent sex differences may be due to endogenous sex steroids, which can be resist arterial stiffness [38]. Testosterone in men gradually decreases between ages 50 and 85 , and estradiol in women rapidly decreases between ages 50 and 60, with further gradual decrease after age 60 [39] Among cardiovascular disease-free participants, men and women showed an increase in CAVI score with age after 20 years of age, and there was no significant sex difference between men and women in the 60's (men: 8.0±0.9 vs. women: 7.8±0.7, $p = 0.09$) and 70's (men: 8.8±0.8 vs. women: 8.8±1.8, $p = 0.97$). However, men showed significantly higher CAVI scores between ages 30 and 50 compared to women [40], which suggests the woman over 60 years old are at significant sex-specific risk of arterial stiffness. Investigating these mechanisms behind the higher perceived psychological stress and significantly lower level of anti-arterial stiffness sex hormones in women aged 60 years and older may help explain why arterial stiffness in response to higher sAA levels was more evident among older women in the present study.

This study used a large and well-characterized sample population with data for sAA activity as well as a large number of lifestyle variables and cardiometabolic biomarkers. However, a major limitation of this study is its cross-sectional design, which limits our ability to draw causal inferences. Second, smoking is a well-known risk factor for arterial stiffness, and this study had fewer individuals who were smokers compared to the general population (12.8% of men and 2.8% of women in our study vs. 27.1% of men and 7.6% of women in Japan in 2019) [41], which suggest that this study population may be heathier and have less risk for arterial stiffness. Moreover, although we adjusted for various possible confounding factors, there is a possibility for residual confounding by unmeasured variables such as genetic factors [42-43], which may influence arterial stiffness. The difference between men

and women might be a question of power as the number of men was relatively small compared to women in the present study. This may be one of the reasons why we could not detect the significant association between sAA and arterial stiffness in men. Lastly, sAA's weak association with arterial stiffness and increased CAVI in all population may be due to the limited sample size. Further longitudinal studies with larger sample sizes are needed to understand the causal association between sAA and increased CAVI (CAVI≥9) in men.

Nonetheless, sAA is a well-validated and reliable stress maker [12] compared to other stress markers in saliva, such as cortisol and CgA, because sAA can reflect not only exposure to acute but also chronic psychological stress and is stable in collected saliva samples [15]. It has been studied in various populations, including healthy populations [44-45], occupational field populations [46-47], patients [15, 48-49] and students [50]. Therefore, sAA can serve as a versatile biomarker for stress in human studies and has the independent association with arterial stiffness particularly older women, as suggested in this study.

Conclusion

We found a significant independent association between sAA activity and arterial stiffness, not mediated via cardiometabolic diseases associated with arterial stiffness, particularly in the subgroup of older women. We also found a significant association between sAA activity and CAVI ≥ 7 in all participants and women, not with increased CAVI defined CAVI ≥ 9.

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Conflict of Interests

All authors report no conflict of interest.

Ethical Approval

This study was approved by the ethics committees of Juntendo University (Reference number: 2014003) and Ehime University (Reference number: 20-2).

Contributorship

T. Tajima, KT, AI and T. Tanigawa had the original idea and developed the study design. T. Tanigawa, IS, KM, TK, and KT recruited study participants and collected data. T. Tajima performed the statistical analyses. T. Tajima wrote the first draft of the manuscript and all authors contributed to the critical revision of the manuscript. All authors read and approved the final manuscript.

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Table 1. Salivary α-amylase activity[a] and demographic, behavioral, and biomedical factors separated by sex

	Total (n = 839)				Men (n = 237)				Women (n = 602)			
	n	Mean	SD	*p* for difference	n	Mean	SD	*p* for difference	n	Mean	SD	*p* for difference
Age, y												
30–39	84	2.72	0.98	<0.001	20	2.43	1.15	<0.01	64	2.81	0.92	<0.001
40–49	206	2.75	0.88		56	2.42	0.84		150	2.88	0.86	
50–59	202	2.92	0.86		45	2.53	0.85		157	3.04	0.83	
60–69	226	3.12	0.85		67	2.70	0.82		159	3.30	0.80	
70–79	121	3.30	1.00		49	3.00	0.94		72	3.50	1.00	
Education attainment level												
<College education	616	3.06	0.90	<0.001	130	2.77	0.94	0.01	486	3.13	0.87	0.03
≥College education	223	2.72	0.92		107	2.48	0.84		116	2.94	0.93	
Marital status												
Married	698	2.95	0.89	0.14	209	2.59	0.90	0.02	489	3.10	0.85	0.89
Non-married	139	3.07	1.02		28	3.01	0.90		111	3.08	1.05	
Employment Status												
Unemployed	321	3.14	0.91	<0.001	64	2.84	0.92	0.05	257	3.21	0.89	<0.001
Full-time	139	2.50	0.87		82	2.43	0.93		57	2.60	0.78	
Part-time	258	3.04	0.90		25	2.71	0.85		233	3.08	0.90	

Self-employed			113	2.86	0.84		66	2.68	0.84		47	3.11	0.78	
BMI														
<25			694	2.97	0.93	0.26	179	2.67	0.94	0.23	515	3.07	0.90	0.25
≥25			145	2.97	0.87		58	2.56	0.80		87	3.24	0.81	
Smoking status														
Non-smoker			787	2.99	0.91	0.01	202	2.65	0.88	0.74	585	3.11	0.89	0.11
Current smoker			52	2.65	0.97		35	2.60	1.06		17	2.76	0.76	
Alcohol intake														
Non-drinker			386	3.09	0.91	<0.01	62	2.70	0.97	0.58	324	3.17	0.88	0.04
Current drinker			453	2.86	0.91		175	2.62	0.88		278	3.01	0.89	
Physical activity METs, quartiles; totals (in men), [in women]														
<32.8	(<31.8)	[<33.1]	209	2.91	0.96	0.78	59	2.86	0.81	0.25	150	3.02	1.02	0.25
32.8–35.2	(31.8–34.4)	[33.1–35.3]	210	2.97	0.91		59	2.60	1.05		151	3.05	0.82	
35.2–37.9	(34.4–38.1)	[35.3–37.8]	211	3.02	0.92		59	2.54	0.87		150	3.18	0.88	
>37.9	(>38.1)	[>37.8]	209	2.97	0.87		60	2.57	0.86		151	3.14	0.82	
Sleep hours														
<7			384	2.94	0.93	0.38	95	2.49	0.90	0.04	289	3.08	0.90	0.71
≥7			455	3.00	0.90		142	2.74	0.90		313	3.11	0.88	
Total cholesterol, mg/dL														
<150			20	2.80	0.77	<0.01	11	2.78	0.81	0.67	9	2.82	0.76	<0.01
150–199			298	2.84	0.93		102	2.68	0.91		196	2.92	0.92	

200–219	206	2.90	0.95		65	2.42	0.95		141	3.11	0.87	
>220	310	3.16	0.87		57	2.81	0.82		253	3.24	0.86	
LDL, mg/dL												
<140	613	2.95	0.94	0.01	178	2.64	0.94	0.77	435	3.07	0.91	<0.01
≥140	226	3.02	0.84		59	2.63	0.78		167	3.15	0.82	
HDL, mg/dL												
<40	17	2.87	1.18	0.30	12	2.62	1.26	0.17	5	3.49	0.75	0.38
≥40	822	2.97	0.91		225	2.64	0.89		597	3.09	0.89	
TG, mg/dL												
<150	728	2.99	0.89	0.98	180	2.65	0.85	0.25	548	3.09	0.88	0.21
≥150	111	2.86	1.05		57	2.60	1.06		54	3.12	0.97	
Blood glucose, mg/dL												
<110	822	2.97	0.91	0.56	226	2.64	0.90	0.38	596	3.10	0.89	<0.01
≥110	17	2.76	0.98		11	2.71	0.97		6	2.84	1.10	
Menopause												
No									230	2.92	0.90	<0.01
Yes									372	3.21	0.86	

a. log-transformed and divided by total saliva volume

Table 2. Association of salivary α-amylase[a] with CAVI (continuous value)

	Total (n = 839)				Male (n = 237)				Female (n = 602)			
	Coefficient	95% CI	Standardized coefficient	*p* value	Coefficient	95% CI	Standardized coefficient	*p* value	Coefficient	95% CI	Standardized coefficient	*p* value
Crude	0.155	(0.089 – 0.220)	0.159	<0.001	0.148	(0.015 - 0.280)	0.142	0.03	0.233	(0.157 - 0.308)	0.240	<0.001
Multivariate model 1[b]	0.052	(0.001 – 0.103)	0.053	0.05	-0.002	(-0.102 - 0.098)	-0.002	0.97	0.074	(0.014 - 0.134)	0.076	0.02
Multivariate model 2[c]	0.050	(-0.001 – 0.101)	0.051	0.05	-0.004	(-0.108 - 0.100)	-0.004	0.94	0.081	(0.022 - 0.140)	0.084	0.01

a. log-transformed and divided by total saliva volume

b. adjusted by age and sex

c. adjusted by age, sex, education attainment level, marital status, employment status, BMI, smoking status, alcohol intake, physical activity, sleep, LDL, HDL, TG, blood glucose, and menopause status (female only). Missing data were addressed with specific dummy variables in the multivariate analyses.

Table 3. Association of salivary α-amylase[a] with CAVI (continuous value) by age and sex groups

Age group	n	Coefficient	95% CI			Standardized coefficient	*p* for difference	*p* for interaction
			Total (n=839)					
<60 years[b]	492	0.033	(-0.025	-	0.090)	0.046	0.26	0.50
≥60 years[b]	347	0.051	(-0.043	-	0.144)	0.046	0.29	
			Male (n=237)					
	n	Coefficient	95% CI			Standardized coefficient	*p* for difference	*p* for interaction
<60 years[b]	121	0.095	(-0.036	-	0.226)	0.125	0.15	0.29
≥60 years[b]	116	-0.114	(-0.287	-	0.060)	-0.121	0.20	
			Female (n=602)					
	n	Coefficient	95% CI			Standardized coefficient	*p* for difference	*p* for interaction
<60 years[b]	371	0.031	(-0.033	-	0.095)	0.044	0.34	0.18
≥60 years[b]	231	0.137	(0.023	-	0.252)	0.140	0.02	

a. log-transformed and divided by total saliva volume

b. adjusted by age, sex, education attainment level, marital status, employment status, BMI, smoking status, alcohol intake, physical activity, sleep, LDL, HDL, TG, blood glucose, and menopause status (female only). Missing data were addressed with specific dummy variables in the multivariate analyses.

Table 4. Odds ratios (95% CI) of CAVI≥7 and ≥9 associated with 1-SD increment of natural log-transformed sAA

	Total (n=839)	Male (n=237)	Female (n=602)
CAVI≥7, n (%)	647 (77.1%)	199 (84.0%)	448 (69.2%)
Crude	1.42 (1.21 – 1.67)	1.23 (0.86 – 1.75)	1.69 (1.38 – 2.06)
Multivariate model 1_a	1.30 (1.07 – 1.58)	1.00 (0.67 – 1.48)	1.42 (1.13 – 1.78)
Multivariate model 2_b	1.36 (1.10 – 1.69)	1.04 (0.66 – 1.66)	1.53 (1.19 – 1.96)
CAVI≥9, n (%)	101 (12.0%)	47 (19.8%)	54 (9.0%)
Crude	1.47 (1.20 – 1.80)	1.50 (1.09 – 2.08)	1.90 (1.42 – 2.53)
Multivariate model 1_a	1.30 (1.02 – 1.65)	1.22 (0.84 – 1.76)	1.35 (0.98 – 1.87)
Multivariate model 2_b	1.26 (0.98 – 1.63)	1.32 (0.88 – 1.97)	1.32 (0.92 – 1.89)

a. adjusted by age and sex

b. adjusted by age, sex, education attainment level, marital status, employment status, BMI, smoking status, alcohol intake, physical activity, sleep, LDL, HDL, TG, blood glucose, and menopause status (female only). Missing data were addressed with specific dummy variables in the multivariate analyses.

Appendix 1. Association of salivary α-amylase (not log-transformed value) with CAVI (continuous values)

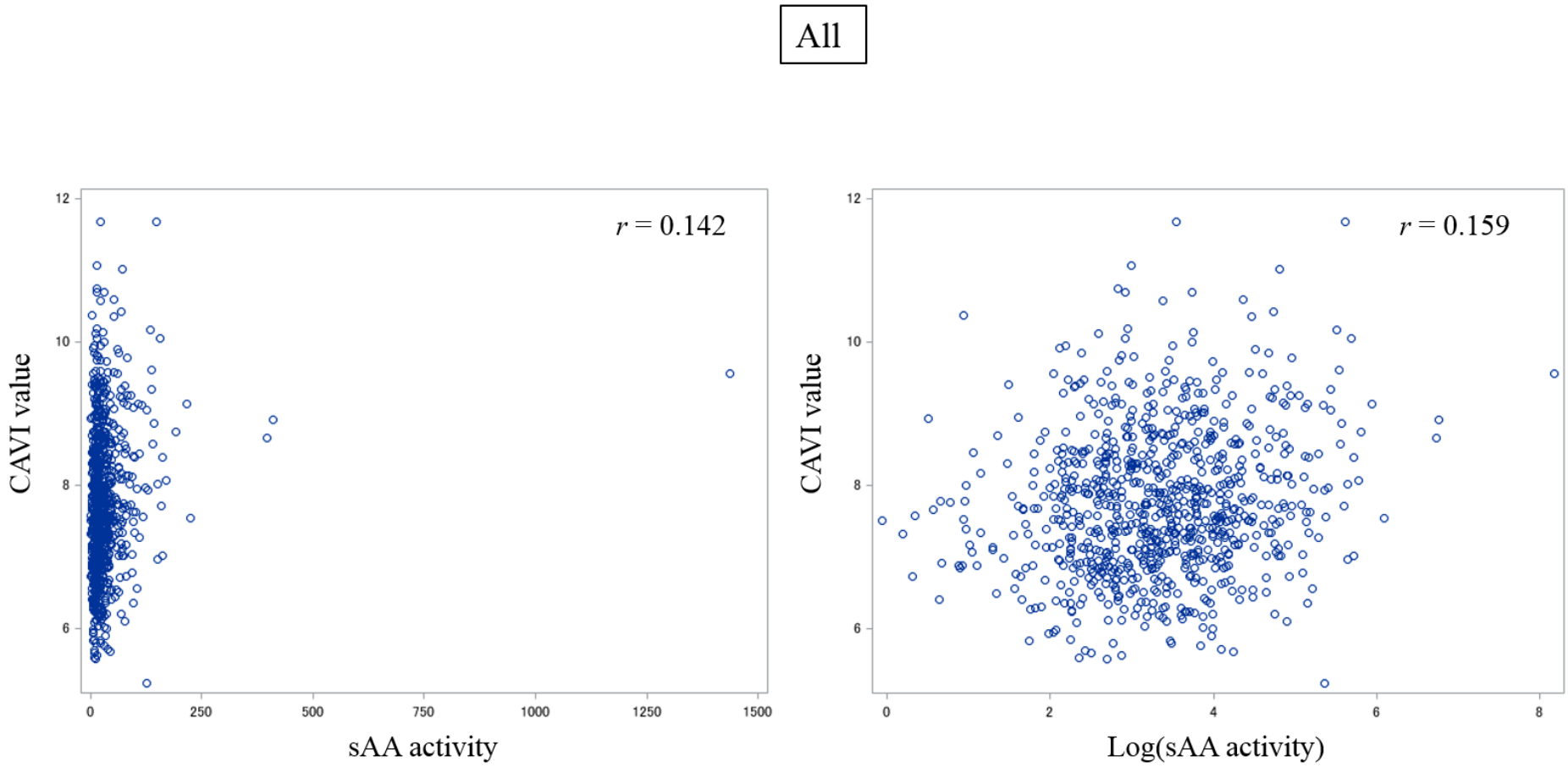
	Total (n = 839)				Male (n = 237)				Female (n = 602)			
	Coefficient	95% CI	Standardized coefficient	*p* value	Coefficient	95% CI	Standardized coefficient	*p* value	Coefficient	95% CI	Standardized coefficient	*p* value
Crude	0.002	(0.001 - 0.004)	0.143	<0.001	0.003	(-0.001 – 0.008)	0.093	0.16	0.003	(0.002 – 0.004)	0.186	<0.001
Multivariate model 1[a]	0.001	(0.000 - 0.002)	0.059	0.02	0.000	(-0.004 – 0.003)	-0.005	0.92	0.001	(0.000 – 0.002)	0.076	0.01
Multivariate model 2[b]	0.001	(0.000 - 0.002)	0.048	0.06	0.000	(-0.004 – 0.003)	-0.011	0.82	0.001	(0.000 – 0.002)	0.060	0.05

a. adjusted by age and sex

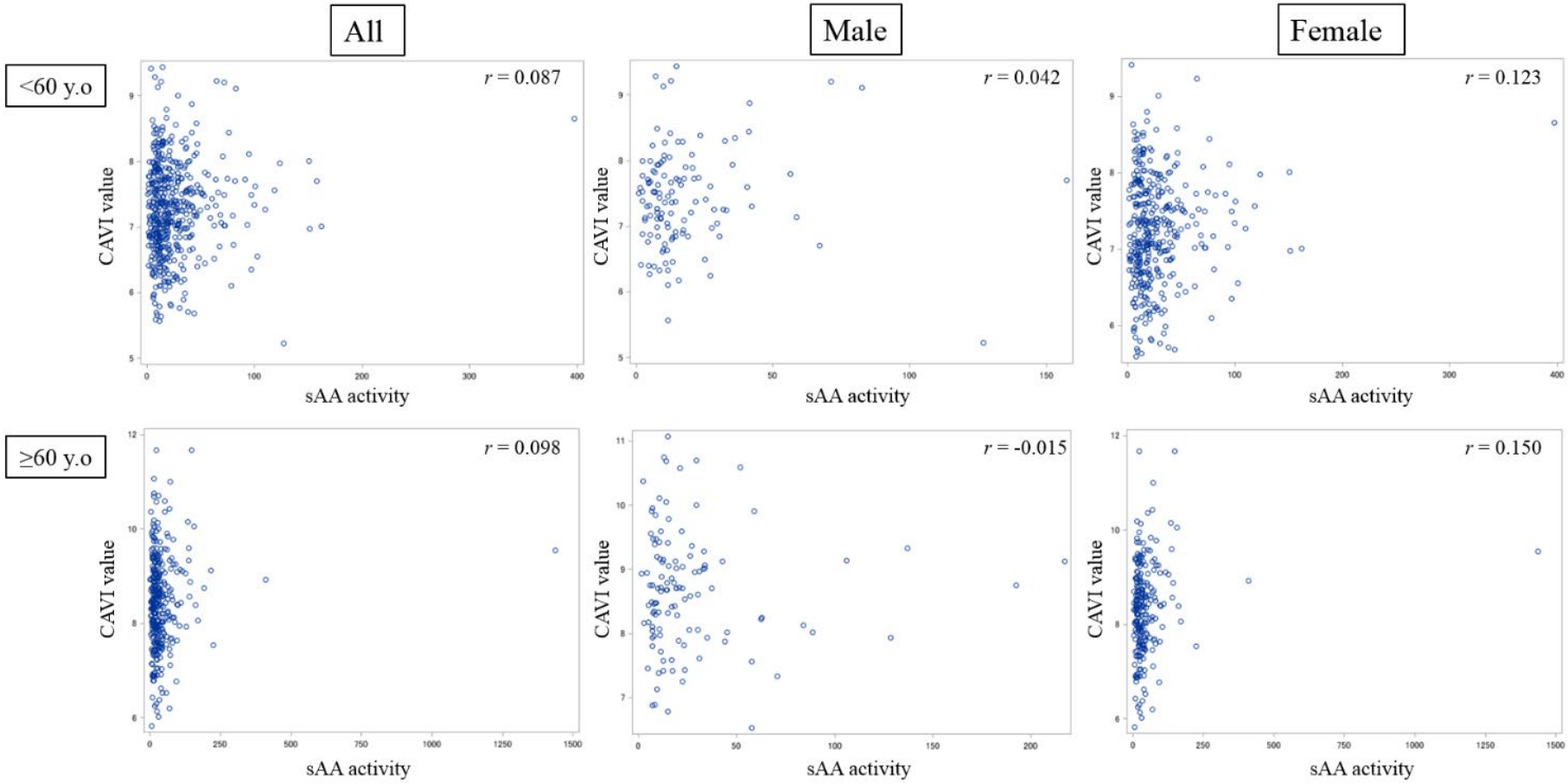
b. adjusted by age, sex, education attainment level, marital status, employment status, BMI, smoking status, alcohol intake, physical activity, sleep, LDL, HDL, TG, blood glucose, and menopause status (female only). Missing data were addressed with specific dummy variables in the multivariate analyses.

Appendix 2. Scatter plot of sAA activity and CAVI value for sex and age subgroups

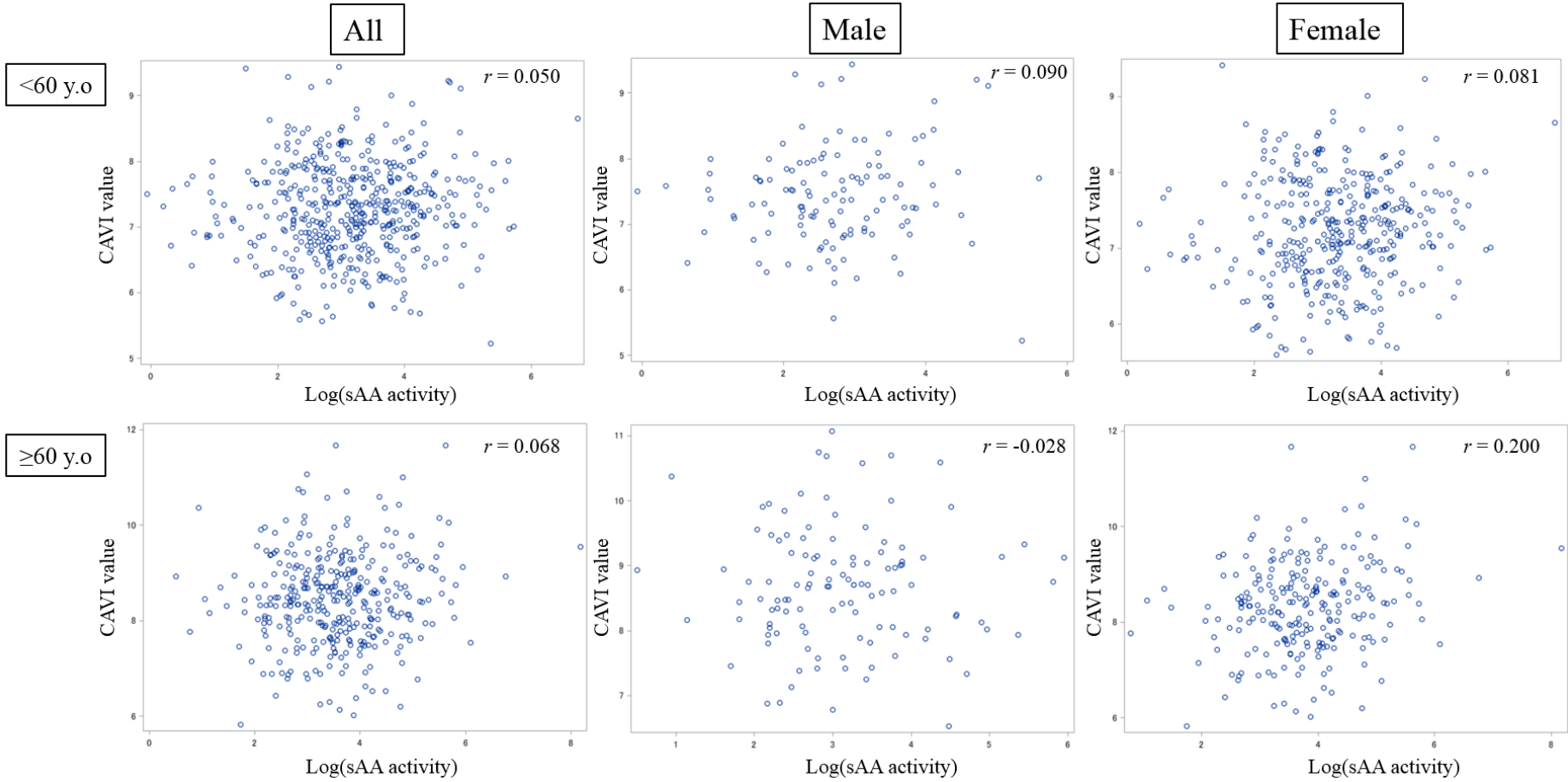
(A) Comparison between sAA (not log-transformed value) and natural log transformed sAA for all participants



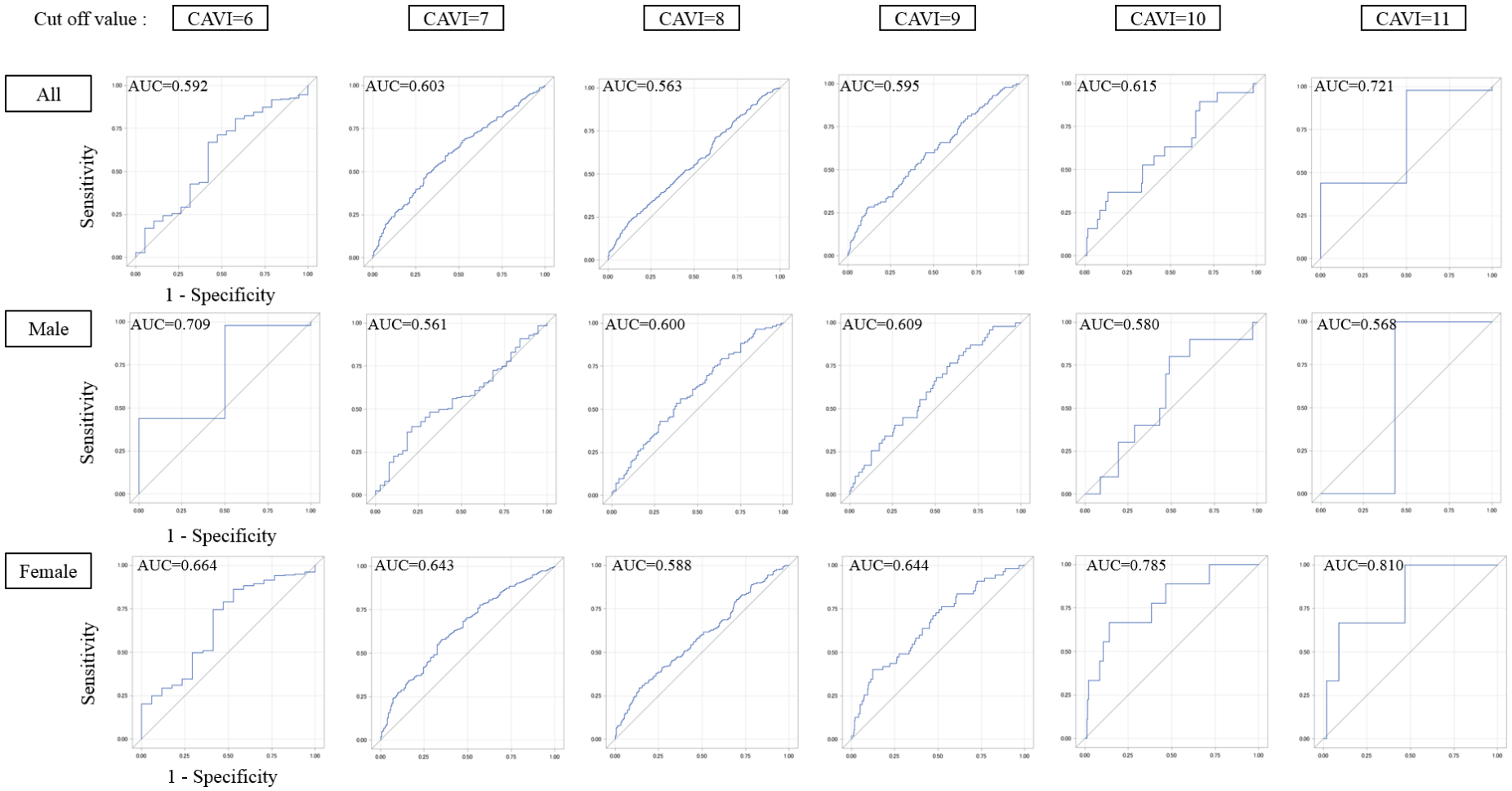
(B) Sex and age subgroup using sAA activity (not log-transformed value)



(C) Sex and age subgroup using natural log-transformed sAA activity



Appendix 3. ROC analysis on various CAVI cut-off values



Appendix 4. Odds ratios (95% CI) of various CAVI cut-off values associated with 1-SD increment of natural log-transformed sAA

	Total (n=839)			Male (n=237)			Female (n=602)		
	Case, n	Case, %	Odds ratio (95% CI)	Case, n	Case, %	Odds ratio (95% CI)	Case, n	Case, %	Odds ratio (95% CI)
CAVI ≥ 6	818	97.5	1.31 (0.77 - 2.24)	235	99.2	-	583	96.8	1.79 (0.98 – 3.30)
CAVI ≥ 7	646	77.0	1.36 (1.10 - 1.69)	199	84.0	1.04 (0.66 – 1.66)	447	74.3	1.53 (1.19 – 1.96)
CAVI ≥ 8	310	36.9	1.09 (0.90 - 1.33)	112	47.3	1.16 (0.78 1.74)	198	32.9	1.13 (0.89 – 1.43)
CAVI ≥ 9	101	12.0	1.26 (0.98 - 1.63)	47	19.8	1.32 (0.88 1.97)	54	9.0	1.32 (0.92 – 1.89)
CAVI ≥ 10	18	2.1	1.24 (0.73 - 2.11)	10	4.2	0.91 (0.36 2.26)	8	1.3	1.46 (0.59 – 3.60)
CAVI ≥ 11	4	0.5	2.50 (0.40 - 15.51)	1	0.4	-	3	0.5	-

adjusted by age, sex, education attainment level, marital status, employment status, BMI, smoking status, alcohol intake, physical activity, sleep, LDL, HDL, TG, blood glucose, and menopause status (female only). Missing data were addressed with specific dummy variables in the multivariate analyses.