Cut-off value of serum homocysteine in relation to increase of coronary artery calcification

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ABSTRACT
A recent study reported that coronary artery calcification (CAC) and serum homocysteine were well associated; however, no report is available for the cut-off value of serum homocysteine according to increase of coronary-artery calcification volume score (CVS). The data of 469 out of 777 subjects in 1 health promotion center located in Seoul were selected after exclusion of the missing data of serum homocysteine and CVS. CVS was categorized into 2 groups: CVS=0 and CVS>0. Serum homocysteine according to the CVS groups was compared, and the cut-off value of serum homocysteine according to the increase of CVS (>0) was calculated using the receiver operating characteristic curve. Mean age was 54.5 years and the proportion of females was 22.2%. Mean serum homocysteine concentration and CVS were 11.2 μmol/L and 50.4, respectively. After adjustments for age and sex, serum homocysteine was associated with CVS (r=0.167, p=0.001), and Log(Homocysteine) also showed a significant difference according to the CVS groups. The cut-off value of serum homocysteine according to the increase of CVS (>0) was 9.45 μmol/L (area under the curve=0.569 (95% CI 0.512 to 0.625), p=0.015). The cut-off value of serum homocysteine was 9.45 μmol/L according to the increase of coronary-artery CVS.

INTRODUCTION
Coronary artery calcification (CAC) in the asymptomatic population has been recognized as a marker for subclinical coronary atherosclerosis.1 The presence of calcification in the coronary artery suggests atherosclerosis, and the progression or severity of calcification is closely related to the total coronary plaque burden and clinical cardiac events.2-4 The prevalence and severity of CAC differ according to ethnicity, age, and comorbidities, such as diabetes or chronic kidney disease.5

In histopathologic studies, calcification is easily found in atherosclerotic plaques in coronary vessels, and unlike non-calcified plaques, calcified plaques are highly related to the level of homocysteine.6 Being a well-known independent risk factor for atherosclerotic vascular disease and cardiac mortality,7 a high level of homocysteine plays a role in the pathophysiology of developing calcified atherosclerosis. Although a positive correlation of the degree of CAC according to the homocysteine level is recognized,8 no studies have been carried out to specify how homocysteine affects CAC.

For the elevation of homocysteine with vascular calcification and the possibility of its function as a predictor for CAC, CT is a useful tool for detecting macrocalcification of atherosclerotic plaques.9 The role of homocysteine in coronary artery plaque formation leading to CAC is being increasingly recognized, yet no study has revealed the cut-off value of homocysteine for the presence of CAC. Therefore, we tried to find the cut-off value for homocysteine...
regarding CAC using the coronary artery volume score (CVS) in a Korean asymptomatic population.

METHODS
Data source and study subjects
This was a cross-sectional study including Koreans who had not been diagnosed with cardiovascular diseases (CVD), such as cardiac arrest, coronary heart disease, stroke, and other definite diseases of cardiovascular origin. The 777 participants had undergone a medical check-up including a cardiac CT scan in a single health promotion center in Seoul, Republic of Korea, from January 2010 to December 2019. The medical check-up included all items in the Korean national health screening program, which contains a lifestyle assessment about exercise, nutrition, obesity, alcohol intake, smoking, measurement of automated blood pressure, height, weight, and blood sample for hemoglobin, fasting glucose, lipid panel, creatinine, estimated glomerular filtration rate, aspartate amino transferase, and alanine amino transferase. In addition, serum fasting insulin and homocysteine were analyzed. A total of 469 participants were included in the study after we excluded 308 participants who had not met the criteria (no data of CVS and serum homocysteine). All participants attended voluntarily without any reward and informed consent was obtained from all subjects.

Anthropometric and laboratory data
The data of each patient’s medical history and lifestyle were recorded on self-administered questionnaires. Smoking status was answered either ‘I am a current smokers’ or ‘I have never been a smoker’. Blood pressure was measured with an automated sphygmomanometer in a sitting position. Body measurements included height, weight, and waist circumference. Subjects’ height and weight were measured by an automated digital height and weight scale. Body mass index (BMI) (kg/m²) was calculated as one’s weight (in kilograms) divided by the square of one’s height (in meters). Waist circumference was measured mid-way with a tape-line from just below the bottom rib to the superior border of the iliac crest while in standing position. Fasting blood samples were obtained from each subject in the morning. They were stored at 4°C, analyzed within 1 day of sampling, and then used for assessing serum glucose, insulin, lipid panels, liver enzymes, and homocysteine. Serum homocysteine was analyzed by a fluorescence polarization immunoassay with IMx Analyzers (AxSYM Abbott, Abbott Park, Illinois, USA).

Measurement of coronary calcification
We have gathered the coronary-artery calcification volume score (CVS) data from January 2010 to December 2019, which data were from the health check-up of general population in 1 health promotion center. We only gathered all checked CVS data during the period followed by the routine manual and CVS was calculated automatically after CT scan. The cardiac CT scan is a 64-slice multiple detector computed tomography (MDCT) scanner (GE LightSpeed VCT; GE Imatron, San Francisco, California, USA), and scan parameters were 3 mm slice thickness, 120 kV tube voltage, and 110 mAs tube current. We used a standard definition of coronary-artery CVS, that is, a hyperattenuating lesion in the coronary artery that is more than 3 pixels in size and more than 130 Hounsfield units.

RESULTS
General characteristics of study subjects are presented in table 1. The mean age was 54.5 years old, and the mean serum homocysteine level was 11.2 μmol/L. Of the 469 subjects, 104 were female, and 158 were current smokers. There were no participants with diabetes in this study. Neither low-density lipoprotein cholesterol (LDL-C) nor systolic or diastolic blood pressure was correlated with coronary-artery CVS increase. Only homocysteine was being significantly correlated with CVS increase (r=0.167, p<0.001) after age and sex adjustments (table 2, figure 1). The mean value of serum homocysteine concentration of the CVS>0 group was 18.17 μmol/L, which was higher than that of the CVS=0 group (10.18 μmol/L). After the log transformation, the Log(Homocysteine) value was

| Values | Mean (SD) |
|--------|-----------|
| n (% of female) | 104 (22.2) |
| Age (y) | 54.5 (9.7) |
| BMI (kg/m²) | 24.5 (2.9) |
| Waist circumference (cm) | 87.5 (8.4) |
| Glucose (mg/dL) | 91.3 (10.6) |
| Total cholesterol (mg/dL) | 209.2 (35.8) |
| Triglyceride (mg/dL) | 143.8 (86.7) |
| HDL-cholesterol (mg/dL) | 51.8 (13.3) |
| LDL-cholesterol (mg/dL) | 131.3 (32.2) |
| AST (mg/dL) | 26.4 (9.1) |
| ALT (mg/dL) | 26.3 (14.6) |
| Homocysteine (μmol/L) | 11.2 (4.4) |
| CVS | 50.4 (91.8) |
| Current smoking, n (%) | 158 (34.0) |
| Hypertension, n (%) | 157 (34.0) |
| Type 2 diabetes, n (%) | 0 (0%) |

Values represent mean and SD values by descriptive method.
ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; CVS, calcification volume score; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Data management and statistical analysis
We represented all data results with mean and SD values by a descriptive method and used a χ² test to evaluate the prevalence of hypertension, diabetes, and smoking status. After age and sex adjustment, we used partial correlation to evaluate the relationship between CVS and the other metabolic parameters, including serum homocysteine. Serum homocysteine showed skewed pattern, so the difference of Log(Homocysteine) between CAC groups (CVS=0 vs CVS>0) was compared by independent t-test and a general linear model (analysis of covariance test) after adjusting for age, smoking, and hypertension. Finally, we calculated the cut-off value for serum homocysteine according to increase in coronary-artery CVS (CVS>0), as shown in the receiver operating characteristic (ROC) curve. A p value <0.05 was considered statistically significant. We did complex sample analysis with SPSS V.25.0 (SPSS).
significantly different between the 2 groups, and the higher CVS group (CVS>0) showed significantly higher value than the normal CVS group (CVS=0), even after age, smoking, and hypertension adjustments (Table 3). Figure 2 shows the ROC curve and cut-off value of the serum homocysteine for the presence of CAC (CVS>0). The area under the curve (AUC) was 0.569 (95% CI 0.512 to 0.625). There were no points on the curve that went below the diagonal line, and the results achieved statistical significance (p=0.015). The arrow on the ROC curve indicates the exact cut-off value of serum homocysteine according to the increase in coronary-artery CVS (CVS>0), and the cut-off value was 9.45 μmol/L (Table 4).

**DISCUSSION**

In this cross-sectional study, serum homocysteine was an independent factor for the increase of coronary-artery CVS, and its correlation remained significant even after adjustments for the risk factors of coronary artery disease, such as age, smoking, and hypertension. In addition, the cut-off value of serum homocysteine was 9.45 μmol/L in relation to the presence of the CAC.

CAC has been used as a marker of coronary atherosclerosis and is associated with major cardiovascular events and mortality. CAC can be found in a broad spectrum of cardiovascular states, from severe CVD to asymptomatic early diagnosed coronary disease. Presence of CAC was positively associated with CVD, unlike absence of CAC, and increasing CAC leads to greater cardiac events and all-cause mortality. Furthermore, the degree of CAC and its progression is correlated with coronary vascular disease events and mortality. Coronary atherosclerotic plaques consist of fatty substance, LDL-C, inflammatory cells, smooth muscle cells (SMC), connective tissue, fibrin, thrombi, and deposits of calcium phosphate. CAC initially begins as microcalcification, which is a small aggregation of extracellular matrix vesicles (EV) from vascular SMCs (vSMC) or macrophages in the intimal lipid core, and grows into a larger calcified plaque by accumulating and aggregating EV, which can be seen as a form of macrocalcification or calcium sheet. A cardiac CT scan cannot detect microcalcification, because of its spatial resolution and modality. However, it is a very effective tool for finding out the coronary plaque burden or coronary inflammation beyond the traditional Framingham

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**Table 2** Partial correlation of coronary-artery calcification volume score (CVS) and other factors after age and sex adjustments

| Values                        | r   | P value |
|-------------------------------|-----|---------|
| Body mass index (kg/m²)       | 0.041 | 0.432 |
| Waist circumference (cm)      | 0.040 | 0.439 |
| Systolic blood pressure (mm Hg) | 0.012 | 0.177 |
| Diastolic blood pressure (mm Hg) | 0.027 | 0.607 |
| Fasting glucose (mg/dL)       | 0.070 | 0.173 |
| Insulin (µIU/mL)              | −0.017 | 0.736 |
| AST (mg/dL)                   | −0.003 | 0.954 |
| ALT (mg/dL)                   | 0.033 | 0.526 |
| Total cholesterol (mg/dL)     | 0.054 | 0.295 |
| Triglyceride (mg/dL)          | 0.034 | 0.507 |
| HDL (mg/dL)                   | 0.011 | 0.831 |
| LDL (mg/dL)                   | 0.034 | 0.505 |
| Homocysteine (µmol/L)         | 0.167 | 0.001 |

Values represent partial correlation coefficient. ALT, alanine amino transferase; AST, aspartate amino transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

**Table 3** Serum homocysteine concentration according to coronary-artery calcification volume score (CVS)

| Value                        | CV$S=0$ | CV$S>0$ | P value | P value* |
|------------------------------|---------|---------|---------|----------|
| Log (homocysteine)           | 2.32 (0.03) | 2.39 (0.02) | 0.028   | 0.035    |

Values represent mean (SE) serum homocysteine according to the CVS by independent t-test after log transformation of serum homocysteine before adjustment (p value).

*P<0.05 by general linear model after age, smoking, and hypertension adjustments.
homocysteine plays pivotal part in the genesis of calcified plaque. where increased serum homocysteine is correlated to increased vascular calcifications. Clinical studies have shown the same outcomes that homocysteine can facilitate osteogenic cell differentiation (conversion of vSMCs to incorporate an osteoblastic feature), which can be proved by calcium deposition, alkaline phosphatase (ALP) activity, and the family of bone-related proteins. Clinical studies have shown the same outcomes that homocysteine is associated with vascular calcification, and there is a positive correlation between its elevated level and the progression of calcification.

Elevated homocysteine alters the endothelial integrity and tone by endothelial damage, vSMC proliferation, increased ROS, and reduced glutathione peroxidase activity, eventually leading to vascular inflammation. There are several in vitro studies about homocysteine evoking vascular inflammation by reducing the nitrogen monoxide and increasing ROS in endothelial cells. The physiological level of homocysteine induces oxidation of LDL, and an elevated homocysteine level leads to the production of homocysteine thioacetone, a highly reactive molecule. An increased homocysteine level accumulates lipid deposits and oxidized LDL in the vessel, triggering the depositing of atherosclerotic plaque and vascular inflammatory pathway. In short, a high level of serum homocysteine is associated with endothelial damage. Homocysteine induces calcification by modulating bone-related markers, such as OPN and OPG. Also, homocysteine promotes the proliferation of vSMC, especially calcifying vSMC, and increases calcium and phosphorous uptake rates in vSMCs, which are associated with elevated ALP activity. Furthermore, a high level of homocysteine makes the vessels thicken and stiffen by altering the vascular coagulation and thrombosis pathway and stimulating the vSMCs to proliferate. As mentioned above, debris of apoptosis produces a lot of lipid core and circulating matrix vesicles, and these serve as a calcium phosphate nucleating core. This process is triggered and accelerated in the increased homocysteine state, which is how elevated serum homocysteine level is involved in vascular calcifications. Similar clinical studies have shown that increased serum homocysteine is correlated to increased

Table 4  AUC and its cut-off value for serum homocysteine according to increase in coronary-artery calcification volume score (CVS)

| AUC (95% CI) | Homocysteine | Sensitivity | 1-Specificity | P value |
|-------------|--------------|-------------|---------------|---------|
| 0.569 (0.512 to 0.625) | 9.45 | 0.711 | 0.578 | 0.015 |

Homocysteine represents serum concentration correspondent to the cut-off value. AUC, area under the curve.
CAC. Kullo et al. showed that serum homocysteine is associated with CAC independent of other CVD risk factors, being possible as a clinical CVS risk marker. In the Multi-Ethnic Study of Atherosclerosis cohort, homocysteine was associated with the presence of CAC and the progression of CAC. The study concluded that elevated homocysteine is a risk factor for severe vascular calcification progression. Bearing in mind that homocysteine induces vascular inflammation, we assumed that there would be a trigger point where vascular calcification accelerates. Our result is similar to the cross-sectional study of Kim et al that looked into the relationship of homocysteine to CAC in Korean men, but their mean homocysteine level was higher than that of our study and included diabetes and current smokers. Other studies on CAC presented the valuable CAC to be over 400 calculated by the Agatston method. However, this CAC level is for those who already have cardiac symptoms or who have existing coronary disease requiring secondary prevention and treatment measures, such as medication and invasive coronary angiography. For primary prevention, the lower point of CAC should be clarified. Based on our findings, we suggest there is a point of homocysteine that is the threshold value of the presence of macrocalcification in the coronary artery for diagnosing asymptomatic subclinical coronary disease. For those whose homocysteine level is over 9.45 μmol/L, without chronic illness, there is plausible vascular inflammation present, and coronary CT scan is recommended to evaluate a possible cardiovascular problem.

This study has several limitations. First, the AUC is relatively low (0.569). Even though AUC was not high enough, the main focus of the current study was to determine the cut-off value of representing the serum homocysteine concentration in relation to the increase of coronary-artery CVS. In clinics, we frequently observed that serum homocysteine concentrations above 10 μmol/L were significantly associated with increased coronary artery calcification score (CACS)>0 as well as abnormal metabolic parameters. Therefore, we conducted the current study to understand the cut-off value of serum homocysteine in relation to the increase of CVS. It is well known that the CACS and CVS are associated with various factors such as age, BMI, calcium, vitamin D and K metabolism, smoking, and so on. The CVS, not CACS, was chosen in this study, since the CVS was to represent active growing calcification status, whereas CACS was the passive score to represent the real calcification in coronary artery. In addition, serum homocysteine has been reported to be an inflammatory marker to show the current inflammation in blood vessels and it can be related to the CAC increase, CVS. Considering serum homocysteine itself can be a minor contributor in comparison with other various confounding factors, it is not surprising to see the AUC of 0.569. In addition, there was a tight relationship of serum homocysteine with CVS. Therefore, we concluded that the cut-off value has a significant meaning despite of the low AUC. We believe that the increased serum homocysteine is one of the factors to have a relation with the increased CVS, coronary artery inflammation, and possible development of coronary artery diseases. We understand that the number of study subjects (n=469) in this study may not be enough for fancy AUC. However, we believe that the cut-off value of serum homocysteine above 9.45 μmol/L can be informative for many clinicians trying to prevent coronary diseases and to control heart health. Furthermore, such cut-off value can serve as a point of recommendation to evaluate the CVS or CACS by coronary artery imaging study in a near future, especially in high-risk patients or subjects. Second, the result cannot address the factors that might have affected the homocysteine level. For example, vitamin intake (vitamins B6, B12, and folate) and smoking cessation were not considered as factors in this study. Third, well-known risk factors for developing coronary calcification, such as LDL-C, total cholesterol, and systolic hypertension, were not related to CVS. In another study for risk factors for predicting subclinical coronary calcified artery disease, LDL-C alone was positively related to coronary calcification, because our subjects were relatively healthy, with well-controlled blood pressure and a normal range of lipid profile (mean total cholesterol was 209.2 mg/dL (SD 35.8)). It is probable that the relatively healthy subjects and small number of subjects in this study lead to no association between metabolic biomarkers and CAC. In case of blood pressure, subjects with high systolic or diastolic blood pressure (>140 or 90 mm Hg) were 8.9% and 11.5%, respectively. Subjects with high total cholesterol, high triglyceride, high LDL-C, or low high-density lipoprotein were 14.9%, 37.7%, 49.8%, and 14.9%, respectively. In addition, it should not be overlooked that the metabolic biomarkers such as blood pressure and cholesterol were obtained from regular health screenings, whereas CVS was the result of chronic exposure of blood vessel inflammation. The regression analysis between CVS and age, BMI, blood pressure, and cholesterol parameters indicates only significance with age (data were not known). Third, our cut-off value of homocysteine cannot be generalized to all populations. CAC scoring has a disparity according to ethnicity, age, and gender; so our cut-off value of homocysteine should be considered valid only for healthy Korean people who had not been diagnosed with hyperlipidemia and diabetes. Nonetheless, this was the first study to clarify the cut-off value of serum homocysteine for predicting the presence of CAC in Korean adults. A further prospective and well-designed large study should be done.

CONCLUSIONS

In summary, the findings of this study are that serum homocysteine was associated with coronary-artery CVS; its correlation remained significant even after adjustments for the risk factors of coronary artery disease, such as age, smoking, and hypertension. In addition, the cut-off value of serum homocysteine was 9.45 μmol/L in relation to the presence of the CAC.

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