December 20, 2016

Dear Editor:

Thank you for considering our paper entitled "Association between high cystatin C levels and carotid atherosclerosis."

We are very pleased to resubmit the revised version of our manuscript for publication. We greatly appreciate the time and effort taken by you and the three reviewers to review our paper. We have addressed all issues indicated in the review, and we offer detailed responses to your comments and those of the referees. The changes are also highlighted in yellow in the revised manuscript.

Responses to the Editor's comments:

Thank you very much for all your valuable guidance in the edited manuscript. Following your comments, we have now added the postcode required in the affiliations of all authors and more details of the address of the corresponding author. This is highlighted in yellow in the revised manuscript. As you requested, we have also provided signed PDF documents of statements related to the institutional review board, informed consent, conflict-of-interest, and data sharing, as well as an audio core tip.

We corrected the numerical errors in Table 2. Please review Table 2.

We added a statement indicating that a biostatistician reviewed the

study and Peer-review section. Please see **Page 8, Line 28-29 and Page 13, Line 19-21.**

Response to comments from Reviewer: 00058872

#1. "Authors should deeply clarify in the Introduction section the role of CysC in the inflammatory process, otherwise it is unclear the rationale of the study.

Response to Reviewer:

We have revised our manuscript as per your recommendations. We clarified in the Discussion section the role of CysC in the inflammatory process. Please refer to it.

Introduction section

Page 6, Line 1-3

"Furthermore, high CysC levels are indicated as a useful marker for identifying an elevated risk of cardiovascular disease and a higher total mortality among patients assessed as being at low risk by both creatinine (Cr) and estimated glomerular filtration rate (eGFR) values[16,17]. A previous study revealed that atherosclerotic changes associated with inflammation could be one mechanism by which cystatin C is associated with CVD[16]. However, the association between CysC and atherosclerotic disorders remains controversial, the cut-off

values of CysC for atherosclerosis are unknown, and previous reports on this association as well as the association between CysC and MCPT are limited[18-20]."

#2. "It is useful to present also the positive and negative predictive values in the Results section."

Response to Reviewer:

We have revised our manuscript as per your recommendations.

Results section

Page 9, Line 20-22

"Regarding lifestyle habits, only the exercise level was lower in the high CysC group than in the low CysC group. In addition, sensitivity, specificity, positive-predictive value, and, negative-predictive value as calculated from the data in Table 2 were 83%, 53%, 54%, and 82% respectively.

Next, we compared differences in demographic and clinical variables between subjects with MCPTs of ≥2 mm or <2 mm in Table 3."

#3. "Furthermore, they should emphasize in the Discussion section that specificity of their test was a little bit low."

Response to Reviewer:

We have revised our manuscript as per your recommendations.

Discussion section

Page 12, Line 4-5

In conclusion, higher CysC levels were correlated with carotid atherosclerosis as defined by an MCPT of ≥2 mm among middle-aged and elderly Japanese

subjects. Higher CysC levels have a low specificity but a high sensitivity and can therefore help exclude atherosclerosis. The CysC cut-off value of 0.73 mg/L appears to aid in the diagnosis of atherosclerosis.

Response to Reviewer #1's Comments: 02454185:

#1. "1. In statistical analysis, the authors used ROC curve to determine the cutoff value. Do you employ Youden index? Please specify."

Response to Reviewer

Thank you for sharing your comments with us and providing us the opportunity to revise the manuscript.

We have revised our tables and manuscript according to your recommendation.

Methods section

Statistical analysis

Page 8, Line 5-6

"The Youden index, a point on the receiver operating characteristic (ROC) curve, was used to determine the diagnostic value of serum CysC levels that were indicative of atherosclerosis."

#2. " In the second paragraph of statistical analysis, the authors used "Wilcoxon rank-sum test for continuous variables." I feel that data should be

first tested for normality and then to choose the statistical methods. For normally distributed data, it is best to use parametric analysis such as t test."

Response to Reviewer

Thank you for your comments. We have revised our manuscript and Table according to your recommendation.

Methods section

Page 8, Line 11

"Their demographic characteristics were then compared using the t test for continuous variables and chi-square test for categorical variables."

Tables

Please review our tables.

#3. "A simple linear regression analysis with adjustments for age and sex was conducted to determine the correlations between an MCPT of ≥2 mm and metabolic components including CysC level."-----all independent variables should be fully described, are there any other metabolic components other than CysC level, included in the model? The response variable appears like a binary outcome (MCPT of ≥2 mm) as described in the text. If it was a linear model, the response variable should be a continuous one."

Response to Reviewer

Thank you for sharing your comments with us and providing us the opportunity to revise the manuscript.

We have revised our manuscript according to your recommendation.

Methods section

Statistical analysis

Page 8, Line 12

"~~simple linear~~ Multiple logistic regression analysis with adjustments for age and sex was conducted to determine the correlations between an MCPT of ≥2 mm and the metabolic variables, including CysC."

#4. "Because this is a cross sectional study, there are numerous confounding factors should be considered. I feel that the model building strategy is a little weak and not technically sound. For a multiple regression model to be meaningful, the variable selection, test of linearity assumption, interaction, model fitting, and model diagnostics should be considered. But this paper did not describe any details in this regard. Several references can be considered at this point: Ann Transl Med. 2016 Mar;4(6):111. doi: 10.21037/atm.2016.02.15. Ann Transl Med. 2016 May;4(10):195. doi: 10.21037/atm.2016.03.36."

Response to Reviewer

Thank you for sharing your comments with us and providing us the opportunity to revise the manuscript. Unfortunately, our study included only 128 subjects, only 52 of whom had arteriosclerosis. Because there is a limit to the number of adjusted variables, we combined several metabolic variables in one item. Therefore, it was difficult for us to evaluate the variable selection and interaction in detail. While we could not conduct the Hosmer-Lemeshow test using our statistical analysis software JMP, we added the coefficient of determination, and Akaike's Information Criterion (AICc) to Table 4.

We have revised our manuscript and table 4 according to your recommendation.

Methods section

Page 8, Line 14-17

Statistical analysis

…and the metabolic variables, including CysC. Our study included only 128 subjects, of whom 52 had arteriosclerosis. Because there is a limit to the number of adjusted variables, we combined several metabolic variables in one item.

Variables that were significantly associated with an MCPT of ≥2 mm were then investigated with multiple logistic regression analysis.

Tables

Please review Table 4.

#5." Thus, future large-scale randomized studies are required."----in the limitation section, the authors described a randomized controlled trial to validate this finding. I am interested in how to conduct this RCT? What is the intervention? Please specify it."

Response to Reviewer

Thank you for sharing your comments with us and providing us the opportunity to revise the manuscript.

We have revised our manuscript according to your recommendation.

Limitations section

Page 11, Line 23

"Thus, future large-scale ~~randomized~~ cohort studies are required."

#6. "I also suggest to present another table comparing differences in demographic and many other variables between patients with MCPT of ≥2 mm

and <2 mm. for the most of time, this is the basic for building a logistic regression model."

Response to Reviewer

Thank you for sharing your comments with us and providing us the opportunity to revise the manuscript.

We have created Table 3 and revised our manuscript according to your recommendation. Please review our tables.

RESULTS section

Page 9, Line 23-28

In addition, sensitivity, specificity, positive-predictive value, and negative-predictive value as calculated from the data in Table 2 were 83%, 53%, 54%, and 82%, respectively.

Next, we compared differences in demographics and clinical variables between subjects with MCPTs of ≥2 mm or <2 mm (Table 3). Age, visceral fat area, hypertension, diabetes mellitus, creatinine, estimated glomerular filtration rate, and CysC were significantly higher in the MCPT of ≥2 mm group than the <2 mm group. Furthermore, the eGFR was significantly lower in the MCPT of ≥2 mm group. The two groups did not differ with regard to lifestyle habits.

The factors associated with an MCPT of ≥2 mm are shown in Table 4.

Response to comments from Reviewer: 00233953

We appreciate your acknowledgement.

I confirm that all authors have approved the above changes. Correspondence should be directed to me at the address below.

Sincerely yours,

Toshiyuki Kobayashi

Department of General Medicine, Zama General Hospital, Kanagawa, 252-0011, Japan

Tel: +81(46)-233-1311

Fax: +81(46)-232-8934

E-mail: kfujiba@juntendo.ac.jp