PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Soluble C-Type Lectin-Like Receptor 2 in Stroke (CLECSTRO) study: protocol of a multicentre, prospective cohort of a novel platelet activation marker in acute ischaemic stroke and transient ischaemic attack
AUTHORS	Uchiyama, , Shinichiro; Suzuki-Inoue, Katsue; Wada, Hideo; Okada, Yasushi; Hirano, Teruyuki; Nagao, Takehiko; Kinouchi, Hiroyuki; Itabashi, Ryo; Hoshino, Haruhiko; Oki, Koichi; Honma, Yutaka; Ito, Nobuo; Sugimori, Hiroshi; Kawamura, Masahide

VERSION 1 – REVIEW

REVIEWER	Zhang, Xia Soochow University
REVIEW RETURNED	29-Mar-2023

GENERAL COMMENTS	It is an interesting protocol to explore the association between plasma CLEC-2 levels and ischemic stroke. This manuscript is well- written with an interesting topic and rigorous logic. However, the main concern is the inclusion of the contemporary patient controls. How to diagnose the AIS or TIA mimics, especially TIA mimics for no lesion in the brain is the diagnostic criteria of TIA. The second concern is why sCLEC-2 is the differential biomarker for cardioembolic and non-cardioembolic AIS/TIA, which should be explained in the background part.

REVIEWER	Suades, Rosa Hospital de la Santa Creu i Sant Pau, Cardiovascular Program ICCC
REVIEW RETURNED	17-Apr-2023

GENERAL COMMENTS	Uchiyama et al. reported the study protocol for the multicentre, prospective ' Soluble C-Type Lectin-Like Receptor 2 (sCLEC-2) in Stroke' (CLECSTRO) cohort study to evaluate sCLEC-2 as a potential biomarker of platelet activation to distinguish acute ischaemic stroke and transient ischaemic attack, and other similar clinical presentations. While the goal of the study is clear and of interest, the following suggestions might improve the quality of the protocol: - The emergence of sCLEC-2 as a novel platelet activation marker associated to distinct outcomes should be properly referenced. - How recorded data will be obtained? - Will multiple regression analyses be used for analyses assessing confounding factors? - How biomarker results will be statistically validated?

	- There is no information about how authors address gender issues, data management plan and data availability, and public dissemination of the findings. - When analysing a biomarker released to the bloodstream either as soluble form or embedded in extracellular vesicles, caution with pre-analytical conditions for blood collection and processing is key. How this will be handled? Please be specific when stating the anticoagulant, the venepuncture method, the processing time, and all the factors that could affect ex vivo platelet activation and, therefore, the results of the study. Please follow up-to-date recommendations from International Society of Thrombosis and Haemostasis. - At what extent the CLEIA method really measures soluble sCLEC-2 or sCLEC2 bound to platelet-derived extracellular vesicles? - Is it planned to evaluate whether sCLEC-2 marker could be used for other disorders? Minor comments: - Abstract should include all the relevant information. - A study flow chart diagram should summarising the study design would be desirable. - Informed consent and related patient information documents should be added. - The term 'microparticle' should be replaced by 'extracellular vesicle' following the International Society of Extracellular Vesicles guidelines.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1 Dr. Xia Zhang

Comment: It is an interesting protocol to explore the association between plasma CLEC-2 levels and ischaemic stroke. This manuscript is well-written with an interesting topic and rigorous logic. However, the main concern is the inclusion of the contemporary patient controls. How to diagnose the AIS or TIA mimics, especially TIA mimics for no lesion in the brain is the diagnostic criteria of TIA.

Response: Thank you for your favourable comment. The definitions of AIS, TIA, AIS mimics, and TIA mimics were additionally described as follows in the "Inclusion/exclusion criteria and outcome measures" subsection of the Methods and Analysis section: "AIS was defined as the abrupt onset of focal neurological deficits with responsible lesions in the brain, which was confirmed on brain MRI or CT. TIA was defined as a transient episode of focal neurological symptoms such as hemiparesis, hemi-sensory deficit, aphasia, hemianopia, or monocular blindness, which meet the criteria by the National Institute of Neurological Diseases III (NINDS III) [6] and without responsible lesions on brain MRI or CT. [7] AIS mimics (unlikely AIS) were defined as acute neurological symptoms, which require differentiation from true AIS but do not meet the criteria by the NINDS III, and without new ischaemic lesions in the brain. TIA mimics (unlikely TIA) were defined as transient episodes of acute neurological symptoms that do not meet the criteria or meet the exclusion criteria for TIA according to the NINDS III guidelines. Additionally, these episodes were identified as not having new ischaemic lesions detected on brain MRI or CT scans. [6,7] Differential diagnoses of AIS and TIA from their mimics were confirmed by the consensus of 2 certified stroke specialists in each stroke centre." (Page 8, Line 75-87 in the revised manuscript).

Comment: The second concern is why sCLEC-2 is the differential biomarker for cardioembolic and non-cardioembolic AIS/TIA, which should be explained in the background part.

Response: We added the following sentences with 3 references regarding this issue in the Discussion section: “D-dimer was reported to be higher in patients with cardioembolic stroke than in those with other subtypes of AIS, [24, 25] and we reported that platelet activation markers such as beta-thromboglobulin and platelet factor 4 were more pronounced in atherothrombotic stroke.[26] Therefore, we inferred that the sCLEC-2/DD ratio could be a sensitive marker for differentiating cardioembolic AIS/TIA from non-cardioembolic AIS/TIA, which was suggested in the previous report.[23]” (Page17, Line 218-223 in the revised manuscript)

Reviewer 2 Dr. Rosa Suades

Comment: The emergence of sCLEC-2 as a novel platelet activation marker associated to distinct outcomes should be properly referenced.

Response: Thank you for your valuable feedback. We acknowledge the importance of referencing the emergence of sCLEC-2 as a novel platelet activation marker associated with distinct outcomes. We have already cited that plasma CLEC-2 was associated with stroke outcomes in the original manuscript (references 21 and 22 in the revised manuscript) but not described associations of sCLEC-2 with distinct outcomes in other diseases. Therefore, we included this information in the revised manuscript with additional references as follows in “C-type lectin-like receptor 2”: “Additionally, in patients with disseminated intravascular coagulation and traumatic brain injury, survivors showed lower levels of sCLEC-2 than non-survivors.[4,5]” (Page 7, Lines 57-58 in the revised manuscript)

Comment: How recorded data will be obtained?
Response: Thank you for this important observation. As for obtaining recorded data, we added the following sentences to the “Baseline and follow-up data” subsection of the Methods and Analysis section: “Data will be entered by investigators into Case Report Form and reviewed by the principal investigator and the investigators in the stroke centres. After confirming that there are no omissions or errors in the content, the principal investigator will sign and complete the case report.” (Page 13, Lines 143-145 in the revised manuscript)

Comment: Will multiple regression analyses be used for analyses assessing confounding factors?

Response: According to your important suggestion, we added the following sentence in the “Statistical analysis” subsection of the Methods and Analysis section: “Multivariate regression analyses will be used for analyses assessing confounding factors possibly affecting the outcomes, which were selected from background variables with $p<0.10$ by univariate regression analysis.” (Page 14, Line 159-161 in the revised manuscript)

Comment: How biomarker results will be statistically validated?

Response: Thank you for your helpful comment. According to the reviewer's suggestion, we corrected the expression of the corresponding sentence as follows in "Statistical analysis": "To validate the diagnostic ability of biomarker results statistically, receiver operating characteristic curve (ROC) analysis will be performed, and sensitivity and specificity at appropriate cut-off values will be determined." (Page 13, Line 154-156 in the revised manuscript)

Comment: There is no information about how authors address gender issues, data management plan and data availability, and public dissemination of the findings.

Response: Thank you for your helpful comment. Gender differences will be examined because it was stated that gender data would be obtained in "Baseline and follow-up data" in the original manuscript (Page 12, Lines 131-134 in the revised manuscript). We mentioned data management and data availability in the original manuscript (Page 19, Lines 254-256 in the revised manuscript). Regarding the public dissemination of the findings, we added the following sentence in "Ethics and dissemination". "The results of this study will be presented in international and domestic conferences and submitted for publication in peer-reviewed journals" (Page 15, lines 182-184 in the revised manuscript).

Comment: When analysing a biomarker released to the bloodstream either as soluble form or embedded in extracellular vesicles, caution with pre-analytical conditions for blood collection and processing is key. How this will be handled? Please be specific when stating the anticoagulant, the venipuncture method, the processing time, and all the factors that could affect ex vivo platelet activation and, therefore, the results of the study. Please follow up-to-date recommendations from International Society of Thrombosis and Haemostasis.

Response: Kazama F. et al. have reported that sCLEC-2 can be measured under normal sampling conditions of citrate or EDTA plasma (citrated plasma in this study) (reference 1 in the revised manuscript). Briefly, we added as follows in the "Measurement of C-type lectin-like receptor 2" subsection of the Methods and Analysis section of the revised manuscript: "The conditions for blood collection in this study were in accordance with recommendations for sample preparation for clotting time of the Japanese Society of Laboratory Haematology (JSLH),[12] which were based on the Clinical and Laboratory Standards Institute (CLSI) H21-A5.[13]" (Page 11, Lines 117-119 in the revised manuscript). Unfortunately, we could not find up-to-date recommendations from ISTH.

Comment: At what extent the CLEIA method really measures soluble sCLEC-2 or sCLEC2 bound to platelet-derived extracellular vesicles?

Response: Thank you for this important comment. We have no data about what extent the CLEIA method really measures sCLEC-2 because true values (concentrations) of sCLEC-2 in each fraction are not known. However, we have previously confirmed that ELISA detected both shed type and platelet-derived extracellular vesicle type using ultracentrifuge fractionation. Shed type and extracellular vesicle type were separated by ultracentrifugation and detected by Western blotting. We observed that ELISA detected both fractions, as reported in reference 1. Since we use the same combination of monoclonal antibodies in sCLEC-2 CLEIA reagents, we believe that CLEIA can detect both types of sCLEC-2, although precise extents of measurement are not known. Therefore, we added the following sentences in the "Measurement of C-type lectin-like receptor 2" subsection of the Methods and Analysis section of the revised manuscript: "We have previously confirmed that ELISA detected shed and platelet-derived extracellular vesicle types using ultracentrifuge fractionation.[1] Shed type and extracellular vesicle type were separated by ultracentrifugation and detected by

Western blotting. We will use the same combination of monoclonal antibodies in sCLEC-2 CLEIA reagents for this study." (Page 12, Lines 123-127)

Comment: Is it planned to evaluate whether sCLEC-2 marker could be used for other disorders?

Response: Thank you for your comment. In accordance with the reviewer's comment, we added as follows in the "Discussion" section of the revised manuscript: "The relationships with sCLEC-2 has also been reported in disseminated intravascular coagulation [4,17], thrombotic microangiopathy [18], COVID-19 [19], traumatic brain injury [5], venous thromboembolism [20] and acute coronary syndrome.[2] However, it should be elucidated whether sCLEC-2 can be a predictor of outcome in these diseases by prospective observational studies." (Page 16, Lines 203-207) in the revised manuscript)

Comment: Abstract should include all the relevant information.

Response:, It was very difficult to include all the relevant information in the Abstract since the number of words is limited to only 300, but we changed the sentences in the "Introduction" and "Methods and Analysis" subsection of "Abstract" section as follows:

"**Introduction**

Soluble C-type lectin-like receptor 2 (sCLEC-2) is a new biomarker for platelet activation, which can be easily measured by usual blood collection. We conducted the CLECSTRO, a prospective, observational cohort study, to evaluate the clinical implications of sCLEC-2 in patients with acute ischaemic stroke (AIS) and transient ischaemic attack (TIA).

Methods and analysis

The participants are patients with AIS/TIA and control patients required for differentiation from AIS/TIA. The target population is 600, including the patients and controls, who would be recruited from eight stroke centres across Japan. The inclusion criteria are AIS within 24 hours of onset and a modified Rankin Scale (mRS) score of 0– 2, TIA within 7 days of onset, and contemporary patients required for differentiation from AIS/TIA. Plasma sCLEC-2 will be measured by high-sensitive chemiluminescent enzyme immunoassay using residual blood samples from routine laboratory examinations at the first visit in all patients and 7 days later or at discharge in patients with AIS/TIA. The outcomes include plasma levels of sCLEC-2 in patients with AIS/TIA and controls, sCLEC-2/D-dimer ratio in non-cardioembolic and cardioembolic AIS/TIA, correlation of sCLEC-2 with recurrence or worsening of stroke, severity of stroke, infarct size, $ABCD^2$ score in TIA, and outcome (mRS) at 7 days and 3 months. " (Page 4, Line 2-18 in the revised manuscript)

Comment: A study flow chart diagram should summarising the study design would be desirable.

Response: Thank you for your insightful comment. In accordance with the reviewer's suggestion, we created the flow chart of the CLECSTRO Study as Figure 2 with its legend in the revised manuscript)

Comment: Informed consent and related patient information documents should be added.

Response: Regarding informed consent, we had described that it would be obtained via the opt-out method, according to the Japanese Ethical Guidelines, in the original manuscript. To explain this in more detail, the following sentences were added in the “Ethics and dissemination” section: “Written informed consent will not be obtained due to the measurement in blood samples collected from residual blood in usual clinical practice; however, detailed information about the study has been made available on a website to ensure that participants are fully informed and have the option to decline participation. The research secretariat confirmed compliance with opt-out procedures at each study site according to the guidelines.” (Page 15, Line 178-182 in the revised manuscript)

Comment: The term ‘microparticle’ should be replaced by ‘extracellular vesicle’ following the International Society of Extracellular Vesicles guidelines.

Response: Thank you for your advice. The term ‘microparticles’ was replaced throughout with ‘extracellular vesicles.’

Once again, I would like to thank the editor and the two reviewers for their comments, and I look forward to the publication of this paper in your journal.

VERSION 2 – REVIEW

REVIEWER	Zhang, Xia Soochow University
REVIEW RETURNED	14-Aug-2023

GENERAL COMMENTS	None

REVIEWER	Suades, Rosa Hospital de la Santa Creu i Sant Pau, Cardiovascular Program ICCC
REVIEW RETURNED	07-Aug-2023

GENERAL COMMENTS	No further comments. Many thanks.