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# 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



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**Title:**

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

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## ABSTRACT

### Introduction

Platelet function tests have not been widely used in patients with acute ischaemic stroke (AIS) or transient ischaemic attack (TIA) due to concerns about reproducibility, rapidity, economic performance, and simplicity. Soluble C-type lectin-like receptor 2 (sCLEC-2) is a new marker for platelet activation, which can be easily measured by usual blood collection. We planned sCLEC-2 in Stroke (CLECSTRO) study, a prospective, observational cohort to evaluate the clinical usefulness of sCLEC-2 in patients with AIS and TIA.

### Methods and analysis

The participants are patients with AIS or TIA and control patients required for differentiation from AIS or 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 modified Rankin Scale (mRS) score of 0–

2, TIA within 7 days of onset, and contemporary patients required for differentiation from AIS or TIA. The exclusion criteria include platelet or coagulation abnormalities, haemorrhagic stroke, head or other trauma, post-surgery, haemorrhagic tendency, and severe infection. The outcomes include plasma levels of sCLEC-2 in patients with AIS or TIA and controls, sCLEC-2/D-dimer ratio in non-cardioembolic and cardioembolic AIS or 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.

**Ethics and dissemination**

This study was approved by the Ethical Committee of the University of Yamanashi as the central ethical committee in agreement with the ethical committees of all collaborative stroke centres. Informed consent will be obtained by an opt-out form from the patients at each stroke centre according to the Ethical Guidelines for Medical and Biological Research Involving Human Subjects by the Japanese Ministry of Health, Labour and Welfare.

**Registration number**

ClinicalTrials.gov NCT05579405, UMIN Clinical Trial Registry UMIN000048954

**STRENGTHS AND LIMITATIONS of this study**

- This study is the first multicentre, prospective, observational cohort for soluble C-type lectin-like receptor 2 (sCLEC-2) in patients with acute ischaemic stroke (AIS) and transient ischaemic attack (TIA).
- Measuring sCLEC-2 is expected to be useful for differentiating true AIS and TIA from their mimics and non-cardioembolic AIS/TIA from cardioembolic AIS/TIA, predicting severity and outcome of AIS and TIA, decision-making of antithrombotic therapy, and monitoring antiplatelet therapy.
- sCLEC-2 can be measured rapidly by easy collection of residual blood in routine clinical practice, and thus expected broad application in the frontline of AIS and TIA.
- It is difficult to recruit contemporary patient controls, who have to be diagnosed with AIS or TIA mimics, to achieve the target population during the planned recruitment period. If the target population is not achieved, we may have to extend the end of recruitment.

## INTRODUCTION

### Background

Platelet function tests conducted to date include platelet aggregometry, measurement of platelet-specific proteins, such as β-thromboglobulin and platelet factor 4, point-of-care testing, such as Verify Now$^{R}$, and measurement of the expression or binding of adhesion molecules on platelets using flow cytometry. However, these tests are limited by concerns about reproducibility, rapidity, economic performance, and simplicity. Therefore, they have not been widely used in clinical practice for patients with acute ischaemic stroke (AIS) or transient ischaemic attack (TIA).

**C-type lectin-like receptor 2**

Soluble C-type lectin-like receptor 2 (sCLEC-2) is a new marker for platelet activation that can be easily measured by usual blood collection. [1,2] Plasma levels of sCLEC-2 have been studied for elucidating the correlations with various thrombotic and inflammatory diseases. [3] Plasma sCLEC-2 levels were reported to be associated with death, poor outcome, severity, disease risk, regulation of inflammatory response, neovascularisation, and tumour growth and metastasis in patients with ischaemic stroke, head trauma, atherosclerosis, deep vein thrombosis, sepsis, and cancer. [3]

**Study Aims**

This study aims to investigate whether sCLEC-2 is useful for differentiating true AIS or TIA from AIS or TIA mimics, classifying AIS and TIA subtypes, decision-making of

antithrombotic therapy (selection of antiplatelet or anticoagulant therapy), monitoring antiplatelet therapy, and predicting severity and outcome, in order to contribute to the progress of precision medicine in the diagnosis and management of AIS and TIA.

## METHODS AND ANALYSIS

### Study design, organisation, and recruitment of participants

The CLECSTRO is a multicentre, prospective cohort study in eight stroke centres across Japan (Supplemental Table), which adheres to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. The study organisation is shown in Supplemental Table. This study was registered in ClinicalTrials.gov NCT05579405 and UMIN Clinical Trial Registry UMIN000048954. Recruitment of patients started on October 11, 2022, and will end on December 1, 2023. The study will be terminated on December 31, 2024.

### Inclusion/exclusion criteria and outcome measures

The inclusion and exclusion criteria are listed in Table 1. The primary outcome measures are (1) plasma levels of sCLEC-2 at baseline in the AIS/TIA and control groups to compare intergroup differences, (2) plasma levels of sCLEC-2 after starting treatment in the AIS/TIA patients for comparison between patients with and without

worsening or recurrence, (3) plasma sCLEC-2 /D-dimer ratios in cardiogenic and non-cardiogenic AIS/TIA classified by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [4] for comparison between the two groups, and (4) correlation of the plasma levels of sCLEC-2 on admission with the modified Rankin Scale (mRS) score [5] at discharge and 3 months following the onset of AIS/TIA. The secondary outcome measures are (1) correlations of sCLEC-2 levels on admission with stroke severity (National Institutes of Health Stroke Scale, NIHSS [6]), size of infarct, and $ABCD^2$ score [7] in TIA, and (2) plasma levels of sCLEC-2 in patients with TOAST subtypes of AIS for comparison of intergroup differences. These data will be used for marketing authorisation application to the Pharmaceuticals and Medical Device Agency for the reagent coded LM22-01, a reagent for determining sCLEC-2.

**Sample size calculation**

The target number of patients is 600, including 400 patients with AIS, 100 with TIA, and 100 control patients. The minimum sample size of cardiogenic or non-cardiogenic AIS is 81 for each group when the sensitivity and specificity are 70%, the level of significance is 5%, and the L value is 0.1 in analysing the diagnostic performance. When patients with cardiogenic AIS were estimated to be approximately 25% of all patients with AIS, the number of AIS was set as 400 so that approximately 100 patients

with cardiogenic AIS would be included (to ensure a minimum of 81 patients is reached). For the TIA and control groups, the minimum sample size is 81 when the sensitivity and specificity are both 70%, the level of significance is 5%, and the L value is 0.1 in analysing the diagnostic performance. The target number of patients in both groups was set at 100 to ensure that more than 81 patients would be included. If the planned analysis changes after database locking, the justification will be described in the completed report.

**Measurement of C-type lectin-like receptor 2**

Residual blood samples from usual laboratory examinations will be used at the first visit, 7 ± 1 days later, and possibly at the time of discharge from patients with AIS or TIA. For the patients in the control group, residual blood samples will be used only during the first visit. Plasma sCLEC-2 will be measured by high-sensitive chemiluminescent enzyme immunoassay (CLEIA). Soluble fibrin, thrombin-antithrombin complex, and D-dimer levels will be measured simultaneously in these samples.

**Baseline and follow-up data**

Baseline characteristics, including age, sex, hypertension, diabetes, dyslipidaemia, current cigarette smoking, habitual alcohol drinking, atrial fibrillation, history of stroke,

myocardial infarction, peripheral artery disease, chronic kidney disease, and use of antiplatelet drugs or anticoagulants, will be documented. Body weight, body mass index, systolic and diastolic blood pressure, complete blood count, liver enzymes, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, blood glucose, haemoglobin A1c, creatinine, uric acid, C-reactive protein, and brain natriuretic peptide (BNP) or NT-pro BNP will also be recorded.

The NIHSS and mRS scores will be assessed on admission, at 7 days or discharge, and at 3 months after the onset. The sizes of infarcts on diffusion-weighted magnetic resonance imaging would be classified into small, large, and medium infarcts. Small infarcts are defined as <2 cm, large infarcts as a half or more in the middle cerebral artery territory, and medium infarcts as sizes between small and large infarcts.

**Statistical analysis**

Statistical analyses are as follows. In the comparison between the two groups, normal distribution in each group will be evaluated using the Kolmogorov–Smirnov test. When a normal distribution is observed, the Welch's test will be used, whereas when a normal distribution is not observed, the logarithmic transformation will be performed. If a normal distribution is observed after the logarithmic transformation, the Welch's test will be used for values after the logarithmic transformation. When a normal distribution

is not observed even after the logarithmic transformation, the Mann–Whitney U test will be used. The Dunnett's, Steel-Dwass, Tukey's, or Kruskal–Wallis test will be used for comparisons of three or more groups. Diagnostic performance will be assessed using the receiver operating characteristic analysis to calculate the area under the curve and sensitivity/specificity at an appropriate cut-off value. Correlations between two variables will be assessed using Pearson's or Spearman's correlation coefficient. Data with missing values for the variables necessary in the analyses will be excluded from the analysis dataset. The level of statistical significance is set at $p< 0.05$. All the statistical analyses will be performed using StatFlex version 7 (Artec Co., Osaka, Japan).

**Ethics and dissemination**

This study was approved by the Ethical Committee of the University of Yamanashi (CS0011) as the central ethical committee in agreement with the ethical committees of all collaborative stroke centres. Informed consent will be obtained by an opt-out form from the patients at each stroke centre according to the Ethical Guidelines for Medical and Biological Research Involving Human Subjects by the Japanese Ministry of Health, Labour and Welfare.

## DISCUSSION

CLEC-2 is a platelet receptor for podoplanin, which is expressed on certain types of tumour and lymphatic endothelial cells (Figure 1). [8,9] The CLEC-2/podoplanin interaction facilitates tumour metastasis, blood/lymphatic vessel separation, and normal lung formation during embryonic development. sCLEC-2 is released from platelets activated by agonists, such as collagen and thrombin; thus, it can be considered as a new biomarker for platelet activation. [1,2,10]

In CLEC-2-deficient mice prepared by the administration of the anti-CLEC-2 antibody, which can abolish CLEC-2 in plasma, platelet adhesion was reported to be preserved; however, platelet aggregation did not occur, and thus the bleeding time was prolonged, and arterial obstruction was not induced. [11]

Suzuki-Inoue et al. and LSI Medience Corporation have established the method to measure sCLEC-2 by enzyme-linked immunosorbent assay and CLEIA using an anti-CLEC-2 antibody. [1] CLEIA method can detect sCLEC-2 with high sensitivity, which can be measured using usual citrated blood in routine clinical practice. When platelets are activated, sCLEC-2 in plasma is measured as a shed form of 25kD molecule, and 32 kD and 40kD molecules bound to microparticles released from the platelets.

sCLEC-2 was reported to be a predictor of death or vascular events in patients with ischaemic stroke, [12] and was associated with stroke progression and outcome. [13] The plasma levels of sCLEC-2 were also reported to be higher in patients with AIS, TIA, and acute myocardial infarction than in healthy controls and those with deep vein thrombosis, syncope, gastrointestinal disease, heart failure, anaemia, thrombotic thrombocytopenic purpura, and indefinite compliant syndrome. [14] Additionally, in this study, the plasma levels of sCLEC-2 were higher in patients with atherothrombotic or lacunar stroke than in those with cardioembolic stroke, while the D-dimer level was higher in patients with cardioembolic stroke than in those with atherothrombotic or lacunar stroke; hence, sCLEC-2/D-dimer ratios were higher in atherothrombotic or lacunar stroke than in those with cardioembolic stroke, which suggests that sCLEC-2/D-dimer ratio is useful for the differential diagnosis of non-cardioembolic stroke (platelet-dependent disease state) and cardioembolic stroke (thrombin-generated disease state). [14]

The CLECSTRO study is the first multicentre, prospective, observational cohort of patients with AIS and TIA across Japan. This study aims to investigate whether sCLEC-2 is useful for differentiating true AIS or TIA from AIS or TIA mimics, classifying AIS and TIA subtypes, decision-making of antithrombotic therapy (selection

of antiplatelet or anticoagulant therapy), monitoring antiplatelet therapy, and predicting severity and outcome. sCLEC-2 could be a new, widely usable biomarker, thereby contributing to the progress of precision medicine in the pathophysiology, diagnosis, and management of AIS and TIA.



**Author Contributions**

SU planned the study, designed the protocol, wrote the original draft of the manuscript and revisions, and chaired the protocol and publication committees as the study chair. K S-I applied the protocol to and obtained approval from the central ethical committee, registered the study to ClinicalTrials.gov and UMIN as the principal investigator, and revised the manuscript. MK acquired funding from LSI Medience Corporation, reviewed and revised the protocol and the manuscript, and submitted the manuscript. HW, YO, TH, and TN discussed the study plan and reviewed and revised the protocol and the manuscript as members of the protocol and publication committees. HK, RI, HH, KO, YH, NI, and HS reviewed the manuscript and are participating in recruiting patients, template recording, management of blood collection, storing, and measurement as the heads or the responsible investigators of the institutions.

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**Competing Interest**

SU reports consultant fees from LSI Medience Corporation. K S-I has a patent related to sCLEC-2 assay (JP-6078845). MK is an employee of LSI Medience Corporation. HW and MK are inventors of a patent application related to sCLEC-2 measurement in AIS and TIA (JP application 2021-091606). The other authors report no disclosure relevant to the present study.

**Patients and public involvement**

Participants and the public were neither involved in the study design, recruitment, or conduct nor in the selection of research questions or study outcomes. All research facilities of the CLECSTRO team post the significance and content of this research on their respective websites and are actively working to make this research known. The CLECSTRO team is fully aware of and committed to the importance of involving the public as active stakeholders in its research activities. We aim to submit the research results to the Japanese authorities (PMDA) for approval as a diagnostic reagent for sCLEC-2. CLECSTRO researchers lead diverse research-related communications, and

public awareness initiatives focused on increasing awareness about AIS/TIA and the research of sCLEC-2 that is being developed.

**Data Availability Statement**

We will make the de-identified participant data from this research available to the scientific community with as few restrictions as possible, while retaining exclusive use until the publication of the major output.

**Supplemental Material**

Supplemental material for this article is available online.

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**Table 1.** Inclusion and exclusion criteria

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| Inclusion criteria:<br><br>1. ≥20 years of age<br><br>2. Male or female<br><br>3. Inclusion criteria by group<br><br>Patient group: Patients with ischaemic stroke within 24 hours of onset and modified Rankin Scale score of 0−2 before the onset or transient ischaemic attack within 7 days after the onset<br><br>Control group: Contemporary patients with neurological symptoms, who are required for differentiating from ischaemic stroke or transient ischaemic attack and ruled out by the final diagnosis at discharge |
| Exclusion criteria:<br><br>1. Concomitant conditions that may affect platelets or blood coagulation, such as acute thrombosis of other organs, haematologic disorders, or pregnancy<br>2. Cerebral haemorrhage, subarachnoid haemorrhage, traumatic brain injury, other trauma, postoperative cases, and bleeding disorders<br>3. Severe infectious disease<br>4. Patients whose onset time is unknown except for those for whom the onset occurred during sleep<br>5. Patients who are deemed inappropriate for this study by a physician<br>6. Inadequate condition of the collected specimens |

**Figure legend**

**Figure 1.** Mechanism of platelet activation by CLEC-2

Abbreviations: CLEC-2; C-type lectin-like receptor 2, Syk; spleen tyrosine kinase, LAT; linker for activation of T cells, SLP-76; SH2 domain-containing leukocyte phosphoprotein of 76kDa, Btk; Bruton's tyrosine kinase, PLCγ2; phospholipase Cγ2, PIP2; phosphatidylinositol 4,5-bisphosphate, DG; diacyl-glycerol, IP3; inositol 1 4 5-trisphosphate.

Figure 1. Mechanism of platelet activation by CLEC-2

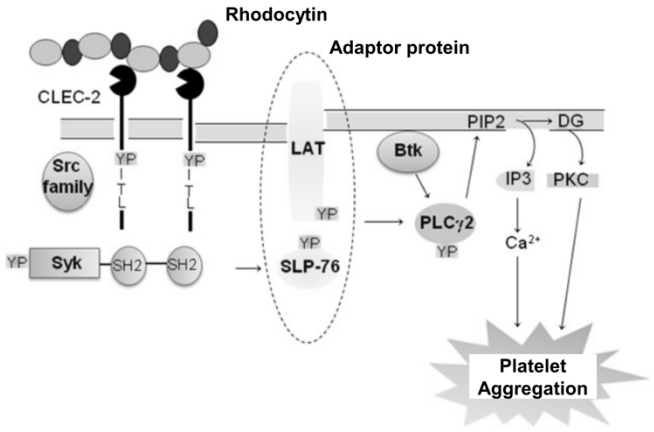


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Table. Study organization

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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

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## ABSTRACT

### 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.

## Ethics and dissemination

This study was approved by the Ethical Committee of the University of Yamanashi as the central ethical committee in agreement with the ethical committees of all collaborative stroke centres. Informed consent will be obtained by an opt-out form from the patients at each stroke centre according to the Ethical Guidelines for Medical and Biological Research Involving Human Subjects by the Japanese Ministry of Health, Labor and Welfare.

## Registration numbers

ClinicalTrials.gov NCT05579405, UMIN Clinical Trial Registry UMIN000048954

## STRENGTHS AND LIMITATIONS

- This study is the first multicentre, prospective, observational cohort for soluble C-type lectin-like receptor 2 (sCLEC-2) in patients with acute ischaemic stroke (AIS) and transient ischaemic attack (TIA).
- Measuring sCLEC-2 is expected to be useful for differentiating true AIS and TIA from their mimics and non-cardioembolic AIS/TIA from cardioembolic AIS/TIA, predicting severity and outcome of AIS and TIA, decision-making of antithrombotic therapy, and monitoring antiplatelet therapy.

- sCLEC-2 can be measured rapidly by easy collection of residual blood in routine clinical practice, and thus expected broad application in the frontline of AIS and TIA.
- It is difficult to recruit contemporary patient controls, who must be diagnosed with AIS or TIA mimics, to achieve the target population during the planned recruitment period. If the target population is not achieved, we may have to extend the end of recruitment.

## INTRODUCTION

### Background

Platelet function tests conducted to date include platelet aggregometry, measurement of platelet-specific proteins, such as β-thromboglobulin and platelet factor 4, point-of-care testing, such as Verify Now$^{R}$, and measurement of the expression or binding of adhesion molecules on platelets using flow cytometry. However, these tests are limited by concerns about reproducibility, rapidity, economic performance, and simplicity. Therefore, they have not been widely used in clinical practice for patients with acute ischaemic stroke (AIS) or transient ischaemic attack (TIA).

### C-type lectin-like receptor 2

Soluble C-type lectin-like receptor 2 (sCLEC-2) is a new marker for platelet activation that can be easily measured by usual blood collection. [1, 2] Plasma levels of sCLEC-2 have been studied for elucidating the correlations with various thrombotic and inflammatory diseases. Plasma sCLEC-2

levels were reported to be associated with death, poor outcome, severity, disease risk, regulation of inflammatory response, neovascularisation, and tumour growth and metastasis in patients with ischaemic stroke, head trauma, atherosclerosis, deep vein thrombosis, sepsis, and cancer. [3] Additionally, in patients with disseminated intravascular coagulation and traumatic brain injury, survivors showed lower levels of sCLEC-2 than non-survivors. [4, 5]

**Study Aims**

This study aims to investigate whether sCLEC-2 is useful for differentiating true AIS or TIA from AIS or TIA mimics, classifying AIS and TIA subtypes, decision-making of antithrombotic therapy (selection of antiplatelet or anticoagulant therapy), monitoring antiplatelet therapy, and predicting severity and outcome, in order to contribute to the progress of precision medicine in the diagnosis and management of AIS and TIA.

## METHODS AND ANALYSIS

### Study design, organisation, and recruitment of participants

The CLECSTRO is a multicentre, prospective cohort study in eight stroke centres across Japan (Supplemental Table), which adheres to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. The study organisation is shown in Supplemental Table. This study was registered in ClinicalTrials.gov NCT05579405 and UMIN Clinical Trial Registry

UMIN000048954. Recruitment of patients started on October 11, 2022, and will end on December 1, 2023. The study will be terminated on December 31, 2024.

**Inclusion/exclusion criteria and outcome measures**

Flow chart of the CLECSTRO study is shown in Figure 1. Inclusion and exclusion criteria are listed in Table 1. 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 scan. [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.

**Table 1.** Inclusion and exclusion criteria

| Inclusion criteria: |
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| 1. ≥20 years of age<br>2. Male or female<br>3. Inclusion criteria by group<br>Patient group: Patients with ischaemic stroke within 24 hours of onset and modified Rankin Scale score of 0−2 before the onset or transient ischaemic attack within 7 days after the onset<br>Control group: Contemporary patients presenting with neurological symptoms, requiring differentiation from ischaemic stroke or transient ischaemic attack and subsequently ruled out by the final diagnosis at discharge |
| Exclusion criteria:<br>1. Concomitant conditions that may affect platelets or blood coagulation, such as acute thrombosis of other organs, haematologic disorders, or pregnancy<br>2. Cerebral haemorrhage, subarachnoid haemorrhage, traumatic brain injury, other trauma, postoperative cases, and bleeding disorders<br>3. Severe infectious disease<br>4. Patients whose onset time is unknown except for those for whom the onset occurred during sleep<br>5. Patients who are deemed inappropriate for this study by a physician |

6. Inadequate condition of the collected specimens

The primary outcome measures are (1) plasma levels of sCLEC-2 at baseline in the AIS/TIA and control groups to compare intergroup differences, (2) plasma levels of sCLEC-2 after starting treatment in the AIS/TIA patients for comparison between patients with and without worsening or recurrence, (3) plasma sCLEC-2/D-dimer ratios in cardiogenic and non-cardiogenic AIS/TIA classified by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [8] for comparison between the two groups, and (4) correlation of the plasma levels of sCLEC-2 on admission with the modified Rankin Scale (mRS) score [9] at discharge and 3 months following the onset of AIS/TIA. The secondary outcome measures are (1) correlations of sCLEC-2 levels on admission with stroke severity (National Institutes of Health Stroke Scale, NIHSS [10]), size of the infarct, and $ABCD^2$ score [11] in TIA, and (2) plasma levels of sCLEC-2 in patients with TOAST subtypes of AIS for comparison of intergroup differences. To validate the diagnostic ability of biomarkers statistically, receiver operating characteristic curve (ROC) analysis will be performed, and sensitivity and specificity at appropriate cut-off values will be determined.

These data will be used for marketing authorisation application to the Pharmaceuticals and Medical Device Agency for the reagent coded LM22-01, a reagent for determining sCLEC-2.

**Sample size calculation**

The target number of patients is 600, including 400 patients with AIS, 100 with TIA, and 100 control patients. The minimum sample size of cardiogenic or non-cardiogenic AIS is 81 for each group when the sensitivity and specificity are 70%, the level of significance is 5%, and the L value is 0.1 in analysing the diagnostic performance. When patients with cardiogenic AIS were estimated to be approximately 25% of all patients with AIS, the number of AIS was set as 400 so that approximately 100 patients with cardiogenic AIS would be included (to ensure a minimum of 81 patients is reached). For the TIA and control groups, the minimum sample size is 81 when the sensitivity and specificity are both 70%, the level of significance is 5%, and the L value is 0.1 in analysing the diagnostic performance. The target number of patients in both groups was set at 100 to ensure that more than 81 patients would be included. If the planned analysis changes after database locking, the justification will be described in the completed report.

**Measurement of C-type lectin-like receptor 2**

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] Residual blood samples from routine laboratory examinations will be used at the first visit, 7 ± 1 days later, and possibly at the time of discharge from patients with AIS or TIA. For the patients in the control group, residual blood samples will be used only during the first visit. Plasma sCLEC-2 will be measured by

high-sensitive chemiluminescent enzyme immunoassay (CLEIA). 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.

Soluble fibrin, thrombin-antithrombin complex, and D-dimer levels will be measured simultaneously in these samples.

**Baseline and follow-up data**

Baseline characteristics, including age, sex, hypertension, diabetes, dyslipidaemia, current cigarette smoking, habitual alcohol drinking, atrial fibrillation, history of stroke, myocardial infarction, peripheral artery disease, chronic kidney disease, and use of antiplatelet drugs or anticoagulants, will be documented. Body weight, body mass index, systolic and diastolic blood pressure, complete blood count, liver enzymes, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, blood glucose, haemoglobin A1c, creatinine, uric acid, C-reactive protein, and brain natriuretic peptide (BNP) or NT-pro BNP will also be recorded.

The NIHSS and mRS scores will be assessed on admission, at 7 days or at discharge, and at 3 months after the onset. The sizes of infarcts on diffusion-weighted magnetic resonance imaging would be classified into small, large, and medium infarcts. Small infarcts are defined as <2 cm, large infarcts

as a half or more in the middle cerebral artery territory and medium infarcts as sizes between small and large infarcts.

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.

**Statistical analysis**

Statistical analyses are as follows. In the comparison between the two groups, the normal distribution in each group will be evaluated using the Kolmogorov–Smirnov test. When a normal distribution is observed, Welch's test will be used, whereas when a normal distribution is not observed, the logarithmic transformation will be performed. If a normal distribution is observed after the logarithmic transformation, Welch's test will be used for values after the logarithmic transformation. When a normal distribution is not observed even after the logarithmic transformation, the Mann–Whitney U test will be used. The Dunnett's, Steel-Dwass, Tukey's, or Kruskal–Wallis test will be used for comparisons of three or more groups. To validate the diagnostic ability of biomarker results statistically, receiver operating curve (ROC) analysis will be performed to calculate, and sensitivity and specificity at appropriate cut-off values will be determined. Correlations between two variables will be assessed using Pearson's or Spearman's correlation coefficient. Data with missing values for the variables necessary in the analyses will be excluded from the analysis dataset. The level of

statistical significance is set at $p< 0.05$. Multivariate regression analyses will be used for analyses assessing confounding factors possibly affecting the outcomes, which were selected from background variables by univariate regression analysis. All the statistical analyses will be performed using StatFlex version 7 (Artec Co., Osaka, Japan).

**Patients and public involvement**

Participants and the public were neither involved in the study design, recruitment, or conduct nor the selection of research questions or study outcomes. All research facilities of the CLECSTRO team post the significance and content of this research on their respective websites and are actively working to make this research known. The CLECSTRO team is fully aware of and committed to the importance of involving the public as active stakeholders in its research activities. We aim to submit the research results to the Japanese authorities (PMDA) for approval as a diagnostic reagent for sCLEC-2. CLECSTRO researchers take the lead in diverse research-related communications and public awareness initiatives aimed at increasing awareness about AIS/TIA as well as the ongoing development of sCLEC-2.

**ETHICS AND DISSEMINATION**

This study was approved by the Ethical Committee of the University of Yamanashi (CS0011) as the central ethical committee in agreement with the ethical committees of all collaborative stroke centres. Informed consent will be obtained by an opt-out form from the patients at each stroke centre according

to the Ethical Guidelines for Medical and Biological Research Involving Human Subjects by the Japanese Ministry of Health, Labour, and Welfare. 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. The results of this study will be presented at international and domestic conferences and submitted for publication in peer-reviewed journals.

## DISCUSSION

CLEC-2 is a platelet receptor for podoplanin, which is expressed on certain types of tumour and lymphatic endothelial cells (Figure 2). [14, 15] The CLEC-2/podoplanin interaction facilitates tumour metastasis, blood/lymphatic vessel separation, and normal lung formation during embryonic development. sCLEC-2 is released from platelets activated by agonists, such as collagen and thrombin; thus, it can be considered a new biomarker for platelet activation. [1, 2]

In CLEC-2-deficient mice prepared by the administration of the anti-CLEC-2 antibody, which can abolish CLEC-2 in plasma, platelet adhesion was reported to be preserved; however, platelet

aggregation did not occur, and thus the bleeding time was prolonged, and arterial obstruction was not induced. [16]

Suzuki-Inoue et al. and LSI Medience Corporation have established the method to measure sCLEC-2 by enzyme-linked immunosorbent assay and CLEIA using anti-CLEC-2 antibodies. [1] CLEIA method can detect sCLEC-2 with high sensitivity, which can be measured using usual citrated blood in routine clinical practice. When platelets are activated, sCLEC-2 in plasma is measured as a shed form of 25 kD molecule, and 32 kD and 40 kD molecules bound to extracellular vesicles released from the platelets, which was confirmed by ELISA and Western Blotting using the same combination of monoclonal antibodies as CLEIA. [1]

The relationship between sCLEC-2 and several diseases has 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 outcomes in these diseases by prospective observational studies. sCLEC-2 was reported to be a predictor of death or vascular events in patients with ischaemic stroke [21] and was associated with stroke progression and outcome. [22] The plasma levels of sCLEC-2 were also reported to be higher in patients with AIS, TIA, and acute myocardial infarction than in healthy controls and those with deep vein thrombosis, syncope, gastrointestinal disease, heart failure, anaemia, thrombotic thrombocytopenic purpura, and indefinite compliant

syndrome. [23] Additionally, in this study, the plasma levels of sCLEC-2 were higher in patients with atherothrombotic or lacunar stroke than in those with cardioembolic stroke, while the D-dimer level was higher in patients with cardioembolic stroke than in those with atherothrombotic or lacunar stroke; hence, sCLEC-2/D-dimer ratios were higher in atherothrombotic or lacunar stroke than in those with cardioembolic stroke, which suggests that sCLEC-2/D-dimer ratio is useful for the differential diagnosis of non-cardioembolic stroke (platelet-dependent disease state) and cardioembolic stroke (thrombin-generated disease state). 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 can be a sensitive marker for differentiating cardioembolic AIS/TIA from non-cardioembolic AIS/TIA, which was suggested in the previous report. [23]

The CLECSTRO study is the first multicentre, prospective, observational cohort of patients with AIS and TIA across Japan. This study aims to investigate whether sCLEC-2 is useful for differentiating true AIS or TIA from AIS or TIA mimics, classifying AIS and TIA subtypes, decision-making of antithrombotic therapy (selection of antiplatelet or anticoagulant therapy), monitoring antiplatelet therapy, and predicting severity and outcome. sCLEC-2 has the potential to serve as a

novel and broadly applicable biomarker, thereby contributing to the progress of precision medicine in the pathophysiology, diagnosis, and management of AIS and TIA.

**Contributorship statement:**

**Author Contributions**

SU planned the study, designed the protocol, wrote the original draft of the manuscript and revisions, and chaired the protocol and publication committees as the study chair. K S-I applied the protocol to and obtained approval from the central ethical committee, registered the study to ClinicalTrials.gov and UMIN as the principal investigator, and revised the manuscript. MK acquired funding from LSI Medience Corporation, reviewed and revised the protocol and the manuscript, and submitted the manuscript. HW, YO, TH, and TN discussed the study plan and reviewed and revised the protocol and the manuscript as members of the protocol and publication committees. HK, RI, HH, KO, YH, NI, and HS reviewed the manuscript and are participating in recruiting patients, template recording, management of blood collection, storing, and measurement as the heads or the responsible investigators of the institutions.

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**Competing Interest**

SU reports consultant fees from LSI Medience Corporation. K S-I has a patent related to the sCLEC-2 assay (JP-6078845). MK is an advisor of LSI Medience Corporation. HW and MK are inventors of a patent application related to sCLEC-2 measurement in AIS and TIA (JP application 2021-091606). The other authors report no disclosure relevant to the present study.

**Data Availability Statement**

We will make the de-identified participant data from this research available to the scientific community with as few restrictions as possible while retaining exclusive use until the publication of the major output.

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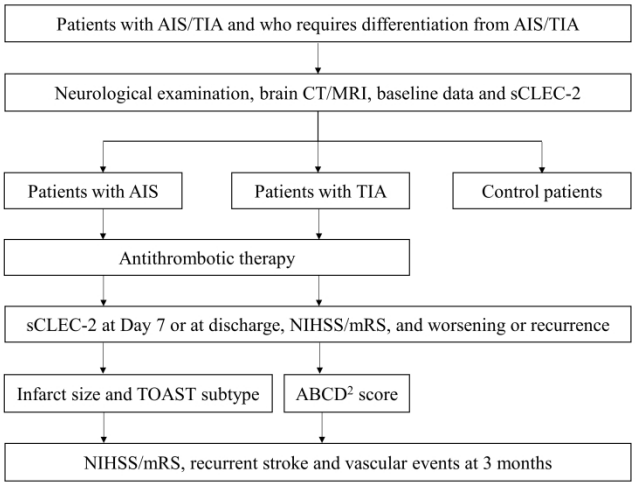
**Figure legend**

**Figure 1.** Flow chart of the CLECSTRO study design

Abbreviations: AIS, acute ischaemic stroke; TIA, transient ischaemic attack; CT, computed tomography; sCLEC-2, soluble C-type lectin-like receptor 2; NIHSS, National Institute Health Stroke Scale; mRS, modified Rankin Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment.

**Figure 2.** Mechanism of platelet activation by CLEC-2

Abbreviations: CLEC-2; C-type lectin-like receptor 2, Syk; spleen tyrosine kinase, LAT; linker for activation of T cells, SLP-76; SH2 domain-containing leukocyte phosphoprotein of 76 kDa, Btk; Bruton's tyrosine kinase, PLCγ2; phospholipase Cγ2, PIP2; phosphatidylinositol 4,5-bisphosphate, DG; diacyl-glycerol, IP3; inositol 1 4 5-trisphosphate.

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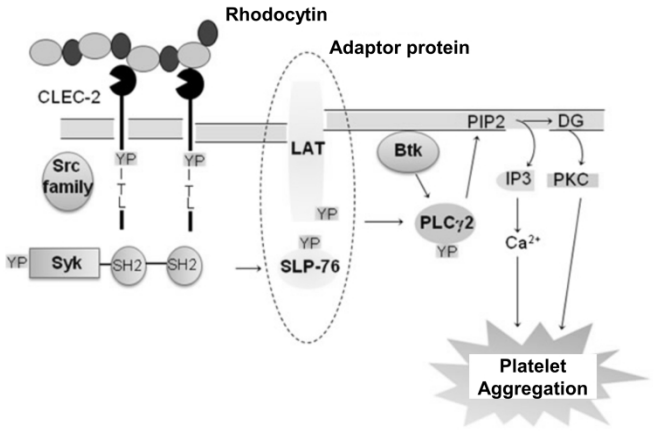


Figure 2. Mechanism of platelet activation by CLEC-2
Abbreviations: CLEC-2; C-type lectin-like receptor 2, Syk; spleen tyrosine kinase, LAT; linker for activation of T cells, SLP-76; SH2 domain-containing leukocyte phosphoprotein of 76 kDa, Btk; Bruton's tyrosine kinase, PLCγ2; phospholipase Cγ2, PIP2; phosphatidylinositol 4,5-bisphosphate, DG; diacyl-glycerol, IP3; inositol 1 4 5-trisphosphate.

338x190mm (300 x 300 DPI)

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