Harboring Cnm-expressing \textit{Streptococcus mutans} in the oral cavity relates to both deep and lobar cerebral microbleeds

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Abstract

**Background:** Cerebral microbleeds (CMBs) influence long-term prognoses of stroke patients. \textit{Streptococcus mutans} expressing the collagen-binding protein Cnm induces cerebrovascular inflammation, impairing blood brain barrier integrity and causing cerebral bleeding. Here, we examine the association of Cnm-positive \textit{S. mutans} with CMBs.

**Methods:** Acute stroke patients were selected from a single-center registry database. Oral carriage of Cnm-positive or Cnm-negative \textit{S. mutans} was determined using polymerase chain reaction assays. The associations of Cnm-positive \textit{S. mutans} with CMB number and specifically the presence of $>10$ CMBs were examined using quasi-Poisson and logistic regression models, respectively.

**Results:** This study included 3154 stroke patients, of which 428 patients (median [interquartile range] age, 73.0 [63.0–81.0] years; 269 men [62.9%]) underwent oral bacterial examinations. In total, 326 patients harbored \textit{S. mutans}. After excluding four patients without imaging data, we compared patients with Cnm-positive ($n=72$) and Cnm-negative ($n=250$) \textit{S. mutans}. Harboring Cnm-positive \textit{S. mutans} was independently associated with...
Cerebral microbleeds (CMBs), which are independent predictors of dementia and stroke [1–4], are radiologically defined constructs found on magnetic resonance imaging (MRI) [5, 6]. The underlying pathology of most CMBs consists of iron-positive focal or dispersed accumulations of siderophages corresponding to previous hemorrhages from small cerebral vessels [7, 8]. However, CMBs are also accompanied by various pathological lesions, such as inflammatory vasculitis and fibrinoid necrosis [7]. Therefore, clarifying the pathogenesis of CMBs remains challenging [5].

Hypertensive arteriopathy and cerebral amyloid angiopathy (CAA) are the two most common causes of CMBs. Previous studies have suggested that deep CMBs are mostly associated with hypertensive arteriopathy, whereas lobar CMBs reflect CAA [9]. However, growing evidence has shown that hypertensive arteriopathy induces both deep and lobar CMBs [9]. One pathological investigation showed that lobar CMBs were related to hypertensive arteriopathy in the absence of CAA [8].

We previously reported that oral carriage of Streptococcus mutans that expresses Cnm protein is related to an increased risk of deep CMBs [10–12]. Streptococcus mutans is a major cariogenic bacterium that is detected in the oral cavity of approximately 90% of the general population [13]. Cnm, encoded by the cnm gene, is a cell surface 120-kDa collagen-binding protein of S. mutans [14]. Intravenous administration of Cnm-expressing S. mutans (Cnm-positive S. mutans) aggravates cerebral bleeding in both cortical and deep gray matter in stroke-prone spontaneously hypertensive rats (SHRs) [15]. Nevertheless, the contribution of Cnm-positive S. mutans to lobar CMBs in humans remains unclear [10, 13, 16]. Infective endocarditis is a critical consequence of dental bacteremia, including that caused by Cnm-positive S. mutans [17], and lobar CMBs precede intracerebral hemorrhage (ICH) in infective endocarditis [18]. We therefore hypothesized that Cnm-positive S. mutans is associated with the development of lobar and deep CMBs. This cross-sectional study investigated the involvement of Cnm-positive S. mutans in deep and lobar CMBs in stroke patients.

**KEYWORDS**
dental caries, microbleeds, risk factor, Streptococcus mutans, stroke

**INTRODUCTION**

Cerebral microbleeds (CMBs), which are independent predictors of dementia and stroke [1–4], are radiologically defined constructs found on magnetic resonance imaging (MRI) [5, 6]. The underlying pathology of most CMBs consists of iron-positive focal or dispersed accumulations of siderophages corresponding to previous hemorrhages from small cerebral vessels [7, 8]. However, CMBs are also accompanied by various pathological lesions, such as inflammatory vasculitis and fibrinoid necrosis [7]. Therefore, clarifying the pathogenesis of CMBs remains challenging [5].

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**METHODS**

**Study design**

The significance of oral carriage of Cnm-positive S. mutans with regard to CMBs was evaluated in a cross-sectional study in accordance with the Declaration of Helsinki standards and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. Approval was obtained from the Ethics Committee of the National Cerebral and Cardiovascular Center (M23-073-8, M27-015-5).

Acute stroke patients were selected from the National Cerebral and Cardiovascular Center Stroke Registry database (https://www.clinicaltrials.gov; unique identifier: NCT02251665) who fully satisfied the following criteria: (1) patients who underwent oral bacterial assessments from February 2014 to May 2016 or from May 2017 to October 2018; (2) patients who developed ischemic stroke or ICH during the above-mentioned period; (3) patients older than 40 years old; and (4) patients or their legal representatives who provided written informed consent for the current study. We did not perform oral bacterial assessments for any patients between June 2016 and April 2017. Patients without MRI data were excluded from the analyses. Patients with Cnm-positive and Cnm-negative S. mutans (the Cnm [+ ] and Cnm [−] groups, respectively) were compared. Acute stroke patients that did or did not undergo an oral bacterial examination were compared to uncover potential sources of bias.

**Clinical characteristics**

Clinical information, except for MRI findings, was obtained from the National Cerebral and Cardiovascular Center Stroke Registry database. Hypertension was defined as systolic blood pressure ≥140mmHg, diastolic blood pressure ≥90mmHg, or a history of antihypertensive medication use. Diabetes mellitus was considered present when a patient used antidiabetic drugs or insulin, the fasting plasma glucose level was ≥126mg/dL, or the glycosylated hemoglobin A1c level was ≥6.5%. The definition of dyslipidemia...
was a low-density lipoprotein cholesterol level of ≥140 mg/dL, a high-density lipoprotein cholesterol level of ≤40 mg/dL, a triglyceride level of ≤150 mg/dL, or use of lipid-lowering drugs. National Institutes of Health Stroke Scale (NIHSS) scores were recorded upon admission. The diagnosis of possible or probable CAA was based on the modified Boston criteria [19].

Detection of Cnm-positive S. mutans

Dental plaque specimens were collected, inoculated in Mitis-Salivarius medium with bacitracin (Sigma-Aldrich) and on 15% sucrose agar plates, and anaerobically incubated at 37°C for 48h. Streptococcus mutans strains were identified and isolated based on rough morphological features on the agar plates. The strains were then cultured in brain–heart infusion broth (Becton, Dickinson and Company) at 37°C for 24h. Bacterial genomic DNA was extracted from each strain. A polymerase chain reaction assay was performed using an S. mutans-specific primer set (forward, 5'-GGCACCACAAATGGGAAGCTCAAGT-3'; reverse, 5'-GGAATGGCCGATCACTTCACAGTGAACAGGAT-3') following a method described previously [20]. The presence or absence of the cnm gene was determined using primers designed to amplify the entire length of the cnm gene (forward, 5'-GACAAAAAGAAAATGAAAGGTGACCCG-3'; reverse, 5'-GCAAAGACTCTTTGCCTGAC-3') [20]. Experiments were conducted by researchers who were blinded to the clinical information.

Evaluation of cerebral microbleeds

In this study, CMBs were illustrated on T2*-weighted MRI. We evaluated the “certain” brain microbleeds as CMBs according to the Brain Observer MicroBleed Scale [6]. The MRI parameters are summarized in Table S1. We defined deep CMBs as hypointensities on T2*-weighted MRI located in the deep gray matter of the basal ganglia or thalamus or the white matter of the corpus callosum or internal, external, or extreme capsule. Lobar CMBs were defined as those in the cortical gray or subcortical white matter. Infratentorial CMBs were defined as those in the brainstem or cerebellum. Strictly lobar CMBs were defined as those in the lobar region. The number of CMBs was independently determined by two trained neurologists blinded to the clinical data and hypothesis of this study. In the case of disagreement, the opinion of a third neurologist was sought.

Statistical analyses

Variables are presented as medians and interquartile ranges or numbers and percentages. The Mann–Whitney U test was used to analyze continuous data, and the χ² or Fisher exact test was used for categorical data. The numbers of all, deep, lobar, and infratentorial CMBs were stratified into six categories: 0, 1, 2–4, 5–10, 11–20, and ≥21 [1, 2]. We examined the linear trend between Cnm-positive S. mutans and each CMB category using the Cochran–Armitage test. In addition, univariate and multivariate logistic regression models were applied to examine the association of Cnm-positive S. mutans with the presence of >10 CMBs, as harboring >10 CMBs predicts future ICH [1], ischemic stroke [2], and death [4]. The association of Cnm-positive S. mutans with the number of CMBs was analyzed using quasi-Poisson regression models. These models were adjusted for age, sex, hypertension, stroke type (ischemic stroke/ICH), and CAA (none/possible or probable CAA) [22]. A p value <0.05 (two-tailed) was considered statistically significant. Statistical analyses were conducted using SPSS version 27 (IBM Corp.) and SAS version 9.4 (SAS Institute).

RESULTS

Patient selection

Among 3154 stroke patients, 428 patients (13.6%) underwent an oral bacterial examination (Figure 1). The characteristics of these 428 patients are described in Table S2. Patients who underwent oral bacterial examinations had lower ages and NIHSS scores than those who did not undergo examinations (age: 73.0 [63.0–81.0] years vs. 76.0 [67.0–83.0] years, p < 0.001; NIHSS score: 3.0 [1.0–7.0] vs. 4.0 [1.0–14.0], p < 0.001). In addition, higher frequencies of ICH and hypertension and a lower prevalence of atrial fibrillation were observed in patients who underwent a bacterial examination (ICH: 36.9% vs. 20.1%, p < 0.001; hypertension: 89.3% vs. 82.1%, p < 0.001; atrial fibrillation: 17.3% vs. 24.0%, p = 0.002).

The oral bacterial examination revealed that S. mutans was present in 326 patients (76.2%) and absent in 102 patients (23.8%). The clinical profiles were similar between patients with and without S. mutans in the oral cavity (Table S3). We identified 72 patients with Cnm-positive S. mutans and 254 with Cnm-negative S. mutans. Four patients were excluded from the analyses in the Cnm (−) group because no MRI data were available.

Demographics and clinical characteristics

The clinical profiles of the Cnm (+) and Cnm (−) groups are described in Table 1. The age, sex, blood pressure, vascular risk factors, and medication histories were similar between the two groups. The Cnm (+) group showed slightly lower NIHSS scores than the Cnm (−) group (2.0 [1.0–5.0] vs. 3.0 [1.0–7.3], p = 0.125). The prevalence of CAA was comparable between the two groups. CMBs were observed in 55 (76.4%) patients in the Cnm (+) group and 166 (66.4%) patients in the Cnm (−) group (p = 0.107). Strictly lobar CMBs were found in five patients (6.9%) in the Cnm (+) group...
and eight patients (3.2%) in the Cnm (−) group (p = 0.175). The median (interquartile range) numbers of CMBs in the Cnm (+) group were 4.0 (1.0–11.0) for all CMBs, 1.0 (0.0–6.5) for deep CMBs, 0.5 (0.0–4.8) for lobar CMBs, and 0.0 (0.0–1.0) for infratentorial CMBs, while the numbers of CMBs in the Cnm (−) group were 2.0 (0.0–7.0) for all, 1.0 (0.0–3.3) for deep, 0.0 (0.0–2.0) for lobar, and 0.0 (0.0–1.3) for infratentorial CMBs. The inter-rater reliabilities for deep, lobar, and infratentorial CMBs were 0.87, 0.88, and 0.95, respectively.

Cnm-positive S. mutans and cerebral microbleeds

Increasing numbers of CMBs are predictive of poor prognoses [1–4]. When the numbers of CMBs were stratified into six categories, the Cnm (+) group was significantly distributed in the “higher CMB” categories for all and lobar CMBs but not for deep or infratentorial CMBs (all CMBs, p = 0.030; deep, p = 0.178; lobar, p = 0.009; infratentorial, p = 0.721, Figure 2).

Table 2 shows the unadjusted and adjusted odds ratios between the harboring Cnm-positive S. mutans and >10 CMBs. In the unadjusted model, harboring Cnm-positive S. mutans was significantly associated with the presence of >10 of all and lobar CMBs, but not deep and infratentorial CMBs (odds ratio of all CMBs, 2.04 [95% confidence interval (95% CI), 1.11–3.74], p = 0.021; deep CMBs, 2.59 [0.95–7.05], p = 0.064; lobar CMBs, 6.56 [2.30–18.74], p < 0.001; infratentorial CMBs, 2.35 [0.39–14.36], p = 0.354). Statistical significance persisted after adjusting for age, sex, hypertension, stroke type, NIHSS score, and CAA (adjusted odds ratio of all CMBs, 2.20 [95% CI, 1.18–4.10], p = 0.013; deep CMBs, 2.63 [0.91–7.59], p = 0.073; lobar CMBs, 6.86 [2.35–20.06], p < 0.001; infratentorial CMBs, 2.10 [0.31–14.11], p = 0.444).

In the quasi-Poisson regression models, harboring Cnm-positive S. mutans was related to higher numbers of both deep and lobar CMBs, but not infratentorial CMBs, in both unadjusted (risk ratio of all CMBs, 2.84 [95% CI, 1.66–4.87], p < 0.001; deep CMBs, 1.57 [1.07–2.30], p = 0.021; lobar CMBs, 5.44 [2.50–11.85], p < 0.001; infratentorial CMBs, 1.55 [0.72–3.33], p = 0.263. Table 3) and adjusted analyses (risk ratio of all CMBs, 2.73 [95% CI, 1.72–4.33], p < 0.001; deep CMBs, 1.61 [1.14–2.27], p = 0.007; lobar CMBs, 5.14 [2.78–9.51], p < 0.001; infratentorial CMBs, 1.46 [0.72–2.96], p = 0.298). In the present study, 63 (87.5%) patients in the Cnm (+) and 197 (78.8%) patients in the Cnm (−) groups received the same MRI (MAGNETOM Spectra, Siemens) scan (Table S1). The sensitivity of CMBs can be influenced by differences in MRI scans [23]. We therefore excluded patients receiving other MRI scans (MAGNETOM Verio, Siemens; Signa EXCITE, GE; MAGNETOM Sonata, Siemens) (9 [12.5%] in Cnm [+] and 53 [21.2%] in Cnm [−]) and performed sensitivity analyses to confirm the results from the primary analyses. Harboring Cnm-positive S. mutans was still related to higher numbers of all and lobar CMBs both in the unadjusted analyses (risk ratio of all CMBs, 2.73 [95% CI, 1.53–4.87], p < 0.001; deep CMBs, 1.44 [0.96–2.17], p = 0.080; lobar CMBs, 5.26 [2.27–12.17], p < 0.001; infratentorial CMBs, 1.52 [0.68–3.39], p = 0.303) and in the analyses adjusting for age, sex, hypertension, stroke type, NIHSS score, and CAA (risk ratio of all CMBs, 2.49 [95% CI, 1.52–4.07], p < 0.001; deep CMBs, 1.44 [0.99–2.09], p = 0.056; lobar CMBs, 4.71 [2.43–9.16], p < 0.001; infratentorial CMBs, 1.37 [0.66–2.86], p = 0.397).
Representative images showing two patients with a substantial number of deep and lobar CMBs are illustrated in Figure 3.

DISCUSSION

The current study demonstrated that oral carriage of Cnm-positive S. mutans was independently associated with a greater number of all, deep, and lobar, but not infratentorial, CMBs. Cnm-positive S. mutans was significantly related to the presence of >10 CMBs, a predictor of future ICH [1], ischemic stroke [2], and mortality [4].

Streptococcus mutans is an anaerobic Gram-positive coccus [13]. Cnm-positive S. mutans has been reported in many countries, including Japan, the United States [24], Canada [25], and Finland [20]. We previously reported a high prevalence [10] and high incidence [12] of deep CMBs in stroke patients harboring Cnm-positive S. mutans. The cross-sectional [10] and retrospective longitudinal [12] studies showed a strong association between Cnm-positive S. mutans and deep, but not lobar, CMBs. The present study showed that harboring Cnm-positive S. mutans was independently associated with the number of lobar CMBs after adjusting for several vascular risk factors and stroke severity. The seemingly different results regarding lobar CMBs may stem from the different sample sizes between the previous and current studies [10, 12]. In addition, the frequency of hypertension (93.1%) in patients with Cnm-positive S. mutans in the present study was also slightly higher than that in the previous studies (85.7–90.9%), which might have contributed to the higher incidence of lobar CMBs.

Bacteremia caused by S. mutans is almost inevitable in daily life because of toothbrushing, flossing, or tooth extraction [11].
The major sources of *S. mutans* are mothers or caregivers [20]. *S. mutans* is vertically transmitted, colonizes the mouths of infants [20], and is rarely implanted during adulthood [26]. However, it frequently disappears from the oral cavity of edentulous individuals because *S. mutans* resides on the tooth surface [27]. We therefore excluded patients without *S. mutans* and only compared patients with Cnm-positive and Cnm-negative *S. mutans* in this study.
Cnm-positive *S. mutans* is characterized by its binding to components of the vascular basement membrane, such as collagen-IV and laminin [14, 17, 28], whereas Cnm-negative *S. mutans* cannot attach to soft tissues [17, 28]. Aging and vascular risk factors, including hypertension, induce endothelial injury and increase the thickness of basement membranes, resulting in collagen-IV and laminin exposure in small cerebral arteries [29, 30]. Once Cnm-positive *S. mutans* adheres to the basement membrane [28], neutrophil infiltration may aggravate local inflammation, resulting in increased permeability of the blood–brain barrier and increased production of enzymes (e.g., matrix metalloproteinase-9 [15]) that accelerate endothelial damage, leading to CMBs (Figure 4) [12]. However, no clinical studies have shown the effects of Cnm-positive *S. mutans* on the blood–brain barrier or neuroinflammation. Gadolinium-enhanced MRI and positron emission tomography are warranted in future studies.

The "vascular centrencephalon" is a phylogenetically ancient part of the brain that is perfused by short straight arteries with few branches, transmitting pressure directly from large arteries to small arterioles [31, 32]. However, the cortex is supplied by long arteries

### Table 3 Risk ratios and 95% confidence intervals for associations between Cnm-positive *Streptococcus mutans* and the number of CMBs.

|                          | Unadjusted | Model 1<sup>a</sup> | Model 2<sup>b</sup> | Model 3<sup>c</sup> |
|--------------------------|------------|----------------------|----------------------|----------------------|
| RR (95% CI)              | P value    | RR (95% CI)          | P value              | RR (95% CI)          | P value |
| All CMBs                 | 2.84 (1.66–4.87) | <0.001 | 2.78 (1.64–4.70) | <0.001 | 2.86 (1.69–4.86) | <0.001 | 2.73 (1.72–4.33) | <0.001 |
| Deep CMBs                | 1.57 (1.07–2.30) | 0.021 | 1.53 (1.04–2.23) | 0.029 | 1.56 (1.09–2.24) | 0.016 | 1.61 (1.14–2.27) | 0.007 |
| Lobar CMBs               | 5.44 (2.50–11.85) | <0.001 | 5.39 (2.57–11.30) | <0.001 | 5.63 (2.75–11.53) | <0.001 | 5.14 (2.78–9.51) | <0.001 |
| Infra-tentorial CMBs     | 1.55 (0.72–3.33) | 0.263 | 1.50 (0.73–3.07) | 0.272 | 1.50 (0.72–3.10) | 0.277 | 1.46 (0.72–2.96) | 0.298 |

Note: The unadjusted and adjusted risk ratio was estimated by quasi-Poisson regression models.

Abbreviations: CI, confidence interval; CMB, cerebral microbleed; RR, risk ratio.

<sup>a</sup>Adjusted for age, sex, and hypertension.

<sup>b</sup>Adjusted for Model 1 plus stroke type (ischemic stroke or intracerebral hemorrhage) and National Institutes of Health Stroke Scale score.

<sup>c</sup>Adjusted for Model 2 plus cerebral amyloid angiopathy.

**Figure 3** Representative images of cerebral microbleeds in two patients with Cnm-positive *Streptococcus mutans*. Numerous microbleeds are demonstrated on T2*-weighted magnetic resonance images from (a) a 74-year-old man and (b) a 55-year-old man harboring Cnm-positive *S. mutans* in their oral cavity.
with many branches, resulting in a large blood pressure decrement in the brain [33]. The marked differences in the arterial pressures between the deep and cortical regions could explain why lacunar infarcts related to high blood pressure-induced vasculopathies preferentially occur in the vascular centrencephalon rather than in the cortex [32]. Conversely, stroke-prone SHRs (i.e., a rat model of systemic hypertension) exhibit endothelial damage not only in deep but also in cortical arteries [30]. Furthermore, cerebrovascular integrity is more severely damaged in stroke-prone SHRs than SHRs, even though stroke-prone SHRs and SHRs show similar degrees of hypertension [34]. These findings suggest that factors other than high blood pressure also contribute to endothelial injury in patients with systemic hypertension [11], which may explain the increased numbers of lobar and deep CMBs in patients with Cnm-positive S. mutans.

This study had certain limitations. First, only 428 patients (13.6%) of the total 3154 stroke patients underwent an oral bacterial evaluation. We attempted to recruit a wide range of stroke patients; however, older and more severe stroke patients tended not to participate in the study, largely because of difficulties in explaining the research to those with impaired consciousness or other disabilities, including dementia and advanced frailty. As a result, the patients that underwent bacterial assessments were younger and had lower NIHSS scores. Most of the enrolled participants were therefore mild-to-moderate stroke patients, raising a potential risk of selection bias, and limiting the ability to generalize our results to more severely affected patients. Additionally, this cross-sectional study was performed retrospectively, and outcome data were not obtained. To this end, we are currently performing a prospective multicenter observational study to evaluate the effects of Cnm-positive S. mutans [35]. Second, the proportion of ICH patients and the number of patients with a history of hypertension or atrial fibrillation differed between those who did and did not undergo oral bacterial examination. We previously reported that Cnm-positive S. mutans is more closely associated with hypertensive ICH than ischemic stroke [10], which might have influenced participation in the study or the success rate of informed consent acquisition. Third, we found no evidence that Cnm-positive S. mutans accelerates the pathophysiology of CAA, a strong risk factor for lobar CMBs. Considering the close association of Cnm-positive S. mutans with deep CMBs, it is challenging to diagnose CAA in a patient harboring Cnm-positive S. mutans. The sensitivity of the modified Boston criteria for the diagnosis of CAA was 94.7% [19]. However, the association between Cnm-positive S. mutans and CAA should be further clarified by newly proposed criteria such as the Edinburgh criteria [36] and the Boston criteria version 2.0 [37]. Fourth, no country other than Japan has reported the effects of Cnm-positive S. mutans on stroke and CMBs. Thus, we cannot extrapolate our findings to other countries, and further multinational validation studies are necessary.

In conclusion, we found that Cnm-positive S. mutans was associated with a higher number of both lobar and deep CMBs. Transmission of Cnm-positive S. mutans can be prevented by improving oral hygiene in early childhood. In addition, several strategies to target Cnm-positive S. mutans, such as immunotherapy, probiotics, and prebiotics, are being innovated [38]. Reducing Cnm-positive S. mutans in the oral cavity may serve as a novel therapeutic approach to improve the long-term prognoses of stroke patients.

AUTHOR CONTRIBUTIONS
Study conception: SS, RN, KN, and MI. Data acquisition: SI, SS, SH, ST, Hajime I, and Hiroyuki I. Analysis and interpretation of data: SI, SS, Yumi Y, RN, MT, and KN. Drafting the manuscript: SI, SS, and MI. Revising the manuscript critically for intellectual content: TT, YH, RPF, ROC, NK, Yusuke Y, HH, MK, and KT. Supervision of the study: SS, HH, and MI.
ACKNOWLEDGMENTS
We are indebted to Ms Yuko Kiyama and Ms Natsuki Hanada for technical assistance. We thank Lisa Kreiner, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

FUNDING INFORMATION
This study was funded by JSPS KAKENHI to Dr Saito (JP19J00106, JP21K16944), to Dr Ihara (JP16K14573, JP19K22610), AMED to Dr Ihara (JP22ek0109516), Mitsui Sumitomo Insurance Welfare Foundation to Dr Ihara, and SENS shin Medical Research Foundation to Dr Ihara.

CONFLICT OF INTEREST
Tomotaka Tanaka reports lecturer fees from Daiichi Sankyo, Eisai, and UCB Japan. Yusuke Yakushiji reports lecturer fees from Daiichi Sankyo and research funds from Eisai. Masatomo Koga reports honoraria from Daiichi Sankyo; and research funds from Daiichi Sankyo, Nippon, Boehringer Ingelheim, and Shionogi. Kazunori Toyoda reports lecture honoraria from Bayer, Bristol-Myers Squibb, Daiichi Sankyo, Otsuka, Novartis, and Abbott Medical, outside the submitted work. Masafumi Ihara reports lecturer fees from Daiichi Sankyo and Eisai, and grant support from Panasonic, GE Precision Healthcare LLC, Bristol-Myers Squibb, and Shimadzu Corporation.

DATA AVAILABILITY STATEMENT
Raw data were generated and stored at the National Cerebral and Cardiovascular Center. Derived data supporting the findings of this study are available from the corresponding authors on request.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.