Stroke

CLINICAL AND POPULATION SCIENCES

Oral Carriage of *Streptococcus mutans* Harboring the *cnm* Gene Relates to an Increased Incidence of Cerebral Microbleeds

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BACKGROUND AND PURPOSE: Cerebral microbleeds (CMB) are associated with stroke and cognitive impairment. We previously reported a high prevalence of CMB in people with *Streptococcus mutans* expressing Cnm, a collagen-binding protein in the oral cavity. *S. mutans* is a major pathogen responsible for dental caries. Repeated challenge with *S. mutans* harboring the *cnm* gene encoding Cnm induced cerebral bleeding in stroke-prone spontaneously hypertensive rats. The purpose of this longitudinal study is to examine the relationship of *cnm*-positive *S. mutans* to the development of CMB.

METHODS: We retrospectively investigated patients with stroke receiving oral microbiological examination and head 3T magnetic resonance imaging evaluations twice in the period 2014 to 2019, allowing >180-day interval. Patients with *cnm*-positive *S. mutans* were compared with those without. Quasi-Poisson regression models were used to explore associations between *cnm*-positive *S. mutans* and the increase in number of CMB between the 2 magnetic resonance imaging scans.

RESULTS: A total of 111 patients were identified; 21 (19%) with *cnm*-positive *S. mutans* and 90 (81%) without. Clinical history, including blood pressure and the use of antithrombotic agents, were comparable between the 2 groups. New CMB were more commonly observed in patients with *cnm*-positive *S. mutans* (52% versus 23%; *P*=0.008). The incidence of CMB was significantly higher in the group with *cnm*-positive *S. mutans*, especially in deep areas, (incidence rate ratios [95% CI], 5.1 [1.9–13.6] for CMB in any brain region; 15.0 [5.4–42.0] for deep CMB), which persisted after adjusting for age, sex, hypertension, and renal impairment (4.7 [1.8–11.9] for CMB in any brain region; 13.9 [4.3–44.5] for deep CMB).

CONCLUSIONS: This study demonstrates that *cnm*-positive *S. mutans* is associated with an increased incidence of CMB. Treatment for *cnm*-positive *S. mutans* infection may be a novel microbiota-based therapeutic approach for stroke and cognitive impairment.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: blood pressure ▪ dental caries ▪ hemorrhage ▪ risk factor ▪ *Streptococcus mutans*

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Nonstandard Abbreviations and Acronyms

any CMB	cerebral microbleeds in any brain region
BM	basement membranes
CRP	C-reactive protein
DWMH	deep white matter hyperintensities
ICH	intracerebral hemorrhage
IL	interleukin
IRR	incidence rate ratios
IS	ischemic stroke
MRI	magnetic resonance imaging
PVH	periventricular hyperintensities
SVD	small vessel disease
TIA	transient ischemic attack
WMH	white matter hyperintensities

Small vessel disease (SVD) is a collective term for pathological changes in cerebral small vessels, which contribute to lacunar infarcts, white matter lesions, and cerebral microbleeds (CMB).[1] Increasing evidence has identified CMB as an independent risk factor for dementia[2] and stroke.[3] CMB are associated with an almost 2-fold increased risk of ischemic stroke (IS) and 4-fold risk of intracerebral hemorrhage (ICH).[4] On gradient-echo T2*-weighted magnetic resonance imaging (MRI), CMB are described as small, round foci with hypointensities.[5–7] The underlying histopathology of CMB is not uniform, representing recent or old hemorrhages, vasculopathies, or hemorrhagic microinfarcts. These heterogeneous pathological substrates likely reflect different causes.[8,9]

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We previously reported a role of systemic inflammation in CMB development.[10] Higher circulating levels of high-sensitivity CRP (C-reactive protein), IL (interleukin)-6, and IL-18 are associated with CMB.[10] Experimental models for CMB include mice subcutaneously injected with lipopolysaccharide.[9,11] CMB are known to be induced by infective endocarditis[12] or bacterial sepsis.[6]

Streptococcus mutans is a Gram-positive bacterium and a major pathogen responsible for dental caries.[13] Several cross-sectional studies have shown that oral infection with *S. mutans* expressing Cnm protein is associated with an increased prevalence of CMB.[14,15] Cnm is a cell-surface 120-kDa collagen-binding protein of *S. mutans*, and its coding gene is *cnm*.[16–19] *S. mutans* resides on the surface of teeth and frequently induces bacteremia through brushing, flossing, or tooth extraction.[20,21] Once in the bloodstream, *cnm*-positive *S. mutans* attaches to cerebrovascular basement membranes (BM)[17–19] inducing local blood-brain barrier inflammation, resulting in ICH.[19] Experimental intravenous administration of *cnm*-positive *S. mutans* in stroke-prone spontaneously hypertensive rats and a mouse model of cerebral hemorrhage exacerbates cerebral bleeds.[19] Epidemiological studies from many countries have shown ≈20% to 30% of patients with ICH[15,22] and 7% to 20% of the general population[14,23–25] have *cnm*-positive *S. mutans* in their oral cavity. Clarifying the effects of *cnm*-positive *S. mutans* on the cerebral vasculature is, therefore, both necessary and urgent.

In this study, we hypothesize that *cnm*-positive *S. mutans* contributes to the development of CMB. We investigated the association between *cnm*-positive *S. mutans* and incidence of CMB in a longitudinal retrospective study.

METHODS

Data Availability Statement

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

Study Design

The current study was approved by the Ethical Committee of the National Cerebral and Cardiovascular Center (M23-073, M25-111 and M27-015) and conducted in accordance with Declaration of Helsinki standards.

Subjects who fully satisfied the following criteria were selected from the database of the National Cerebral and Cardiovascular Center Stroke Registry (https://www.clinicaltrials.gov; Unique identifier: NCT02251665) and included in the analysis: (1) subjects who developed acute IS, transient ischemic attack (TIA), or ICH from February 15, 2014 to April 8, 2018; (2) subjects who signed an informed consent form for the current research, including receiving oral bacterial assessments from February 15, 2014 to April 30, 2018; and (3) subjects receiving 3T-MRI scans for clinical purposes twice, with more than a 180-day interval between examinations, from February 15, 2014 to February 15, 2019. The first MRI scan was used for baseline evaluation and the second for follow-up. If >2× of 3T-MRI scans were performed, the oldest and the latest MRI data were selected. The observational period was defined as the period from baseline to follow-up MRI scans. Subjects with *cnm*-positive *S. mutans* (*cnm* [+] group) were compared to those without (*cnm* [−] group) unless otherwise noted. The *cnm* (−) group comprised patients with *cnm*-negative *S. mutans* and those without *S. mutans*.

Clinical Characteristics

Clinical information, including incidence of symptomatic stroke and TIA, was collected from medical records. The clinical laboratory results nearest to baseline MRI were used as baseline data. Blood pressure was examined at baseline and follow-up visits. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or history of antihypertensive medication use. Diabetes was considered present through a history of antidiabetic drug or insulin use, a fasting plasma glucose level of ≥126 mg/dL, or glycated hemoglobin A1c level of ≥6.5%. Dyslipidemia was defined as low-density lipoprotein cholesterol level ≥140 mg/dL, high-density lipoprotein cholesterol level ≤40 mg/dL, triglyceride level ≥150 mg/dL, or use of lipid-lowering drugs. Renal impairment was defined as <60 mL/min/1.73 m^2 of estimated glomerular filtration rate, according to previous reports.[26,27] The presence of atrial fibrillation and current smoking pattern were also noted. Previous IS, TIA and ICH were defined according to the presence of each disease >3 months before the baseline MRI scan, whereas events within 3 months before the baseline MRI were described as recent IS, TIA, or ICH.

Detection of *cnm*-Positive *S. mutans*

Dental plaque specimens were collected and inoculated on Mitis-Salivarius medium with bacitracin (Sigma-Aldrich, St. Louis, MO) and 15% sucrose agar plates and anaerobically incubated at 37 °C for 48 hours. *S. mutans* strains were identified and isolated based on rough morphological features on agar plates, and all strains were cultured in brain heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37 °C for 24 hours. Bacterial genomic DNA of each strain was extracted, and *S. mutans* and *cnm* genes screened using polymerase chain reaction. MKD primer sets for *S. mutans* and *cnm* were used to identify *cnm*-positive and *cnm*-negative *S. mutans*.[23] Experiments were conducted by researchers blind to clinical information.

MRI Evaluation

Fluid-attenuated inversion recovery and gradient-echo T2*-weighted images were obtained at baseline and follow-up MRI (3T, Magnetom Verio or Spectra; Siemens Medical Solutions, Erlangen, Germany). The presence of CMB on T2*-weighted images was noted according to the Brain Observer MicroBleed Scale.[7] CMB were categorized into 3 groups: (1) deep CMB in the deep gray matter in the basal ganglia or thalamus, or white matter in the corpus callosum, internal, external, or extreme capsule, (2) lobar CMB in the cortical gray or subcortical white matter, and (3) subtentorial CMB in the cerebellum or brain stem. CMB in any brain region (any CMB) were also recorded. Newly developed CMB were recorded at follow-up, but not baseline, MRI. All slices were taken parallel to the orbitomeatal line from the base of the skull to the vault. The sequence parameters of T2*-weighted images were as follows: slice thickness, 4.0 mm; interslice gap, 2.0 mm; echo time, 12 ms; repetition time, 550 ms; and flip angle, 20 degrees.

Lacunar infarcts and white matter hyperintensities (WMH) were evaluated by fluid-attenuated inversion recovery images. Lacunar infarcts were defined as supratentorial hypointense lesions of 3 to 15 mm in diameter with a hyperintense rim. Periventricular hyperintensities (PVH) and deep WMH (DWMH) were scored by the Fazekas scale.[28] Sequence parameters of fluid-attenuated inversion recovery images were as follows: slice thickness, 5.0 mm; interslice gap, 1.0 mm; echo time, 94 to 114 ms; and repetition time, 12 000 ms.

Severity of SVD

Total severity of SVD was rated as described previously.[29] Briefly, 1 point was added if each SVD feature was present: ≥1 of any CMB, ≥1 of lacunar infarcts, irregular PVH extending into deep white matter (Fazekas score 3), and confluent DWMH (Fazekas score 2 or 3). Sum of ratings was used as a total SVD severity (range, 0–4).[29]

Ratings

SVD markers were independently rated by 2 neurologists. Interrater correlation coefficients were 0.87 for any CMB, 0.94 for deep CMB, 0.94 for lobar CMB, 0.93 for subtentorial CMB, 0.79 for lacunar infarcts, 0.70 for DWMH, and 0.91 for PVH.

Statistical Analyses

Variables were presented as median and interquartile range or numbers and percentages. Mann-Whitney *U* or Kruskal-Wallis test for continuous data and χ^2 or Fisher exacts test for categorical data was used. Quasi-Poisson regression models were applied for associations between *cnm*-positive *S. mutans* and number of newly developed CMB during the observational period. The incidence rate ratios (IRR) and their 95% CI were estimated. Based on previous reports,[15,27,30] age, sex, hypertension, and renal impairment were set as adjustment factors. We estimated hazard ratios by applying Cox proportional hazard models for associations between *cnm*-positive *S. mutans* and symptomatic ICH, IS, and TIA incidence. A $P<0.05$ (2-tailed) was considered statistically significant. Statistical analysis was conducted using SPSS version 26 (IBM, Armonk, NY) and SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

From the 3782 patients with acute stroke, 404 patients (11%) received oral bacterial examination (Figure I in the Data Supplement). The clinical profiles of subjects with and without bacterial assessment were similar apart from age, the National Institutes of Health Stroke Scale, and modified Rankin Scale (Table I in the Data Supplement).

We identified 111 subjects fulfilling all the criteria and found that *cnm*-positive *S. mutans* was present in 21 (19%), and absent in 90 (81%), patients. Among the 90 patients in the *cnm* (−) group, *cnm*-negative *S. mutans* was detected in 69 and no *S. mutans* in 21. Characteristics of subjects at baseline MRI are described in Table 1. Age, sex, blood pressure, and vascular risk factors were similar between *cnm* (+) and *cnm* (−) groups (systolic blood pressure: 126 mm Hg [116–134] versus 130 mm Hg [118–147], $P=0.267$; diastolic blood pressure: 76 mm Hg [64–85] versus 74 mm Hg [65–84], $P=0.946$). The 2 groups also exhibited equivalent blood pressure at follow-up evaluation (systolic blood pressure: 125 mm Hg [117–135] versus 122 mm Hg [115–135]; diastolic blood pressure: 75 mm Hg [70–80] versus 70 mm Hg [64–80]). The *cnm* (+) group showed higher, but

Table 1. Clinical Characteristics at the Baseline Evaluation

	cnm (+) group (n=21)	*cnm* (−) group (n=90)	*P* value
Age, y	73.0 (63.0–78.0)	71.5 (64.0–81.0)	0.564
Male, n (%)	14 (67)	54 (60)	0.572
Hypertension, n (%)	18 (86)	72 (80)	0.759
SBP, mm Hg	126 (116–134)	130 (118–147)	0.267
DBP, mm Hg	76 (64–85)	74 (65–84)	0.946
Diabetes, n (%)	3 (14)	22 (24)	0.396
Dyslipidemia, n (%)	13 (62)	52 (58)	0.730
Renal impairment, n (%)	10 (48)	41 (46)	0.864
Atrial fibrillation, n (%)	2 (10)	13 (14)	0.732
ATA use, n (%)	15 (71)	73 (81)	0.372
Antiplatelet agents, n (%)	12 (57)	54 (60)	0.810
Anticoagulants, n (%)	5 (24)	23 (26)	0.868
≥ 2 ATA use, n (%)	2 (10)	15 (17)	0.520
Recent IS, n (%)	7 (33)	31 (34)	1.000
Recent TIA, n (%)	1 (5)	10 (11)	0.687
Recent ICH, n (%)	1 (5)	7 (8)	1.000
Previous IS, n (%)	13 (62)	42 (47)	0.234
Previous TIA, n (%)	0 (0)	5 (6)	0.581
Previous ICH, n (%)	5 (24)	10 (11)	0.155
Smoking, n (%)	10 (48)	42 (47)	0.937
mRS	1.0 (0–3.5)	1.0 (0–3.0)	0.542
CRP, mg/dL*	0.15 (0.04–0.79)	0.08 (0.04–0.23)	0.250
Fibrinogen, mg/dL†	344 (274–415)	308 (273–358)	0.185
CMB, n (%)	12 (57)	38 (42)	0.216
Lacunar infarcts, n (%)	13 (62)	28 (31)	0.008
PVH=3, n (%)	8 (38)	13 (14)	0.026
DWMH ≥2, n (%)	16 (76)	49 (54)	0.069
Total SVD severity	3.0 (1.0–3.0)	1.0 (0–2.0)	0.004

Data represent median (interquartile range) or number (percent). ATA indicates antithrombotic agents; CMB, cerebral microbleeds; CRP, C-reactive protein; DBP, diastolic blood pressure; DWMH, deep white matter hyperintensities; ICH, intracerebral hemorrhage; IS, ischemic stroke; mRS, modified Rankin Scale; PVH, periventricular hyperintensities; SBP, systolic blood pressure; SVD, small vessel disease; and TIA, transient ischemic attack.

*CRP data was missing in 1 patient in the *cnm* (−) group.

†Fibrinogen was obtained 18 subjects in the *cnm* (+) and 70 in the *cnm* (−) group.

not significant, levels of CRP and fibrinogen than the *cnm* (−) group (CRP: 0.15 mg/dL [0.04–0.79] versus 0.08 mg/dL [0.04–0.23], *P*=0.250; fibrinogen: 344 mg/dL [274–415] versus 308 mg/dL [273–358], *P*=0.185).

CMB were detected in 12 (57%) of the *cnm* (+), and 38 (42%) of the *cnm* (−), group. Lacunar infarcts, PVH, and DWMH were commonly observed in the *cnm* (+) group (lacunar infarcts: 62% versus 31%, *P*=0.008; PVH: 38% versus 14%, *P*=0.026; DWMH: 76% versus 54%, *P*=0.069). Consequently, total SVD severity was significantly more advanced in the *cnm* (+) than *cnm* (−) group (3.0 [1.0–3.0] versus 1.0 [0–2.0], *P*=0.004).

Cerebral Microbleeds

The numbers of CMB are summarized in Table 2. The *cnm* (+) group showed a marginally increased number of CMB versus the *cnm* (−) group at baseline, especially in the deep region, but comparable in lobar and subtentorial regions (any CMB: 2.0 [0–10.5] versus 1.0 [0–5.3], *P*=0.094; deep CMB: 1.0 [0–7.5] versus 0 [0–2.0], *P*=0.091) and follow-up (any CMB: 4.0 [0.5–13.5] versus 1.0 [0–6.0], *P*=0.067; deep CMB: 2.0 [0–10.0] versus 0 [0–2.0], *P*=0.039).

We assessed the development of new CMB from baseline to follow-up MRI. The observational period was similar between the *cnm* (+) and *cnm* (−) group (509 [279–584] versus 482 [364–732] days, *P*=0.405). CMB development was significantly higher in the *cnm* (+) than *cnm* (−) group (52% versus 23%, *P*=0.008; Table 3). In particular, newly developed CMB were more frequent in deep regions (48% versus 9%, *P*<0.001) in the *cnm* (+) than *cnm* (−) group. Mean numbers of new CMB in the *cnm* (+) and *cnm* (−) groups were 2.2 versus 0.5 for any

Table 2. The Number of CMB at the Baseline and the Follow-Up MRI Scans

	Baseline MRI	Follow-up MRI
Any CMB		
cnm (+) group	2.0 (0–10.5)	4.0 (0.5–13.5)
cnm (–) group	1.0 (0–5.3)	1.0 (0–6.0)
cnm-negative *S. mutans* (+)	1.0 (0–6.0)	2.0 (0–6.5)
S. mutans (–)	0 (0–3.0)	0 (0–3.0)
Deep CMB		
cnm (+) group	1.0 (0–7.5)	2.0 (0–10.0)
cnm (–) group	0 (0–2.0)	0 (0–2.0)
cnm-negative *S. mutans* (+)	0 (0–2.0)	0 (0–3.0)
S. mutans (–)	0 (0–1.0)	0 (0–1.0)
Lobar CMB		
cnm (+) group	1.0 (0–3.5)	1.0 (0–4.5)
cnm (–) group	0 (0–1.3)	1.0 (0–2.0)
cnm-negative *S. mutans* (+)	1.0 (0–1.5)	1.0 (0–2.0)
S. mutans (–)	0 (0–1.5)	0 (0–2.5)
Subtentorial CMB		
cnm (+) group	0 (0–1.5)	0 (0–1.5)
cnm (–) group	0 (0–1.0)	0 (0–1.0)
cnm-negative *S. mutans* (+)	0 (0–1.0)	0 (0–1.0)
S. mutans (–)	0 (0–0)	0 (0–0)

Data represent median (interquartile range). Any CMB indicates CMB in any brain region; CMB, cerebral microbleeds; and MRI, magnetic resonance imaging.

CMB, 1.4 versus 0.1 for deep, 0.4 versus 0.4 for lobar, and 0.4 versus 0.1 for subtentorial.

We estimated the IRR considering newly developed CMB and observational period. IRR for CMB in deep and subtentorial, but not lobar, regions were significantly higher using unadjusted analysis (any CMB: IRR, 5.1 [95% CI, 1.9–13.6], P=0.001; deep CMB: IRR, 15.0 [95% CI, 5.4–42.0], P<0.001; subtentorial CMB: IRR, 6.4 [95% CI, 1.3–30.9], P=0.020; lobar CMB: IRR, 1.3 [95% CI 0.2–7.7], P=0.808). Statistical significance for any and deep CMB was confirmed after adjusting for age, sex, hypertension, and renal impairment (any CMB: IRR, 4.7 [95% CI, 1.8–11.9], P=0.001; deep CMB: IRR, 13.9 [95% CI, 4.3–44.5], P<0.001; Table 4). Representative images showing the increase in deep CMB are illustrated in Figure II in the Data Supplement.

Table 3. The Frequency of New CMB Development

	cnm (+) group	*cnm* (–) group	
	(n=21)	(n=90)	*P* value
Any CMB, n (%)	11 (52)	21 (23)	0.008
Deep CMB, n (%)	10 (48)	8 (9)	<0.001
Lobar CMB, n (%)	4 (19)	13 (14)	0.736
Subtentorial CMB, n (%)	4 (19)	5 (6)	0.064

Any CMB indicates CMB in any brain region; and CMB, cerebral microbleeds.

Table 4. IRRs of Newly Developed CMB

	Unadjusted		Adjusted*	
	IRR (95% CI)	*P* value	IRR (95% CI)	*P* value
Any CMB	5.1 (1.9–13.6)	0.001	4.7 (1.8–11.9)	0.001
Deep CMB	15.0 (5.4–42.0)	<0.001	13.9 (4.3–44.5)	<0.001
Lobar CMB	1.3 (0.2–7.7)	0.808	1.2 (0.2–5.7)	0.826
Subtentorial CMB	6.4 (1.3–30.9)	0.020	5.8 (0.9–35.5)	0.060

Any CMB indicates CMB in any brain region; CMB, cerebral microbleeds; and IRR, incidence rate ratios.

*Adjusted for age, sex, hypertension, and renal impairment.

Progression of Other SVD Markers

We next evaluated progression of SVD features other than CMB. Frequency of lacunar infarcts (*cnm* [+] versus *cnm* [–]: 67% versus 36%), PVH (38% versus 17%), and DWMH (76% versus 57%) on follow-up MRI was subtly increased from baseline. The change in frequency of each SVD feature other than CMB during the observation period was equivalent between *cnm* (+) and *cnm* (–) groups (lacunar infarcts: 5% versus 4%, P=1.000; PVH: 0% versus 2%, P=1.000; DWMH: 0% versus 2%, P=1.000).

Stroke and TIA

Symptomatic stroke and TIA frequency during the observation period was investigated. ICH, IS, and TIA incidence was similar in *cnm* (+) and *cnm* (–) groups (ICH: 2 [10%] versus 3 [3%], hazard ratios, 5.3 [95% CI, 0.7–38.8]; IS: 6 [29%] versus 23 [26%], hazard ratios, 1.4 [95% CI, 0.6–3.4]; TIA, 1 [5%] versus 3 [3%], hazard ratios, 1.7 [95% CI, 0.2–16.8]).

Comparison Between the 3 Groups

S. mutans, whether *cnm* positive or not, may contribute to mycotic aneurysms and cerebral hemorrhage.[31] We, therefore, compared the 3 groups: (1) subjects with *cnm*-positive *S. mutans*, (2) those with *cnm*-negative *S. mutans*, and (3) those without *S. mutans*. Background profiles were similar among the 3 groups, except for some imaging markers of SVD, such as lacunar infarcts, WMH, and total SVD severity (Table II in the Data Supplement). Development of CMB was most prominent in *cnm*-positive *S. mutans* subjects (Table III in the Data Supplement). No significant difference was observed between subjects with *cnm*-negative *S. mutans* and those without *S. mutans*.

DISCUSSION

We found harboring *cnm*-positive *S. mutans* was closely related to an increased incidence of CMB, especially in the deep area, together with a high prevalence of lacunar infarcts and WMH.

The strong linkage of *cnm*-positive *S. mutans* and deep CMB aligns with previous cross-sectional studies.[14,15] Estimated IRR for deep CMB was high in comparison with other known risk factors in previous reports.[30,32,33] Deep CMB were considered as biomarkers for hypertensive arteriopathy,[3,34] but their pathogenesis cannot be fully explained by hypertension, as they are occasionally found in subjects without high blood pressure.[14,32]

An important hallmark of *S. mutans* expressing Cnm protein[13] is its binding activity to components of vascular BM, such as collagen-IV[17] and laminin.[18] Collagen-binding activity is positively correlated with *cnm* mRNA expression in *S. mutans.*[23] Conversely, neither *cnm*-negative *S. mutans* nor *cnm* knockout strains of *S. mutans* can attach to soft tissues such as vessel walls.[17] Aging and hypertension induce endothelial injury and BM thickening, resulting in collagen-IV and laminin exposure in cerebral small arteries.[35,36] Once *cnm*-positive *S. mutans* adheres to BM, infiltration of neutrophils may activate local inflammation, increasing blood-brain barrier permeability, and production of enzymes, such as matrix metalloproteinase-9,[19] inducing ICH or CMB (Figure). Endothelial injury related to aging and hypertension is prominent in the deep cerebral vessels,[37] likely contributing to an increase in the deep CMB rather than lobar CMB, by *cnm*-positive *S. mutans*.

Furthermore, unlike *cnm*-negative *S. mutans,* the *cnm*-positive *S. mutans* can suppress collagen-induced platelet aggregation.[19] All *S. mutans*, whether *cnm*-positive or not, have negative zeta potential values, an indicator of cell-surface charge, although *cnm*-positive *S. mutans* possesses lower zeta potential values.[19] Since platelets also possess negative potentials, *cnm*-positive *S. mutans* may inhibit platelet adhesion and aggregation,

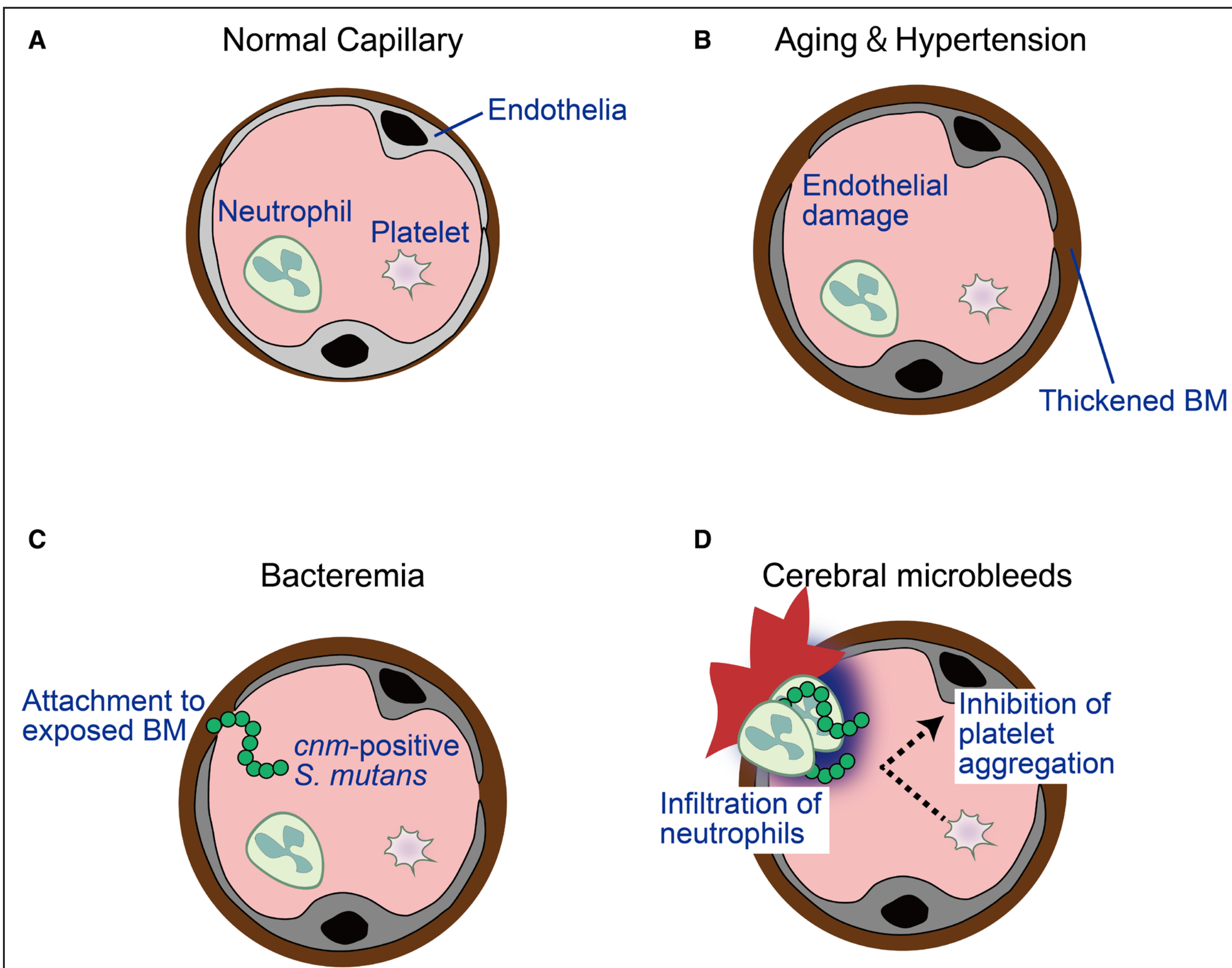

Figure. Hypothetical model of the mechanism contributing to develop cerebral microbleeds (CMB) by the infection of *cnm*-positive Streptococcus mutans *(S. mutans)*.

A, Normal vessel. Cerebral bleeding may occur at the level of arterioles and capillaries. **B**, Aging and hypertension results in endothelial damage and thickened basement membranes (BM). **C**, Bacteremia of *S. mutans* are induced by brushing, flossing or tooth extraction. Unlike *cnm*-negative *S. mutans, cnm*-positive *S. mutans* can attach to the BM. **D**, Once *cnm*-positive *S. mutans* binds to the vessel wall, infiltration of neutrophils results in local inflammation. The negative charges on the surface of *cnm*-positive *S. mutans* inhibit aggregation of platelets, which also possess negative charges on the surfaces. CMB are eventually induced.

accelerating thus cerebral bleeding. Zeta potential values differ among strains of *cnm*-positive *S. mutans* and lower zeta potential values significantly correlate with decreased collagen-induced platelet aggregation.[19]

We previously reported *cnm*-positive *S. mutans* is significantly associated with severe dental caries.[23] *S. mutans* expressing collagen-binding protein can strongly bind to the type-I collagen-composed dentin tooth layer, accelerating development of carious lesions.[17] The increased predisposition of *cnm*-positive *S. mutans* to invade dental caries provides opportunities for *S. mutans* to enter the bloodstream and cerebral circulation. Poor oral health could facilitate dental bacteremia and cerebrovascular health.[17,38] The collagen-binding activity of *cnm*-positive *S. mutans* to type-I collagen in teeth and type-IV collagen in cerebrovascular BM facilitates CMB.

S. mutans, including *cnm*-positive, are commonly transmitted by vertical infection, colonizing mouths of infants at around 2 years.[23,39] Mothers and caretakers of children are the major sources of *S. mutans*,[40] which generally remain after colonization[16] but are not easily implanted again in adulthood.[41,42] Therefore, preventing vertical *cnm*-positive *S. mutans* infection could represent a major preventative factor in SVD and CMB.

Here, overall prevalence of CMB was 45%, higher than previous IS cohorts,[9,43] and equivalent to IS and ICH mixed stroke cohorts.[43] The current study included about 20% of subjects with a history of ICH. Additionally, high magnetic field strength of MRI may have affected CMB frequency. Only patients receiving 3T-MRI scans were included, which is suitable for CMB detection and superior to 1.5T-MRI.[5]

Although CMB may predict future ICH,[3,4,44] the incidence of symptomatic ICH in the *cnm* (+) group was similar to the *cnm* (−) group. Circulating inflammatory marker level was increased, but nonsignificantly, in the *cnm* (+) group, which may be a consequence of a small sample size. Thus, to definitively establish an association between *cnm*-positive *S. mutans*, symptomatic ICH, and inflammatory marker levels, a large-scale prospective investigation is warranted. This study leads to new hypotheses and provides useful data to guide power calculations and effect sizes for future larger-scale investigations.

There are some limitations to this study. First, it involved Japanese subjects only, making predictions for other countries uncertain and demonstrating the need for multinational validation studies. Second, this was a retrospective study, posing potential risk of selection bias. Only 11% of the total stroke patients had oral bacterial evaluation due to age and factors, leading to difficulty providing informed consent, such as impaired consciousness, cognitive impairments, and advanced frailty. This resulted in the lower age and scores of National Institutes of Health Stroke Scale and modified Rankin Scale in patients receiving bacterial assessments (Table I in the Data Supplement). Finally, all patients had a history of stroke and the effect of *cnm*-positive *S. mutans* on CMB development should be examined in a population-based cohort in any future study.

In conclusion, *cnm*-positive *S. mutans* was associated with increased CMB incidence. Though the results should be verified by large-scale prospective studies, a close association between *cnm*-positive *S. mutans* and CMB development suggests treatments targeting *cnm*-positive *S. mutans* may act as novel therapeutic approaches for dementia and stroke.

ARTICLE INFORMATION

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Supplemental Materials

Tables I–III
Figures I–II

REFERENCES

1. Ihara M, Yamamoto Y. Emerging evidence for pathogenesis of sporadic cerebral small vessel disease. *Stroke*. 2016;47:554–560. doi: 10.1161/STROKEAHA.115.009627
2. Akoudad S, Wolters FJ, Viswanathan A, de Bruijn RF, van der Lugt A, Hofman A, Koudstaal PJ, Ikram MA, Vernooij MW. Association of cerebral microbleeds with cognitive decline and dementia. *JAMA Neurol*. 2016;73:934–943. doi: 10.1001/jamaneurol.2016.1017
3. Pasi M, Cordonnier C. Clinical relevance of cerebral small vessel diseases. *Stroke*. 2020;51:47–53. doi: 10.1161/STROKEAHA.119.024148
4. Debette S, Schilling S, Duperron MG, Larsson SC, Markus HS. Clinical significance of magnetic resonance imaging markers of vascular brain injury:

a systematic review and meta-analysis. *JAMA Neurol*. 2019;76:81–94. doi: 10.1001/jamaneurol.2018.3122

5. Nandigam RN, Viswanathan A, Delgado P, Skehan ME, Smith EE, Rosand J, Greenberg SM, Dickerson BC. MR imaging detection of cerebral microbleeds: effect of susceptibility-weighted imaging, section thickness, and field strength. *AJNR Am J Neuroradiol*. 2009;30:338–343. doi: 10.3174/ajnr.A1355
6. Corrêa DG, Cruz Júnior LC, Bahia PR, Gasparetto EL. Intracerebral microbleeds in sepsis: susceptibility-weighted MR imaging findings. *Arq Neuropsiquiatr*. 2012;70:903–904. doi: 10.1590/s0004-282x2012001100017
7. Cordonnier C, Potter GM, Jackson CA, Doubal F, Keir S, Sudlow CL, Wardlaw JM, Al-Shahi Salman R. improving interrater agreement about brain microbleeds: development of the brain observer microbleed scale (BOMBS). *Stroke*. 2009;40:94–99. doi: 10.1161/STROKEAHA.108.526996
8. van Veluw SJ, Biessels GJ, Klijn CJ, Rozemuller AJ. Heterogeneous histopathology of cortical microbleeds in cerebral amyloid angiopathy. *Neurology*. 2016;86:867–871. doi: 10.1212/WNL.0000000000002419
9. Pétrault M, Casolla B, Ouk T, Cordonnier C, Bérézowski V. Cerebral microbleeds: beyond the macroscope. *Int J Stroke*. 2019;14:468–475. doi: 10.1177/1747493019830594
10. Miwa K, Tanaka M, Okazaki S, Furukado S, Sakaguchi M, Kitagawa K. Relations of blood inflammatory marker levels with cerebral microbleeds. *Stroke*. 2011;42:3202–3206. doi: 10.1161/STROKEAHA.111.621193
11. Sumbria RK, Grigoryan MM, Vasilevko V, Krasieva TB, Scadeng M, Dvornikova AK, Paganini-Hill A, Kim R, Cribbs DH, Fisher MJ. A murine model of inflammation-induced cerebral microbleeds. *J Neuroinflammation*. 2016;13:218. doi: 10.1186/s12974-016-0693-5
12. Okazaki S, Sakaguchi M, Hyun B, Nagano K, Tagaya M, Sakata Y, Sakaguchi T, Kitagawa K. Cerebral microbleeds predict impending intracranial hemorrhage in infective endocarditis. *Cerebrovasc Dis*. 2011;32:483–488. doi: 10.1159/000331475
13. Ito S, Misaki T, Naka S, Wato K, Nagasawa Y, Nomura R, Otsugu M, Matsumoto-Nakano M, Nakano K, Kumagai H, et al. Specific strains of *Streptococcus mutans*, a pathogen of dental caries, in the tonsils, are associated with IgA nephropathy. *Sci Rep*. 2019;9:20130. doi: 10.1038/s41598-019-56679-2
14. Watanabe I, Kuriyama N, Miyatani F, Nomura R, Naka S, Nakano K, Ihara M, Iwai K, Matsui D, Ozaki E, et al. Oral Cnm -positive Streptococcus mutans expressing collagen binding activity is a risk factor for cerebral microbleeds and cognitive impairment. *Sci Rep*. 2016;6:38561.
15. Tonomura S, Ihara M, Kawano T, Tanaka T, Okuno Y, Saito S, Friedland RP, Kuriyama N, Nomura R, Watanabe Y, et al. Intracerebral hemorrhage and deep microbleeds associated with cnm-positive *Streptococcus mutans*; a hospital cohort study. *Sci Rep*. 2016;6:20074. doi: 10.1038/srep20074
16. Nomura R, Ogaya Y, Nakano K. Contribution of the collagen-binding proteins of *Streptococcus mutans* to bacterial colonization of inflamed dental pulp. *PLoS One*. 2016;11:e0159613. doi: 10.1371/journal.pone.0159613
17. Nomura R, Naka S, Nemoto H, Otsugu M, Nakamura S, Ooshima T, Nakano K. Potential high virulence for infective endocarditis in *Streptococcus mutans* strains with collagen-binding proteins but lacking PA expression. *Arch Oral Biol*. 2013;58:1627–1634. doi: 10.1016/j.archoralbio.2013.06.008
18. Sato Y, Okamoto K, Kagami A, Yamamoto Y, Igarashi T, Kizaki H. *Streptococcus mutans* strains harboring collagen-binding adhesin. *J Dent Res*. 2004;83:534–539. doi: 10.1177/154405910408300705
19. Nakano K, Hokamura K, Taniguchi N, Wada K, Kudo C, Nomura R, Kojima A, Naka S, Muranaka Y, Thura M, et al. The collagen-binding protein of *Streptococcus mutans* is involved in haemorrhagic stroke. *Nat Commun*. 2011;2:485. doi: 10.1038/ncomms1491
20. Lockhart PB, Brennan MT, Sasser HC, Fox PC, Paster BJ, Bahrani-Mougeot FK. Bacteremia associated with toothbrushing and dental extraction. *Circulation*. 2008;117:3118–3125. doi: 10.1161/CIRCULATIONAHA.107.758524
21. Fernandes CP, Oliveira FA, Silva PG, Alves AP, Mota MR, Montenegro RC, Burbano RM, Seabra AD, Lobo Filho JG, Lima DL, et al. Molecular analysis of oral bacteria in dental biofilm and atherosclerotic plaques of patients with vascular disease. *Int J Cardiol*. 2014;174:710–712. doi: 10.1016/j.ijcard.2014.04.201
22. Inenaga C, Hokamura K, Nakano K, Nomura R, Naka S, Ohashi T, Ooshima T, Kuriyama N, Hamasaki T, Wada K, et al. A potential new risk factor for stroke: *streptococcus mutans* with collagen-binding protein. *World Neurosurg*. 2018;113:e77–e81. doi: 10.1016/j.wneu.2018.01.158
23. Nomura R, Nakano K, Taniguchi N, Lapirattanakul J, Nemoto H, Grönroos L, Alaluusua S, Ooshima T. Molecular and clinical analyses of the gene encoding the collagen-binding adhesin of *Streptococcus mutans*. *J Med Microbiol*. 2009;58(pt 4):469–475. doi: 10.1099/jmm.0.007559-0
24. Momeni SS, Ghazal T, Grenett H, Whiddon J, Moser SA, Childers NK. *Streptococcus mutans* serotypes and collagen-binding proteins Cnm/Cbm in children with caries analysed by PCR. *Mol Oral Microbiol*. 2019;34:64–73. doi: 10.1111/omi.12254
25. Lamba GS, Dufour D, Nainar SMH, Cioffi I, Lévesque CM, Gong SG. Association of *Streptococcus mutans* collagen binding genes with severe childhood caries. *Clin Oral Investig*. 2020;24:3467–3475. doi: 10.1007/s00784-020-03217-4
26. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A; Collaborators developing the Japanese equation for estimated GFR. Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis*. 2009;53:982–992. doi: 10.1053/j.ajkd.2008.12.034
27. Kim SH, Shin DW, Yun JM, Lee JE, Lim JS, Cho BL, Kwon HM, Park JH. Kidney dysfunction and cerebral microbleeds in neurologically healthy adults. *PLoS One*. 2017;12:e0172210. doi: 10.1371/journal.pone.0172210
28. Fazekas F, Chawluk JB, Alavi A, Hurtig HI, Zimmerman RA. MR signal abnormalities at 1.5 T in Alzheimer's dementia and normal aging. *AJR Am J Roentgenol*. 1987;149:351–356. doi: 10.2214/ajr.149.2.351
29. Hatate J, Miwa K, Matsumoto M, Sasaki T, Yagita Y, Sakaguchi M, Kitagawa K, Mochizuki H. Association between cerebral small vessel diseases and mild parkinsonian signs in the elderly with vascular risk factors. *Parkinsonism Relat Disord*. 2016;26:29–34. doi: 10.1016/j.parkreldis.2016.02.011
30. Poels MM, Ikram MA, van der Lugt A, Hofman A, Krestin GP, Breteler MM, Vernooij MW. Incidence of cerebral microbleeds in the general population: the Rotterdam Scan Study. *Stroke*. 2011;42:656–661. doi: 10.1161/STROKEAHA.110.607184
31. Alawieh A, Chaudry MI, Turner RD, Turk AS, Spiotta AM. Infectious intracranial aneurysms: a systematic review of epidemiology, management, and outcomes. *J Neurointerv Surg*. 2018;10:713–721.
32. Liu W, Liu R, Sun W, Peng Q, Zhang W, Xu E, Cheng Y, Ding M, Li Y, Hong Z, et al; CASISP Study Group. Different impacts of blood pressure variability on the progression of cerebral microbleeds and white matter lesions. *Stroke*. 2012;43:2916–2922. doi: 10.1161/STROKEAHA.112.658369
33. Akoudad S, Aarts N, Noordam R, Ikram MA, Tiemeier H, Hofman A, Stricker BH, Vernooij MW, Visser LE. Antidepressant use is associated with an increased risk of developing microbleeds. *Stroke*. 2016;47:251–254. doi: 10.1161/STROKEAHA.115.011574
34. Vernooij MW, van der Lugt A, Ikram MA, Wielopolski PA, Niessen WJ, Hofman A, Krestin GP, Breteler MM. Prevalence and risk factors of cerebral microbleeds: the Rotterdam Scan Study. *Neurology*. 2008;70:1208–1214. doi: 10.1212/01.wnl.0000307750.41970.d9
35. Farrall AJ, Wardlaw JM. Blood-brain barrier: ageing and microvascular disease–systematic review and meta-analysis. *Neurobiol Aging*. 2009;30:337–352. doi: 10.1016/j.neurobiolaging.2007.07.015
36. Burns EM, Kruckeberg TW, Gaetano PK. Changes with age in cerebral capillary morphology. *Neurobiol Aging*. 1981;2:283–291. doi: 10.1016/0197-4580(81)90037-3
37. Lammie GA. Pathology of small vessel stroke. *Br Med Bull*. 2000;56:296–306. doi: 10.1258/0007142001903229
38. Meurman JH, Hämäläinen P. Oral health and morbidity–implications of oral infections on the elderly. *Gerodontology*. 2006;23:3–16. doi: 10.1111/j.1741-2358.2006.00102.x
39. Caufield PW, Cutter GR, Dasanayake AP. Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity. *J Dent Res*. 1993;72:37–45. doi: 10.1177/00220345930720010501
40. Lapirattanakul J, Nakano K. Mother-to-child transmission of mutans streptococci. *Future Microbiol*. 2014;9:807–823. doi: 10.2217/fmb.14.37
41. Krasse B, Edwardsson S, Svensson I, Trell L. Implantation of caries-inducing streptococci in the human oral cavity. *Arch Oral Biol*. 1967;12:231–236. doi: 10.1016/0003-9969(67)90042-8
42. Berkowitz RJ, Jordan HV, White G. The early establishment of *Streptococcus mutans* in the mouths of infants. *Arch Oral Biol*. 1975;20:171–174. doi: 10.1016/0003-9969(75)90005-9
43. Cordonnier C, Al-Shahi Salman R, Wardlaw J. Spontaneous brain microbleeds: systematic review, subgroup analyses and standards for study design and reporting. *Brain*. 2007;130(pt 8):1988–2003. doi: 10.1093/brain/awl387
44. Wilson D, Charidimou A, Ambler G, Fox ZV, Gregoire S, Rayson P, Imaizumi T, Fluri F, Naka H, Horstmann S, et al. Recurrent stroke risk and cerebral microbleed burden in ischemic stroke and TIA: A meta-analysis. *Neurology*. 2016;87:1501–1510. doi: 10.1212/WNL.0000000000003183