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ORIGINAL RESEARCH

Association of Subgingival Microbiota Composition With Risk, Severity, and Outcome of Cryptogenic Ischemic Stroke in Young Adults

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BACKGROUND: Oral and gut dysbiosis has been linked to stroke pathogenesis and its prognosis. However, the relationship between the subgingival microbiota and cryptogenic ischemic stroke (CIS) remains unclear. We compared the subgingival microbiota of patients with CIS and their age-and sex-matched stroke-free controls to identify the specific microbiota associated with CIS, stroke symptom severity, and clinical outcome.

METHODS: This multicenter, case–control study was conducted between 2013 and 2019 as part of a screening protocol for the SECRETO (Searching for Explanations for Cryptogenic Stroke in the Young: Revealing the Etiology, Triggers, and Outcome) study (NCT01934725). Stroke severity was assessed using the National Institutes of Health Stroke Scale score. After thorough clinical and radiographic oral examinations, subgingival samples were collected and analyzed using 16S rRNA gene sequencing.

RESULTS: A total of 272 participants (134 patients and 138 controls) were included. There were no differences in the following clinical characteristics between patients and controls: diabetes, hypertension, smoking, alcohol use, abdominal obesity, physical activity, and chronic multiorgan disease. Beta diversity differed significantly between patients and controls ($P<0.05$). The abundance of *Spirochaetota* and *Treponema* was higher and *Pseudomonadota*, *Veillonella*, and *Capnocytophaga* were lower in patients than in controls ($P<0.05$). The abundance of *T. denticola* was associated with an increased risk of CIS (odds ratio [OR], 1.002 [95% CI, 1.000–1.003], $P=0.023$), and this association persisted after adjusting for relevant comorbidities and medications (OR, 1.002 [95% CI, 1.000–1.003], $P=0.021$).

CONCLUSIONS: The subgingival microbiota is associated with CIS, suggesting a possible link between oral health and stroke pathophysiology. Although causality cannot be proven, oral microbiota may be a modifiable treatment target for the prevention of CIS and improving its outcome.

Key Words: 16S rRNA gene sequencing ■ modified Rankin Scale ■ stroke severity ■ subgingival microbiome ■ young-onset stroke

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CLINICAL PERSPECTIVE

What Is New?

- Young adults with cryptogenic ischemic stroke have distinct subgingival microbiota, including a greater abundance of *Treponema denticola*, which is associated with increased stroke risk, cognitive impairment, and poorer clinical outcomes.

What Are the Clinical Implications?

- Assessment and management of oral health, including periodontal evaluation and plaque control, may be a modifiable target to reduce stroke risk, improve recovery, and support secondary prevention strategies in young adults.

Nonstandard Abbreviations and Acronyms

CIS	cryptogenic ischemic stroke
SVD	small-vessel disease
WMH	white matter hyperintensities

Cryptogenic ischemic stroke (CIS) accounts for 15% to 30% of all ISs in general, despite intensive diagnostic workup.[1] The cause, incidence, and risk factors of IS in young adults differ from those in older adults, with an estimated 10% to 20% of cases occurring in individuals aged 18 to 50 years.[2] Hypertension, dyslipidemia, and cigarette smoking are frequent risk factors in young patients with IS.[2–4] In particular, abdominal obesity,[5] migraine,[6] and heavy alcohol consumption[7] have recently emerged as important risk factors for young-onset CIS. Additionally, emerging evidence links oral health and periodontal disease to CIS. We and others previously found that young patients with CIS more often had periodontal disease compared with stroke-free controls and that severity of periodontal disease gradually increases stroke risk.[8,9]

Recent studies have further highlighted a potential link between the oral microbiota and stroke risk. We previously found that dysbiosis of the salivary microbiome is associated with CIS in young adults.[10,11] Similarly, increased salivary microbial diversity in patients with IS predicts stroke severity.[12] Additionally, among older Chinese women, a higher abundance of oral *Corynebacterium*, *Lautropia*, and *Selenomonas* was associated with an elevated risk of stroke.[13] Furthermore, an animal model revealed that periodontitis-associated salivary microbiota exacerbates IS outcomes through IL-17A (interleukin-17A)-mediated neuroinflammation.[14]

Despite emerging evidence of an oral-systemic connection, the relationship between the subgingival microbiome and CIS remains largely unexplored.

We hypothesized that the composition and diversity of the subgingival microbiota in young CIS patients may differ from that in stroke-free controls, potentially influencing stroke severity and functional outcomes and contributing to systemic inflammation. To test this hypothesis, we analyzed the subgingival microbial communities in patients with CIS and identified bacterial taxa potentially associated with CIS.

METHODS

Data Availability

The raw sequence files for the 16S rRNA gene are deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB82979.

Ethical Approval, Compliance, and Participant Consent

The study was conducted in accordance with the Declaration of Helsinki and the Strengthening the Reporting of Observational Studies in Epidemiology reporting guideline.[15] This clinical study received approval from the Helsinki and Uusimaa Hospital District (institutional review board number 362/13/03/00/2012) and the local ethics committees at each recruiting center before its initiation. All participants were fully informed about the study and gave written informed consent.

Study Population

SECRETO Oral was a substudy of the SECRETO (Searching for Explanations for Cryptogenic Stroke in the Young: Revealing the Etiology, Triggers, and Outcome) study, an international, prospective, multicenter, case–control study. All participants were examined using a standardized protocol as previously described.[16] This study enrolled adults aged 18 to 49 years who were hospitalized due to a first-ever imaging-positive IS of undetermined cause. SECRETO Oral participants were prospectively recruited between November 2013 and December 2019 at the University Hospitals of Helsinki and Turku, Finland.

In brief, the SECRETO Oral cohort[9,10] included data from 169 patients who underwent extensive diagnostic workup. This included brain magnetic resonance imaging, imaging of intracranial and extracranial brain-supplying vessels using either computed tomography angiography or magnetic resonance angiography, echocardiography, 12-lead electrocardiography, continuous ECG monitoring for at least 24 hours,

transthoracic echocardiography, transesophageal echocardiography, laboratory testing per protocol, and screening for coagulopathies. Ancillary testing was performed according to the discretion of the attending physician.

Stroke severity was assessed using the National Institutes of Health Stroke Scale score, which ranges from 0 to 42 (higher scores indicate greater severity). Stroke severity on hospital admission was classified into the following 3 categories: (1) score 0, no symptoms on admission; (2) score 1 to 4, mild symptoms; and (3) score ≥5, moderate-to-severe symptoms. CIS was defined according to the Atherosclerosis, Small Vessel Disease, Cardiac Source, Other Cause classification.[17] Pathologies were categorized as absence of disease (grade 0), grade II (causality uncertain), or grade III (unlikely a direct cause), using diagnostic tests with the highest level of evidence.

The modified Rankin Scale was used to evaluate functional outcomes in patients with CIS at 3 months post stroke, with a cutoff of modified Rankin Scale score 0 to 1 (favorable outcome) versus >1 (unfavorable outcome).[18] The Montreal Cognitive Assessment was used to evaluate cognitive function in patients, categorizing cognitive problems at 3 months into the following 2 groups: scores<26 (cognitive impairment) or scores between 26 and 30 (normal cognitive function). Anxiety and depressive symptoms were measured using the Hospital Anxiety and Depression Scale, with scores categorized as <8 (normal) or ≥8 (indicative of anxiety/depression).[19]

To evaluate whether microbial diversity and composition are associated with adverse stroke outcomes through the presence of cerebral small-vessel disease (SVD), neuroimaging studies were performed at the Imaging Core Laboratory at the Medical University of Graz by 3 experienced raters blinded to demographic and clinical information according to a prespecified protocol.[20,21] SVD features on brain magnetic resonance imaging were rated according to the Standards for Reporting Vascular Changes on Neuroimaging-1 criteria.[22] Any sign of SVD was defined as the presence of a recent small subcortical infarct in the supply area of a single penetrating artery (compatible with acute "lacunar" stroke), a (chronic) lacunar lesion, or white matter hyperintensities (WMH), and was categorized as absent or present. No patients had cerebral microbleeds, and enlarged perivascular spaces were not assessed.

Stroke-Free Control Group

To identify the microbial risk factors associated with patients with CIS compared with the general population, each CIS case was matched to a control at each recruitment site following the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.[15] Patients were age and sex matched in a 1:1 ratio to stroke-free controls. The matching criteria included sex, age (±5 years), and ethnicity to ensure comparability between cases and controls. Controls met the following specific criteria to be eligible for participation: age 18 to 49 years; absence of prior stroke (assessed by reviewing all statewide electronic medical records and with the Questionnaire for Verifying Stroke-Free Status)[23]; underwent clinical oral examinations; and refrained from antibiotic usage within 1 month before subgingival sample collection.

Identification of Relevant Comorbidities and Lifestyle Factors

At the time of recruitment, all participants underwent a thorough structured interview, and a detailed clinical history was obtained from the medical records. Relevant comorbidities were identified, including diabetes, hypertension, current or former tobacco smoking, heavy alcohol consumption, abdominal obesity, chronic multiorgan disease (any of inflammatory bowel disease or autoimmune disease), depression, and migraine with aura (assessed using a validated migraine screener).[6] Antibiotic use within the preceding 3 months and illicit drug use within the past 12 months were also recorded. Physical inactivity was defined using the short version of the International Physical Activity Questionnaire; participants were categorized as physically inactive if they did not meet the criteria for moderate or high levels of physical activity.[24] The presence of a patent foramen ovale (PFO) was determined either by echocardiography or by detecting a right-to-left shunt using a transcranial Doppler ultrasound with a bubble screen. All available information from transthoracic echocardiography, transesophageal echocardiography, and transcranial Doppler bubble studies was used to identify and define high-risk PFO, which was defined as PFO with an atrial septal aneurysm or a large-sized shunt (≥25 microbubbles crossing the atrial septum on transesophageal echocardiography or detected by transcranial Doppler).

Clinical Oral Examination and Sample Collection

Clinical oral examinations were conducted by the same periodontal specialist (S.P.) in a standard dental office setting at the Universities of Helsinki and Turku between April 2014 and February 2020 to avoid interexaminer differences.[9] Dental health indicators, including number of missing teeth, mucosal lesions or conditions, and frequency of dental visits, were recorded. Caries lesions were diagnosed in clinical settings using the complete International Caries Detection and Assessment System

code. Periodontitis staging and grading were determined based on (dental) panoramic radiographs.[25]

Subgingival plaque samples were collected from the deepest periodontal pocket in each quadrant, using a periodontal curette, and were pooled together. The collected samples were suspended in 500 μL of sterile water and immediately stored at −20 °C. Samples were then transported to the laboratory in a frozen state and stored at −80 °C until processing. The median time between CIS (for cases) or recruitment (for controls) and oral examination, which included subgingival sample collection, was 112 days (interquartile range, 92–140 days) for cases and 68 days (interquartile range, 43–135 days) for controls.

Subgingival Microbiota Analysis

DNA extraction was performed as previously described.[26] Subgingival samples were collected, and 500 μL of Milli-Q water was added to the samples. A 300 μL aliquot of the resulting mixture was then combined with 300 μL of lysis buffer. The samples were then homogenized and DNA extraction was performed using the Chemagic 360 instrument (PerkinElmer). The V1–V2 hypervariable region of the 16S rRNA gene was amplified using the Phusion Green Hot Start II High-Fidelity PCR Master Mix and a Bio-Rad C1000 Touch thermal cycler. DNA concentration was quantified using a PicoGreen dsDNA Assay. Library preparations were performed following the 16S protocol and sequenced on an Illumina MiSeq platform (250 bp paired-end) at the QMUL Genome Center.

Postsequencing data processing, including trimming, filtering, and chimera removal, was conducted using the DADA2 (v.1.14.1).[27] Taxonomy was then assigned to amplicon sequence variants using the Human Oral Microbiome Database (www.homd.org/;HOMD;v.15.1).

Statistical Analysis

Statistical analysis was performed using R software (v.4.2.2). Baseline characteristics are summarized with descriptive statistics. Data normality was assessed using the Shapiro–Wilk test. Statistical tests included the Mann–Whitney *U* test, Kruskal–Wallis test, χ^2 test, and independent *t* test as appropriate for the data type.

Alpha diversity was assessed using the total number of observed amplicon sequence variants, the Shannon and the inverse Simpson diversity indexes. Beta diversity was evaluated using Bray–Curtis dissimilarity and Jaccard distances, and the results were visualized using principal coordinate analysis plots. To identify significantly different taxa between groups, linear discriminant analysis effect size (LEfSe)[28] analysis was conducted at various taxonomic levels (phylum, genus, and species). Taxa were considered significantly different if the linear discriminant analysis score exceeded 4.

Stratified analyses by periodontitis were conducted using logistic regression for alpha diversity indices. Differences in alpha diversity between participants with and without periodontitis were tested using the Wilcoxon rank-sum test. Trends in subgingival taxa across stroke severity groups were assessed using the Kruskal–Wallis test based on centered log ratio-transformed abundances. Taxa with a *P* value <0.05 were considered significant.

Subgingival Microbial Dysbiosis Index Computation

The genus-level subgingival microbial dysbiosis index[29] indices were calculated for each sample as the difference in the mean centered log ratio bacterial abundance of dysbiotic and normobiotic taxa using the subgingival microbial dysbiosis index online calculator (https://bioinformatics.forsyth.org/smdi/index.php). A summary variable was calculated as the per-sample sum of the relative abundances of significant species identified as enriched in CIS. Spearman correlations were computed to assess the relationships between the summary variables and diversity metrics. We assessed the association between the summed relative abundance of significant subgingival taxa and CIS using conditional logistic regression. Models were adjusted for age and antibiotic use.

RESULTS

Clinical Characteristics

A total of 272 participants (134 patients with CIS and 138 stroke-free controls) were included (Figure SI). The characteristics of patients with CIS and controls are summarized in Table. Age, sex, diabetes, hypertension, smoking, heavy alcohol use, abdominal obesity, physical activity, depression, chronic multiorgan disease, antibiotic use in the preceding 3 months, statin use, and periodontitis did not differ between patients with CIS and controls. However, migraine with aura (*P*<0.001), binge drinking (*P*=0.016), carious lesions (*P*=0.016), and gingival bleeding on probing (*P*=0.004) were more frequent in patients than controls.

Compared with controls, patients had fewer regular dentist visits and less often had postsecondary nontertiary education or tertiary education than controls (*P*=0.041 and *P*=0.014, respectively). Among participants, 145 (53.3%) had PFO, including 71 (26.1%) with high-risk PFOs, with significant differences observed between the groups (*P*<0.001) (Table). During the 3-month follow-up, 6 patients experienced recurrent

Table. Characteristics of Study Populations

	Patients (n=134)	Controls (n=138)	*P* value
Sex, male	81 (60.4)	85 (61.6)	0.846
Mean age±SD, y	39.4 (7.7)	40.1 (7.6)	0.462
Education*	66 (49.3)	86 (62.3)	0.014
Diabetes diagnosis	4 (3.0)	6 (4.3)	0.550
Hypertension	31 (23.1)	25 (18.1)	0.306
Smoking	66 (49.3)	57 (41.3)	0.188
Heavy alcohol use	23 (17.2)	13 (9.4)	0.060
Abdominal obesity	71 (53)	65 (47.1)	0.332
Physical activity†	28 (20.9)	27 (19.6)	0.855
Depression‡	37 (27.6)	29 (21.6)	0.257
Chronic multiorgan disease	22 (16.4)	18 (13.0)	0.432
Migraine with aura§	59 (44.0)	15 (10.9)	<0.001
Antibiotics (preceding 3 mo)	9 (6.7)	6 (4.3)	0.392
Any illicit drug use (past 12 mo)	7 (5.2)	6 (4.3)	0.735
Statin use	3 (2.2)	10 (7.2)	0.053
Binge drinking	21 (15.7)	9 (6.5)	0.016
Periodontitis‖	39 (29.1)	30 (21.7)	0.162
Caries	66 (49.3)	48 (34.8)	0.016
Bleeding on probing	42.1 (15.3)	37.0 (13.1)	0.004
Mucosal lesions and changes#	17 (12.7)	18 (13.0)	0.949
Regular dentist visit**	65 (48.5)	85 (61.6)	0.041
Number of missing teeth			0.032
0	101 (75.4)	105 (76.1)	
1–2	14 (10.4)	25 (18.1)	
3–5	16 (11.9)	8 (5.8)	
>5	3 (2.2)	0	
Any PFO***	93 (69.4)	52 (37.7)	<0.001
High-risk PFO***	55 (41.0)	16 (11.6)	<0.001

Data are n (%) unless otherwise indicated. Education: postsecondary nontertiary education or tertiary; chronic multiorgan disease: any of IBD, autoimmune disease (excluding IBD), chronic kidney/liver disease, or hematologic disease/thrombophilia; abdominal obesity: men ≥0.9, women ≥0.85; migraine with aura (based on migraine screener); depression: feeling depressed for 2 wk in the prior 12 mo; physical activity: total metabolic equivalent task/wk <1500, missing information: *Education=4, †Physical activity=5, ‡Depression=4, §Migraine with aura=1, ‖Periodontitis=2, #Mucosal lesions and changes=1, **Regular dentist visit=2, ***Based on information from both echocardiography and transcranial Doppler with bubble screen. IBD indicates inflammatory bowel disease; and PFO, patent foramen ovale.

cerebrovascular events: 3 ISs, 1 intracerebral hemorrhage, and 2 transient ischemic attack.

Differences in Subgingival Microbiome Between the Group With CIS and Control Group

No significant differences were observed in alpha diversity between the patient and control groups (observed richness: *P*=0.880, Shannon index: *P*=0.740, and inverse Simpson index: *P*=0.520; Figure 1A). For beta diversity, a significant difference was observed in the Bray–Curtis (*P*=0.003) and Jaccard (*P*=0.007; Figure 1B; Table S1) distances. Bar plots displaying the relative abundance of the top phyla and genera were created to visualize the differences in microbial composition between cases and controls (Figure 1C). Although the predominant subgingival microbiota were comparable between patients and controls, specific differences in abundance were noted at both the phylum and genus levels (Figure S2A,B). At the phylum level, the abundance of *Spirochaetota* was significantly higher (*P*=0.002), whereas *Pseudomonadota* was lower (*P*=0.041) in patients than in controls (Figure S2C). At the genus level, the subgingival microbiota was dominated by *Fusobacterium*, *Treponema*, *Actinomyces*, *Prevotella*, and *Leptotrichia*. *Treponema* was significantly more abundant in patients, whereas *Veillonella* and *Capnocytophaga* were significantly less abundant in patients (Figure S2D; Table S2). The bacterial species that showed the highest increase in abundance in patients were *T. denticola* (*P*=0.029), *Treponema socranskii* (*P*=0.006), and *Tannerella forsythia* (*P*=0.016). Conversely, the bacterial species *Veillonella parvula* (*P*=0.017) and *Haemophilus parainfluenzae* (*P*=0.025) were less abundant in patients than in controls (Figure S2E; Table S2).

Stratified analyses by periodontitis showed that alpha diversity indices were not associated with CIS in participants with or without periodontitis (Table S3). We also compared microbial diversity between the periodontal disease groups and found no significant differences in alpha diversity metrics between participants with and without periodontitis (Table S4).

Differences in Subgingival Microbiome by Admission Stroke Symptom Severity

Forty-four (32.8%) patients had no symptoms on hospital admission, 65 (48.5%) had mild symptoms, and 25 (18.7%) had moderate-to-severe symptoms. Alpha diversity analysis by stroke severity categories showed no significant differences across the observed richness, Shannon, and inverse Simpson indices (Figure 2A; Table S5). Similarly, beta diversity analysis revealed no significant differences when using the Bray–Curtis or Jaccard metrics. No differences were observed between those with no symptoms versus mild symptoms, whereas a nonsignificant trend was found between those with no symptoms and moderate-to-severe symptoms (*P*=0.066 for Bray–Curtis and *P*=0.054 for Jaccard; Figure 2B; Table S6).

Differences in microbial composition between stroke severity groups are shown in Figure 2C. The abundances of *Spirochaetota*, *Treponema*, and *T. denticola* exhibited an increasing trend in severe stroke cases (*P*<0.05), whereas the *Actinomycetota*, *Lautropia, Peptidiphaga*,

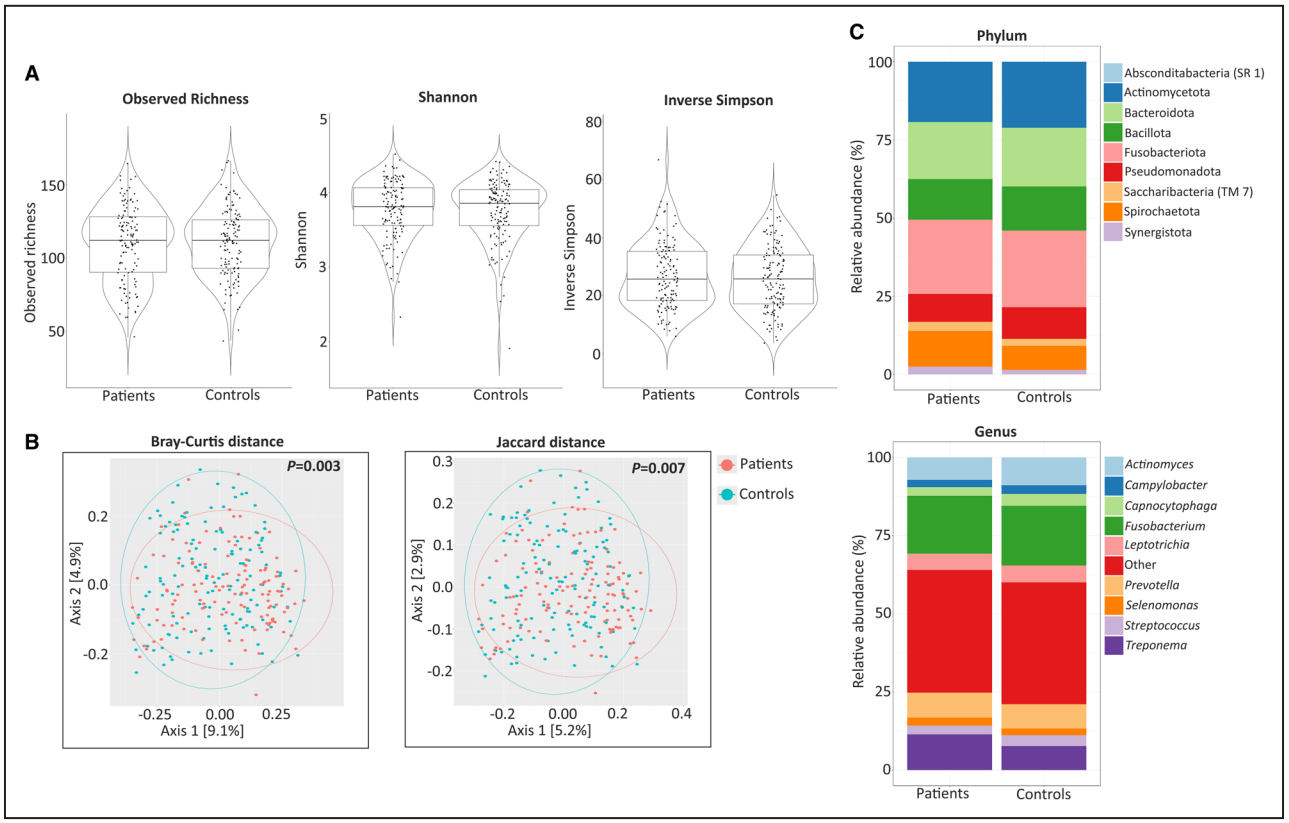

Figure 1. Microbial diversity and taxonomical composition of the subgingival microbiome in 272 participants (134 patients with cryptogenic ischemic stroke and 138 stroke-free controls).
A, Box plots showing the alpha diversity indices (observed richness, Shannon, and inverse Simpson index) of the subgingival microbiota in patients and controls. **B**, Principal coordinates analysis plots illustrating beta diversity based on Bray–Curtis and Jaccard distance metrics between patients and controls. **C**, Stacked bar plots depicting the mean relative abundance of bacterial taxa at the phylum and genus levels in patients and controls.

Neisseria flava, and *Actinomyces naeslundii* showed a decreasing trend ($P<0.05$) (Figure 3). LEfSe analysis revealed significant differences in the abundance of specific taxa between stroke severity groups. Notably, when comparing the no symptoms group to the mild symptoms group, *Capnocytophaga* was significantly enriched in the no symptoms group ($P=0.013$). Further analysis comparing the no symptoms group with the moderate-to-severe symptoms group revealed additional taxonomic features associated with stroke severity. *Treponema* sp. HMT 237 ($P=0.019$) and *Fretibacterium* sp. HMT 359 ($P=0.013$) were more abundant in the moderate-to-severe symptoms group (Table S7).

Subgingival Microbiomes in Patients with Favorable Versus Unfavorable Outcomes

Of the patients with CIS, 81(60.4%) had a favorable outcome and 52 (38.8%) had an unfavorable outcome. When comparing the subgingival microbiomes between these groups, alpha diversity tended to be higher in the favorable outcome group (observed species, $P=0.550$; Shannon index, $P=0.700$; inverse Simpson index, $P=0.950$; Figure 4A). Bray–Curtis ($P=0.199$) and Jaccard ($P=0.177$; Figure 4B; Table S8) also revealed no significant differences between the 2 groups. However, many predominant phyla and genera differed between patients with favorable and unfavorable outcomes (Figure 4C). According to LEfSe analysis, the phyla *Pseudomonadota* and *Capnocytophaga* were predominant in the favorable outcome group. In contrast, the abundance of the phylum *Spirochaetota*, genus *Treponema*, and species *T. denticola* and *Treponema* sp. HMT 237 was increased in the unfavorable outcome group ($P<0.05$) (Table S9).

Subgingival Microbiomes in Patients With Cognitive Impairment Versus Normal Cognitive Function

Cognitive impairment was present in 27 (20.1%) patients at 3 months. Alpha diversity was not significantly different between the cognitive impairment and normal cognitive function groups (observed

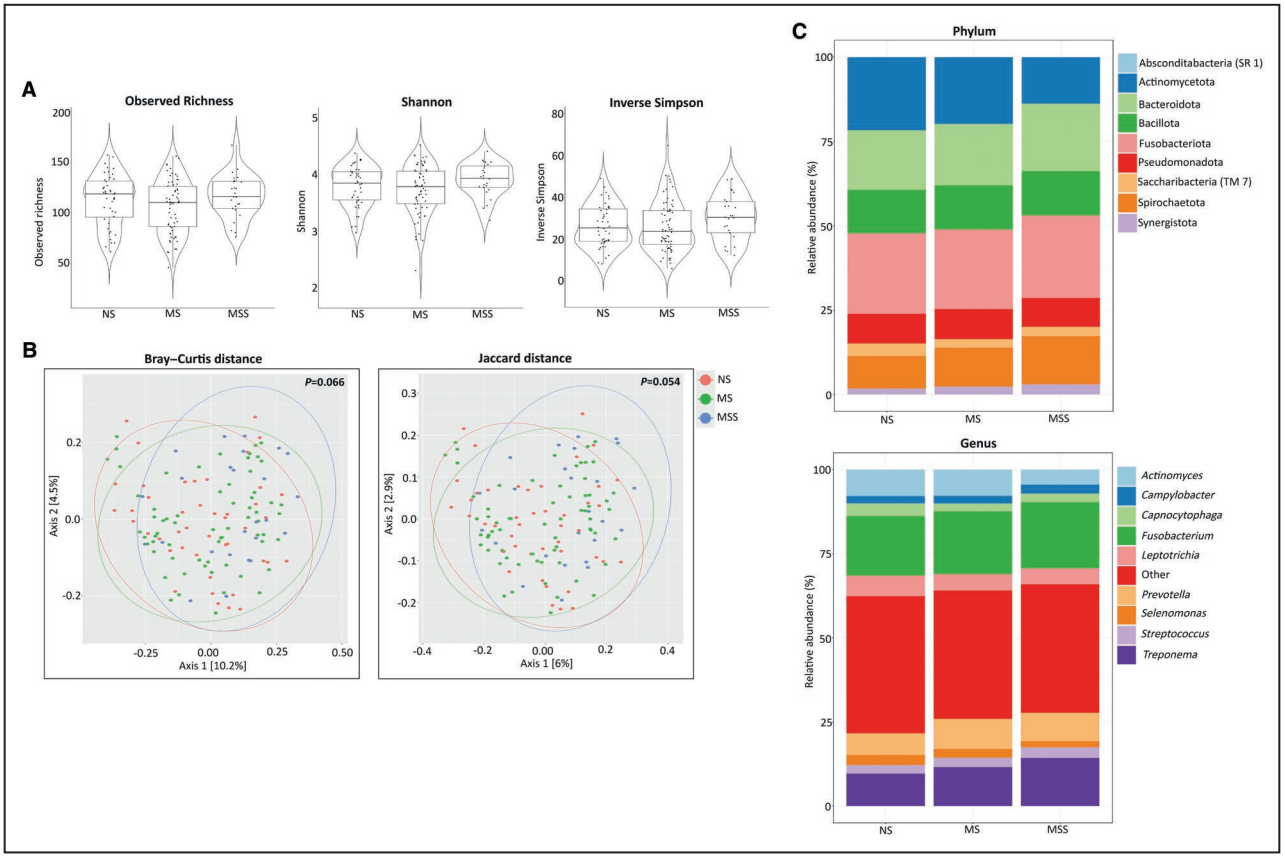

Figure 2. Subgingival microbiome based on stroke severity in 134 patients with cryptogenic ischemic stroke (no scorable symptoms on admission=44, mild symptoms=65, and moderate-to-severe symptoms=25).
A, Box plots displaying the alpha diversity indices (observed richness, Shannon, and inverse Simpson index) among different National Institutes of Health Stroke Scale groups. **B**, Principal coordinates analysis plots illustrating beta diversity among the different severity groups. **C**, Stacked bar plots showing the mean relative abundance of bacterial taxa at the phylum and genus levels across the different National Institutes of Health Stroke Scale score groups. MS indicates mild symptoms; MSS, moderate-to-severe symptoms; and NS, no scorable symptoms.

species, *P*=0.840; Shannon index, *P*=0.970; inverse Simpson index, *P*=0.790; Figure 5A). Bray–Curtis (*P*=0.142) and Jaccard (*P*=0.155) indices revealed no significant differences between the cognitive impairment versus normal cognitive function groups (Figure 5B; Table S10). Many phyla and genera differed between patients with cognitive impairment versus normal cognitive function (Figure 5C). At the phylum level, *Actinomycetota* was enriched in patients with normal cognitive function (*P*=0.004), whereas *Spirochaetota* (*P*=0.003) and *Saccharibacteria* (TM7) (*P*=0.032) were more abundant in those with cognitive impairment. At the genus level, *Actinomyces* (*P*=0.031) and *Capnocytophaga* (*P*=0.012) were associated with normal cognitive function, whereas *Treponema* (*P*=0.003) was significantly enriched in the cognitive impairment group. At the species level, *Peptidiphaga* sp. HMT 183 (*P*=0.020) was predominant in those with normal cognitive function, whereas *T. denticola* (*P*=0.033) was enriched in patients with cognitive impairment (Table S11).

Subgingival Microbiomes in Patients With White Matter Hyperintensities

WMH were present in 58 (43.3%) patients. There were no significant differences in alpha diversity between the patients with and without WMH (observed species, *P*=0.481; Shannon index, *P*=0.662; inverse Simpson index, *P*=0.873). Regarding beta diversity, Bray–Curtis distance and Jaccard matrices did not reveal significant differences between patients with and without WMH (*P*=0.528 and *P*=0.480, respectively; Figure S3; Table S12). LEfSe analysis revealed that only 1 taxon, *Bacillota*, was significantly more abundant in patients with WMH than in those without (*P*=0.021).

Subgingival Microbiomes in Patients With Any Sign of Small-Vessel Disease

To explore the potential role of the subgingival microbiome in cerebrovascular pathology, we analyzed the microbiome of patients with any sign of SVD, including recent small subcortical (lacunar) infarction, lacunes, or

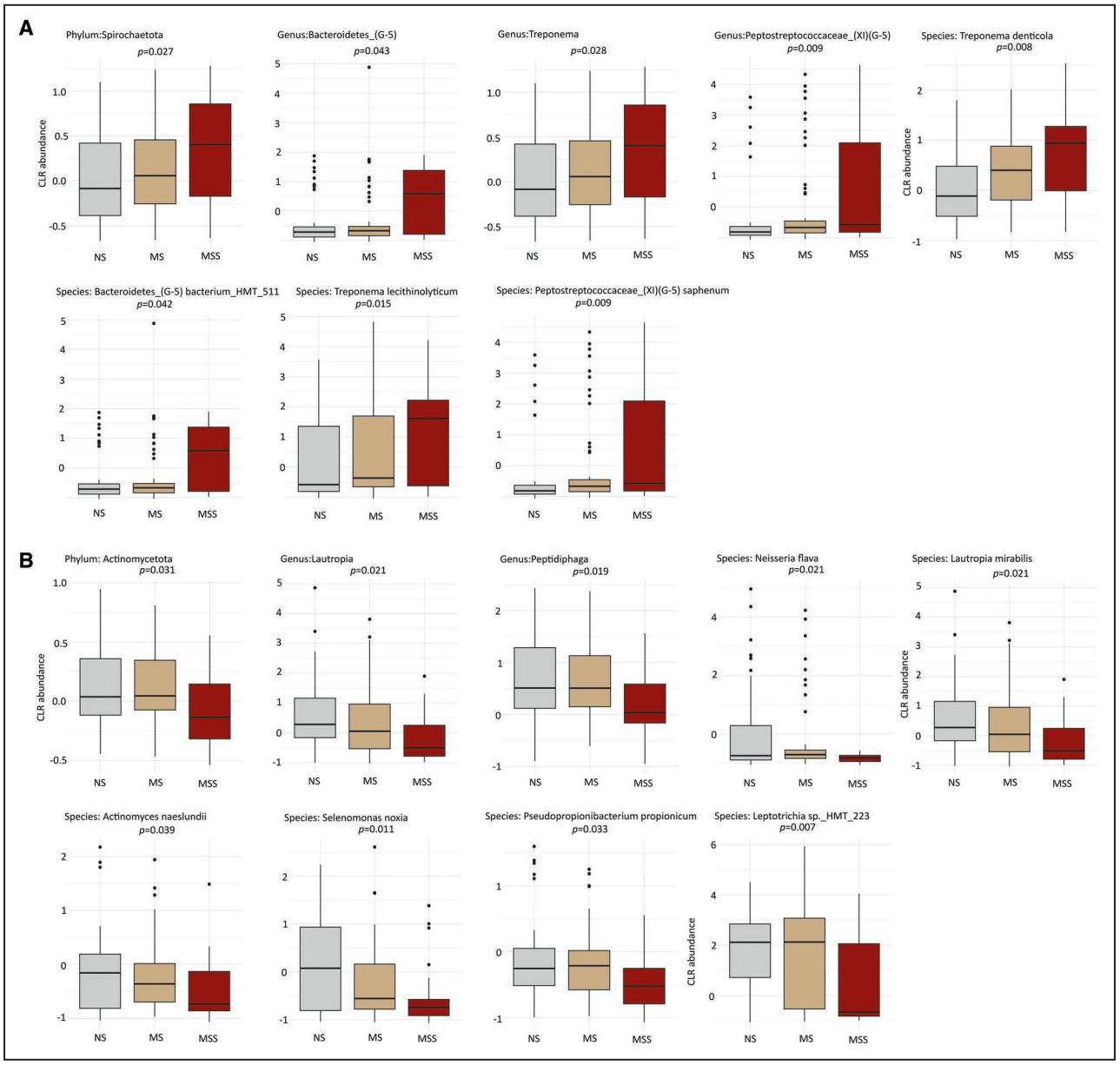

Figure 3. Boxplots showing the distribution of the centered log ratio-transformed abundance of subgingival bacterial taxa based on National Institutes of Health Stroke Scale stroke severity (no scorable symptoms on admission =44, mild symptoms =65 and moderate-to-severe symptoms=25).
A, Prevailing bacterial taxa with increasing stroke severity. **B**, Bacterial taxa declining with increasing stroke severity. CLR indicates centered log ratio; MS indicates mild symptoms; MSS, moderate-to-severe symptoms; and NS, no scorable symptoms.

WMH. Among patients with CIS, 73 (54.5%) had at least 1 indicator of SVD. There were no significant differences in alpha diversity between groups with SVD (observed species, *P*=0.991; Shannon index, *P*=0.932; inverse Simpson index, *P*=0.869; Figure S4A). Similarly, permutational multivariate ANOVA revealed no significant differences in microbial community composition between patients with and without SVD (Bray–Curtis, *P*=0.656; Jaccard, *P*=0.730; Figure S4B; Table S12). LEfSe analysis revealed that the genera *Rothia* and species *Rothia aeria* (*P*=0.044) were significantly more abundant in patients without any signs of SVD than in those with any SVD.

Subgingival Microbiomes in Patients With Anxiety/Depression Versus Those Without Anxiety/Depression

At 3 months, 26 (19.5%) patients exhibited symptoms of anxiety/depression. Alpha diversity showed no significant differences between the group with anxiety/

depression and the normal group across the observed species (P=0.240), Shannon index (P=0.210), and inverse Simpson index (P=0.290) (Figure 6A). Beta diversity using Bray–Curtis (P=0.652) and Jaccard (P=0.650) distances revealed no significant differences between the anxiety/depression and normal groups (Figure 6B; Table S13). Many predominant phyla and genera differed between the anxiety/depression and normal groups (Figure 6C). LEfSe analysis revealed that only 1 genus, *Cardiobacterium*, was significantly more abundant in patients with anxiety/depression than in the normal group (P=0.049).

Dysbiosis Patterns in Patients and Controls

The subgingival microbial dysbiosis index showed a statistically significant difference in subgingival microbial dysbiosis between the control and patient groups (P=0.036). However, the linear trend test in ANOVA (P=0.165) showed no significant trend in dysbiosis with increasing stroke severity (Figure S5).

We constructed a summary variable representing the combined relative abundance of 3 significant species identified as enriched in CIS. The summary variable showed weak positive correlations with standard alpha diversity metrics (Observed ρ=0.38, Shannon ρ=0.32, inverse Simpson ρ=0.28, Spearman), indicating that it captures distinct subgingival microbial shifts not fully reflected by overall diversity. In a matched conditional logistic regression model adjusted for age and antibiotic use, higher summed relative abundance of the significantly different taxa was associated with increased odds of CIS (P=0.002). Age and recent antibiotic use were not significantly associated with CIS in this model.

Association Between *T. denticola* Abundance and CIS

We performed multiple logistic regression analyses to evaluate the association between *T. denticola* abundance and stroke severity, unfavorable outcomes, and cognitive impairment in patients. The abundance of *T. denticola* was associated with an increased risk of stroke (odds ratio [OR], 1.002 [95% CI, 1.000–1.003], P=0.023). The association was independent of education, body mass index, smoking, diabetes, hypertension, antibiotic use, alcohol consumption, periodontitis,

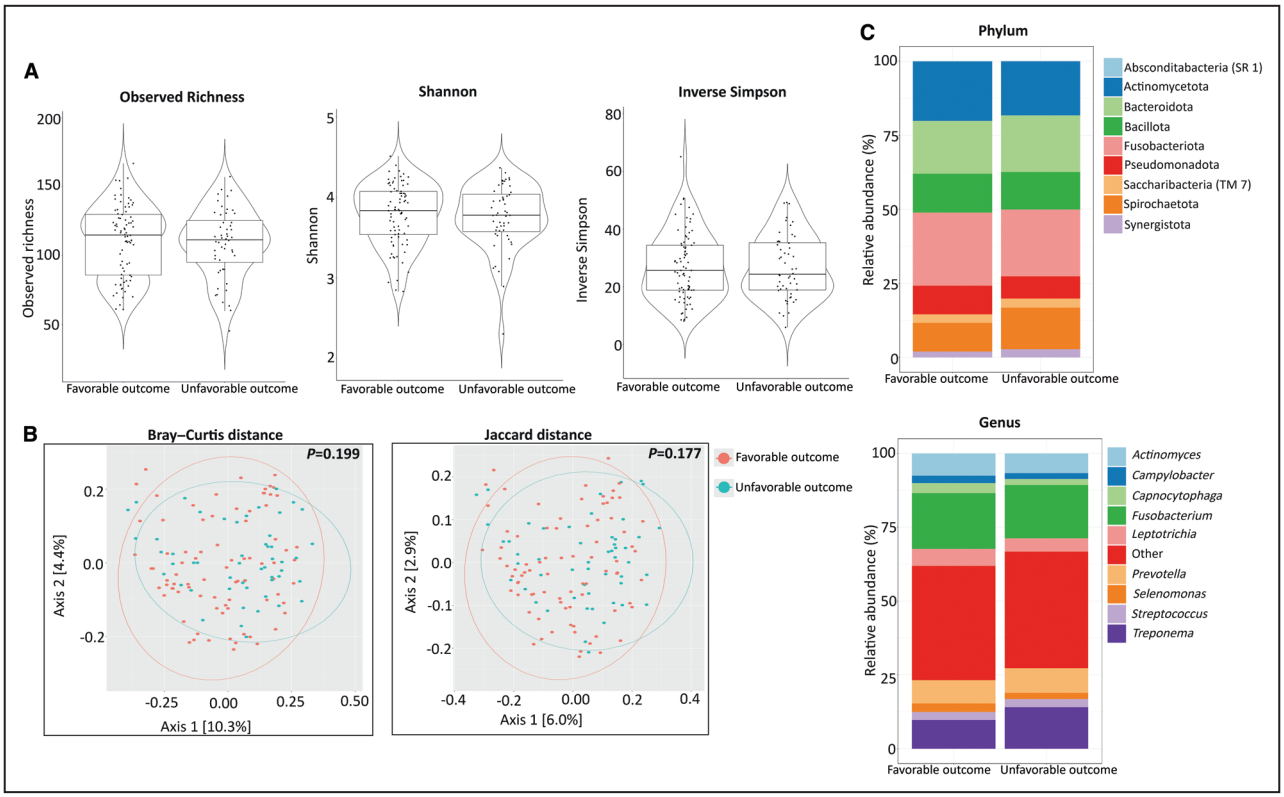

Figure 4. Subgingival microbiomes in patients with favorable (n=81) and unfavorable (n=52) outcomes.
A, Box plots showing the alpha diversity indices (observed richness, Shannon, and inverse Simpson index) of the subgingival microbiota in patients with cryptogenic ischemic stroke with favorable and unfavorable outcomes. **B**, Principal coordinates analysis plots illustrating beta diversity based on Bray–Curtis and Jaccard distance metrics between patients with cryptogenic ischemic stroke with favorable and unfavorable outcomes. **C**, Stacked bar plots depicting the mean relative abundance of bacterial taxa at the phylum and genus levels in patients with favorable and unfavorable outcomes.

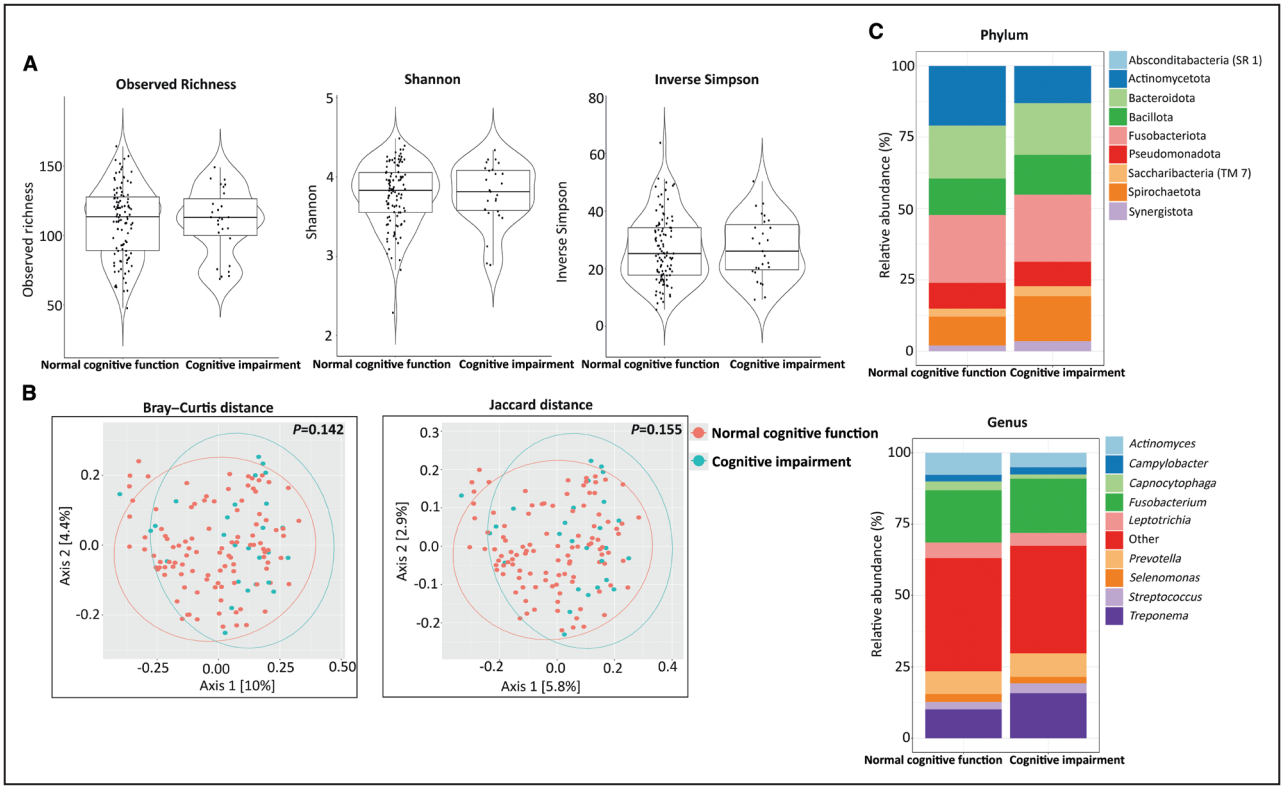

Figure 5. Subgingival microbiomes in patients with cognitive impairment (n=27) versus normal cognitive function (n=106). **A**, Box plots showing the alpha diversity indices (observed richness, Shannon, and inverse Simpson index) of the subgingival microbiota in patients with cognitive impairment vs normal cognitive function. **B**, Principal coordinates analysis plots illustrating beta diversity based on Bray–Curtis and Jaccard distance metrics between patients with cryptogenic ischemic stroke with cognitive impairment vs normal cognitive function. **C**, Stacked bar plots depicting the mean relative abundance of bacterial taxa at the phylum and genus levels in patients with cognitive impairment vs normal cognitive function.

caries, and statin use (OR, 1.002 [95% CI, 1.000–1.003], *P*=0.021). After further adjustment for migraine with aura, binge drinking, and PFO, the association was attenuated (OR, 1.002 [95% CI, 1.000–1.003], *P*=0.059: Table S14).

However, no association was found between *T. denticola* and stroke severity (OR, 1.000 [95% CI, 0.999–1.002], *P*=0.631, Table S15) or outcomes (OR, 1.001 [95% CI, 1.000–1.003], *P*=0.158, Table S16). *T. denticola* abundance was associated with the risk of cognitive impairment (OR, 1.002 [95% CI, 1.000–1.004], *P*=0.037, Table S17).

DISCUSSION

In this study, we investigated the subgingival microbiome of patients with young-onset CIS and their age- and sex-matched stroke-free controls. Although, there was no differences in alpha diversity, beta diversity analyses revealed distinct clustering, indicating variations in overall microbial composition. We identified a higher abundance of *Spirochaetota* and a lower abundance of *Pseudomonadota* in patients with CIS compared with controls, suggesting a potential role of *Spirochaetota* in the pathogenesis of CIS. Moreover, our findings suggest that specific bacterial taxa in the subgingival microbiome are associated with CIS severity and outcomes, potentially through mechanisms such as systemic inflammation, immune dysregulation, endothelial dysfunction, and bacterial translocation,[30] thereby emphasizing the role of oral health in CIS pathophysiology and recovery.

Regardless of the diversity metric used, we did not observe significant differences in alpha diversity of the subgingival microbiome between patients with CIS and controls, which is consistent with our previous findings on the salivary microbiome.[10] However, a recent study examining subgingival plaque revealed that patients with stroke and their spouses had higher alpha diversity than controls.[31] We observed significant beta diversity between the groups, although no differences were observed when stroke severity and outcomes were compared. Sun et al.[12] observed a progressive increase in oral microbial diversity from healthy controls to high-risk individuals and patients with acute stroke,

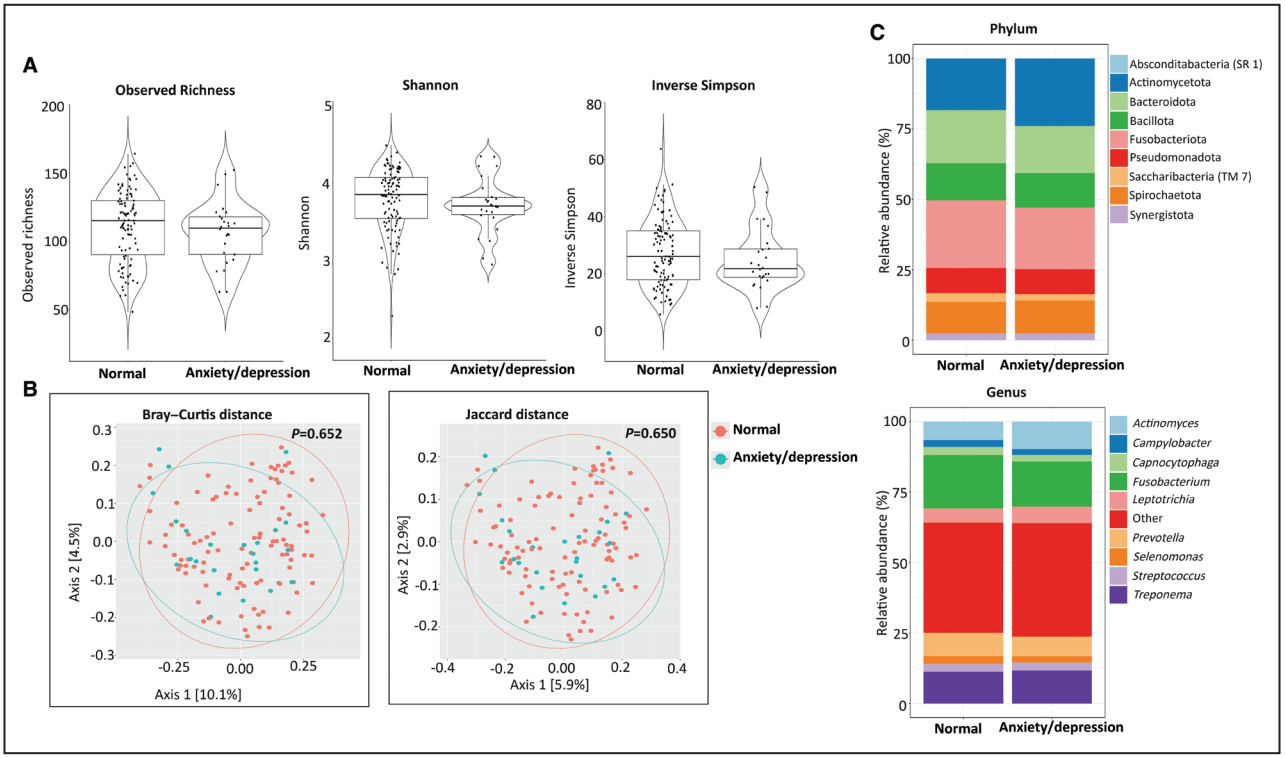

Figure 6. Subgingival microbiomes in patients with anxiety/depression (n=26) versus those without any anxiety/depression (n=107).

A, Box plots showing the alpha diversity indices (observed richness, Shannon, and inverse Simpson index) of the subgingival microbiota in patients with anxiety/depression vs normal. **B**, Principal coordinates analysis plots illustrating beta diversity based on Bray–Curtis and Jaccard distance metrics between patients with cryptogenic ischemic stroke with anxiety/depression vs normal. **C**, Stacked bar plots depicting the mean relative abundance of bacterial taxa at the phylum and genus levels in patients with anxiety/depression vs normal.

which is inconsistent with our findings. Significant differences in beta diversity between groups suggest a shift in the subgingival microbial community, whereas previous saliva metagenome findings showed no such distinction.[10] This indicates that the subgingival microbiome may better differentiate patients with CIS from controls, likely because of its association with periodontal conditions and dysbiosis.

The abundance of *Spirochaetota* was significantly higher in our patients than in controls. Other studies have suggested that the presence of oral *Spirochaetota* in plaques from patients with healthy periodontal tissues is associated with an increased susceptibility to developing periodontitis.[32] Consistent with our findings, Sun et al.[12] also reported that the relative abundances of *Treponema* and *Prevotella* in saliva samples were higher in all-age high-risk patients with IS. *Porphyromonas*, *Fusobacterium*, *Treponema*, and *Prevotella* are significantly enriched in periodontal diseases,[33] which is consistent with the concept that periodontitis is an independent risk factor for IS.[34] Our recent case–control study further supports this finding, which revealed that severe periodontitis and a high periodontal inflammation burden were strongly associated with CIS in this population, with ORs of 7.48 (95% CI, 1.24–44.9) and 10.48 (95% CI, 3.18–34.5), respectively.[9] Further species-level analysis confirmed that periodontitis-associated species, such as *T. denticola*, and *T. forsythia*, were significantly abundant in patients. Additionally, certain species within the genus *Treponema* reflected the severity of stroke symptoms and were associated with recovery and functional outcomes. Most *Treponema* species produce various virulence factors that cause significant tissue damage and contribute to systemic inflammation, which in turn play a role in the pathogenesis of adverse systemic effects, including cardiovascular disease, atherosclerosis, and stroke.[35] Serological evidence from our previous study indicated that elevated serum antibody levels against periodontal pathogens, particularly *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, are associated with an increased risk of future stroke.[36,37]

We also evaluated the severity and recovery of CIS. We observed that stroke severity does not have a distinct impact on oral microbiome diversity. However,

specific microbial taxa were associated with both stroke severity and functional outcomes. For example, *Capnocytophaga* was associated with no symptoms on admission and favorable outcomes, whereas *Treponema* sp. HMT_237, and *T. lecithinolyticum* were associated with more severe symptoms and unfavorable outcomes, indicating a possible connection between poor recovery and unfavorable outcomes. Previous findings on gut microbiota showed that dysbiosis is associated with functional outcomes in patients with acute IS and nonalcoholic fatty liver disease.[38] Interestingly, a Mendelian randomization study linked the gut microbiota to IS outcomes, showing that *Enterobacteriales* and *Enterobacteriaceae* (phylum Pseudomonadota) were associated with motor weakness.[39] Similarly, we observed a reduction in *Pseudomonadota* abundance with increasing stroke severity.

Although the concept of a relationship between the oral microbiota and stroke is recent,[10–12] several mechanisms may explain the link between dysbiosis of the subgingival microbiota and CIS. The oral microbiota can easily disseminate to the distal gut via enteral or hematogenous pathways,[40] contributing to gut dysbiosis and systemic inflammation. In chronic periodontitis, oral dysbiosis induces endothelial dysfunction and proatherogenic conditions and promotes prothrombotic states and vascular injury.[41] Our previous findings demonstrated that periodontitis contributes to cardiometabolic risk through persistent endotoxemia,[42] which influences atherogenesis via translocation, endotoxins, cross-reactive antibodies, and altered lipoprotein metabolism.[30]

WMHs are neuroimaging findings observed in magnetic resonance imaging scans that are associated with cognitive impairment and an increased risk of CIS and dementia.[43] In this study, no significant differences in the oral microbiota were found between patients with CIS with and without WMH or other signs of SVD. However, *Bacillota* was more abundant in patients with WMH, whereas *Rothia* was more abundant in patients without any signs of SVD. In patients with acute IS, higher levels of gut microbiome-derived metabolites correlate with more severe WMH.[44] Further studies are warranted to assess the relationships among WMHs, oral microbiome, functional pathways, and cognitive outcomes.

According to the LEfSe analysis, abundance of *T. denticola* is associated with stroke severity, unfavorable outcomes, and cognitive impairment in CIS. Multiple logistic regression analyses further confirmed that *T. denticola* abundance is significantly associated with an increased risk of stroke. However, no significant association was found between *T. denticola* and stroke severity or outcomes, whereas *T. denticola* abundance was significantly associated with the risk of cognitive impairment. In this study, the abundance of *T. denticola* was associated with CIS after adjusting for demographics and comorbidities; however, the association was attenuated after the inclusion of migraine with aura, binge drinking, and PFO. Our previous findings showed that oral microbiome dysbiosis was linked to high-risk PFO in patients with CIS, supporting a potential interaction between the microbiome and PFO-related stroke mechanisms.[11] We also showed that migraine with aura has a strong association with CIS in this population, independent of vascular risk factors and the presence of PFO.[6] *T. denticola* has been identified in brain tissue of patients with Alzheimer disease,[45] suggesting it is associated with neurodegeneration. In an animal study, Chen et al.[14] demonstrated that periodontitis-associated salivary microbiota can exacerbate IS and neuroinflammation by increasing IL-17A-producing immune cell migration in the gut and facilitating migration to the brain. These findings suggest bidirectional communication between the oral-gut-microbiota and the brain–immune system, highlighting the role of the oral microbiota in systemic vascular pathologies and their impact on stroke outcomes and cardiovascular risk.

This study has several limitations. First, as a retrospective case–control study, our findings do not allow for the causal inferences or the consideration of other risk factors for CIS, such as genetics, metabolomics, and environmental factors. Second, younger patients often present with milder clinical symptoms despite similar neuroimaging findings, likely due to healthier compensatory mechanisms. There may also be a selection bias toward milder stroke cases, as participation in a demanding research program requires greater engagement and functional ability.[46,47] Although our study specifically focused on young adults with CIS, further research is needed to explore the link between subgingival microbiome dysbiosis and different stroke subtypes, as well as in other age groups, including older adults, to determine whether these associations are unique to young-onset stroke or also occur in older populations.

In our study, there were no significant differences between the patients and controls in terms of key clinical characteristics, including age, sex, diabetes, hypertension, smoking, alcohol use, obesity, physical activity, depression, and the use of antibiotics. However, migraine with aura, binge drinking, and the presence of PFO were more common among patients than among controls, consistent with our previous findings.[6,11] We excluded participants with antibiotic use in the past month and instructed them to abstain from brushing, eating, or drinking 30 minutes before sampling. However, we did not restrict the use of antimicrobial mouthwash, and some participants may have used this before attending the clinic.

Oral examinations and subgingival sample collection were conducted once for patients, with a median

of 112 days post CIS. We minimized confounding by excluding recent antibiotic use, adjusting for comorbidities and medications, and using standardized protocols across sites with identically assessed and matched controls. Because the oral microbiome may be influenced by various poststroke factors, particularly neglected oral hygiene[48] and other behavioral changes, future studies should assess microbiome alterations at multiple time points after stroke to better clarify their relationship with the subgingival microbiome and cerebrovascular health. Additionally, data on oral hygiene practices, brushing, diet, and plaque amount were not available, which could potentially contribute to oral microbiome dysbiosis.[49,50] Future studies should assess microbiome changes at multiple time points in order to better understand their relationship with cerebrovascular health. Moreover, the inherent variability in periodontal conditions among participants made it challenging to achieve complete uniformity in the amount of subgingival plaque collected, which may have contributed to the differences in microbial community representation between patients and controls. A limitation of this study is the absence of inflammatory biomarker data (eg, IL-10 and C-reactive protein), which could help clarify how systemic inflammation mediates the relationship between periodontal disease, oral microbiota alterations, and the risk of CIS. Metagenomic sequencing, as demonstrated in our previous analyses,[10,11] provides a broader perspective by capturing the microbial communities linked to CIS. Finally, because this study was conducted at 2 centers, ethnic and regional differences should also be considered when comparing our results with those of other studies. Therefore, longitudinal studies with larger sample sizes are necessary to establish cause–effect relationships between the subgingival microbiome and CIS.

CONCLUSIONS

Our study revealed distinct differences in the subgingival microbiota in young patients with CIS compared with stroke-free controls, emphasizing the role of the microbiota as a potentially modifiable risk factor. Further studies are warranted to examine such associations. Routine oral health assessments and periodontal treatments including professional mechanical plaque control, may support prevention of CIS.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Tables S1–S17
Figures S1–S5

REFERENCES

1. Li L, Yiin GS, Geraghty OC, Schulz UG, Kuker W, Mehta Z, Rothwell PM; Oxford Vascular Study. Incidence, outcome, risk factors, and long-term prognosis of cryptogenic transient ischaemic attack and ischaemic stroke: a population-based study. *Lancet Neurol.* 2015;14:903–913. doi: 10.1016/S1474-4422(15)00132-5
2. Putaala J, Metso AJ, Metso TM, Konkola N, Kraemer Y, Haapaniemi E, Kaste M, Tatlisumak T. Analysis of 1008 consecutive patients aged 15 to 49 with first-ever ischemic stroke: the Helsinki young stroke registry. *Stroke.* 2009;40:1195–1203. doi: 10.1161/STROKEAHA.108.529883
3. Putaala J, Yesilot N, Waje-Andreassen U, Pitkäniemi J, Vassilopoulou S, Nardi K, Odier C, Hofgart G, Engelter S, Burow A, et al. Demographic and geographic vascular risk factor differences in European young adults with ischemic stroke: the 15 cities young stroke study. *Stroke.* 2012;43:2624–2630. doi: 10.1161/STROKEAHA.112.662866
4. George MG. Risk factors for ischemic stroke in younger adults: a focused update. *Stroke.* 2020;51:729–735. doi: 10.1161/STROKEAHA.119.024156
5. Jaakonmäki N, Zedde M, Sarkanen T, Martinez-Majander N, Tuohinen S, Sinisalo J, Ryödi E, Autere J, Hedman M, Junttola U, et al. Obesity and the risk of cryptogenic ischemic stroke in young adults. *J Stroke Cerebrovasc Dis.* 2022;31:106380. doi: 10.1016/j.jstrokecerebrovasdis.2022.106380
6. Martinez-Majander N, Artto V, Ylikotila P, von Sarnowski B, Waje-Andreassen U, Yesilot N, Zedde M, Huhtakangas J, Numminen H, Jäkälä P, et al. Association between migraine and cryptogenic ischemic stroke in young adults. *Ann Neurol.* 2021;89:242–253. doi: 10.1002/ana.25937
7. Martinez-Majander N, Kutal S, Ylikotila P, Yesilot N, Tulkki L, Zedde M, Sarkanen T, Junttola U, Nordanstig A, Fromm A, et al. Association between heavy alcohol consumption and cryptogenic ischaemic stroke in young adults: a case-control study. *J Neurol Neurosurg Psychiatry.* 2025;96:114–121. doi: 10.1136/jnnp-2024-333759
8. Lee YT, Tsai CF, Yen YC, Huang LK, Chao SP, Hu LY, Shen CC, Lee HC. Periodontitis is a potential risk factor for transient ischemic attack and minor ischemic stroke in young adults: a nationwide population-based cohort study. *J Periodontol.* 2022;93:1848–1856. doi: 10.1002/JPER.21-0528

9. Leskelä J, Putaala J, Martinez-Majander N, Tulkki L, Manzoor M, Zaric S, Ylikotila P, Lautamäki R, Saraste A, Suihko S, et al. Periodontitis, dental procedures, and young-onset cryptogenic stroke. *J Dent Res.* 2024;103:494–501. doi: 10.1177/00220345241232406
10. Manzoor M, Leskelä J, Pietiäinen M, Martinez-Majander N, Ylikotila P, Könönen E, Niiranen T, Lahti L, Sinisalo J, Putaala J, et al. Multikingdom oral microbiome interactions in early-onset cryptogenic ischemic stroke. *ISME Commun.* 2024;4:ycae088. doi: 10.1093/ismeco/ycae088
11. Manzoor M, Leskelä J, Pietiäinen M, Martinez-Majander N, Könönen E, Sinisalo J, Putaala J, Pussinen PJ, Paju S. Oral microbiome dysbiosis in cryptogenic ischemic stroke patients with high-risk patent foramen ovale. *Sci. Rep.* 2025;15:11535. doi: 10.1038/s41598-025-95728-x
12. Sun W, Huang S, Yang X, Luo Y, Liu L, Wu D. The oral microbiome of patients with ischemic stroke predicts their severity and prognosis. *Front Immunol.* 2023;14:1171898. doi: 10.3389/fimmu.2023.1171898
13. Wang C, Yang Y, Cai Q, Gao Y, Cai H, Wu J, Zheng W, Long J, Shu XO. Oral microbiome and ischemic stroke risk among elderly Chinese women. *J Oral Microbiol.* 2023;15:2266655. doi: 10.1080/20002297.2023.2266655
14. Chen YL, Bai L, Dilimulati D, Shao S, Qiu C, Liu T, Xu S, Bai XB, Du LJ, Zhou LJ, et al. Periodontitis salivary microbiota aggravates ischemic stroke through IL-17A. *Front Neurosci.* 2022;16:876582. doi: 10.3389/fnins.2022.876582. PMID: 35663549; PMCID: PMC9160974
15. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. The strengthening the reporting of observational studies in epidemiology (STROBE) statement: guidelines for reporting observational studies. *Lancet.* 2007;370:1453–1457. doi: 10.1016/S0140-6736(07)61602-X
16. Putaala J, Martinez-Majander N, Saeed S, Yesilot N, Jäkälä P, Nerg O, Tsivgoulis G, Numminen H, Gordin D, von Sarnowski B, et al. Searching for explanations for cryptogenic stroke in the young: revealing the triggers, causes, and outcome (SECRETO): rationale and design. *Eur Stroke J.* 2017;2:116–125. doi: 10.1177/2396987317703210
17. Amarenco P, Bogousslavsky J, Caplan LR, Donnan GA, Hennerici MG. New approach to stroke subtyping: the A-S-C-O (phenotypic) classification of stroke. *Cerebrovasc Dis.* 2009;27:502–508. doi: 10.1159/000210433
18. Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. *Stroke.* 2007;38:1091–1096. doi: 10.1161/01.STR.0000258355.23810.c6
19. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand.* 1983;67:361–370. doi: 10.1111/j.1600-0447.1983.tb09716.x PMID: 6880820
20. Fazekas F, Enzinger C, Schmidt R, Dichgans M, Gaertner B, Jungehulsing GJ, Hennerici MG, Heuschmann P, Holzhausen M, Kaps M, et al. MRI in acute cerebral ischemia of the young: the Stroke in Young Fabry Patients (SIFAP1) study. *Neurology.* 2013;81:1914–1921. doi: 10.1212/01.wnl.0000436611.28210.ec
21. Rolfs A, Martus P, Heuschmann PU, Grittner U, Holzhausen M, Tatlisumak T, Böttcher T, Fazekas F, Enzinger C, Ropele S, et al. Protocol and methodology of the Stroke in Young Fabry Patients (SIFAP1) study: a prospective multicenter European study of 5,024 young stroke patients aged 18-55 years. *Cerebrovasc Dis.* 2011;31:253–262. doi: 10.1159/000322153
22. Wardlaw JM, Smith EE, Biessels GJ, Cordonnier C, Fazekas F, Frayne R, Lindley RI, O'Brien JT, Barkhof F, Benavente OR, et al. Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. *Lancet Neurol.* 2013;12:822–838. doi: 10.1016/S1474-4422(13)70124-8
23. Jones W, Williams L, Meschia J. Validating the questionnaire for verifying stroke-free status (QVSFS) by neurological history and examination. *Stroke.* 2001;32:2232–2236. doi: 10.1161/hs1001.096191
24. Craig CL, Marshall AL, Sjöström M, Bauman AE, Booth ML, Ainsworth BE, Pratt M, Ekelund U, Yngve A, Sallis JF, et al. International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc.* 2003;35:1381–1395. doi: 10.1249/01.MSS.0000078924.61453.FB
25. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: framework and proposal of a new classification and case definition. *J Periodontol.* 2018;89:S159–S172. doi: 10.1002/JPER.18-0006
26. Manzoor M, Leskelä J, Pietiäinen M, Martinez-Majander N, Könönen E, Niiranen T, Lahti L, Sinisalo J, Putaala J, Pussinen PJ, et al. Shotgun metagenomic analysis of the oral microbiome in gingivitis: a nested case-control study. *J Oral Microbiol.* 2024;16:2330867. doi: 10.1080/20002297.2024.2330867
27. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13:581–583. doi: 10.1038/nmeth.3869
28. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. *Genome Biol.* 2011;12:R60. doi: 10.1186/gb-2011-12-6-r60
29. Chen T, Marsh PD, Al-Hebshi NN. SMDI: an index for measuring subgingival microbial Dysbiosis. *J Dent Res.* 2022;101:331–338. doi: 10.1177/00220345211035775
30. Pietiäinen M, Liljestrand JM, Kopra E, Pussinen PJ. Mediators between oral dysbiosis and cardiovascular diseases. *Eur J Oral Sci.* 2018;126(Suppl 1):26–36. doi: 10.1111/eos.12423 PMID: 30178551
31. Wang H, Yang M, Cheng S, Ren Y, Deng Y, Liang J, Lin X, Li J, Yin J, Wu Q. The spouses of stroke patients have a similar oral microbiome to their partners with an elevated risk of stroke. *Microorganisms.* 2022;10:2288. doi: 10.3390/microorganisms10112288
32. Riviere GR, DeRouen TA, Kay SL, Avera SP, Stouffer VK, Hawkins NR. Association of oral spirochetes from sites of periodontal health with development of periodontitis. *J Periodontol.* 1997;68:1210–1214. doi: 10.1902/jop.1997.68.12.1210
33. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol.* 1998;25:134–144. doi: 10.1111/j.1600-051x.1998.tb02419.x
34. Dörfer CE, Becher H, Ziegler CM, Kaiser C, Lutz R, Jörss D, Lichy C, Buggle F, Bültmann S, Preusch M, et al. The association of gingivitis and periodontitis with ischemic stroke. *J Clin Periodontol.* 2004;31:396–401. doi: 10.1111/j.1600-051x.2004.00579.x
35. Bale BF, Doneen AL, Vigerust DJ. High-risk periodontal pathogens contribute to the pathogenesis of atherosclerosis. *Postgrad Med J.* 2017;93:215–220. doi: 10.1136/postgradmedj-2016-134279
36. Pussinen PJ, Alfthan G, Rissanen H, Reunanen A, Asikainen S, Knekt P. Antibodies to periodontal pathogens and stroke risk. *Stroke.* 2004;35:2020–2023. doi: 10.1161/01.STR.0000136148.29490.fe
37. Pussinen PJ, Alfthan G, Jousilahti P, Paju S, Tuomilehto J. Systemic exposure to *Porphyromonas gingivalis* predicts incident stroke. *Atherosclerosis.* 2007;193:222–228. doi: 10.1016/j.atherosclerosis.2006.06.027
38. Yu G, Chen Q, Chen J, Liao X, Xie H, Zhao Y, Liu J, Sun J, Chen S. Gut microbiota alterations are associated with functional outcomes in patients of acute ischemic stroke with non-alcoholic fatty liver disease. *Front Neurosci.* 2023;17:1327499. doi: 10.3389/fnins.2023.1327499
39. Qu D, Jiang D, Xin Y, Yang G, Liang H, Wang L. Gut microbiota and functional outcome after ischemic stroke: a Mendelian randomization study. *Front Immunol.* 2024;15:1414653. doi: 10.3389/fimmu.2024.1414653
40. Elghannam MT, Hassanien MH, Ameen YA, Turky EA, ELattar GM, ELRay AA, ELTalkawy MD. Oral microbiome dysbiosis and gastrointestinal diseases: a narrative review. *Egypt. Liver J.* 2024;14:32. doi: 10.1186/s43066-024-00340-9
41. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol.* 2015;15:30–44. doi: 10.1038/nri3785
42. Pussinen PJ, Kopra E, Pietiäinen M, Lehto M, Zaric S, Paju S, Salminen A. Periodontitis and cardiometabolic disorders: the role of lipopolysaccharide and endotoxemia. *Periodontol 2000.* 2022;89:19–40. doi: 10.1111/prd.12433
43. West NA, Windham BG, Knopman DS, Shibata DK, Coker LH, Mosley TH Jr. Neuroimaging findings in midlife and risk of late-life dementia over 20 years of follow-up. *Neurology.* 2019;92:e917–e923. doi: 10.1212/WNL.0000000000006989
44. Yu F, Feng X, Li X, Luo Y, Wei M, Zhao T, Xia J. Gut-derived metabolite Phenylacetylglutamine and white matter hyperintensities in patients with acute ischemic stroke. *Front Aging Neurosci.* 2021;13:675158. doi: 10.3389/fnagi.2021.675158
45. Riviere GR, Riviere KH, Smith KS. Molecular and immunological evidence of oral *Treponema* in the human brain and their association with Alzheimer's disease. *Oral Microbiol Immunol.* 2002;17:113–118. doi: 10.1046/j.0902-0055.2001.00100.x
46. Putaala J. Ischemic stroke in the young: current perspectives on incidence, risk factors, and cardiovascular prognosis. *Eur Stroke J.* 2016;1:28–40. doi: 10.1177/2396987316629860
47. Ekker MS, Verhoeven JI, Schellekens MMI, Boot EM, van Alebeek ME, Brouwers PJAM, Arntz RM, van Dijk GW, Gons RAR, van Uden

IWM, et al. Risk factors and causes of ischemic stroke in 1322 young adults. *Stroke*. 2023;54:439–447. doi: 10.1161/STROKEAHA.122.040524

48. Leonov G, Salikhova D, Starodubova A, Vasilyev A, Makhnach O, Fatkhudinov T, Goldshtein D. Oral microbiome dysbiosis as a risk factor for stroke: a comprehensive review. *Microorganisms*. 2024;12:1732. doi: 10.3390/microorganisms12081732
49. Li X, Liu Y, Yang X, Li C, Song Z. The Oral microbiota: community composition, influencing factors, pathogenesis, and interventions. *Front Microbiol*. 2022;13:895537. doi: 10.3389/fmicb.2022.895537
50. Rajasekaran JJ, Krishnamurthy HK, Bosco J, Jayaraman V, Krishna K, Wang T, Bei K. Oral microbiome: a review of its impact on oral and systemic health. *Microorganisms*. 2024;12:1797. doi: 10.3390/microorganisms12091797