ORIGINAL RESEARCH

Oral Bacterial Signatures in Cerebral Thrombi of Patients With Acute Ischemic Stroke Treated With Thrombectomy

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***Background*—**Chronic infections have been reported to be risk factors for both coronary heart disease and ischemic stroke. DNA of oral bacteria, mainly from the viridans streptococci group, has been detected in coronary thrombus aspirates of myocardial infarction and cerebral aneurysms. Viridans streptococci are known to cause infective endocarditis and possess thrombogenic properties. We studied the presence of oral bacterial DNA in thrombus aspirates of patients with acute ischemic stroke treated with mechanical thrombectomy.

***Methods and Results*—**Thrombus aspirates and arterial blood were taken from 75 patients (69% men; mean age, 67 years) with acute ischemic stroke. The presence of *Streptococcus* species, mainly the *Streptococcus mitis* group, belonging to viridans streptococci as well as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in samples were determined using a quantitative polymerase chain reaction with specific primers and probes. The relative amount of bacterial DNA in a sample was determined with the comparative threshold cycle method. Bacterial DNA was detected in 84% (n=63) of aspired thrombi, and 16% (n=12) of samples were considered bacterial DNA negative. DNA of *Streptococcus* species, mainly the *S mitis* group, was found in 79% (n=59) of samples. The median relative amount of *Streptococcus* species DNA was 5.10-fold higher compared with the control blood samples from the same patients. All thrombi were negative for both *P gingivalis* and *A actinomycetemcomitans.*

***Conclusions*—**This is the first study showing the common presence of bacterial DNA from viridans streptococci in aspired thrombi of patients with acute ischemic stroke. Streptococcal bacteria, mostly of oral origin, may contribute to the progression and thrombotic events of cerebrovascular diseases. **(*J Am Heart Assoc*. 2019;8:e012330. DOI: 10.1161/JAHA.119.012330.)**

Key Words: acute stroke • arterial thrombosis • atherogenesis • atherosclerosis • cerebral ischemia

Cardiovascular and cerebrovascular diseases are major causes of death, and stroke is the leading cause of adult long-term disability in western countries.[1] Stroke can be divided into intracerebral hemorrhage, subarachnoid hemorrhage, and cerebral ischemia. In the Unites States of America, ≈795 000 people experience stroke each year, of which ≈692 000 are acute ischemic strokes (AISs).[2] Treatment of AIS has undergone major developments during the past 2 decades: first, the cerebral computed tomography made it possible to identify patients for medical thrombolysis; second, the intravascular catheter-based revascularization of cerebral vessels began to evolve. The modern intravascular revascularization techniques are stent retriever thrombectomy and direct aspiration of cerebral arterial clots.[3–5]

In addition to traditional causative factors, such as hypertension, hypercholesterolemia, diabetes mellitus, smoking, and obesity, bacterial inflammation has been suggested to contribute directly or indirectly to the development of the atherosclerosis and atherothrombotic events.[6]

Levels of CRP (C-reactive protein) seem to have correlation with the incidence for cardiovascular events and stroke.[7,8] CRP increases the adhesiveness of platelets for endothelium.[9] Inflammation of atherosclerotic plaque may affect its growth and contribute to plaque rupture, leading to thrombosis.[10,11] Inflammation at nonvascular sites may also affect progression

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Received February 19, 2019; accepted April 5, 2019.

Clinical Perspective

What Is New?

- We found DNA of viridans streptococci in most aspired thrombi of patients with acute ischemic stroke, which suggests that oral bacteria may have a role in the cause of cerebrovascular disease.

What Are the Clinical Implications?

- Repeated transient bacteremia, caused by poor dental care or bacterial infections, may trap pathogens in atherosclerotic plaques and promote rupture of the plaques; therefore, regular dental care should be emphasized in the primary prevention of acute ischemic stroke.

of atherosclerotic lesions via circulating chemical mediators.[12] Severe periodontitis increased 2-fold the risk of ischemic stroke among middle-aged men but not in women after adjusting for age, sex, number of teeth, vascular risk factors and diseases, childhood and adult socioeconomic conditions, and lifestyle factors.[13] In the Indian population, periodontitis was even a stronger risk factor compared with hypertension and smoking.[14] According to recent studies, periodontitis and tooth loss have an independent direct association with stroke.[15,16]

We have earlier reported that bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombi, and periodontal pathogens were measured in 34.7% of thrombus aspirates from coronary arteries in patients with acute myocardial infarction. The median value for the total amount of bacterial DNA in coronary thrombus was 16 times higher than that found in their blood samples.[17] Furthermore, we were able to detect these bacteria in ruptured and nonruptured cerebral aneurysm samples[18] and from thrombus aspirates of patients with lower limb vascular disorders.[19] The presence of odontogenic bacteria, such as *Porphyromonas gingivalis* and *Streptococcus sanguinis*, has been shown in the atherosclerotic plaque of human carotid artery histologically and by polymerase chain reaction (PCR).[20,21]

In this study, we used a real-time quantitative PCR (qPCR) to detect bacterial DNA from thrombi that have been collected by stent retriever technique from the cerebral arteries of patients with AIS. To our knowledge, there are no earlier studies depicting the role of oral bacteria in the thromboembolic events of cerebral arteries among patients with AIS. We hypothesized that oral bacterial DNA can be found in cerebral arterial thrombi similarly as from coronary or inferior extremity peripheral vessel thrombi.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

The series comprises 75 patients with AIS who were treated by intra-arterial thrombectomy between November 2013 and January 2017 in the Acute Stroke Unit of Tampere University Hospital, Tampere, Finland (Table). This series is a subset from a larger Tampere BMG (Brain Microbes and Genetics) study, focusing on the role of inflammation and genetics in ischemic stroke. A neurologist (J.O.) examined all patients when they arrived to the hospital and evaluated the possibility of revascularization using thrombectomy together with a neurointerventional radiologist (J.-P.P.). The cause of the brain large-vessel occlusions of patients treated with endovascular thrombectomy during the study period in Tampere University Hospital was cardioembolic in 38% and atherosclerotic in 62% of the patients (oral communication, 6th February, J.O.). The only excluding criterion for recruiting patients was that if the thrombus was not retrieved successfully using mechanical thrombectomy. The median delay time between onset of an

Table. Patients' Characteristics

Characteristics	Data for All Patients (N=75)
Age, mean±SD, y	66.9±12.4
Men, n (%)	52 (69.3)
Diabetes mellitus, n (%)	12 (16.0)
Dyslipidemia, n (%)	29 (38.7)
Arterial hypertension, n (%)	40 (53.3)
Coronary heart disease, n (%)	15 (20.0)
Cerebrovascular disease, n (%)*	20 (26.7)
Pulmonary disease, n (%)	4 (5.33)
Renal insufficiency, n (%)	7 (9.33)
Atrial fibrillation, n (%)	48 (64.0)
Heart failure, n (%)	10 (13.3)
Smoking status, %*	34.9
Location of thrombus aspirate, n (%)	
ICA	25 (33.3)
MCA	72 (97.3)
ACA	6 (8.00)
PCA	1 (1.33)
VA	6 (8.00)
AB	1 (1.33)
Arrival time to the hospital (quartile 1, median, quartile 2), h	1.20, 2.30, 3.80

AB indicates basilar artery; ACA, anterior cerebral artery; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; VA, vertebral artery.
*Smoking status was only available from 43 patients.

ischemic stroke and hospital arrival was 2 hours 20 minutes (range, 0-16 hours). Medical history was collected from the Tampere University Hospital digital patient archives. Criteria for dyslipidemia were fP-kol (cholesterol levels measured from plasma after 12 hours fasting (f=fasting, P=plasma)) low-density lipoprotein >3.0 mmol/L, fP-kol high-density lipoprotein <1.0 mmol/L, or fP triglycerides >2.0 mmol/L.

The study was approved by the ethics committee (R13093) of the Tampere University Hospital. The study was explained to the patients, and informed consent was obtained.

Mechanical Thrombectomy and Thrombus Sample Collection

An introducer sheet was placed on a femoral artery, and a control blood sample for bacterial genetic analysis was collected through the sheet. A guiding catheter up to 9F (Merci; Stryker Neurovascular) with a tip balloon was then navigated into the carotid artery proximal to the occluded site. The microcatheter (0.021 inches) with the guide wire was used to navigate through the occluded site and to deploy the stent retriever (Trevo; Stryker Neurovascular) over the thrombus. An additional distal access catheter was used to access the thrombus if needed. Forceful aspiration through proximal catheters was acquired with a 60-mL syringe while retrieving the deployed stent. In a minority of the cases, only direct thrombus aspiration was used. Different device settings were selected by operator case by case. Thrombectomy was repeated until the angiologic result was satisfactory. The gathered thrombus was divided into a 1.5-mL Eppendorf microcentrifuge tube for qPCR analysis, and a histological sample was placed in a formalin container.

Quantitative PCR

Bacterial DNA was extracted from samples using a commercially available QIAamp DNA Mini Kit (Qiagen, Germany), according to the instructions provided.

The presence of bacterial DNA in thrombus and blood samples from the same patients was determined by using published oligonucleotide primers and probes for *Streptococcus* species (mainly the *Streptococcus mitis* group[22]), *P gingivalis*, and *Aggregatibacter actinomycetemcomitans*[23]; and universal bacterial primers and probe[24] were determined with RNaseP (Applied Biosystems, Foster City, CA), as a reference.

qPCR assays were performed using specific TaqMan allele hybridization with the AbiPrism 7900 HT Sequence Detection System (TaqMan; Applied Biosystems, Carlsbad, CA) under standard conditions with the following cycle profile: 50°C for 2 minutes, 95°C for 10 minutes, and 60 cycles of 95°C for 15 seconds and 58°C for 1 minutes. MasterMix was prepared using Maxima Probe/ROX qPCR MasterMix (Thermo Fischer Scientific, Waltham, MA), adding at final concentrations of 900 nmol/L of each primer and 250 nmol/L of each fluorescence-labeled probe. All amplifications and detections were performed as duplicates or quadruples (in uncertain cases), depending on test runs in a MicroAmp optical 384-well reaction plate with optical caps (Sarsted, Nümbrecht, Germany) in a reaction volume of 5 μL, with 1 μL of nondiluted DNA.

Human housekeeping gene, RNaseP, was used as a reference measurement to determine the relative amount of bacterial DNA in the sample. The determination was done by the comparative threshold cycle (Ct) method. The critical Ct is the cycle at which a statistically significant increase in ΔRn (normalized reporter) is first detected and at which the fluorescence becomes detectable above background. Ct is inversely proportional to the logarithm of the initial number of template molecules (ie, the initial amount of sample DNA). Calculation with the Ct method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$) was done with a simplification.[25–28] First, the differences of the Ct values between candidate bacteria and reference gene measurement (candidate bacteria–RNaseP [ΔCt]) for each sample were calculated; then, the comparative Ct (thrombus–patients own blood [ΔΔCt]) was calculated. The samples were marked bacterial positive, if $2^{-\Delta\Delta Ct} \geq 2$,[29,30] or if there was amplified bacterial DNA in the thrombus but not in the control sample. DNA was extracted from the entire thrombus in most of the cases. If the aspired thrombus was large, a small part of it was taken and sent for histological analyses, and DNA was extracted from the rest of the thrombus.

Statistical Analysis

Statistical analysis was done using IBM SPSS Statistics 25 (SPSS, Chicago, IL). Associations between the bacterial findings and nominal parameters were analyzed using Pearson's χ^2 test. Age was treated as normally distributed and, therefore, mean and sample SD values were calculated and the Student *t* test was used. The Mann-Whitney *U*-test was used to analyze the associations between bacterial findings and the median arrival time to the hospital; these values were not normally distributed. Statistical significance was set at $P<0.05$.

Results

Patient Characteristics

Of the study population, 69.3% (n=52) were men and 30.7% (n=23) were women. The mean age of the patients was 66.9 years. None of the patients had been treated with antibiotics or experienced severe infections or septicemia

during the stroke. Characteristics of the study population are presented in the Table.

Presence of Bacterial DNA in Thrombus Aspirates

Of the 75 patients who underwent thrombectomy for treatment of acute stroke, 84.0% (n=63) of aspirated thrombi were positive for bacterial DNA in qPCR compared with 16.0% (n=12) of bacteria-negative thrombi. In addition, 78.7% (n=59) of aspirated thrombi were positive for *Streptococcus* species, mainly the *S mitis* group. Bacterial DNA of *P gingivalis* and *A actinomycetemcomitans* was not found in thrombi. Of the arterial blood samples that were collected during the thrombectomy procedure, 9.33% (n=7) were positive for both bacteria and *Streptococcus* species, mainly the *S mitis* group, and 1.33% (n=1) were positive for both *P gingivalis* and *A actinomycetemcomitans*.

The presence of *Streptococcus* species, mainly the *S mitis* group DNA, was 5.10-fold in median and total bacteria DNA was 7.93-fold in median, compared with the control blood sample from the same patients. N-fold values for qPCR findings are presented in Figure 1. Patients positive for any bacterial DNA were more often men (P=0.067) and more often had diabetes mellitus (P=0.074) and previous cerebrovascular disease (P=0.046) compared with patients negative for any bacterial DNA. However, there were no differences in patients' demographic parameters between those positive or negative for the *S mitis* group bacterial DNA (Figure 2).

Discussion

The present concept is that atherosclerosis is a complex chronic inflammatory disorder driven by oxidized or otherwise modified low-density lipoprotein.[31–33] However, increasing knowledge suggests that the atherosclerotic process may be accelerated by bacterial infection.[34,35]

In our study, we found that thrombi collected from patients with AIS were 84% positive for bacterial DNA and especially for the *S mitis* group, whereas we could not detect DNA from *P gingivalis* and *A actinomycetemcomitans*. To our knowledge, this is the first study in which bacterial signatures of the thrombi of patients with AIS have been analyzed using qPCR.

Streptococcus species are mostly found in the oral cavity of healthy individuals and in patients with periodontal disease.[36,37] The *S mitis* group comprises 20 species.[38] Oral bacteria can gain access to the bloodstream after trauma or dental procedures (eg, root canal treatment or tooth extraction) and cause transient bacteremia. In tooth extraction, most bacteria translocated into the circulation were viridans streptococci.[39] Viridans streptococci are the most common cause of infective endocarditis and sepsis.[40] It is possible that oral bacteria can be trapped in atrial thrombosis in patients with atrial fibrillation. Moreover, it has been found that bacteria can be found in the circulation without any clinical symptoms.[41]

There are several ways how bacteria can enter the atherosclerotic plaque. Bacteria can be phagocytosed and end up in the atherosclerotic plaque via circulating macrophages. Bacteria can also directly drift in the bloodstream through the vasa vasorum or the neovasculature vessels developing inside the atherosclerotic plaque. Amoxicillin medication in connection with tooth extraction decreased the frequency of positive streptococci findings in peripheral blood samples of the patients.[42] A recent study confirmed that regular dental care lowers the risk for ischemic stroke.[43]

It has been shown that oral pathogens, such as viridans group streptococci, can stimulate endothelial cells to produce various proinflammatory cytokines, such as interleukin 6 and interferon γ.[44,45] These cytokines are involved in the pathogenetic pathway of atherosclerosis and may promote the rupture of the plaque.[46] Oral pathogens seem to activate toll-like receptors, which mediate inflammation responses to pathogens and cause endothelial dysfunction. Endothelial toll-like receptors initiate inflammation and are an important component in the establishment of plaque.[47,48]

In addition to the regular inflammatory pathway, bacteria can straightforwardly interact with a platelet, causing its activation.[49] Bacterial surface proteins of *S mitis* can directly bind to various platelet receptors.[50,51] There can also be indirect activation via plasma immunoglobulin G.[52] Moreover, odontogenic bacteria can secrete gingipains proteinase, which can persuade an increase in platelet intracellular [Ca^{2+}] and lead to the activation and aggregation of platelets.[53,54] Activated platelets induce the recruitment of proatherosclerotic cells and speed up the development of

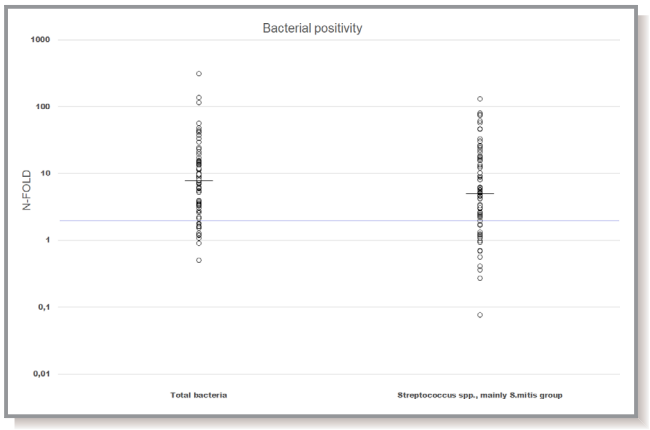

Figure 1. N-fold values >2.0 are significantly positive for bacterial DNA. Line at the 2.0 *y*-axis level indicates that most thrombi collected during thrombectomies of patients with acute stroke are positive for bacterial DNA. Median N-fold values are illustrated with black lines.

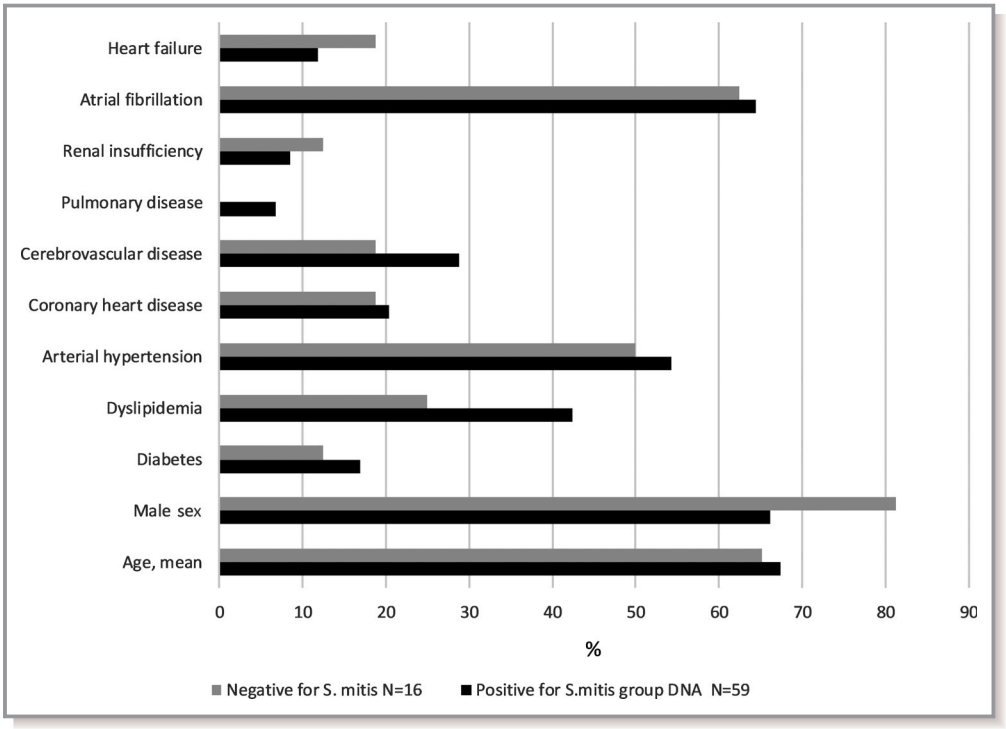

Figure 2. Clinical characteristics of patients with negative or positive thrombus aspirates for the *Streptococcus mitis* group DNA.

atherothrombotic lesions.[55] Activated platelets can take part in the formation of cardioembolic stroke if the hemodynamic conditions are altered, such as in atrial fibrillation.[56,57]

One of the limitations of this study is its small sample size, which prevents further subgroup analyses. The exact impact of living bacteria on thrombosis cannot be declared using PCR. In addition, the PCR method we used detects the presence of bacterial DNA in the examined samples but is unable to separate living bacteria from phagocytized bacterial DNA. Culturing and staining are the most frequently performed techniques when detecting bacterial species. Nonetheless, the PCR method seems to be more accurate and cost-effective in comparison with traditional culturing.[58,59] Although our analysis revealed the absence of *P gingivalis* and *A actinomycetemcomitans* in the thrombi, it does not exclude the possibility of their role in the pathogenesis of AIS. The presence of bacterial DNA was defined by an artificial cutoff value: the sample was considered positive if it contained 2 times more bacterial DNA compared with the control sample from the same patient. In median, the samples contained ≈5 times more *S mitis* DNA than the control samples. However, the validity is also affected by the inhomogeneity of the thrombus material.[60,61]

The findings of this study confirm those of our previous studies,[17,19] suggesting that bacterial infection may be involved in the pathogenesis of coronary and lower limb thrombosis. However, it is not known whether the oral bacteria are one of the causes of atherothrombotic events or whether their role is solely as bystander. The exact role of bacteria in AIS thus remains unclear.

Conclusion

We found DNA of *Streptococcus* species, mainly the *S mitis* group, belonging to viridans streptococci, in most aspirated thrombi of the patients with AIS. This suggests that viridans streptococci may have a role in the cause of cerebrovascular disease. Regular dental care should be emphasized in the primary prevention of AIS.

Sources of Funding

This study was supported with grants from the Competitive Research Funding of the Tampere University Hospital (grant X51001 for Lehtimäki and Karhunen), the Emil Aaltonen Foundation (Lehtimäki), and the Academy of Finland (grant 286284 for Lehtimäki), the Tampere Tuberculosis Foundation, the Finnish Foundation for Cardiovascular Research, the Yrjö Jahnsson Foundation, the Pirkanmaa Cultural Foundation (Tuomisto), the European Union 7th Framework Program (grant 201668 for AtheroRemo (European Collaborative Project on Inflammation and Vascular Wall Remodelling in

Atherosclerosis), and EU (European Union) Horizon 2020 (grant 755320 for TAXINOMISIS Project: A multidisciplinary approach for the stratification of patients with carotid artery disease).

Disclosures

None.

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