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Oral microbiota and atherothrombotic carotid plaque vulnerability in periodontitis patients. A cross-sectional study

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Abstract

Background: An increased risk of atherothrombotic vascular events has been reported in periodontitis patients. Periodontitis is associated with dysbiotic subgingival biofilms and bacteremia.

Objective: We hypothesized (a) that the oral microbiome is associated with the carotid microbiome and (b) that periodontitis could contribute to plaque vulnerability. The aim of this study was to determine the associations between periodontitis, the carotid microbiome, and the local innate immune response in carotid atherothrombotic plaques vulnerable to rupture.

Methods: In this cross-sectional study, 45 patients admitted for carotid endarterectomy underwent a preoperative periodontal examination. The volume of intraplaque hemorrhage reflected by the hemoglobin level released in carotid-conditioned media was considered as a criterion of carotid plaque vulnerability. Levels of antibodies against periodontal bacteria were determined in sera. The signature of the oral microbiota was assessed by microbial whole-genome sequencing, nested PCR, and immunostaining in carotid plaque samples. Markers of neutrophil recruitment (leukotriene B4), neutrophil activation (myeloperoxidase, defensins), and cytokines were measured in carotid-conditioned media and/or plasma.

Results: All patients exhibited periodontitis. One hundred and forty-four bacterial genera were detected in the carotid microbiome. While *Streptococcus* was found in 84% of the carotid samples, periodontitis-associated genera were detected in 21%. *P. gingivalis* DNA and gingipains were also identified in carotid samples. There were significant inverse correlations between periodontal attachment loss/serum anti-*P. gingivalis* Immunoglobulin A and cytokine inhibiting neutrophils (all $P < .01$). There were also significant positive correlations between lipopolysaccharides, myeloperoxidase/human neutrophil peptides1-3, and hemoglobin levels (all $P < .01$).

Conclusions: In patients at risk of stroke, the carotid plaque microbiome was highly diverse and compatible with an oral origin. Periodontitis was significantly associated with neutrophil activation markers and plaque vulnerability to rupture.

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Odontologique (IFRO), from Colgate Palmolive and from Sos-Attaque Cerebrale (a not-for-profit stroke survivor association).

KEYWORDS
atherothrombosis, carotid microbiome, neutrophils, next-generation sequencing, oral microbiota, periodontitis

1 | INTRODUCTION

Despite advances in the prevention and control of traditional risk factors for atherothrombosis, the incidence of clinical events is still high.[1,2] It is thus of interest to explore comorbidities, which may determine the initiation and progression of atherothrombosis; and to better understand the biological relationships between atherothrombosis and other non-traditional modifiable risk factors such as oral diseases. Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic subgingival biofilms and characterized by the progressive destruction of the tooth-supporting apparatus.[3] Although periodontitis is highly prevalent,[4] in most cases, it might be prevented through proper oral hygiene behaviors.[5] If left untreated, periodontitis has a potentially negative impact on general health.[6] Cross-sectional and cohort studies have established a consistent epidemiological link between periodontitis and clinical manifestations of atherothrombosis including stroke.[7-11] Biological evidence is less strong, a direct microbial hypothesis and an indirect immune hypothesis are suggested.

Microbiota of oral origin has been identified in atherothrombotic plaques[12-15] that may be involved in the vascular pathogenesis. Viable low pathogenic bacteria passing through the vessel wall into the bloodstream, that is, bacteremia has been suggested in patients after dental treatment and daily oral activities although accurate results regarding the magnitude, duration, and nature of bacteremia are missing.[16,17] Apart from any specific oral activity, a study even showed that blood from donors self-reported as medically healthy harbored viable mainly anaerobic bacteria from the oral cavity.[18]

In the presence of bacteria, neutrophils, the most abundant leukocytes (60%-70%) in human blood are recruited locally and activated in the atherothrombotic plaque by chemoattractant molecules such as leukotriene B4.[19] Neutrophils are activated in order to kill bacteria by several mechanisms: phagocytosis, degranulation, that is, the release of cytokines (such as interleukins), antimicrobial peptides (such as defensins), and granular enzymes (such as proteases), the release of reactive oxygen species (ROS)/ROS-generating molecules such myeloperoxidase, and the formation of Neutrophil Extracellular Traps into their surroundings.[20] Ultimately, the innate immune response activated, mainly neutrophils, may provoke the destabilization of the carotid atherothrombotic plaque and its rupture, leading to clinical stroke. The vulnerability to rupture of carotid plaque is associated with the presence of intraplaque hemorrhages and clotting.[21,22] Studies in mammals,[23,24] including humans[25] have shown that blood clots are privileged sites for bacterial trapping. Therefore, the oral microbiota may traffic from periodontal pockets to the atherothrombotic plaques where it may induce a disproportionate local innate immune response within the vessels like in the periodontal tissues.[26]

We hypothesize that patients at risk of stroke and with periodontitis: (a) show signatures from the oral cavity in the microbiome of carotid atherothrombotic plaques; (b) show associations between plaque vulnerability to rupture with markers of neutrophil activation. To assess these potential clinico-biological associations, we designed a cross-sectional study of patients scheduled for revascularization of carotid stenosis by endarterectomy.

2 | MATERIALS AND METHODS

2.1 | Patient population, samples, and data collection

Consecutive patients from the CASIMIR study (131 patients), scheduled for revascularization of carotid stenosis by endarterectomy in the departments of Neurology and Vascular Surgery at the Bichat Hospital (Paris, France) between 2011 and 2013 were screened for eligibility in the present cross-sectional study. The inclusion criteria were as follows: (a) age between 35 and 85 years old; (b) having at least 6 teeth; (c) written informed consent obtained; and (d) be affiliated with national social insurance. Patients were excluded if: (a) they could not sustain the dental chair or bedside oral examination position for at least 15 min; and (b); the sample of carotid endarterectomy was not available.

Prior to surgical procedure, the patients' characteristics were obtained via medical questionnaires and interviews. Age, gender, weight in kg, smoking status, and medical history were recorded. According to the glossary of the Centers for Disease Prevention and Control, smoking status was defined as follows: (a) non-smoker if the patient has never smoked or has smoked less than 100 cigarettes in his or her lifetime, (b) current smoker if the patient has smoked 100 cigarettes in his or her lifetime and who currently smokes cigarettes (including every day and someday smokers), and (c) former smoker if the patient has smoked at least 100 cigarettes in his or her lifetime but had quit smoking at the time of interview. Blood was collected to quantify usual biological variables (including HbA1c, total cholesterol, and low-density lipoprotein cholesterol) and antibodies against periodontal bacteria. One extra prechilled EDTA tube of blood was collected for the purpose of the study. No later than 30 minutes after collection, two centrifugations were performed to separate the plasma from the blood cells (2500 rpm, 15 minutes, 12°C; 2500 rpm, 15 minutes, 4°C). Serum and plasma samples were stored at −80°C until use.

A full periodontal examination including the Plaque Control Record[27] used as a Plaque Index (PI), Bleeding on Probing (BOP), Clinical Attachment Level (CAL), and Periodontal Pocket Depth (PD) measurements was performed by 2 calibrated periodontists. The level of agreement among the examiners for the measurement of pocket depth was estimated as good with an intraclass correlation coefficient of 0.853. To explore the microbial profile of the patient, subgingival biofilm was sampled using paper points inserted within the four deepest periodontal pockets.

Immediately after surgery, vascular samples were kept in sterile saline solution at 4°C until laboratory processing.

This study was conducted according to the ethical standards of the Committee on Human Experimentation of our institution (CPP 110434) and conformed to the principles outlined in the Declaration of Helsinki. Informed written consent was obtained from all the participating patients.

2.2 | Periodontal data analysis and study outcomes

Periodontal clinical and microbiological parameters were compared for each patient to clinical and biological parameters.

DNA from subgingival samples was treated with the micro-Ident® *plus* test (Biocentric company, Bandol, France) to detect *Porphyromonas gingivalis (P. gingivalis)* and *Tannerella forsythia (T. forsythia)*. The four plaque samples were pooled for analysis.

Carotid plaque vulnerability to rupture was the study outcome, assessed through the presence of highly hemorrhagic plaque, and a high level of neutrophil activation markers in the carotid plaque.[28] The median concentration of carotid intraplaque hemoglobin in the study population (2500 µg/g) was used as a cut-off to discriminate highly hemorrhagic plaques from low hemorrhagic plaques.

2.3 | Carotid-conditioned medium and post-incubation carotid tissues

Human carotid endarterectomy samples were processed within a few hours after surgery. They were dissected as previously reported.[28,29] Shortly after, samples were incubated for 24 hours at 37°C in a standardized volume (6 mL/g of sample wet weight) of Roswell Park Memorial Institute medium (RPMI) with antibiotics. Tissue and conditioned medium samples were then frozen and stored at −80°C.

2.4 | Bacterial DNA extraction from carotid samples

Human carotid samples were pulverized using a freezer mill (Spex Certiprep Ltd). DNA extraction from the carotid endarterectomy samples was performed using the QiAmp DNA blood Mini Kit (Qiagen®) and for calcified samples, with the PrepFiler BTA forensic DNA extraction kit (Life Technologies®). Extracted DNA was analyzed using a NanoDrop 2000 spectrophotometer. A PCR to amplify the GAPDH gene was performed to confirm DNA integrity (Appendix S1).

2.5 | Microbial whole-genome sequencing

Extracted DNA from randomly selected carotid samples (n = 19) was sent to GATC (Eurofins GATC Biotech, Constance, Germany) for Microbial whole-genome sequencing (INVIEW Microbiome High Specificity), using a PacBio platform (Pacific Biosciences). Amplification of full-length 16S rRNA gene by PCR was performed using validated primers (V1-V8). The sequence data were checked for chimeras. Non-chimeric, unique sequences were then subjected to BLASTn. Reference 16S rRNA gene sequences were obtained from the Ribosomal Database Project. Only good quality and unique 16S rRNA gene sequences which had a taxonomic assignment were considered and used to assign operational taxonomic unit (OTU) status to the sequences. The best hits with 97% similarity were analyzed to distinguish bacteria at the genus level (Appendix S1).

2.6 | Amplification of Porphyromonas gingivalis by nested PCR and sequencing

Detection of *P. gingivalis* in carotid samples was done with nested PCR.[30] Amplification of the bacterial 16S ribosomal-RNA gene was carried out with broad-range eubacterial primers (forward fD1: 5′-AGAGTTTGATCCTGGCTCAG −3′; reverse rP2: 5′-ACGGCTACCTTGTTACGACTT-3′).[31] The products of this first PCR were then purified (Illustra Exoprostar 1Step®, Duster) and a second PCR was carried out on using specific sets of primers corresponding to a sequence encoding the 16S rRNA gene of *P. gingivalis* (forward 5′-AGGCAGCTTGCCATACTGC-3′; reverse 5′-ACTGTTAGCAACTACCGATGT-3′).[32] Amplification products were then analyzed by electrophoresis with 1% agarose gel stained by ethydium bromide. Genomic DNA samples extracted from dental plaque samples were used as positive controls and PCR grade H2O was used as negative control. Positive samples were sent for sequencing (GATC Biotech, France; Appendix S1).

2.7 | Optical Density measurement of specific immune response against periodontal bacteria in serum

The determination of immunoglobulins (Ig)A against *P. gingivalis* and *T. forsythia* was performed in serum, as previously published.[25,33] Briefly, a suspension of *P. gingivalis* or *T. forsythia* was centrifuged (10 000 g for 30 minutes at 4°C). The pellet was washed by 0.05 mol/L sodium carbonate buffer and then resuspended in the same buffer to reach an optical density of 1.0 at 640 nm. The bacteria were heated at

60°C for 45 minutes, diluted 1:10 in sodium carbonate buffer, and dispensed in 96-well plates. The plates were then incubated at 37°C for 4 hours and then overnight at 4°C. The excess bacteria were removed by washing in 0.005% Tween 20 in PBS and the plates were allowed to air-dry before storage at −20°C until use. Each serum sample (dilution 1:100, in 1% BSA-PBS) was added to the *P. gingivalis* or *T. forsythia* -coated plate and incubated at 37°C for 2 hours. After washing, 100 mL of peroxidase-labeled rabbit anti-human Immunoglobulin A (dilution 1:500 in 1% BSA-PBS) was added to each well and incubated for 2 hours at 37°C. After washing, 3, 30, 5, 50-tetramethylbenzidine (TMB) was used as substrate for peroxidase and the reaction was stopped with 0.5N H_2SO_4 before reading at 450 nm. The results (ELISA units, EU) were calculated as continuous variables.

2.8 | Measurements of proteins and hemoglobin in carotid-conditioned medium

Total protein concentrations in conditioned media were quantified using the BIO-RAD Protein Assay. 50 µL of samples (1:40) and standard (BSA 120–7,5 µg/mL) were diluted in distilled water. BIO-RAD reagent (30%) was added and absorbance was read at 590 nm after incubation for 5 minutes. Hemoglobin concentration in conditioned media was quantified by the indirect biochemical quantification of heme. The standard (Hemoglobin Bovine Erythrocyte 5000 µg/mL – 19,7 µg/mL) was diluted in distilled water. Formic acid (70 µL) was added to the samples (30 µL) and standard (30 µL) and absorbance were read immediately at 405 nm.

2.9 | Measurement of myeloperoxidase and human neutrophil peptides 1-3 in carotid-conditioned medium

Myeloperoxidase (MPO) was quantified using the "human MPO Elisa kit" (Hycult Biotechnology) and Human Neutrophil Peptides (HNP)1-3 were quantified using the "Human HNP1-3 Elisa kit" (Hycult Biotechnology) according to the supplier's instructions. Briefly, samples diluted at 1:10 (MPO) or 1:20 (HNP1-3) were incubated with a monoclonal antibody for 1 hour at room temperature (RT). After washing, a biotinylated tracer antibody and then a solution with streptavidin-peroxidase were added and incubated for 1 hour at RT. After washing the peroxidase substrate TMB was added. Absorbance at 405nm was measured after 30 min of incubation at RT and the addition of STOP solution.

2.10 | Measurement of Leukotriene B4 in carotid-conditioned medium

Leukotriene (LT)B4 was quantified using the "LTB4 Elisa Kit" (Cayman Chemical) following the manufacturer's instructions. Briefly, samples diluted at 1:10 were incubated with a mouse anti-rabbit IgG, an acetylcholinesterase (AChE) tracer, and an ELISA antiserum overnight at 4°C. After washing, Ellman's Reagent was added. Absorbance at 410 nm was measured after 100 minutes of incubation at RT.

2.11 | Measurement lipopolysaccharides in carotid-conditioned medium

Lipopolysaccharides released by the plaque into the conditioned medium were quantified using the Limulus Amebocyte Lysate (LAL) chromogenic endpoint assay (Hycult Biotechnology) according to the supplier's instructions. Briefly, samples diluted at 1:5 were incubated with LAL reagent (50 mL) for 30 minutes at room temperature and a stop solution was added before reading on a spectrophotometer at 405 nm.

2.12 | Measurement of cytokines in plasma and in carotid-conditioned medium

Levels of interleukin (IL)-1b; IL-1 receptor antagonist (IL-1ra); IL-2; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-10; IL-12; IL-13; IL-15; IL-17; eotaxin; fibroblast growth factor-basic (FGF-basic); granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon gamma (IFN)-γ; interferon gamma-induced protein 10 (IP-10); monocyte chemoattractant protein-1 (MCP-1); macrophage inflammatory protein (MIP)-1α; MIP-1β; regulated on activation, normal T cell expressed and secreted (RANTES); tumor necrosis factor-alpha (TNF-α); vascular endothelial growth factor (VEGF); and platelet-derived growth factor-bb (PDGF-bb) were determined in plasma and carotid-conditioned medium with the Bio-Plex Pro™ Human Cytokines kit (Biorad, Ca, USA) according to the manufacturer's instructions, and by Luminex xMap technology. The samples were diluted fourfold with Bio-Plex sample diluents.

2.13 | Immunofluorescence

Atherothrombotic carotid paraffin sections (n = 6, randomly selected among samples confirmed positive for *P. gingivalis* with nested PCR) were incubated with an antibody directed against gingipains from *P. gingivalis* (4 µg/mL, 61BG1.3, DHSB, Iowa, USA). To stain nuclei, all slides were incubated with 4, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA). They were visualized using a ZEISS Axioskop 2 Plus fluorescence microscope (Appendix S1).

2.14 | Statistical analysis

Quantitative data were reported as medians (interquartile range) and compared using the Mann-Whitney U tests and Spearman correlation as appropriate. Qualitative data were reported as the number of patients (percentages) and compared using Pearson chi-square tests or Fisher exact tests, depending on the sample size. All the tests were two-sided. Missing data were not analyzed or estimated.

P-values < .05 were considered to be significant. The methods of Benjamini and Hochberg[34] were used to control the false discovery rate. The covariable smoking status was dichotomized in non-smokers versus current and former smokers.

Analyses were performed using R statistical software, version 3.6.2 (R Project for Statistical Computing). The data file received approval from the French data protection agency. The study was reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[35] (Appendix S1).

3 | RESULTS

3.1 | Characteristics of the study population

Demographic, periodontal, medical, and clinical biomarkers are indicated in Table 1. All the 45 included patients presented a moderate to severe periodontitis according to the CDC-AAP classification,[36] associated with a high frequency of *Porphyromonas gingivalis (P. gingivalis) and Tannerella forsythia (T. forsythia)* in subgingival biofilm samples. Major cardiovascular risk factors (age, hypertension, hypercholesterolemia, tobacco smoking) were frequently reported and three quarters of the study population had carotid stenosis > 70%.

TABLE 1 Demographic, medical, carotid, periodontal, and clinical laboratory data

Demographics		
Age, y, median (IQR)	71	(64-77) [45]
Men, n (%)	32	(71%) [45]
BMI, kg/m^2, median (IQR)	25	(23-28) [45]
Medical history		
Diabetes, n (%)	6	(19%) [32]
Hypertension, n (%)	22	(69%) [32]
Current or former smoker, n (%)	20	(63%) [32]
Carotid data		
Symptomatic carotid plaque, n (%)	24	(75%) [32]
Stenosis > 70%, n (%)[a]	19	(73%) [26]
Presence of *P. gingivalis* , n (%)	11	(24%) [45]
Periodontal data		
Mean PI, %		81% [44]
Mean BOP, %		26% [45]
%CAL ≥ 5 mm		36% [45]
%PD ≥ 6 mm		7% [45]
Presence of *P. gingivalis* , n (%)	23	(52%) [44]
Presence of *T. forsythia* , n (%)	39	(89%) [44]
Clinical laboratory data		
HbA1c, %, median (IQR)	6.2	(5.7-6.4) [25]
Total cholesterol, mmol/L, median (IQR)	5	(3.6-5.4) [25]
LDLc, mmol/L, median (IQR)	2.7	(2.1-3.4) [25]

Note: Data are reported as percentages of the available data [n].

Abbreviations: BMI, body mass index; BOP, bleeding on probing; CAL, clinical attachment level; HbA1c, glycated hemoglobin; IQR, interquartile range; LDLc, low-density lipoprotein cholesterol; PD, pocket depth; *P. gingivalis*, *Porphyromonas gingivalis*; PI, periodontal plaque index; *T. forsythia*, *Tannerella forsythia*.

[a]Measured by Doppler.

3.2 | Atherothrombotic plaque microbiome

The atherothrombotic microbiota, identified by microbial whole-genome sequencing in 19 samples, was dominated by *Proteobacteria* (85.8%), followed by *Actinobacteria* (7.6%), *Firmicutes* (5.6%), and *Bacteroidetes* (0.8%) (Figure 1). One hundred and forty-four bacterial genera were detected (Table S1) and 55, 20, and 72 bacterial genera found in the carotid microbiome were shared with the aerodigestive upper tract, the oral cavity, and the gut, respectively (Table 2). The most frequently detected genera (>1%) in the carotid biopsies were: *Variovorax* (46.2%), *Ochrobactrum* (11.2%), *Acidovorax* (9.3%), *Propionibacterium* (5.7%), *Undibacterium* (4.1%), *Stenotrophomonas* (4.0%), *Massilia* (2.1%), *Staphylococcus* (2.1%), *Pseudomonas* (1.5%), *Burkholderia* (1.2%), *Streptococcus* (1.2%) *and Corynebacterium* (1.0%) (Figure 2). More specifically, *Streptococcus* was found in 84% of carotid plaques. 21% of the samples were positive for at least one genus associated with periodontitis, that is, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Campylobacter*, and *Capnocytophaga*.

3.3 | Periodontitis microbial signature and biological activity of the carotid plaque

P. gingivalis DNA was found in 24% of the samples by nested PCR and gingipains from *P. gingivalis* were identified by immunofluorescence surrounded by cells (Figure 3).

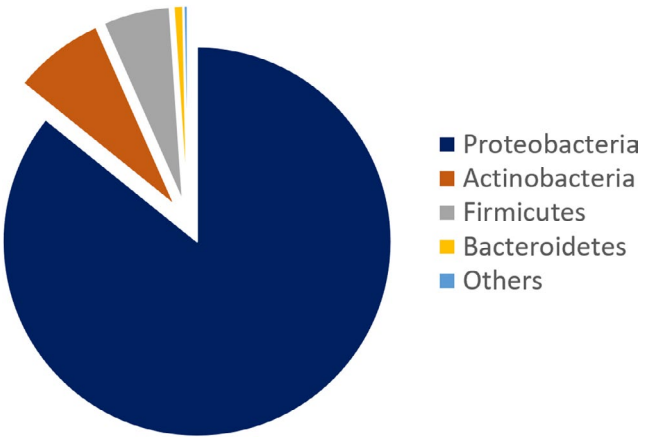

FIGURE 1 The atherothrombotic microbiota, identified by microbial whole-genome sequencing in 19 samples, was dominated by *Proteobacteria* (85.8%), followed by *Actinobacteria* (7.6%), *Firmicutes* (5.6%), and *Bacteroidetes* (0.8%)

TABLE 2 Bacterial genera identified in carotid atherothrombotic samples by whole bacterial genome sequencing

Genus	n						
Acetobacter‡	2	*Dermacoccus*	1	*Limnobacter*	1	*Pseudarthrobacter*	1
Achromobacter ‡	8	*Desemzia*	2	*Luteimonas*	1	*Pseudochrobactrum*	3
Acidovorax ‡	16	*Devosia*	2	***Lysinibacillus*** ‡	1	***Pseudomonas*** ‡	16
Acinetobacter ‡	10	***Dialister*** ‡	1	*Lysobacter*	1	*Psychrobacter*‡	5
Actinomyces ‡	2	***Dietzia***	1	*Massilia*	15	*Qipengyuania*	2
Actinoplanes	1	*Duganella*	2	*Methylibium*‡	2	*Rathayibacter*	1
Aerococcus ‡	6	*Dyella*	3	*Methylobacterium*‡	8	*Reyranella*	4
Afipia ‡	8	***Enhydrobacter*** ‡	2	***Microbacterium***	1	*Rhizobacter*	1
Agrobacterium	15	***Enterococcus*** ‡	1	***Micrococcus*** ‡	3	*Rhizobium*‡	5
Alkanindiges	1	*Exiguobacterium*‡	3	*Microcoleus vaginatus*	1	*Rhodanobacter*	2
Altererythrobacter	1	*Faecalibacterium*‡	1	*Microlunatus*	2	***Rhodobacter*** ‡	1
Amnibacterium	1	***Finegoldia*** ‡	4	*Micromonospora*	1	*Rhodococcus*‡	2
Anaerococcus ‡	7	*Flavobacterium*‡	1	*Microvirga*	1	*Rhodopseudomonas*‡	1
Arthrobacter‡	6	*Friedmanniella*	2	*Modestobacter*	1	***Roseomonas*** ‡	2
Atopobium ‡	1	*Frigoribacterium*	1	***Mycobacterium*** ‡	1	*Rubellimicrobium*	1
Bacillus ‡	7	***Fusobacterium*** ‡	1	*Nakamurella*‡	1	*Ruminiclostridium*	1
Beijerinckia‡	3	*Geobacillus*‡	1	*Naumannella*	1	*Shinella*	1
Blastococcus	4	*Georgenia*	1	***Neisseria*** ‡	2	*Skermanella*	3
Brachybacterium‡	1	*Gordonia*‡	1	*Neorhizobium*	2	*Sphingobacterium*‡	4
Bradyrhizobium ‡	3	***Granulicatella*** ‡	1	*Nocardioides*‡	1	*Sphingobium*‡	3
Brevibacterium ‡	1	***Hemophilus*** ‡	1	***Novosphingobium*** ‡	11	***Sphingomonas*** ‡	12
Brevundimonas	5	*Haloanella*	2	***Ochrobactrum*** ‡	10	*Sphingopyxis*‡	3
Burkholderia ‡	16	*Herminiimonas*‡	15	***Paenibacillus*** ‡	1	***Staphylococcus*** ‡	18
Campylobacter ‡	1	*Humibacillus*	1	***Paracoccus*** ‡	8	***Stenotrophomonas*** ‡	9
Candidatus Phytoplasma	5	*Hymenobacter*	3	*Parageobacillus*	1	*Steroidobacter*	1
Capnocytophaga ‡	1	*Ideonella*	2	***Pedobacter*** ‡	3	***Streptococcus*** ‡	15
Carnobacterium	1	***Janibacter***	1	*Pelomonas*	4	*Tepidimonas*	5
Chelatococcus	1	*Janthinobacterium*	3	***Peptoniphilus*** ‡	3	*Tepidiphilus*	1
Chroococcus	1	*Kaistobacter*	1	*Phreatobacter*	3	*Terrisporobacter*	1
Chryseobacterium‡	4	***Kocuria*** ‡	3	*Planomicrobium*	2	*Thermoactinomyces*	1
Cloacibacterium	3	***Kytococcus*** ‡	1	*Plantibacter*	2	*Undibacterium*	17
Corynebacterium ‡	17	***Lactobacillus*** ‡	15	*Polaromonas*‡	3	*Unknown*	14
Curtobacterium	3	***Lactococcus*** ‡	12	***Porphyromonas*** ‡	2	***Variovorax*** ‡	18
Curvibacter	12	***Lautropia***	1	***Prevotella*** ‡	3	***Veillonella*** ‡	1
Daeguia	1	*Leucobacter*	2	*Prolinoborus*	2	*Vibrionimonas*	4
Delftia ‡	10	*Lihuaxuella*	1	***Propionibacterium*** ‡	19	*Xylophilus*	1

Note: Identification by body origin (aerodigestive upper tract, oral cavity, and gut). Bold, underlined, ‡ = bacterial general respectively shared with aerodigestive upper tract, oral cavity, and gut[49,50]

Abbreviation: n, n vascular biopsies positive (total n = 19).

There was an inverse significant correlation between levels of immunoglobulin (Ig)A against *P. gingivalis* in serum and interleukin (IL)-4 ($r = -.481$, $P = .011$) in carotid-conditioned medium. There was also an inverse correlation between levels of IgA against *T. forsythia* in serum and IL-4 ($r = -.492$, $P = .010$) and IL-5 ($r = -.485$, $P = .011$) in carotid-conditioned medium. Finally, there was a significant correlation between levels of IgA against *P. gingivalis* in serum and leukotriene (LT)B4 ($r = .457$, $P = .041$) in carotid-conditioned medium (Figure 4).

3.4 | Biological activity of intraplaque hemorrhage

Levels of proteins, myeloperoxidase (MPO), human neutrophil peptide (HNP)1-3, LTB4, and lipopolysaccharides (LPS) were significantly higher in carotid-conditioned medium of high-volume carotid intraplaque hemorrhage compared to low volume hemorrhagic ones (Table 3).

In addition, in carotid-conditioned medium, there was a significant correlation between LPS and proteins ($r = .418$, $P = .050$),

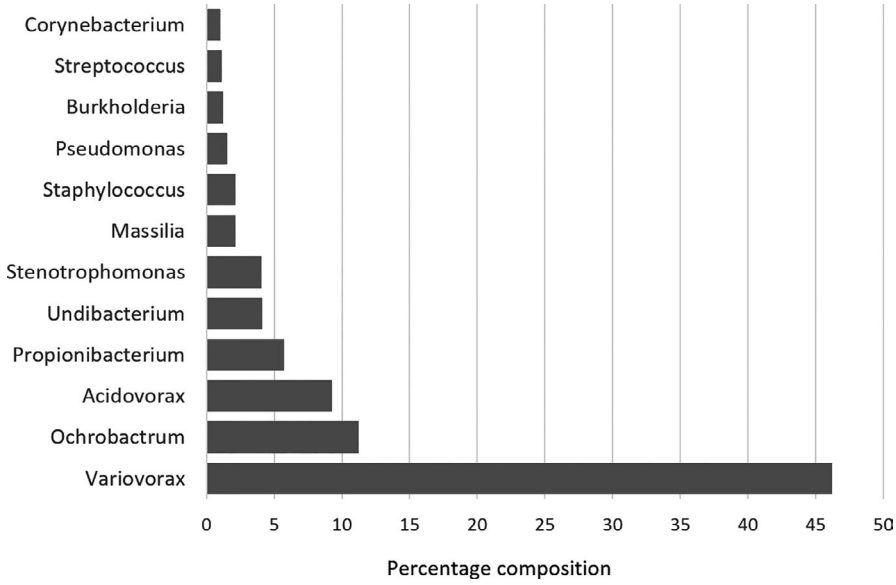

FIGURE 2 Genus distribution plot. The most frequent (>1%) bacterial genera detected in carotid atherothrombotic samples were *Variovorax* (46.2%), *Ochrobactrum* (11.2%), *Acidovorax* (9.3%), *Propionibacterium* (5.7%), *Undibacterium* (4.1%), *Stenotrophomonas* (4.0%), *Massilia* (2.1%), *Staphylococcus* (2.1%), *Pseudomonas* (1.5%), *Burkholderia* (1.2%), *Streptococcus* (1.2%) and *Corynebacterium* (1.0%)

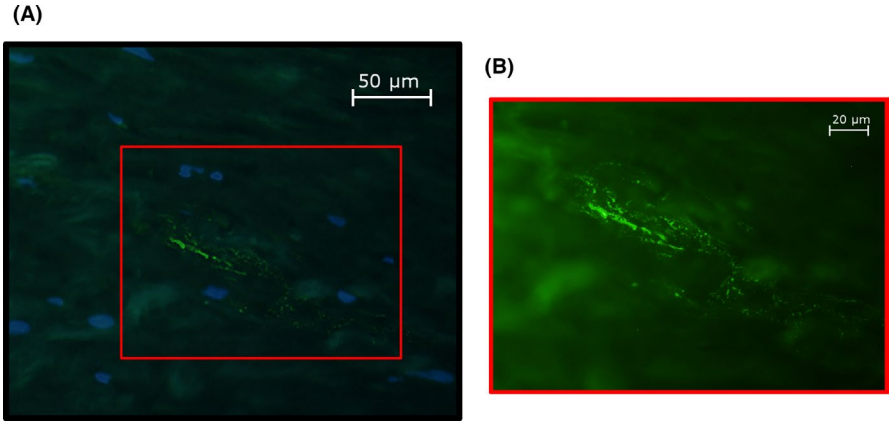

FIGURE 3 Cross-section of a complicated human carotid atheroma plaque. Gingipains from *Porphyromas gingivalis* (*P. gingivalis*) were shown by immunofluorescence. A, Merge with 4',6-diamidino-2-phénylindole (DAPI) staining. Gingipains from *P. gingivalis* appear in bright green and nuclei in blue [16 mm ~ 50 µm]. B, Magnification focused on *P. gingivalis* (bright green) [8 mm ~ 20 µm]

hemoglobin (r = .515, P = .022) and HNP1-3 levels (r = .484, P = .022). There was also a significant correlation between hemoglobin and proteins (r = .597, P < .001), MPO (r = .347, P = .050) and HNP1-3 (r = .410, P = .024; Figure 5).

3.5 | Periodontitis endpoints and serum markers

In our population, there was a strong association between the percentage of pocket depth ≥ 6 mm, total cholesterol (r = .628, P = .007), and low-density lipoprotein cholesterol (r = .618, P = .008) in serum.

There was also a significant inverse correlation between the percentage of clinical attachment loss ≥ 5 mm and IL-4 (r = −0.418, P = .041) and IL-5 in plasma (r = −.446, P = .025; Figure 4).

4 | DISCUSSION

The present study indicates that patients at risk of stroke with a moderate to severe periodontitis presented a highly diverse carotid microbiome with a high frequency of samples showing positive for bacterial genera from the oral cavity, thus confirming our research hypothesis. Neutrophil activation markers were associated with levels of LPS in carotid-conditioned medium and a high-volume of carotid intraplaque hemorrhage. Finally, levels of cytokines that inhibit neutrophils were inversely correlated with clinical markers of periodontitis. These findings support a link between periodontitis and the activation of the local innate immune response, mainly through neutrophils in carotid atherothrombotic plaques.

There is consistent epidemiological evidence that periodontitis increases the risk of atherothrombotic stroke.[11] Many in vitro and pre-clinical studies have shown a plausible role for specific periodontal bacteria such as *P. gingivalis* in atherogenesis.[12,37] Recently, a polyinfection murine model of periodontitis, triggering a unique oral microbiome and virome[38] has been successfully used to induce a modified inflammatory pattern that accelerated aortic atherosclerotic plaque formation.[39] No observational study has been conducted in humans to investigate the relationship between periodontitis, the carotid microbiome, and the innate immune response in atherothrombotic plaques.

Several studies examined bacterial diversity in human atherothrombotic plaques using broad-range PCR (based on 16S rRNA gene sequencing) or next-generation sequencing.[40-48] Using the metagenomic approach, we found more than a hundred and forty

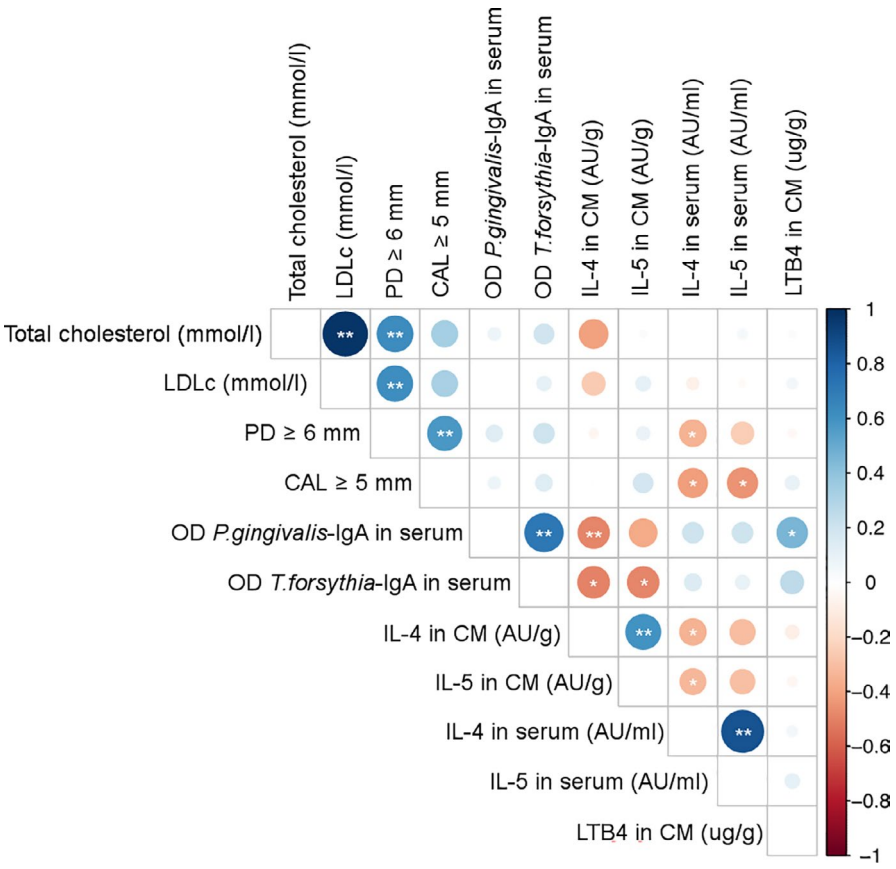

FIGURE 4 Spearman correlation between cholesterol levels, clinical and biological markers of periodontitis, cytokine levels, and neutrophil recruitment marker in serum or carotid-conditioned medium. Data are reported according to the available data. CAL, clinical attachment level; CM, conditioned medium; IL, interleukin; LDLc, low-density lipoprotein cholesterol; LTB4, leukotriene B4; OD, optical density; PD, pocket depth; *P. gingivalis*, *Porphyromonas gingivalis*; *T. forsythia*, *Tannerella forsythia*. **Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed). Blue = positive correlation. Red = negative correlation

(144) different bacterial genera in the carotid microbiome. According to the Human Oral Microbiome Database,[49] 38.2% of the bacterial genera that we identified in the atherothrombotic plaque biopsies were shared with the aerodigestive upper tract (including at least 13.8% with the oral cavity). In addition, 50.0% of the 144 identified genera were shared with the gut.[50] 58.1% of the bacterial genera found in the aerodigestive upper tract were shared with the gut microbiome. The percentage of the bacterial genera found in the carotid atherothrombotic samples that are shared neither with the aerodigestive upper tract, nor with the gut was 45.8%. This high percentage highlights the complexity of the interrelationships between the microbiomes from different body sites. *Ott et al*,[48] were the first to investigate the highly diverse bacterial DNA signatures in atherosclerotic lesions. Their outcomes clearly suggest that atherothrombotic plaques are associated with polyinfection rather than monoinfection.

A study on vascular biopsies (aortic aneurysm, carotid, femoral arteries) showed a higher load and diversity of the vascular microbiome in periodontitis patients compared with periodontally healthy patients.[43] However, *P. gingivalis* was detected in only one of the vascular biopsies of periodontitis patients with no correlation between the compositions of the subgingival microbiota (DNA hybridization) and the vascular microbiome (16S rRNA) in periodontitis patients. Similarly, in our study, no correlation was found between the periodontal microbiota checked by commercial specific PCR in the subgingival dental plaque samples, the carotid microbiome explored by NGS, or plaque vulnerability to rupture. In addition, periodontal bacteria DNA signatures in carotid plaques were scarce (21%) while 80% of the carotid biopsies were positive for at least one *Streptococcus*, a major commensal bacterial genus from the oral cavity. This result is consistent with a previous study that reported the detection of bacterial DNA in most of the endarterectomy specimens but the absence of DNA from periodontal pathogens.[51] *Streptococcus mutans*, a cariogenic strain of *Streptococcus*, has been associated with stroke.[52,53] The discrepancy between the high frequency of detection of commensal versus periodontal bacteria in the carotid samples can be explained by: (a) differential temporality between oral bacteremia, bacterial trapping in intraplaque hemorrhages and sampling; (b) easier elimination by the immune system of periodontal bacteria (*P. gingivalis*, *T. forsythia*) compared with commensal species such as *Streptococcus* that are less antigenic; (c) differential capacity to invade endothelial cells between different strains of bacteria.

Our results showed a high presence of commensals alongside a moderate presence of periodontal bacteria in carotid samples in patients with periodontitis, and a high risk of stroke.

In this study, our aim was also to determine whether periodontitis, measured clinically and biologically was associated with the activation of the local innate immune response involved in vulnerability to rupture of the atherothrombotic plaque. We found that the specific immune response (IgA) directed against *P. gingivalis* in serum was linked to the presence of LTB4, a mediator of neutrophil recruitment[54] in the conditioned medium. This suggested that systemic exposure to *P. gingivalis* may be linked to the recruitment

TABLE 3 Demographic, medical, carotid, periodontal, and clinical laboratory data according to carotid plaque hemoglobin concentration

	Low HB (<2500 µg/g)			High HB (>2500 µg/g)			
Demographics							
Age, y, median (IQR)	70	(62-76)	[19]	73	(65-79)	[18]	ns
Men, n (%)	13	(68%)	[19]	13	(72%)	[18]	ns
BMI, kg/m^2, median (IQR)	23	(22-26)	[13]	25,52	(24-29)	[14]	ns
Medical history							
Diabetes, n (%)	4	(31%)	[13]	2	(14%)	[14]	ns
Hypertension, n (%)	9	(69%)	[13]	9	(64%)	[14]	ns
Current or former smoker, n (%)	9	(69%)	[13]	7	(50%)	[14]	ns
Carotid data							
Symptomatic carotid plaque, n (%)	9	(69%)	[13]	11	(79%)	[14]	ns
Stenosis > 70%, n (%)[a]	8	(67%)	[12]	8	(73%)	[11]	ns
Presence of *P. gingivalis* , n (%)	7	(37%)	[19]	4	(22%)	[18]	ns
Periodontal data							
Mean PI, %	77%		[18]	82%		[18]	ns
Mean BOP, %	26%		[19]	26%		[18]	ns
%CAL ≥ 5 mm	47%		[19]	27%		[18]	ns
%PD ≥ 6 mm	10%		[19]	5%		[18]	ns
Presence of *P. gingivalis* , n (%)	9	(47%)	[19]	8	(47%)	[17]	ns
Presence of *T. forsythia* , n (%)	17	(89%)	[19]	15	(88%)	[17]	ns
Clinical laboratory data (carotid-conditioned medium)							
Proteins, µg/g, median (IQR)	10 625	(8169-13072)	[19]	16 235	(11950-18325)	[18]	*P* = .008
HNP1-3, µg/g, median (IQR)	0.055	(0.05-0.08)	[19]	0.099	(0.06-0.22)	[18]	*P* = .007
MPO, µg/g, median (IQR)	0.16	(0.09-1.6)	[19]	1.58	(0.41-6.52)	[18]	*P* = .029
LTB4, µg/g, median (IQR)	0.003	(0.002-0.005)	[14]	0.005	(0.003-0.006)	[15]	ns
LPS, EU/mL, median (IQR)	1.80	(1.78-1.88)	[11]	1.99	(1.89-2.42)	[15]	*P* = .009
Clinical laboratory data (serum)							
HbA1c, %, median (IQR)	6.3	(6.2-6.5)	[8]	5.9	(5.8-6.4)	[10]	ns
Total cholesterol, mmol/L, median (IQR)	5.2	(3.9-6.2)	[11]	4.9	(3.9-5.4)	[10]	ns
LDLc, mmol/L, median (IQR)	3.1	(2.3-4.1)	[11]	2.6	(1.8-3.4)	[10]	ns
Anti- *P. gingivalis* IgA, OD, median (IQR)	0.25	(0.17-0.29)	[19]	0.29	(0.18-0.34)	[18]	ns
Anti-*T. forsythia* IgA, OD, median (IQR)	0.11	(0.09-0.15)	[19]	0.12	(0.08-0.15)	[18]	ns

Note: The distribution is balanced between the two groups. Data are reported as percentages of the available data [n].

Abbreviations: BMI, body mass index; BOP, bleeding on probing; CAL, clinical attachment level; HB, hemoglobin; HbA1c, glycated hemoglobin; HNP1-3, human neutrophil peptides 1-3; IgA, immunoglobulin A; IQR, Interquartile range; LDLc, low-density lipoprotein cholesterol; LPS, lipopolysaccharides; LTB4, leukotriene B4; MPO, myeloperoxidase; PD, pocket depth; *P. gingivalis*, *Porphyromonas gingivalis*; PI, Periodontal plaque Index; *T. forsythia*, *Tannerella forsythia*.

[a]Measured by Doppler.

of neutrophils in the atherothrombotic plaque. There was also an inverse correlation between the specific immune response (IgA) directed against *P. gingivalis* or *T. forsythia* in serum and the level of IL-4 and/or IL-5 in carotid-conditioned medium. IL-4 and IL-5 are known to regulate Th2 lymphocyte response involved in wound repair.[55] Recently, there has been a growing body of evidence that suggests involvement of neutrophils in type 2 immunity.[20] IL-4 and its signaling pathway participate in the inhibition process of several neutrophil effector functions such as the formation of neutrophil extracellular traps to prevent them from damaging healthy tissues. Consequently, we showed that high systemic exposure to major periodontal bacteria was correlated with low cytokine response able to shut off the activation of neutrophils and damage to them in the surrounding vascular tissues. The present data also showed an association in carotid-conditioned medium between bacterial endotoxins (LPS), neutrophil activation markers (MPO, HNP1-3), and

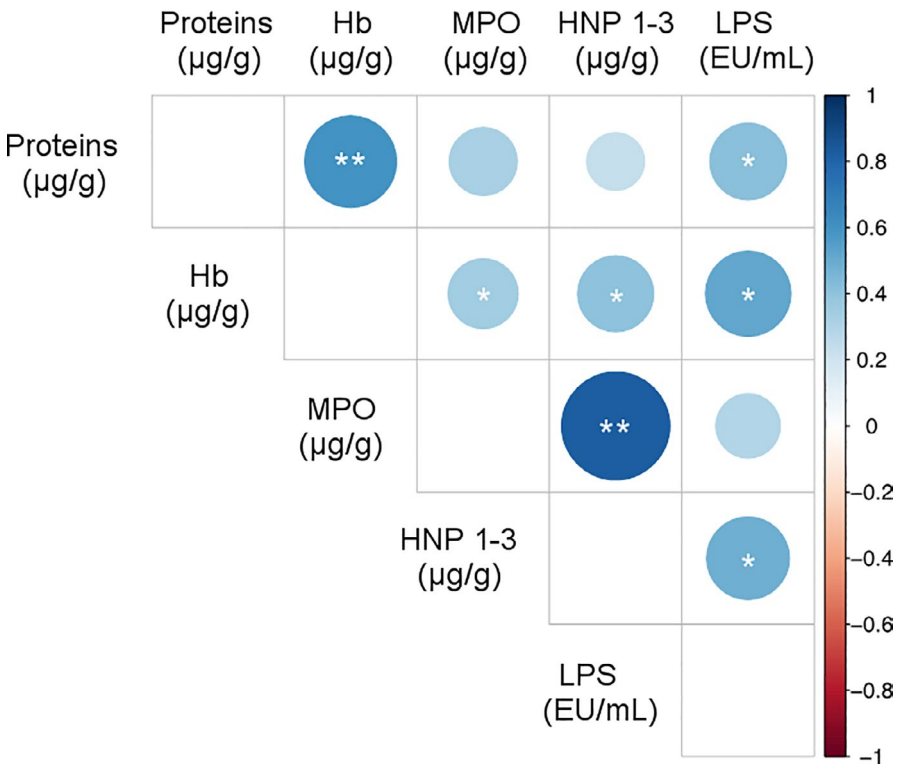

FIGURE 5 Spearman correlation between carotid plaque activity (proteins), neutrophil activation markers (myeloperoxidase, human neutrophil peptides 1-3), markers of carotid plaque vulnerability (hemoglobin), and pathogens (lipopolysaccharides). Data are reported according to the available data. Hb, hemoglobin; HNP 1-3, human neutrophil peptides 1-3; LPS, lipopolysaccharides; MPO, myeloperoxidase. **Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed). Blue = positive correlation. Red = negative correlation

a high-volume carotid intraplaque hemorrhage (hemoglobin). The role of periodontitis in carotid atherothrombosis can be inferred by maintaining a low level of anti-inflammatory cytokines (IL-4, IL-5) and a high level of neutrophil activation markers (LTB4, MPO, HNP1-3) that favor tissue damage and vulnerability to rupture of the carotid atherothrombotic plaque.

Lastly, the present study showed a strong association between high total and LDL cholesterol and deep periodontal pockets, and an inverse correlation between clinical attachment loss and the level of anti-inflammatory cytokines (IL-4, IL-5) in plasma. Studies have repeatedly shown that periodontal therapy decreases serum lipid concentration[56,57] and levels pro-inflammatory cytokines.[58] Thus, we can hypothesize that periodontitis increases LDL cholesterol, leading to the dysregulation of the inflammatory pattern.

To our knowledge, the present observational study is the first to show a relationship between the microbial signature in carotid plaques and immunobiological activity, characterizing their vulnerability to rupture in periodontitis patients. It reinforces the hypothesis that atherothrombosis is associated with polyinfection rather than with specific bacteria, either from the oral cavity or from another ecological site. The results strengthen our hypothesis that oral microbiota in patients with periodontitis may act with microbiota from other sites, in particular the gastrointestinal tract. Those microbiomes may dysregulate the pattern of inflammatory mediators in favor of long-lasting neutrophil-induced breakdown within the atherothrombotic plaque. However, there are some limitations to acknowledge. An important one is that we measured the serum level of IgA only, based on our previous work in coronary artery disease patients showing association between IgA (but not IgG) and a decrease in capacity of atherosclerotic plaque remodeling. However, humoral immunity against periodontal bacteria is driven by several class of immunoglobulins. Associations between levels of serum Ig G in periodontitis patients and markers of carotid plaque vulnerability remain to be determined. Although the study sample size was rather high compared to other studies assessing biological relationships between periodontitis and atherothrombosis, the number of subjects included was too small to highlight clinical inference between both diseases. Last, all the subjects included in the study had periodontitis however that was not an inclusion criterion. This clinical feature can be explained because patients scheduled for carotid endarterectomy at the Bichat hospital, that is, the CASIMIR cohort 1) were elderly (median age: 71 years old), 2) had a high prevalence of periodontal risk factors and comorbidities, such as dental plaque (mean plaque index 81%), smoking (63%), diabetes (19%) The lack of a control group without periodontitis was an unexpected characteristic of the present observational study that should be acknowledged.

5 | CONCLUSION

Atherothrombosis is associated with the presence of a highly diverse bacterial microbiome in the plaque. The oral microbiota, both periodontal and commensal bacteria, is an unneglectable part of the carotid microbiome in patients with periodontitis. Oral microbiota with microbiota from other origins may stimulate a neutrophil response in carotid intraplaque hemorrhages, with a high risk of clinical complications such as stroke. Emerging evidence of a wide range of bacterial species, including those from the oral cavity, such as *Porphyromonas gingivalis* and *Streptococcus species*, justifies the need for more studies with a translational approach.

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CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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