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Periodontal bacteria in human carotid atherothrombosis as a potential trigger for neutrophil activation

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ABSTRACT

Objective: Epidemiological, biological and clinical links between periodontal and cardiovascular diseases are now well established. Several human studies have detected bacterial DNA corresponding to periodontal pathogens in cardiovascular samples. Intraplaque hemorrhage has been associated with a higher risk of atherosclerotic plaque rupture, potentially mediated by neutrophil activation. In this study, we hypothesized that plaque composition may be related to periodontal pathogens.
Methods: Carotid culprit plaque samples were collected from 157 patients. Macroscopic characterization was performed at the time of collection: presence of blood, lipid core, calcification and fibrosis. Markers of neutrophil activation released by carotid samples were quantified (myeloperoxidase or MPO, cell-free DNA and DNA-MPO complexes). PCR analysis using specific primers for *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcommitans*, *Treponema denticola*, *Prevotella intermedia* and *Tannerella forsythia* was used to detect DNA from periodontal pathogens in carotid tissues. In addition, bacterial lipopolysaccharide (LPS) and Immunoglobulins G against *T. forsythia* were quantified in atherosclerotic carotid conditioned medium.
Results: Intraplaque hemorrhage was present in 73/157 carotid samples and was associated with neutrophil activation, reflected by the release of MPO, cell-free DNA and MPO-DNA complexes. LPS levels were also linked to intraplaque hemorrhage but not with the neutrophil activation markers. Seventy-three percent of the carotid samples were positive for periodontal bacterial DNA. Furthermore, hemoglobin levels were associated with the detection of *T. forsythia* and neutrophil activation/inflammation markers.
Conclusion: This study suggests a potential role of periodontal microorganisms, especially *T. forsythia*, in neutrophil activation within hemorrhagic atherosclerotic carotid plaques.

1. Introduction

For over 20 years, epidemiologic data supporting the idea that periodontal disease may impact the progression and, potentially, the onset of cardiovascular disease has been the subject of numerous reports [1,2]. Furthermore, meta-analyses have suggested an association between periodontal and cardiovascular diseases [3–7]. The strength of this association, may, however, be confounded by the fact that the two groups share risk factors such as aging, diabetes, obesity and smoking. Biological evidence suggests a potential causal role for periodontal bacteria that could be

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involved directly in atherogenesis [8,9] or indirectly by increasing circulating cytokines and inflammatory mediators. Periodontal bacteria could migrate from the gingival/dental sites to the vascular wall via the bloodstream and act directly via their virulence factors, such as gingipains, proteinases, fimbriae, and/or lipopolysaccharides (LPS). In addition, the capacity of periodontal bacteria to induce leukocyte recruitment may contribute to this biological process.

Numerous clinical studies have shown the presence of DNA from periodontal pathogens in atherosclerotic plaques (for review [10]) and others have recovered viable, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* from plaque samples [11–13]. The biological evidence underlying the bacterial hypothesis chiefly relies on reports of *in vitro* activation of endothelial cells by periodontal pathogens and LPS (via Toll-like receptors 4 and 2 [14,15]) and the subsequent induction of apoptosis [16]. Animal studies using models of atheroma (apoE−/− mice, rabbits and pigs [10]) or abdominal aortic aneurysm in rats [17] suggest that *P. gingivalis* may promote atherogenesis and thrombus formation. Leukocytes may also directly or indirectly stimulate endothelial cells which produce pro-atherogenic molecules, such as monocyte chemoattractant protein-1, intercellular adhesion molecule-1 or vascular cell adhesion protein-1 (for review [18]).

In cerebrovascular diseases, which represent 16.4% of the global cardiovascular deaths [19] and lead to major sequelae for patients, the role of intraplaque hemorrhage in favoring plaque vulnerability to rupture via neutrophil protease release has been clearly shown [20–23]. A recent study has underlined the biological role of the thrombus for trapping pathogens in order to prevent their dissemination [24].

We thus hypothesize that intraplaque hemorrhage may promote periodontopathogen translocation into atherosclerotic plaques that could in turn trigger neutrophil activation, leading to clinical complications. One hundred and fifty seven human carotid plaque samples were macroscopically and biochemically classified into hemorrhagic versus non-hemorrhagic groups. Markers of neutrophil activation and death (myeloperoxidase −MPO- and neutrophil extracellular traps −NETs-), oxidative stress and inflammation (carbonyl groups and NFκB activity) were quantified using the conditioned medium [20] obtained from each carotid sample. MPO is an enzyme released by neutrophils to kill bacteria and other pathogens whereas the formation of NETs is induced by infection. NETs consist of extracellular, highly decondensed chromatin, including histones and DNA associated with neutrophil granule proteins [25]. DNA extraction and specific amplification of five major periodontopathogens were performed. The biological data obtained for carotid plaque samples were analysed in view of the corresponding clinical characteristics of each patient.

2. Materials and methods

2.1. Tissue sampling

Human carotid endarterectomy samples ($n = 157$) and non-atherosclerotic mammary endarteries ($n = 10$) were collected from patients undergoing surgery at the Centre Cardiologique du Nord (Saint Denis, France). All patients underwent an interview before surgery to collect medical information (Table 1) and absence of objection to using their carotid samples, considered as surgical waste in accordance with French ethical laws (L.1211-3 to L.1211-9) and the INSERM Ethics Committee. Tissue samples were collected in cold RPMI (4 °C) containing antibiotics plus an antimycotic, and processed within 2 h after surgery.

Table 1
Patients, carotid plaque and biological characteristics according to hemoglobin quartiles.

	Hemoglobin, μg/mL				*P* for trend
	<52 *n* = 29	52–94 *n* = 30	95–196 *n* = 29	>196 *n* = 29	
Demographics					
Age, years, mean ± SD	70.8 ± 8.8	71.5 ± 12.8	73.8 ± 8.4	71.6 ± 9.3	0.58[a]
Men, *n*(%)	21 (72.4)	19 (63.3)	19 (65.5)	24 (82.8)	0.38[b]
BMI, kg/m^2, mean ± SD	26.5 ± 3.4	26.1 ± 3.8	26.4 ± 4.5	26.5 ± 4.2	0.97[a]
Medical history					
Diabetes, *n*(%)	9 (31.0)	11 (36.7)	9 (31.0)	8 (27.6)	0.68[b]
Hypertension, *n*(%)	20 (69.0)	22 (73.3)	22 (75.9)	22 (75.9)	0.53[b]
Hypercholesterolemia, *n*(%)	21 (72.4)	20 (66.7)	22 (75.9)	23 (79.3)	0.41[b]
Current or former smoker, *n*(%)	15 (51.7)	14 (46.7)	17 (58.6)	18 (62.1)	0.30[b]
Carotid data					
Symptomatic Carotid Plaque, *n*(%)	9 (31.0)	5 (17.2)	6 (20.7)	9 (31.0)	0.93[b]
Intraplaque hemorrhage, *n*(%)	11 (37.9)	12 (40.0)	10 (34.5)	24 (82.8)	0.002[b]
Intimal fibrin deposition, *n*(%)	8 (27.6)	10 (33.3)	6 (20.7)	7 (24.1)	0.53[b]
Lipid rich plaque, *n*(%)	11 (37.9)	15 (50.0)	10 (34.5)	8 (27.6)	0.25[b]
Calcified plaque, *n*(%)	20 (69.0)	20 (66.7)	21 (72.4)	13 (44.8)	0.10[b]
Biological data					
Carbonyl, nmol, median (IQR)	8.1 (6.6–9.5)	8.1 (6.1–9.3)	9.0 (5.3–9.7)	5.5 (4.8–9.8)	0.84[c]
DNA, ng/mL, median (IQR)	1100 (631–1505)	1308 (654–2771)	1360 (808–2379)	2194 (1258–2631)	0.005[c]
Myeloperoxidase, μg/mL, median (IQR)	61 (33–118)	145 (44–214)	87 (55–186)	230 (127–386)	<0.001[c]
Lipopolysaccharides, EU/mL, median (IQR)	0.30 (0.15–0.84)	0.73 (0.41–1.07)	0.42 (0.28–1.00)	1.50 (0.73–2.29)	<0.001[c]
DNA-MPO complexes, median (IQR)	1.04 (0.49–1.58)	2.19 (0.67–3.37)	1.43 (0.81–3.17)	3.53 (2.69–4.03)	<0.001[c]
NFκB activity, median (IQR)	0.26 (0.20–0.31)	0.28 (0.21–0.31)	0.29 (0.24–0.57)	0.34 (0.27–0.42)	0.035[c]
Presence of ≥1 bacterial species, *n*(%)	19 (65.5)	21 (70.0)	20 (69.0)	24 (82.8)	0.18[b]
Presence of Pg DNA, *n*(%)	11 (37.9)	15 (50.0)	11 (37.9)	10 (34.5)	0.58[b]
Presence of Tf DNA, *n*(%)	8 (27.6)	6 (20.0)	13 (44.8)	14 (48.3)	0.03[b]
Presence of Pi DNA, *n*(%)	9 (31.0)	9 (30.0)	8 (27.6)	9 (31.0)	0.95[b]
Anti-Tf IgG (EU)	0.021 (0.004–0.078)	0.053 (0.023–0.1)	0.080 (0.044–0.106)	0.119 (0.075–0.182)	<0.001[c]

[a] Analysis of variance trend test.
[b] Mantel-Haenszel trend test.
[c] Non-parametric analysis of variance trend test.

2.2. Conditioned media and post-incubation tissues

Carotid endarterectomy samples were dissected as previously reported [20,21,26], separating the culprit part from the adjacent plaque. Culprit plaques (CPs), corresponding to the most stenosed segments, were compared with non-stenosing, adjacent plaques (NPs) and with control non-atherosclerotic mammary endarteries. Culprit plaques were qualitatively classified, according to the macroscopic presence of intraplaque hemorrhage, calcification, lipids and fibrosis. The presence of intraplaque hemorrhage was confirmed by the indirect biochemical quantification of heme (see Supplemental data 1). Conditioned media and corresponding post-incubation tissues were obtained as previously described [20,21]. Briefly, small pieces of tissue (about 5 mm^3) were incubated (24 h at 37 °C) in a standardized volume (6 mL/g of sample wet weight) of RPMI culture medium supplemented with antibiotics and an antimycotic.

2.3. Determination of cell-free DNA concentrations

Cell-free DNA (cf-DNA) concentrations in the conditioned media were estimated using Quant-it™ Picogreen® ds DNA Reagent (Invitrogen). Dilutions of 25 μL of samples and lambda DNA standard (1 ng/mL − 1 μg/mL) in TE buffer (200 mM Tris-HCL, 20 mM EDTA, pH 7.5, 100 μL final) were performed, then 100 μL of Picogreen® ds DNA Reagent was added. Fluorescence was measured (excitation 480 nm, emission 520 nm) after homogenization and incubation for 5 min at room temperature in the dark. The inter-assay coefficient of variation (CV) was estimated at 4.35%.

2.4. Determination of myeloperoxidase (MPO) and MPO-DNA complexes

The concentration of MPO in CP conditioned medium ($n = 157$) was determined using human MPO ELISA kit from Hycult Biotechnology (Uden, The Netherlands, interassay CV: 3.66%). MPO-DNA complexes were quantified in carotid and mammary artery conditioned media by combining two different ELISA tests as previously described [17]. First, MPO was captured in wells of a 96-well plate coated with a monoclonal antibody against MPO (human MPO ELISA kit, Hycult Biotechnology). Diluted samples (1:5) were added to the plate and incubated for 1 h at room temperature. After washing (twice with PBS), a peroxidase-labeled anti-DNA monoclonal antibody (component number 2 of the Cell death detection ELISA kit, Roche) was added and incubated for 2 h with gentle shaking. After washing, the peroxidase substrate 3, 3′, 5, 5′-tetramethylbenzidine or TMB (Sigma–Aldrich) was added, and the absorbance at 405 nm was measured after 30 min of incubation in the dark at 4 °C. The interassay CV was estimated at 4.22%.

2.5. Determination of carbonyl levels

The degree of carbonylation in conditioned medium was determined by ELISA, based on the recognition of protein-bound 2, 4-dinitrophénylhydrazine or DNPH in carbonylated proteins with an anti-DNP antibody as previously described (Supplemental data 2) [27]. Absorbances were read at 490 nm against the blank. A six-point standard using oxidized bovine serum albumin solution (2.5–20 nmol of carbonyl) was included in each plate. The inter-assay CV was estimated at 12.4%.

2.6. Determination of NFκB activity

RAW-Blue™ cells previously cultured in 96-well plates (50,000 cells/well) were incubated with 50 μl of CP conditioned medium at 37 °C in a 5% CO_2 incubator for 24 h. Briefly, 120 μL of p-nitrophenyl-phosphate 1.10−3 M were added to 40 μL of each supernatant at 37 °C for 120 min and converted to para-nitrophenol which is yellow in alkaline medium and assayed to determine secreted embryonic alkaline phosphatase levels using a spectrophotometer at 420 nm.

2.7. Determination of lipopolysaccharide activity

Lipopolysaccharides released by the CPs ($n = 157$) 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 (50 μL) were incubated with LAL reagent (50 μL) for 30 min at room temperature and a stop solution was added before reading on a spectrophotometer at 405 nm. The interassay CV was estimated at 8.58%.

2.8. Bacterial DNA extraction from carotid tissues and amplification by PCR

Samples were cryopulverized using a freezer mill (Spex Certiprep Ltd) and DNA was extracted from the powder by the QIAamp® DNA blood Midi kit (Qiagen) as previously described (Supplemental data 2) [17]. Before amplification, the quality of extracted DNA was controlled by electrophoresis in a 1% agarose gel stained by ethydium bromide. The extracted DNA of the CP samples was amplified using specific sets of primers corresponding to a sequence encoding 16S rRNA of *P. gingivalis* [17], *A. actinomycetemcomitans* [28], *Treponema denticola* [28], *Prevotella intermedia* [28] and *Tannerella forsythia* [29]. The respective amplicon lengths were 218 bp, 557 bp, 316 bp, 575 bp and 65 bp (Supplemental data Table 2). Briefly, PCR was carried out in a mixture containing 5 μL of DNA (50 ng), 9 μL of H_2O, 4 μL of Master Mix and 2 μL of 0.2 mM PCR primer set and amplification (50 cycles) was performed by real time PCR using a LightCycler® system with SYBR green detection (Roche Applied Biosystems). Products of amplification were then analyzed by electrophoresis in a 1% agarose gel stained by ethydium bromide. Genomic DNA samples extracted from bacterial cultures (Supplemental data 4) were used as positive controls and PCR grade H_2O was used as negative control. After checking the band position corresponding to the expected amplicon size for each sample and primer set, DNA sequences were routinely established in order to confirm the identity of the bacteria. Bidirectional fluorescent sequencing of purified PCR products (ExoSAP-IT, GE Healthcare) was carried out with the Big Dye® Terminator v1.1 Cycle Sequencing kit on an ABI-PRISM® 3130XL genetic analyzer (Applied Biosystems, Applera France SA). Sequences were performed through a partnership with La Plate-Forme Séquençage et Génomique (Institut Cochin, Paris, http://cochin.inserm.fr/).

2.9. Determination of anti- Tannerella forsythia antibodies

Determination of immunoglobulins G against *T. forsythia* in carotid conditioned medium was performed as previously published [17,30] (Supplemental data 5). The results (ELISA units, EU) were calculated as continuous variables. The interassay CV was estimated at 7.22%.

2.10. Statistical analysis

Data are expressed as means (±standard deviation) or medians (+interquartile range) for continuous variables and numbers (percentage) for qualitative variables. Since the biological data did not present a normal distribution (checked graphically and by using

Shapiro–Wilks test), between-group comparisons and correlations were performed using non-parametric tests. The Mann–Whitney *U* test was used to compare two groups, the non-parametric analysis of variance to compare three or more groups, with a trend test, when appropriate. Correlations were assessed using the Spearman's rank correlation coefficient. For other continuous variables, the Student *t* test or analysis of variance was used. Differences in qualitative variables were assessed using the Chi-square test (Fisher's exact test was used when the expected cell frequency was<5) or the Mantel-Haenszel trend test. Among the 157 carotid endarterectomy samples, medical records were available for 122 patients. To assess the selection bias related to missing patient information, we compared demographics, carotid data and biological data according to included and non-included patients and found no significant difference (data not shown). Statistical testing was done at a 2-tailed α level of 0.05. Data were analyzed with Prism 5 (GraphPad software) or SAS version 9.3 (SAS Institute, Cary, NC).

3. Results

3.1. Neutrophil activation markers were associated with hemorrhagic atherosclerotic plaques

In conditioned medium, MPO levels were higher in CPs relative to NPs ($p = 0.0021$) and mammary arteries ($p = 0.0006$) (Fig. 1A). Moreover, hemorrhagic CPs released more MPO than those without intraplaque hemorrhage ($p = 0.0028$) (Fig. 1B). There was a positive correlation between MPO and hemoglobin concentrations in the conditioned medium of CPs ($r = 0.335$, $p < 0.0001$) (Fig. 1C and Table 1).

The release of cell-free DNA into the conditioned medium indirectly reflects the presence of NETs. Cell-free DNA was released in higher amounts by CPs than NPs ($p = 0.0008$) and mammary arteries ($p < 0.0001$) (Fig. 2A). The measure of NET levels in conditioned medium by quantification of MPO-DNA complexes is more specific. MPO-DNA complex concentrations were significantly higher in the conditioned medium of carotid CPs than in that of NPs ($p = 0.0117$) or mammary arteries ($p < 0.0001$) (Fig. 2B). Furthermore, hemorrhagic CPs released more MPO-DNA complexes levels than those without hemorrhage ($p = 0.0183$) (Fig. 2C). Finally, a positive correlation between MPO and cf-DNA concentrations was observed ($r = 0.446$, $p < 0.0001$) (Fig. 3A). These results suggest that intraplaque hemorrhages are associated with the presence of activated neutrophils reflected by the release of MPO, cf-DNA and MPO-DNA complexes that were increased in higher hemoglobin quartiles relative to the lower quartile (Table 1). In addition, we provide evidence that carotid samples presenting intraplaque hemorrhage also display an increased pro-inflammatory potential as measured by NFκB activity (Table 1).

3.2. Lipopolysaccharides from gram-negative bacteria were present in conditioned media of carotid CPs

As bacterial material represents one the major triggers for NET formation, we tested the hypothesis that intraplaque hemorrhage could be a substrate for periodontal bacterial retention and therefore participate in carotid plaque progression. LPS levels were positively correlated with the presence of hemoglobin ($r = 0.438$, $p < 0.0001$) (Fig. 3B). Samples that released more hemoglobin (higher quartile) contained 5-times more LPS that those with low levels of hemoglobin (Table 1). There was no correlation between LPS and cf-DNA or MPO levels in conditioned media (Fig. 3A).

3.3. Detection of the five major periodontal pathogens DNA in carotid samples

The presence of five major periodontal bacteria was investigated by PCR on the total DNA extracted from all 157 carotid culprit plaques. Seventy-three percent of these samples were positive for periodontal bacterial DNA; *P. gingivalis*, *T. forsythia* and *P. intermedia* were the most frequent species detected (respectively found in 39%, 35% and 33% of carotid samples, Supplemental data Fig. 1). None of the tissue samples were positive for *A. actinomycetemcomitans*. There was an equilibrated distribution between non-infected (27%), mono-infected (41%) and multi-infected (32%) carotid samples. Interestingly, the presence of *T. forsythia* was positively associated with hemoglobin levels (Table 1, *p* for trend = 0.03; Supplemental Table 5, $p = 0.04$).

Among the 157 patients who underwent carotid endarterectomy, 122 of them presented a complete medical record. Patient and carotid characteristics, as well as biological data, are summarized in Table 2. Interestingly, 83% of carotid samples from diabetic patients were positive for at least one bacterial species and they displayed a higher prevalence of multiple positivity ($p = 0.006$). This trend was not observed for other traditional atherosclerosis risk factors (hypertension, hypercholesterolemia and smoking status) and the number of bacteria detected did not impact on the symptomatology (Supplemental data Table 3). Both *P. gingivalis* and

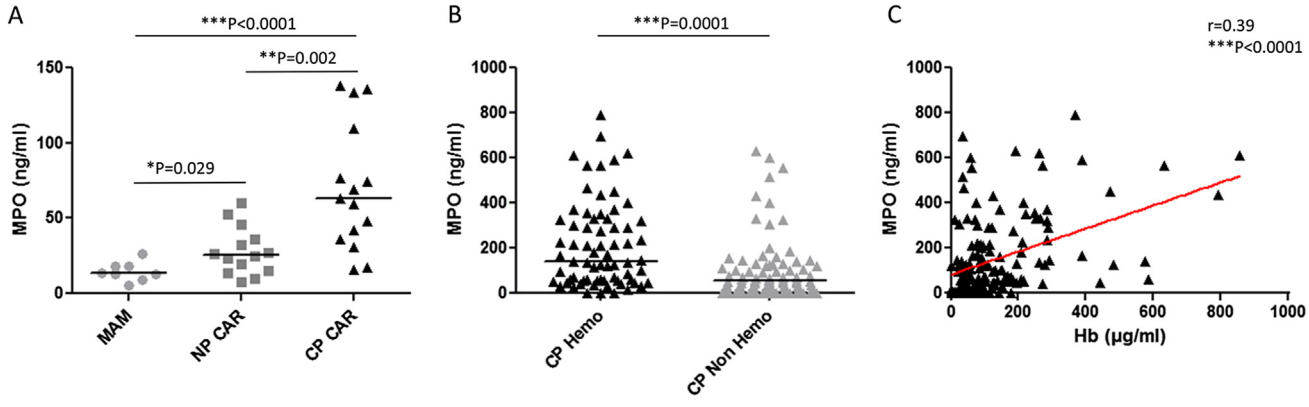


Fig. 1. Increased release of myeloperoxidase (MPO) into the medium conditioned by culprit carotid plaques. A: MPO levels were measured by ELISA in the medium conditioned by mammary endartery (MAM, $n = 9$) or carotid endarteriectomy samples, separated into non-complicated plaques (NP, $n = 15$) and culprit plaques (CP, $n = 15$). Results are presented as scatter plots in which the median is shown. B: MPO concentration was determined in all culprit parts of carotid samples ($n = 157$) separated according to their hemorrhagic status. Hemorrhagic carotid samples ($n = 73$), calcified, lipidic and fibrosed culprit plaques ($n = 84$). C: Correlation between MPO and hemoglobin levels in the conditioned medium.

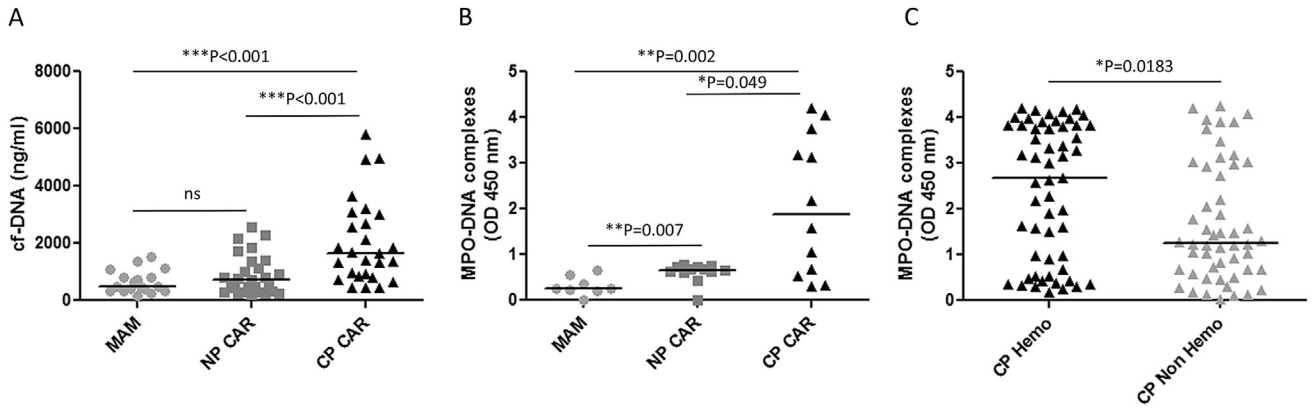


Fig. 2. Increased neutrophil activity markers in the medium conditioned by carotid samples with intraplaque hemorrhage. A: Cell-free DNA concentration was measured in the medium conditioned by CP carotid ($n = 27$), NP carotid ($n = 27$) or mammary artery ($n = 18$) samples. B: Determination of MPO-DNA complex concentration, reflecting the formation of neutrophil extracellular traps (NETs), in the medium conditioned by CP ($n = 15$) and NP ($n = 15$) carotid samples and mammary arteries ($n = 9$). C: MPO-DNA complex concentration was determined in all culprit parts of carotid arteries ($n = 157$) separated according to their hemorrhagic status. Hemorrhagic carotid samples: $n = 73$, calcified, lipidic and fibrosed culprit plaques: $n = 84$.

T. forsythia, but not *P. intermedia*, were more prevalent in diabetic patients (Supplemental data Table 3–6). *P. gingivalis* detection was associated with increased levels of carbonyls (Supplemental Table 4, $p = 0.04$). *T. forsythia* was found more often in hypercholesterolemic (88.1 vs 66.3%, $p = 0.009$) patients and in smokers (69.1 vs 48.8%, $p = 0.03$) (Supplemental data Table 5).

3.4. Anti-T. forsythia titers were associated with hemoglobin concentrations in carotid conditioned medium

T. forsythia was indirectly assessed by quantification of specific antibodies released in the conditioned medium of culprit atherosclerotic carotid plaques. A strong positive association ($p < 0.0002$) was observed between the titers of anti-*T. forsythia* immunoglobulins G in conditioned medium and hemoglobin levels (Fig. 4).

4. Discussion

The nature of the relation between periodontal and cardiovascular diseases needs to be clarified. In particular, new evidence is required to support a possible causal link between periodontal bacterial translocation into vascular tissue and atherothrombotic complications.

Intraplaque hemorrhage is a hallmark of atherosclerotic plaque vulnerability to rupture [22,23]. Our hypothesis is that blood conveys leukocytes, and in particular neutrophils, into the atherosclerotic plaque where they may be activated, leading to plaque weakening due to intense oxidative stress and protease activity [20,21]. Neutrophil activation could, at least in part, be due to the presence of LPS subsequent to repeated bacteriemia, as observed in periodontal conditions [31].

Our results show that in atherosclerotic carotid plaques, the release of neutrophil activation markers is associated with the presence of intraplaque hemorrhage and of *T. forsythia*, one of the more pathogenic periodontal bacterial. The presence of *T. forsythia* has been determined directly within the plaques by PCR and indirectly in the plaque-conditioned medium by the measurement of specific antibodies.

First, neutrophil extracellular traps (NETs), myeloperoxidase and cell-free DNA are released in larger amounts by the culprit atherothrombotic plaques and in particular in those containing an intraplaque hemorrhage. No correlation could be observed between LPS and cf-DNA or MPO in the conditioned medium whereas all these markers were correlated with hemoglobin levels. Blood contained within intraplaque hemorrhages represents a vector for both bacterial material and leukocytes, including neutrophils, in humans. However, LPS retention within the vascular tissue may differ from that of neutrophil activation markers. One of the roles of NETs is to trap bacterial material which may be, at least in part, retained within carotid plaques [32]. The thrombus, and

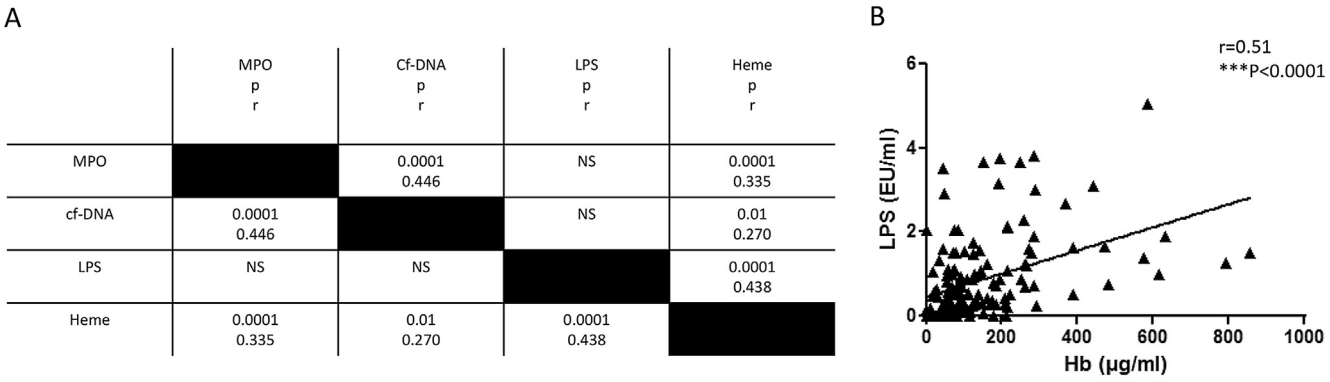


Fig. 3. Heme concentration in medium conditioned by carotid culprit plaques was correlated with levels of markers of neutrophil activity and LPS. A: Correlations were determined among the different markers of neutrophil activity and LPS. NS: not statistically significant. B: LPS levels from gram-negative bacteria were correlated with the hemoglobin concentration. All correlations were calculated using the Spearman test.

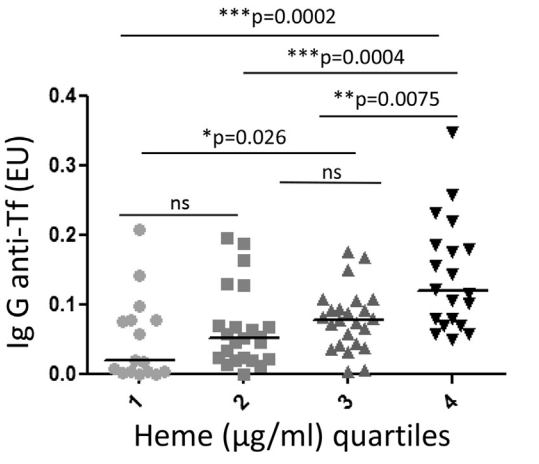
Table 2
Patient characteristics ($n = 122$).

	Intraplaque hemorrhage		*P*
	Absence	Presence	
Number of patients	65	57	
Demographics			
Age, years, mean ± SD	70.1 ± 10.6	74.0 ± 9.2	0.03
Men	41 (63.1)	46 (80.7)	0.03
BMI, kg/m^2, mean ± SD	25.9 ± 4.2	26.6 ± 3.8	0.41
Medical history			
Diabetes	24 (36.9)	16 (28.1)	0.30
Hypertension	49 (75.4)	42 (73.7)	0.83
Hypercholesterolemia	49 (75.4)	41 (71.9)	0.67
Current or former smoker	36 (55.4)	32 (56.1)	0.93
Symptomatic Carotid Plaque	18 (28.1)	11 (19.3)	0.28

Values are numbers (%) unless otherwise indicated.

particularly fibrin, may be a substrate and a source of cofactors for periodontal bacterial retention and activity in atherothrombotic tissue samples [33].

Several lines of evidence suggest that oral bacteria may translocate into vascular tissues and thus promote local recruitment of neutrophils. Koren et al. recently reported a correlation between the abundance of bacteria and their composition in the oral cavity and in atherosclerotic plaques, suggesting that oral bacteria may be transported to the vascular lesion via the bloodstream [34]. In contrast to several studies on carotid atherosclerosis that reported the presence of *A. actinomycetemcomitans* in vascular tissue samples using specific PCR [15], nested-PCR [31] or hybridization [35]. However, this periodontal bacteria, associated with the aggressive form of periodontitis, has been detected in 5–67% of carotid endarterectomy samples in other studies [34,36]. Differences in the type of vascular samples and variations in the ethnic origin of the populations investigated may be an explanation for this discrepancy [30]. Of note, positive controls using cultured bacteria were included in each set of experiments, and PCR products were checked by DNA sequencing. Less than 5% of carotid samples were infected with 3 bacteria (*P. gingivalis, T. forsythia*, *P. intermedia or T. denticola*). Among infected samples, 48% were positive for two or three bacteria, whereas in 42% of carotids, only one periodontal pathogen could be detected. This is consistent with findings in



Quartile 1: Hb <52 µg/ml; quartile 2: 52< Hb <94 µg/ml; quartile 3: 95< Hb <196 µg/ml; quartile 4: Hb >196 µg/ml.

Fig. 4. Specific antibodies against *Tannerella forsythia* in medium conditioned by carotid culprit plaques according to their heme concentration divided into quartiles.

periodontitis patients, where subgingival dental plaques were not always infected by all the major periodontal pathogens belonging to the "red complex" i.e. *P. gingivalis, T. forsythia* and *T. denticola* [37].

Interestingly, we report for the first time that diabetic patients were twice as frequently infected by 2 or more bacteria than non-diabetics. This may be due to i) increased prevalence and severity of periodontal disease in diabetics, ii) differences in periodontal microbiota, iii) an increased neovascularization in atherosclerotic plaques, and/or iv) a dysfunctional endothelium leading to enhanced invasion [38–41]. In particular, almost half of the patients whose carotid samples were positive for *P. gingivalis* and *T. forsythia* were diabetic. Previous studies have shown that the quantity of red complex bacteria positively correlated with HbA1C [42]. Further investigations are needed to clarify the impact of diabetes on atherothrombotic plaque phenotype and the presence of periodontal bacteria. The red complex is associated with the severity of periodontal disease [43]; in contrast, *P. intermedia* is a less virulent periodontal bacteria which can be found in the healthy periodontium. Interestingly, the presence of this less pathogenic periodontal pathogen was associated with a trend towards a decrease in all the neutrophil activation markers (MPO, MPO-DNA, cf-DNA) and NFκB activity ($p = 0.02$) (Supplemental data Table 6). NFκB activation has been reported to be involved in neutrophil extracellular trap formation and could represent a link between oxidative stress and neutrophil activation [44]. Atherothrombotic plaques in which *P. gingivalis* was detected exhibited higher levels of carbonyls (a protein marker reflecting oxidative modifications) (Supplemental Table 4).

Our analysis according to hemoglobin levels indicated that intraplaque hemorrhage is associated with increased neutrophil activation markers (cf-DNA, MPO, DNA-MPO complexes), NFκB activity and LPS levels. The capacity of the conditioned medium to activate NFκB in reporter cells may be due to the presence of hemoglobin, LPS or other pro-inflammatory stimuli [45]. We cannot exclude that LPS from bacteria other than periodontal pathogens may participate in neutrophil activation. Both LPS and DNA from *P. gingivalis* and *T. forsythia* were reported to induce NFκB, via toll-like receptors (TLR-2, 4 and 9) [46]. Interestingly, in our study, *T. forsythia* was the only bacterium associated with the presence of intraplaque hemorrhage attested by high levels of hemoglobin. Recently, Posch et al. have partly elucidated the structure of *T. forsythia* LPS (a complex, rough-type LPS), which may be responsible for the major pathophysiological potential of the bacteria [47]. In the same study, the authors found that *T. forsythia* LPS-induced cytokine production was strictly dependent on the presence of serum components. In contrast, the levels of proinflammatory cytokines upon stimulation with *P. gingivalis* LPS were high, even in the absence of serum. Moreover, *T. forsythia* is reported to express hemagglutinin that can bind and agglutinate red blood cells [48]. Thus, intraplaque hemorrhage could be a substrate for *T. forsythia* and a source of cofactors for proinflammatory cytokine upregulation. Nevertheless, *T. forsythia* is a poorly studied periodontal pathogen relative to *P. gingivalis* (for review [49]), which is well documented in cardiovascular diseases. This is the first study that reports a higher prevalence of *T. forsythia* in more vulnerable, hemorrhagic plaques. Recently, Lee et al. showed induction of foam cell formation and progression of atherosclerotic lesions by *T. forsythia* and BspA lesions in ApoE$^{-/-}$ mice. The mouse serum levels of CRP and LDL were increased, and HDL was decreased by *T. forsythia* and BspA [50]. Moreover, in our study, *T. forsythia* is detected twice as frequently in diabetic versus non-diabetic patients (Supplemental Table 3). *T. forsythia* was recently reported to be positively associated with overweight and obesity [51] and detected more frequently in oral samples from diabetic in comparison with non-diabetic subjects [42]. Further studies are

needed in large cohorts in order to assess the potential role of *T. forsythia* in atherogenesis and cardiovascular complications.

One limitation of our study is that plasma samples were not available; thus, correlations between the presence of bacteria and other clinical parameters (cholesterol levels, C-reactive protein, etc.) could not be analyzed. Only 24% of plaques were from symptomatic patients, suggesting that the bacterial burden may be underestimated, since it could be expected that ruptured, symptomatic plaques may present a more hemorrhagic phenotype. Lastly, the lack of appropriate tools for *in situ* detection of periodontal pathogens did not allow us to provide topological information, such as colocalization within specific areas of the plaque.

5. Conclusions

In this study, intraplaque hemorrhages in culprit carotid samples were associated with neutrophil activation. Lipopolysaccharide levels were also linked to intraplaque hemorrhage. Lastly, hemoglobin levels were associated with the detection of *T. forsythia* and neutrophil activation/inflammation markers. This study raises the possibility of a potential role for periodontal microorganisms, especially *T. forsythia*, in neutrophil activation within hemorrhagic atherosclerotic carotid plaques.

Conflict of interest

The authors have nothing to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.07.034.

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