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Prevotella nigrescens and *Porphyromonas gingivalis* are associated with signs of carotid atherosclerosis in subjects with and without periodontitis

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Background and Objective: Oral microorganisms may be involved in the development of cardiovascular diseases, and *Porphyromonas gingivalis* is one of the periodontal microorganisms that has been found in carotid atheroma. The aim of this work was to study subgingival microorganisms and early carotid lesions in subjects with and without periodontitis.

Material and Methods: Eighty-eight subjects with periodontitis and 40 subjects without periodontitis underwent dental examinations in 2003. The presence of the periodontal microorganisms *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Tannerella forsythia* was analyzed from subgingival plaque using PCR amplification. The common carotid artery was scanned using ultrasound and the calculated intima-media area (cIMA) was measured. The association between periodontitis, the cIMA value and the presence of periodontal microorganisms, together with several confounders, was studied in a multiple logistic regression model.

Results: Smoking [odds ratio (OR) = 5.64; $p = 0.001$), level of education (OR = 5.02; $p < 0.05$) and the presence of *P. gingivalis* (OR = 6.50; $p < 0.05$) were associated with periodontitis. Explanatory factors for the increased cIMA were periodontitis (OR = 4.22; $p < 0.05$), hypertension (OR = 4.81; $p < 0.05$), high body mass index (OR = 5.78; $p < 0.01$), male gender (OR = 3.30; $p < 0.05$) and poor socioeconomic status (OR = 4.34; $p < 0.05$). *P. nigrescens* (OR 4.08; $p < 0.05$) and *P. gingivalis* (OR 7.63; $p < 0.01$) also appeared as explanatory variables associated with increased cIMA values.

Conclusion: This cross-sectional study showed that *P. nigrescens* and *P. gingivalis* were significantly associated with increased cIMA values.

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The results of several studies published during the last two decades have shown that oral diseases, especially periodontitis, may act as risk factors for the development of cardiovascular diseases (CVD) (1–6). A recently published review concluded that periodontitis may indeed contribute to the systemic inflammatory burden and pathogenic

processes that lead to atherosclerosis in otherwise healthy individuals (7). However, a direct causal relationship has not been established between periodontitis and atherosclerotic CVD (8). Longitudinal studies have shown that oral pathogens may be involved in the development of CVD such as coronary thrombosis, stroke (9–11) and bacterial endocarditis (12). *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* have been identified, using PCR amplification, in carotid atheromas (13). Both *A. actinomycetemcomitans* and *P. gingivalis* can invade epithelial cells and induce mechanisms that are detrimental to host cells (14). A systemic antibody response to infections with periodontal microorganisms has been shown, for example by the direct relationship between the serum levels of IgG to *P. gingivalis* and subgingival colonization with *P. gingivalis* (15).

Periodontitis is characterized by infection and inflammation in the periodontal tissue, leading to destruction of the bone surrounding the teeth and, ultimately, to tooth loss (16). The bacterial biofilm on the teeth triggers an immune-inflammatory response in the adjacent host tissues and can initiate periodontitis (17,18). Periodontal pathogens are necessary, but not sufficient, in the pathogenesis of the disease (19,20). The complexity of variations in the composition and virulence of the oral microbiota that contribute to periodontitis has not been explored in detail (21,22). Nevertheless, gram-negative anaerobes, such as *P. gingivalis*, are well-established periodontal pathogens (23,24). *Tannerella forsythia* and *Treponema denticola* also belong to the group of microorganisms categorized as periodontal pathogens. This concept is based on the association of these microorganisms with severe forms of periodontitis, as defined by Socransky *et al.* (21). The bacterial flora of a healthy oral cavity has not been defined; however, the microbiota in the healthy oral cavity is different from that found in connection with oral and dental diseases in that many species particularly linked with periodontitis, such as *P. gingivalis* and *T. forsythia*, are not detected in healthy sites (25).

With this background, we hypothesized that the presence of certain subgingival microorganisms is associated with atherosclerosis. Hence, the aim of this study was to investigate subgingival microorganisms and early carotid lesions in subjects with and without periodontitis.

Material and methods

Study participants

The baseline cohort was selected in 1985 using the registry file of all inhabitants of the Stockholm metropolitan area (Sweden) and consisted of 3273 individuals 30–40 years of age and born on the 20th of any month between 1945 and 1954. All selected individuals were informed about the purpose of the study and were offered a dental examination. In total, 1676 individuals agreed to participate and underwent an initial dental examination (26). This initial dental screening revealed that 256 persons suffered from periodontal disease. Subjects with three teeth (excluding third molars) with a pocket depth of ≥ 5 mm, as well as gingival bleeding on probing were diagnosed as having periodontal disease. After at least 16 years, 150 age- and gender-matched subjects were randomly selected from the cohort using a computer program, and 64 women and 64 men were recalled and re-examined in the present study in 2003. The study profile is shown in Fig. 1.

All subjects fulfilled the following inclusion criteria: no systemic disease, no systemic or local antibiotic therapy during the 6 mo prior to the clinical examination and no periodontal treatment during the last 3 mo. The data analyzed in this report also included subjects from earlier studies (3,4,27). The study was approved by the Ethics Committee of the Karolinska University Hospital at Huddinge. All subjects gave their informed consent to participate.

Examination and analyses

The subjects answered a questionnaire concerning health problems, medication, dental visits, tobacco use, marital status, socioeconomic data, education and occurrence of stroke or coronary artery disease in siblings or parents before the age of 65.

In all subjects the following clinical oral health parameters were recorded in 2003: the number of remaining teeth (28), oral hygiene status (using the dental plaque index) (29), gingival inflammation (using the gingival index) (30) on six surfaces and bleeding on probing. Periodontal pocket depth and attachment level were determined using a periodontal probe (Hu-Friedy PCPUNC 15; Hu-Friedy, Chicago, IL, USA) and the measurements made at six sites of each tooth were rounded up to the nearest millimeter. Wisdom teeth were excluded as described in a previous publication (28). Dental examinations were performed by one of the authors (B.S.). For calibration, 10 patients were examined by the examiner, who recorded pocket depths and attachment levels. The patients were examined on two occasions and two sets of mean values were obtained for each patient.

Radiographs

A full-mouth set of 14 Kodak Ektaspeed periapical radiographs was obtained from each patient in 2003 (Ekta Speed Eastman Kodak, Rochester, NY, USA). An Eggen film-holder (DAB, Stockholm, Sweden) and an Oralix Roentgen apparatus (65 kVp/7.5 mA) (Gendex Dental System, Milano, Italy) equipped with a cone of rectangular section were used, permitting a modified parallel long-cone technique, with a focus-film distance of approximately 30 cm. At each measurable interproximal surface, except on third molars, the alveolar bone height was determined as a percentage of the root length from the radiographs magnified ×5, using a computerized measuring system described earlier (3). The radiographs were evaluated by one of the authors (P.-Ö.S), blinded to the results of the clinical dental examination.

Analyses of periodontal microorganisms

In 2003, subgingival plaque samples were collected from four test sites on each subject, the second premolar in each quadrant. The samples were

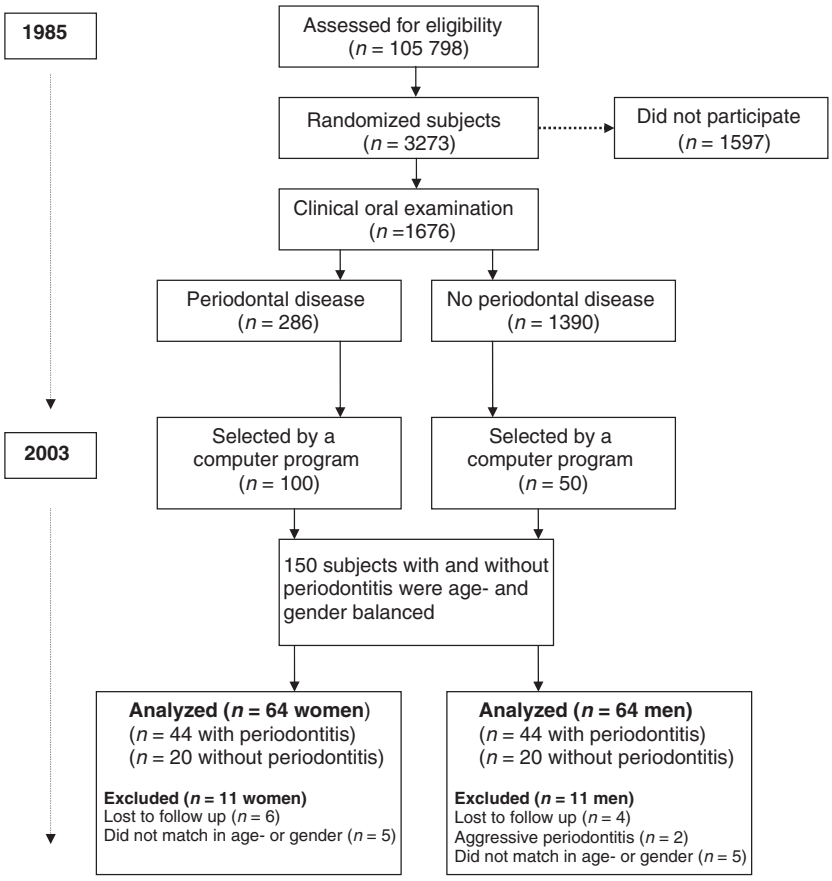

Fig. 1. Flow chart showing the selection of participants in 1985 to re-examination in 2003.

collected with a sterile curette into Eppendorf tubes (Eppendorf Nordic, Copenhagen, Denmark) containing 100 μL of phosphate-buffered saline (pH 7.4). The samples were then frozen to −70°C until required for subsequent analyses. The PCR was used to detect microorganisms in a non-quantitative manner (i.e. present or not present). Specific primers were designed to hybridize to various regions of 165 ribosomal RNA (rRNA) genes. *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens* and *T. forsythia* were analyzed and expressed as a percentage if present in at least one site. The detection limit of the PCR method used was 5–10 cells. The sensitivity and specificity of the PCR method used are described in the original publications of the method (31,32).

Assays of biochemical markers in plasma

At the time of the oral examination in 2003, blood pressure was measured and 12-lead electrocardiography was used to record the electrocardiogram. Blood samples were taken after 12 h of overnight fasting to analyze plasma total cholesterol. The analyses were made using routine methods at the Laboratory of Clinical Chemistry at Karolinska University Hospital, Huddinge, Sweden.

Carotid B-mode ultrasound

Carotid ultrasound was performed in 2003. Carotid arteries were examined bilaterally using a duplex scanner (Aspen; Acuson, Mountain View, CA, USA) using a 7-MHz linear-array transducer. All recordings were made by the same trained sonographer with the subjects in a supine position, and with the head turned slightly away from the sonographer. The scans were videotaped for subsequent analyses by a computer system with automated tracing of the echo interfaces (33). Measurements of distances between the wall echoes within a 10-mm-long section of the common carotid artery (CCA) were made in late diastole defined by a simultaneous electrocardiographic recording. The far wall of the CCA, 0.5–1.0 cm proximal to the proximal delimitation of the carotid bulb, was used to measure the intima-media thickness (IMT) and the lumen diameter. The IMT was defined as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo. The lumen diameter was defined as the distance between the leading edge of the intima-lumen echo of the near wall and the leading edge of the lumen-intima echo of the far wall. The mean values of the IMT and the lumen diameter within each 10-mm-long section were calculated unless the presence of plaques was observed in the region of interest, in which case the measurements of the IMT were abandoned. Carotid plaque was defined as a localized IMT of > 1 mm and at least a 100% increase in thickness compared with adjacent wall segments. In order

to compensate for the stretching effect of arterial distension (secondary to increased arterial pressure) on the wall thickness, the cross-sectional intima-media area (cIMA) (34) was calculated using the formula: 3.14 [(lumen diameter/2 + intima-media thickness)^2 – (lumen diameter/2)^2]. The differences between repeated measurements of IMT and the lumen diameter, using the automated analysis system, were 3.2% and 0.6% (coefficient of variation), respectively (with an IMT of 0.48–1.04 mm and a lumen diameter of 4.34–7.91 mm).

Statistical analyses

The final analyses included 88 patients with periodontitis and 40 subjects without periodontitis. The standard deviation (SD) of a single observation, as derived from the two repeated clinical examinations, was 0.05. The intra-examiner reproducibility was tested using the intraclass correlation coefficient (ICC); the ICC for periodontal pocket depth was 0.849 and the ICC for attachment level was 0.849 (PASW Statistics Inc., Chicago, IL, USA). In addition, the intra-examiner reproducibility was also tested by double-blind re-analysis of all the radiographs of five participants 6 mo after the first analysis. The intra-examiner reproducibility was tested using the ICC, and the ICC value was 0.967.

The Student's unpaired *t*-test and analysis of variance were used for data analysis. The chi-square test with Fisher's exact *p*-value was used to determine the differences between categorical data. Multiple logistic regression analyses were used to compare cIMA values while simultaneously controlling for several potential confounding variables. In the multiple logistic regression analysis models we included confounding variables such as age, sex, education, smoking habits (calculated in pack-years) and periodontitis. The smoking habits were divided into smokers (subjects who had ever smoked) and nonsmokers (subjects who had never smoked). The model with these confounding variables was correlated with cIMA. A backwards elimination method was used to control for multicollinearity (correlation between confounding variables). The model summary was determined according to Cox & Snell R^2 and Nagelkerke R^2. Differences between data sets with a probability of < 0.05 were regarded as significant. All *p*-values are two-tailed, and confidence intervals were calculated at the 95% level. All statistical analyses were performed using the SPSS® software package, version 16.1 (SPSS Inc., Chicago, IL, USA).

Results

In total, 128 subjects participated and underwent a clinical re-examination in 2003. Eighty-eight subjects [44 women, mean age ± SD = 54.7 ± 2.9 years; and 44 men, mean age ± SD = 54.7 ± 2.4 years] had periodontitis in 2003 while 40 subjects (20 women, mean age ± SD = 53.5 ± 2.7 years; and 20 men, mean age ± SD = 53.4 ± 3.0 years) were clinically examined and were documented not to have periodontitis (control group) (Fig. 1).

Demographic data and atherosclerosis risk factors of control subjects and patients are given in Table 1. The percentage of individuals with higher education was greater among controls than among subjects with periodontitis. Smoking habits were also significantly different between patients and controls. There were significantly more smokers in the patient group than in the controls. Patients also had a higher body mass index (BMI) and higher levels of plasma cholesterol than controls. Significant differences were observed between patients and controls in all clinical oral health parameters. When comparing the presence and types of periodontal microorganisms in control subjects and patients, a significant difference was found in the frequency of being infected with certain microorganisms. Patients were more often infected with *P. gingivalis*, *P. intermedia* and *T. forsythia* (Table 1).

Multiple logistic regression analyses of the relationship between periodontitis as the dependent variable and several independent variables were carried out. Smoking and education were the explanatory variables for periodontitis. Furthermore, the occurrence of *P. gingivalis* was associated with a 6.50-fold increased odds for periodontitis. The results are given in Table 2.

Multiple logistic regression analyses for the increased cIMA showed that periodontitis, hypertension, BMI, male gender and poor socioeconomic status were explanatory variables. Also, *P. nigrescens* ($p = 0.0034$) and *P. gingivalis* ($p = 0.008$) were explanatory

Table 1. Demographic data and atherosclerosis risk factors of control subjects and patients

	Controls ($n = 40$)	Patients ($n = 88$)	p[a]
Age (years)	53.4 ± 2.8	54.7 ± 2.7	NS
Education (higher education in %)	92.5	58	< 0.001
Smoking	12.5	40.9	< 0.01
Body mass index (kg/m^2)	23.7 ± 3.0	25.3 ± 4.5	< 0.05
Plasma cholesterol (mmol/L)	5.5 ± 0.85	5.9 ± 0.94	< 0.05
Hypertension[b]	17.1	27.5	NS
Periodontal pocket depth (mm)	1.9 ± 0.3	2.8 ± 0.6	< 0.001
Loss of attachment (mm)	1.9 ± 0.6	3.6 ± 1.1	< 0.001
Gingival index	0.2 ± 0.6	1.5. ± 1.0	< 0.001
Bleeding on probing (%)	14.0 ± 16.7	39.0 ± 25.8	< 0.001
Aggregatibacter actinomycemtemcomitans	3.0	16.1	NS
Porphyromonas gingivalis	7.3	27.6	< 0.01
Prevotella intermedia	9.7	41.4	0.02
Prevotella nigrescens	63.4	70.1	NS
Tannerella forsythia	19.5	52.9	< 0.001

Data are given as a percentage or as mean ± standard deviation.
[a]Fisher's exact test or the Student's *t*-test for unpaired samples was used, as appropriate.
[b]Systolic pressure < 140 mmHg, diastolic pressure > 90 mmHg or ongoing therapy.
NS, not significant.

Table 2. Multiple logistic regression analysis of the relationship between periodontitis as the dependent variable and several independent variables[a]

Dependent variable	Explanatory variable	β	*p*	Odds ratio	95% confidence interval
Periodontitis	Smoking pack-year	1.73	0.001	5.64	1.98–16.12
	Porphyromonas gingivalis	1.87	0.017	6.50	1.40–30.21
	Education	1.61	0.023	5.02	1.25–20.25

[a]Periodontitis, age, gender, heredity for atherosclerotic diseases, calculated intima-media area (cIMA), body mass index (BMI), hypertension, dental visits, smoking, education, income, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Tannerella forsythia*. Cox & Snell $R^2 = 0.351$; Nagelkerke $R^2 = 0.491$.

variables associated with increased cIMA values. The results are given in Table 3.

Discussion

The results of the present study confirmed the hypothesis by showing that two species of subgingival microorganisms are associated with an increased cIMA. Being infected with *P. gingivalis* increased the odds of higher cIMA values by more than seven-fold and being infected with *P. nigrescens* increased the odds of higher cIMA values by more than four-fold. Desvarieux *et al*. reported a positive, independent, relationship between carotid IMT and cumulative periodontal bacterial burden. However, in that study *P. nigrescens* was not measured (35). High BMI is a well-known risk factor for atherosclerosis (36,37). This is in agreement with the result of the present study, in which we found that a high BMI increased the risk for a high cIMA by more than five-fold. Corresponding results were found by Pussinen *et al*., who found that systemic exposure to *P. gingivalis* might predispose to incident stroke, demonstrating the assumption that oral pathogens may indeed contribute to atherogenesis (38). The mechanism of atherogenesis can be explained by the findings of Qi *et al*. (39), who concluded that *P. gingivalis* cells or their vesicles, released from periodontal lesions into the circulation, might affect the arterial wall by virulence compounds (such as lipopolysaccharides) and initiate or promote foam cell formation in macrophages, thus contributing to the growth of atheroma. The effect of *P. gingivalis* on endothelial cells might play an important role, and could be another explanation of atherogenesis because the up-regulation of immunological mediators might also be a factor here (40). *In vitro* studies have shown that *P. gingivalis* invades consecutive epithelial cell layers and multiplies intracellularly (41). In our study, *P. nigrescens* also showed a significant association with increased cIMA. To our knowledge, this study is the first investigation where *P. nigrescens* has been associated with an increase in the cIMA. This microorganism, however, is not regarded as virulent as *P. gingivalis* or *T. forsythia* but nevertheless belongs to the periodontal microbiota of concern.

Table 3. Multiple logistic regression analysis of the relationship between the calculated intima-media area (cIMA, sin, dx/2) as a dependent variable and several independent variables[a]

Dependent variable	Explanatory variable	β	*p*	Odds ratio	95% confidence interval
cIMA	Periodontitis	1.44	0.024	4.22	1.20–14.70
	Hypertension	1.57	0.029	4.81	1.17–19.70
	BMI	1.75	0.004	5.78	1.75–19.05
	P. nigrescens	1.41	0.034	4.08	1.11–15.00
	Gender (male)	1.20	0.035	3.30	1.08–10.04
	Socioeconomic data	1.47	0.037	4.34	1.09–17.30
	P. gingivalis	2.03	0.008	7.63	1.70–34.13

[a]Periodontitis, age, gender, heredity for atherosclerotic diseases, body mass index (BMI), total cholesterol, hypertension, dental visits, smoking, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Tannerella forsythia*. Cox & Snell $R^2 = 0.335$; Nagelkerke $R^2 = 0.453$.

The present study also addressed the issue of periodontitis as a future risk for coronary heart disease. Our results identified periodontitis as an explanatory variable of increased cIMA values. This finding is in agreement with recently published investigations demonstrating that periodontitis is indeed associated with the future development of atherosclerosis (5,42–44). Periodontitis may thus indicate risk for future CVD complications (3,27), and, according to our findings, that risk may be even higher when *P. gingivalis* or *P. nigrescens* is present.

Regarding the reliability of our results, we emphasize that the subjects with and without periodontitis were chosen using a computer program to avoid selection bias. A single, experienced dental examiner (B.S.) performed all dental examinations in 2003, whereas dental radiographs were evaluated by one of the other authors (P.-Ö.S.), the latter being blinded to the results of the clinical examination. The carotid ultrasound was performed and evaluated by the same experienced and blinded sonographer. Hence, the methodological bias was minimized. Furthermore, it can be emphasized that the B-mode-derived carotid cIMA is a well-established variable reflecting early atherosclerosis and its value has been well documented in studies on atherosclerosis at early stages of the disease, and in assessing various vascular risk factors (45–48).

One limitation of the present study was the cross-sectional data. Another main limitation of the study was the qualitative PCR method used.

Nevertheless, PCR is a sensitive method for detecting microorganisms in the gingival crevicular fluid, and it is also a faster, easier and more specific technique than conventional culture. In our laboratory, real-time quantitive PCR was not available at the time of the present analyses. However, the validated method has been successfully used in a number of earlier studies. The present hypothesis was based on the absence or presence of certain strains of microorganisms in the gingival pocket samples and on their association with the study parameters recorded. The samples used for the detection of microorganisms were taken from one test site in each quadrant, which is a further limitation of the study. However, four samples were taken from each subject. Thus, our results provide information on the presence of microorganisms in different quadrants of the mouth. Nevertheless, further studies in this area are needed to explain the link between the multicausal diseases periodontitis and atherosclerosis. It also needs to be investigated if and what role the periodontal microorganisms may have in the pathogenesis of atherosclerosis.

Conclusion

This cross-sectional study showed that the periodontal microorganisms *P. nigrescens* and *P. gingivalis* were significantly associated with increased cIMA.

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