J Periodont Res 2006; 41; 411–417



JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00888.x

# Levels of matrix metalloproteinases-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood

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*Söder B, Airila Månsson S, Söder P-Ö, Kari K, Meurman J. Levels of matrix metalloproteinase-8 and -9 with simultaneous presence of periodontal pathogens in gingival crevicular fluid as well as matrix metalloproteinase-9 and cholesterol in blood. J Periodont Res 2006; 41: 411–417. © Blackwell Munksgaard 2006.*

*Background and Objectives:* To investigate the levels of matrix metalloproteinase (MMP) -8 and -9 with the simultaneous presence of periodontal pathogens in gingival crevicular fluid (GCF) as well as MMP-9 and cholesterol in blood. Although bacterial pathogens are required to initiate the periodontal disease process, in some individuals the reaction to bacteria may lead to an excessive host response, resulting in a general inflammatory response.

*Methods:* MMP-9 and lipids were analyzed from the blood samples of 33 subjects with a 16-year history and oral health records of periodontal disease as well as from 31 periodontally healthy controls. Information was obtained on education, body mass index, and family history of atherosclerosis. GCF was taken to determine MMP-8 and MMP-9 levels, and bacterial samples were simultaneously collected for polymerase chain reaction assessment of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia*, and *Treponema denticola*. Analysis of variance, chi-squared test, and multiple logistic regression analysis were used to analyze the results.

*Results:* Demographic data showed significant differences between patients and controls in smoking ($P < 0.01$), body mass index ($P < 0.05$), family history of atherosclerotic disease ($P < 0.01$), and education ($P < 0.01$). Significant differences were also observed in oral health data, in the detection of *P. gingivalis* ($P < 0.001$), *P. intermedia* ($P < 0.01$), *P nigrescens* ($P < 0.001$), and *T. forsythia* ($P < 0.001$) and in the levels of MMP-8 and MMP-9 in GCF between patients and controls. *T. forsythia* [odds ratio(OR) 10.1; $P = 0.001$] and age (OR 5.54; $P = 0.008$) appeared to be the main independent predictors for high

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Keywords: Periodontitis, gingival crevicular fluid, plasma, matrix metalloproteinases

Accepted for publication January 3, 2006

MMP-8 in GCF. Patients had significantly higher total cholesterol ($P < 0.01$), low-density lipoprotein cholesterol ($P = 0.05$), and triglycerides ($P < = 0.01$) than controls. Plasma levels of MMP-9 were significantly higher in patients than in controls ($P = 0.001$).

*Conclusions:* Specific periodontal microorganisms appeared to induce host response, with increased release of MMP-8 and MMP-9 in gingival pockets as well as of MMP-9 in plasma, possibly triggering its up-regulation in blood.

Periodontal disease is characterized by chronic infection and inflammation in periodontal tissue, leading to destruction of the bone surrounding the teeth and, ultimately, to tooth loss (1). An estimated 15–35% of the adult population in industrialized countries suffers from this multifactorial disease (2–4). Periodontal disease is initiated by a biofilm of bacteria on the teeth, which triggers an immuno-inflammatory response in the adjacent host tissues (1,5). Although bacterial pathogens are required to initiate the disease process, their presence alone is not sufficient to cause the tissue destruction that occurs in periodontitis (4,6). In some individuals, the reaction to bacteria may lead to an excessive host response, resulting in a general inflammatory response. Systemic markers that reflect inflammation include the highly sensitive C-reactive protein (hs-CRP) (7). On the vascular level, inflammatory cells secrete matrix-degrading proteases such as matrix metalloproteinases (MMPs). The MMPs are a group of enzymes responsible for the degradation of most matrix proteins in growth and normal tissue turnover (8). They also play a role in cardiovascular pathology, e.g. in coronary artery disease (9). MMP-8, in conjunction with MMP-9 and functional granulocyte elastase, is involved in tissue destruction in subjects with periodontal disease (10–12). MMP-8 has also been found in periodontal lesions together with *Chlamydia pneumoniae* (13). Evidence indicates that MMP-9 increases during chronic inflammation (9) and may be associated with *C. pneumoniae*-related atherosclerosis (14). Plasma MMP-9 seems to provide a useful marker of inflammation, as it correlates with leukocyte count and is not associated with the lipid profile (15).

Elevated levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides and diminished levels of high-density lipoprotein (HDL) cholesterol are associated with an increased risk of coronary events (16–20). Human cytomegalovirus modifies LDL uptake and may thus be linked to atherosclerotic plaque formation. Human cytomegalovirus occurs with elevated frequency in severe adult periodontitis (21,22).

The relationship between chronic inflammation and atherogenesis has recently been expanded to include other pro-inflammatory processes related to a hyperactive immune response or an autoimmune reaction to microbial or other metabolic stimuli (23,24). Poor oral health may therefore contribute to the pathogenesis of atherosclerosis in several ways, including via inflammatory pathways (25).

Our hypothesis was that periodontal pathogens, which trigger inflammatory response in diseased periodontal pockets, resulting in higher levels of MMPs in gingival crevicular fluid (GCF), may, also cause an increase in plasma MMPs, thus contributing to the pathogenesis of such systemic diseases as atherosclerosis. The aim of this study was to investigate in a representative sample of subjects with long-term periodontal disease the levels of MMP-8 and MMP-9 with the simultaneous presence of periodontal pathogens in GCF as well as the levels of MMP-9, cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and hs-CRP in blood. This study belongs to the oral infection–systemic disease paradigm in periodontal disease and atherosclerosis.

## Materials and methods

### Patients and control subjects

Participants comprised 33 subjects (13 women, 20 men) with periodontal diseases and 31 periodontally healthy controls (17 women, 14 men) with a mean age of 53.6 (± 2.9 SD) years. The subjects were randomly selected from a group of 1676 patients who had volunteered to take part in an epidemiological study of periodontal health that started in 1985 and continued until 2003 (26,27). At the beginning and the end of the study, the subjects answered a structured questionnaire concerning health problems, use of tobacco, socioeconomic data, education, and dental visits.

The presence of periodontal disease was documented in 1985 and confirmed between 2001 and 2003.

At the same time, the 31 periodontally healthy controls were randomly selected from a group of individuals who were found to be free of periodontitis in 1985 and confirmed to be healthy between 2001 and 2003. Nine subjects who were periodontally healthy in 1985 no longer fulfilled the inclusion criteria in 2001/2003 and were excluded from the study. At the time of the oral examination in 2001 and 2003, blood pressure was measured and blood was collected after 12 h of overnight fasting for the analysis of total plasma cholesterol. Blood (20 ml) was drawn from the antecubital vein of the subjects, and plasma was separated and stored at −70°C. Samples were later analyzed to determine the levels of plasma hs-CRP, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and MMP-9.

### Ethical considerations

The Ethics Committee of the Huddinge Hospital, Sweden, had approved the study protocol. Subjects gave their informed written consent to participate. All subjects fulfilled the following inclusion criteria: no systemic disease, no systemic or local antibiotic therapy during the 6 months prior to the start of the clinical examinations, and no periodontal treatment during the last 3 months.

### Clinical and X-ray examination, oral microbiological and gingival crevicular fluid sampling

The clinical examinations performed after the microbial and GCF sampling included measurement of the dental plaque index (28) and determination of gingival inflammation by using a non-invasive modification of the gingival index (29). Dental plaque was determined on buccal and lingual surfaces on all teeth, excluding wisdom teeth. Periodontal pocket depth and loss of attachment were measured at mesio-buccal, mid-buccal, mesio-lingual, disto-buccal, disto-lingual, and mid-lingual sites of the teeth. Bleeding on probing, a clinical sign of gingival inflammation, was assessed by using a periodontal probe with a tip diameter of 1 mm and a probing pressure of 25 g. Bleeding within 60 s was recorded as bleeding on probing. The six sites mentioned above were tested on each tooth; one bleeding site per tooth was registered, and bleeding was expressed as the percentage of bleeding sites per patient. The occurrence of bleeding was expressed as the percentage of bleeding teeth per patient.

Radiographs of the jaws were taken and periodontal bone height was determined as a percentage of root length from radiographs magnified seven times, using a computerized measuring system (30).

The deepest site in each jaw quadrant was selected for microbial and GCF sampling. The sites to be sampled were isolated with cotton rolls, gently air-dried, and supragingival plaque was carefully removed at the place of insertion of the sampling needle into the pocket. GCF was collected using the intracrevicular washing technique (31). The ejection needle of the instrument was carefully inserted into the crevice to a level of approximately 1 mm below the gingival margin. The sulcus or pocket was then flushed by a constant delivery system (10 µl per flushing) with an aliquot of 15 µl phosphate-buffered saline (pH 7.4) and simultaneously drained through the collection needle into Eppendorf tubes by constant suction (flow rate 25 ml/h). The gingival washings in the Eppendorf tubes were diluted up to a final volume of 500 µl by washing the drain-tubes. The samples were immediately centrifuged (8000 *g*) for 5 min at 4°C, and the supernatants and pellets were frozen to − 70°C until analysis.

### Assays of MMP-8 and MMP-9

GCF supernatants (20 µl) were assayed for MMP-8 and MMP-9 (both free and complexed) with an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Amersham Life Science Ltd, Buckinghamshire, UK). The levels of MMP-8 and MMP-9 were determined as a total amount per site (ng/site). MMP-9 was also assayed in plasma and expressed in ng/ml (32).

### Assay of interleukin-1β

GCF supernatants (20 µl) were assayed for Interleukin-1beta (IL-1β) with enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R & D Systems Europe Ltd, Abingdon, UK). The levels of IL-1β were determined as a total amount per site (pg/site).

### Assays of hs-CRP, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides

Hs-CRP, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were assayed in plasma. Hs-CRP was expressed in mg/l, and HDL cholesterol, LDL cholesterol, and triglycerides in mmol/l. The determinations were carried out using routine methods at the Laboratory of Clinical Chemistry in Karolinska University Hospital, Huddinge, Sweden.

### Analyses of microorganisms

Samples were collected from the periodontal test sites to detect the presence or absence of the microorganisms *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia*, and *Treponema denticola*. The specimens were analyzed using the polymerase chain reaction technique by means of specific primers for the periodontal bacteria as described by Wahlfors *et al*. (33) and Meurman *et al*. (34).

### Statistical analysis

Analysis of variance (ANOVA), chi-squared tests, and multiple logistic regression analysis with backwards elimination of non-significant variables were performed using the SPSS® software package, version 13.0 (SPSS Inc., Chicago, IL, USA). All *P*-values are two-tailed, and confidence intervals were calculated at the 95% level.

## Results

Table 1 presents the demographic data and risk factors of the subjects. Oral health data are given in Table 2. Several oral health parameters were poor among the patients in comparison with the controls. MMP-9 levels in plasma of patients and controls are shown in Fig. 1. Twenty per cent of the patients and 19% of the controls had hypertension. According to logistic regression analysis, hypertension or antihypertensive therapy and body mass index were not significantly related to MMP-9 in plasma; hs-CRP values did not differ between the groups. *P. gingivalis*, *P. intermedia*, and *P. nigrescens*, *T. forsythia*, and *T. denticola* were detected significantly more often among subjects in the patient group than in the control group. All of the strains of microorganisms identified were also present in the control group, but in fewer subjects (Fig. 2). In a multiple

*Table 1.* Demographic data and risk factors of subjects

| | Patients ($n = 33$) number, mean ± SD | Controls ($n = 31$) number, mean ± SD | *P*-value |
|---|---|---|---|
| Gender (female/male) | 13/20 | 17/14 | NS |
| Age (years) | 54.0 ± 2.9 | 53.2 ± 2.8 | NS |
| Education (compulsory/higher) | 14/19 | 3/28 | < 0.01 |
| Smoking (yes/no) | 13/20 | 3/28 | < 0.01 |
| Body mass index (kg/m$^2$) | 25.9 ± 5.2 | 23.5 ± 3.0 | < 0.05 |
| Family history of atherosclerotic disease (yes/no) | 15/16 | 4/26 | < 0.01 |
| Diabetes (yes/no) | 1/32 | 0/31 | NS |
| Hypertension (yes/no)[a] | 8/23 | 5/26 | NS |
| Hs-CRP (mg/l) | 2.53 ± 5.93 [b] | 2.41 ± 2.66 | NS |
| Total cholesterol (mmol/l) | 5.98 ± 0.91 [b] | 5.37 ± 0.71 | < 0.01 |
| HDL cholesterol (mmol/l) | 1.46 ± 0.38 [b] | 1.49 ± 0.36 | NS |
| LDL cholesterol (mmol/l) | 3.81 ± 0.97 [b] | 3.40 ± 0.67 | = 0.05 |
| Triglycerides (mmol/l) | 1.57 ± 1.13 [b] | 1.04 ± 0.35 | = 0.01 |

Hs-CRP, highly sensitive C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a] Systolic pressure > 140 mmHg, diastolic pressure > 90 mmHg, or ongoing antihypertensive therapy.

[b] Values from two subjects are missing.

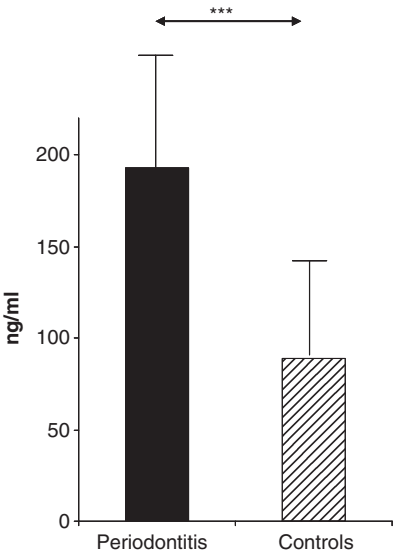


*Fig. 1.* MMP-9 levels (ng/ml ± SD) in plasma of periodontitis patients and controls. ***$P < 0.001$.

*Table 2.* Clinical oral and radiographic data (mean ± SD) of patients with periodontal disease and controls

| | Patients ($n = 33$) | Controls ($n = 31$) | *P*-value |
|---|---|---|---|
| Number of missing teeth | 3.3 ± 5.1 | 0.6 ± 0.8 | < 0.005 |
| Number of teeth with gingival pocket depth = > 5 mm | 9.9 ± 6.4 | 0.1 ± 0.3 | < 0.001 |
| Pocket depth (mm) | 3.2 ± 0.9 | 1.9 ± 0.3 | < 0.001 |
| Loss of attachment (mm) | 4.3 ± 1.8 | 2.1 ± 0.4 | < 0.001 |
| Gingival index | 1.5 ± 1.0 | 0.2 ± 0.2 | < 0.001 |
| Dental plaque index | 0.8 ± 0.8 | 0.2 ± 0.2 | < 0.001 |
| Percentage of bleeding on probing | 51.0 ± 30.5 | 15.1 ± 12.9 | < 0.001 |
| Percentage of remaining alveolar bone on radiographs | 81.1 ± 13.0 | 93.3 ± 1.9 | < 0.001 |

logistic regression model for MMP-9 in plasma, smoking, *P. gingivalis*, and dental plaque appeared to be the main independent predictors of increased MMP-9, associated with 6.45 times the odds for smoking, 6.21 times the odds for *P. gingivalis*, and 5.39 times the odds for dental plaque (Table 3).

In GCF samples, the levels of MMP-8 and MMP-9 were significantly higher in patients with periodontal disease than in controls, while no difference was observed in IL-1β levels (Fig. 3). MMP-8 and MMP-9 levels in the GCF of all subjects studied and the simultaneous presence or absence of the microorganisms investigated are presented in Table 4. The levels of MMP-8 were significantly higher in GCF when *P. intermedia*, *T. forsythia* and *T. denticola* were present than when these microorganisms were absent. Similarly, the levels of MMP-9 in GCF were significantly higher when *P. gingivalis*, *P. intermedia*, *P. nigrescens*, and *T. forsythia* were present than when these microorganisms were absent. Table 5 presents the results of the multiple logistic regression analysis for MMP-8 in GCF, with *T. forsythia* and age appearing to be the main independent predictors, associated with 10 times the odds of increase for *T. forsythia* and 5.5 times that for age. In a multiple logistic regression model for MMP-9, *T. forsythia* was the only independent predictor, associated with 2.9 times the odds for *T. forsythia* (Table 5).

## Discussion

In subjects with periodontal disease, reactions to bacteria in periodontal pockets may lead to an excessive host response, manifesting as a local and general inflammatory response, which can result in chronic inflammation and atherogenesis. Our findings are in agreement with those of earlier studies by our group on carotid artery intima-media thickness and calculated intima media area in subjects with periodontal disease, where periodontal disease was associated with development of early atherosclerotic carotid lesions (35).

Previous studies have shown that serum MMP-9 is elevated in subjects at risk for cardiovascular disease (15,36,37), with a history of cardiovascular disease (38), and peripheral arterial disease (9). Although no differences were present in the levels of hs-CRP between patients and controls in our study, the increased level of circulating MMP-9 in patients with periodontal disease could be an early marker of general inflammation and even an indicator of future cardiovascular disease. MMP-9 expression has been observed to be associated with the presence of *C. pneumoniae* in human coronary atherosclerotic plaque (14), this bacterium has also been found in

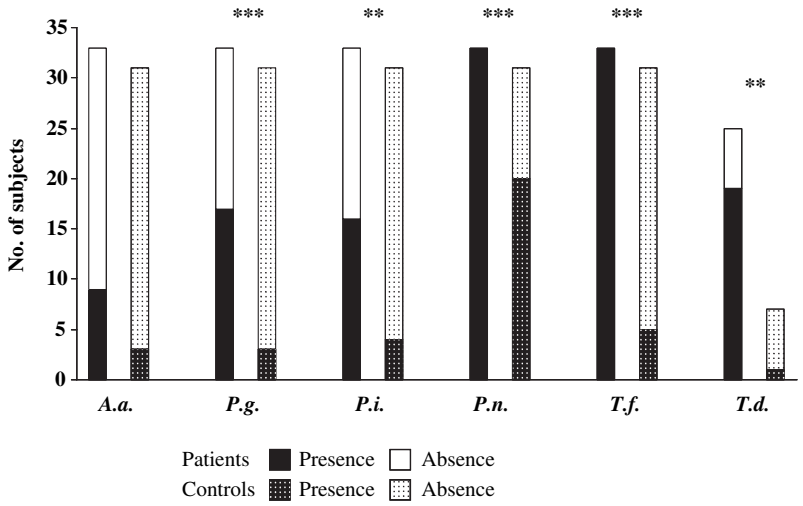


*Fig. 2.* Number of subjects in the periodontitis ($n = 33$) and control ($n = 31$) groups with presence or absence of *Actinobacillus actinomycetemcomitans* (*A. a*), *Porphyromonas gingivalis* (*P. g*), *Prevotella intermedia* (*P.i*), *Prevotella nigrescens* (*P.n*), and *Tannerella forsythia* (*T.f*). For *Treponema denticola* (*T.d*) the number of subjects with periodontitis were 25 and seven, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$.

*Table 3.* Results of multiple logistic regression analysis of the relationship between matrix metalloproteinase-9 (MMP-9) in plasma of subjects with periodontitis (dependent variable) and several independent variables (periodontal disease, age, gender, smoking, education, dental plaque, and periodontal microorganisms)

| | Explaining variable | β | Chi-squared | *P*-value | Odds ratio | 95% confidence interval |
|---|---|---|---|---|---|---|
| MMP-9 | Smoking | 1.867 | 3.90 | 0.048 | 6.45 | 1.01–41.67 |
| | *Porphyromonas gingivalis* | 1.828 | 5.52 | 0.019 | 6.21 | 1.35–28.57 |
| | Dental plaque | 1.685 | 5.36 | 0.021 | 5.39 | 1.30–22.44 |

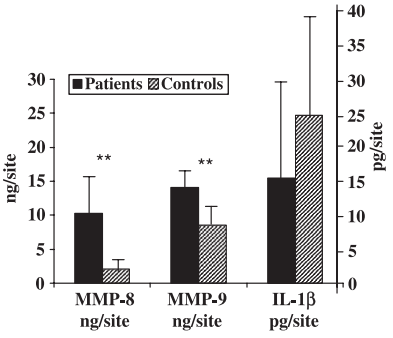


*Fig. 3.* Levels of matrix metalloproteinase-8 (MMP-8), MMP-9, and interleukin-1β (IL-1β) (mean ± SD) in gingival crevicular fluid of patients with periodontal disease and controls. $^{**}P < 0.01$.

deep periodontal lesions together with MMP-9 (13). In multiple logistic regression analysis, *T. forsythia* was a significant predictor for both MMP-8 and MMP-9. *C. pneumoniae,* together with *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*, has also been identified in atheromatous plaque (39).

Our study addressed, for the first time, the issue of the simultaneous presence of MMP-8 and MMP-9 and the presence or the absence of the periodontal microorganisms *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *T. forsythia*, and *T. denticola* in periodontal pockets of subjects with periodontal disease. Both patients and controls were randomly chosen to avoid selection bias and to ensure normal distribution of the sampled variables. The patients had suffered from documented periodontitis for at least 16 years at the time of re-examination, while the controls had stayed periodontally healthy during the same period. This long observation time is a major strength of the study. Both groups had visited their dentists almost every year. However, patients had visited dental hygienists significantly more often than had controls. It seems very difficult for these patients to recover from periodontal disease probably because of poor oral hygiene.

The higher amount of MMP-8 in the GCF of patients than controls is in agreement with earlier studies, which have shown that the major collagenase in periodontitis lesions is MMP-8, accompanied by MMP-9 (10,11). Most cell types found in the gingival periodontal region can be induced to express MMP-8 (40–43). We did not measure MMP-8 in plasma; however, subjects with hypoechogenic plaques have been observed to have higher levels of MMP-8 in plasma than controls (44).

The GCF of patients also contained significantly more MMP-9 than that of controls. It could be speculated that MMP-9 from diseased pockets leaks out, contributing to the increased plasma levels of MMP-9 in periodontitis patients. MMP-9 might be inhibited and removed by protease inhibitors such as $\alpha_2$-macroglobulin and tissue inhibitor of metalloproteinases.

Periodontal bacteria might also enter the bloodstream during transient bacteremias, inducing the systemic up-regulation of MMP-9 by hyperinflammatory monocytes. Beck *et al.* suggested that certain forms of periodontal disease, including refractory periodontitis, possess hyperinflammatory monocytes (45). By analyzing the levels of MMP-8 and MMP-9 in the periodontal pockets and simultaneously determining whether bacteria were present, it was possible to identify which periodontal microorganisms could induce the release of MMP-8 and MMP-9.

The multiple logistic regression analysis for MMP-9 in plasma showed that *P. gingivalis* was one of the main independent predictors for increased levels of the enzyme. Both *P. gingivalis* and *T. forsythia* have recently been found in samples of coronary stenotic artery plaque and dental plaque, with detection rates of 21.6% and 23.3%, respectively. The detection rate of

*Table 4*. Levels of matrix metalloproteinase-8 (MMP-8) and MMP-9 [ng/site (mean ± SD)] in gingival crevicular fluid with the simultaneous presence or absence of periodontal microorganisms

| Microorganism | Present (mean ± SD) | *n* | Absent (mean ± SD) | *n* | *P*-value |
|---|---|---|---|---|---|
| MMP-8 | | | | | |
| *Actinobacillus actinomycetemcomitans* | 8.21 ± 9.1 | 12 | 6.24 ± 10.7 | 52 | NS |
| *Porphyromonas gingivalis* | 9.63 ± 8.8 | 20 | 5.24 ± 10.9 | 44 | NS |
| *Prevotella intermedia* | 12.76 ± 15.4 | 20 | 3.82 ± 5.3 | 44 | 0.001 |
| *Prevotella nigrescens* | 7.45 ± 10.9 | 53 | 2.56 ± 6.5 | 11 | NS |
| *Tannerella forsythia* | 10.9 ± 12.3 | 38 | 1.53 ± 2.1 | 26 | < 0.001 |
| *Treponema denticola* | 15.21 ± 14.5 | 20 | 3.84 ± 5.8 | 12 | 0.015 |
| MMP-9 | | | | | |
| *Actinobacillus actinomycetemcomitans* | 13.55 ± 9.5 | 12 | 10.87 ± 8.4 | 52 | NS |
| *Porphyromonas gingivalis* | 15.45 ± 8.3 | 20 | 9.52 ± 8.1 | 44 | 0.009 |
| *Prevotella intermedia* | 14.61 ± 8.7 | 20 | 9.90 ± 8.2 | 44 | 0.04 |
| *Prevotella nigrescens* | 12.57 ± 8.5 | 53 | 5.61 ± 6.6 | 11 | 0.01 |
| *Tannerella forsythia* | 13.68 ± 8.8 | 38 | 8.00 ± 7.2 | 26 | < 0.008 |
| *Treponema denticola* | 14.58 ± 9.36 | 20 | 9.36 ± 8.9 | 12 | NS |

*Table 5*. Results of multiple logistic regression analysis of the relationship between matrix metalloproteinase-8 (MMP-8) and MMP-9 levels in gingival crevicular fluid (dependent variables) and the independent variables of periodontal disease, age, gender, smoking, education, dental plaque index, and presence of periodontal microorganisms

| Dependent variable | Explaining variable | Beta | Chi-squared | *P*-value | Odds ratio | 95% confidence interval |
|---|---|---|---|---|---|---|
| MMP-8 | *T. f.* | − 2.314 | 11.94 | 0.001 | 10.10 | 2.72–34.04 |
| | Age | 1.712 | 7.01 | 0.008 | 5.54 | 1.56–19.67 |
| MMP-9 | *T. f.* | − 1.063 | 4.04 | 0.044 | 2.90 | 1.03–8.20 |

*T.f.*, *Tannerella forsythia*.

*P. gingivalis* in coronary artery plaque was correlated with its presence in subgingival plaque (46). Human atherosclerotic plaque has been shown to contain viable invasive *A. actinomycetemcomitans*, and *P. gingivalis* (47).

Even small changes in blood lipid profiles can have considerable benefits for public health (48). Since infection and inflammation play a role in the complex interactions of lipid metabolism and in the pathogenesis of atherosclerosis (47), reducing the total inflammatory burden on subjects is critical. Periodontitis patients can be regarded as being at risk for future cardiovascular events because of their chronic infection burden.

In conclusion, periodontal microorganisms appeared to induce a host response, with increased release of MMP-8 and MMP-9 in periodontal pockets. Higher concentrations of MMP-9 were detected in blood samples of periodontitis patients, indicating that this enzyme either seeps into the circulation from inflammatory cells in the periodontal pockets or its upregulation in blood is triggered by periodontal bacteria.

## Acknowledgements

The author B.S. has been supported by the Heart and Lung Foundation and AFA Insurance, Stockholm, Sweden. The author, J.H.M., has been supported by grant TYH 3245 from the Helsinki University Central Hospital, Helsinki, Finland, and by the Ulf Nilsonne Foundation (SalusAnsvar Prize), Stockholm, Sweden.

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