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Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in blood as markers for early atherosclerosis in subjects with chronic periodontitis

P-Ö. Söder[1], J. H. Meurman[2,3], T. Jogestrand[4], J. Nowak[4], B. Söder[1]

[1]Institute of Odontology, Karolinska Institute, Huddinge, Sweden, [2]Institute of Dentistry, University of Helsinki and [3]Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland and [4]Department of Clinical Physiology Karolinska University Hospital, Huddinge, Sweden

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Background and Objectives: An association has been found between periodontal disease and the development of atherosclerosis. We investigated the hypothesis that periodontal disease triggers the expression of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in blood. Increased levels of these parameters might then indicate early atherosclerosis.

Material and Methods: In this cross-sectional study, the material comprised 80 subjects with chronic periodontitis and 31 subjects with no periodontal disease. Sixteen years after diagnosis of periodontal disease ultrasonography revealed a statistically significant difference ($p < 0.001$) of carotid intima–media thickness between the subjects with chronic periodontitis and the periodontally healthy subjects. Matrix metalloproteinase-9 and TIMP-1 were analyzed from blood as periodontal and systemic inflammatory markers. The relationship between MMP-9, TIMP-1 and MMP-9/TIMP-1 as dependent variables and several independent variables (age, sex, smoking, education, body mass index, hypertension, periodontal disease and cholesterol) were analyzed in multiple logistic regression models to assess the value of the inflammatory markers in predicting carotid atherosclerosis.

Results: Matrix metalloproteinase-9 and TIMP-1 were significantly higher in plasma from subjects with periodontal disease and atherosclerosis. Periodontal disease was identified as the principal independent predictor both for atherosclerosis (odds ratio 3.89 for increase in bilateral carotid intima–media thickness) and for increased MMP-9, TIMP-1 and MMP-9/TIMP-1 (odds ratio 2.58, 5.53 and 3.41, respectively). Classical atherosclerosis risk factors, such as increased total cholesterol, age and sex (women), were significant predictors in the model.

Conclusion: Matrix metalloproteinase-9, TIMP-1 and MMP-9/TIMP-1 in blood from subjects with periodontal disease could be useful laboratory markers for increased carotid artery intima–media thickness.

Professor, Dr Birgitta Söder, Karolinska Institutet, Institute of Odontology, Box 4064, 141 04 Huddinge, Sweden
Tel: +46 8 5248 8241
Fax: +46 8 663 03 06
e-mail:birgitta.soder@ki.se

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Inflammation plays a role in both the development of atherosclerosis (1) and periodontal disease (2,3). Chronic periodontitis is characterized by infection and inflammation of the periodontal tissue, leading to the destruction of the bone surrounding the teeth (4). Approximately 15–35% of the adult population in industrialized countries suffer from this multifactorial disease (5,6). Desvarieux *et al.* (7) have reported a relationship between periodontal disease, tooth loss and carotid artery plaque, and studies indicate that periodontal disease is associated with the development of early atherosclerotic lesions in the carotid artery (8) and with increased risk for stroke (9) and myocardial infarction (10). We have also shown that periodontal disease is associated with the development of early atherosclerotic carotid lesions (8).Inflammatory cells secrete matrix-degrading proteases, such as metalloproteinases (MMPs). The MMPs are a group of enzymes responsible for the degradation of most matrix proteins in growth and normal tissue turnover (11). The MMPs are inhibited by a family of naturally occurring specific inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs) which are essential in the regulation of connective tissue metabolism. The MMPs and TIMPs play a role in maintaining blood vessel integrity as well as in the pathology of coronary arteries. The TIMPs bind to active MMPs in a 1:1 relationship, inhibit MMP enzymatic activity and thereby maintain the balance in the metabolism of the extracellular matrix (12,13). If the balance between MMPs and TIMPs is disturbed with increased amount of MMPs, a breaking-down process of the extracellular matrix may begin. Matrix metalloproteinases may allow smooth muscle cells to invade and migrate, which may also happen in atherosclerosis (14,15). Extracellular matrix degradation by MMPs, specifically MMP-9, is involved in the pathogenesis of a wide spectrum of cardiovascular disorders, including atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction and aortic aneurysm (14,15), and in the pathogenesis of periodontal disease (16).

In light of earlier data and the slow development of atherosclerotic lesions, it is important to find markers which could detect the risk early (17). In this regard, we investigated 16 year clinical follow-up data of patients with or without periodontal disease. Our hypothesis was that subjects with periodontal disease have significantly more MMP-9 and TIMP-1 in blood compared with subjects having no periodontal disease and that these inflammatory markers also indicate carotid atherosclerosis.

Material and methods

Study groups

We studied 80 subjects with chronic periodontal disease and 31 individuals with no periodontal disease, as shown in Table 1. The presence of periodontal disease was documented in 1985 and confirmed as chronic periodontitis by clinical examinations in 2003. The 31 periodontally healthy participants were randomly selected from the group of individuals who were found to be free from periodontal disease in 1985 and confirmed to be periodontally healthy in 2003. The cohort and study selection are presented in Fig. 1. Power calculation had been conducted before onset of the investigation. There were significant differences in carotid artery intima–media thickness (IMT) between subjects with chronic periodontitis and periodontally healthy subjects (Table 1).

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

Table 1. Demographic data, risk factors, clinical oral and radiographic data, as well as ultrasonographic B-mode variables, in subjects with and without chronic periodontitis

	Chronic periodontitis (n = 80)	No periodontitis (n = 31)	p-value
Demographic data and risk factors			
Sex (women/men)	41/39	17/14	NS
Age (years)	54.4 ± 3.0	53.2 ± 2. 8	NS
Education (compulsary/higher)	30/47	3/28	< 0.01
Smoking (yes/no)	29/51	3/28	< 0.01
Body mass index (kg/m^2)	25.3 ± 4.3	23.5 ± 3.0	NS
Heredity for atherosclerotic disease (yes/no)	36/42	4/26	< 0.002
Hypertension (yes/no)[a]	19/59	5/26	NS
Clinical oral and radiographic data			
Number of missing teeth	2.3 ± 2.4	0.6 ± 0.8	< 0.001
Pocket depth (mm)	2.8 ± 0.7	1.9 ± 0.3	< 0.001
Loss of attachment (mm)	3.4 ± 1.1	2.1 ± 0.4	< 0.001
Gingival index (18)	1.2 ± 1.0	0.2 ± 0.2	< 0.001
Dental plaque index (19)	0.7 ± 0.7	0.2 ± 0.2	< 0.001
Percentage of remaining bone on radiographs	85.2 ± 6.4	93.3 ± 1.9	< 0.001
Ultrasonographic B-mode variables[b]			
Common carotid artery IMT (mm), right side	0.66 ± 0.12	0.58 ± 0.09	< 0.01
Common carotid artery IMT (mm), left side	0.68 ± 0.12	0.58 ± 0.08	< 0.001
Common carotid artery lumen (mm), right side	6.00 ± 0.63	5.65 ± 0.53	< 0.05
Common carotid artery lumen (mm), left side	5.86 ± 0.64	5.56 ± 0.41	< 0.05
Common carotid artery cIMA (mm^2), right side	13.83 ± 3. 56	11.38 ± 2.16	< 0.001
Common carotid artery cIMA (mm^2), left side	14.15 ± 3.49	11.11 ± 1.87	< 0.001

Values are given as number, mean ± SD. NS, not significant.

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

[b]In two cases, measurements of the B-mode variables listed in the table could not be successfully performed because of a local plaque formation or other technical reasons.

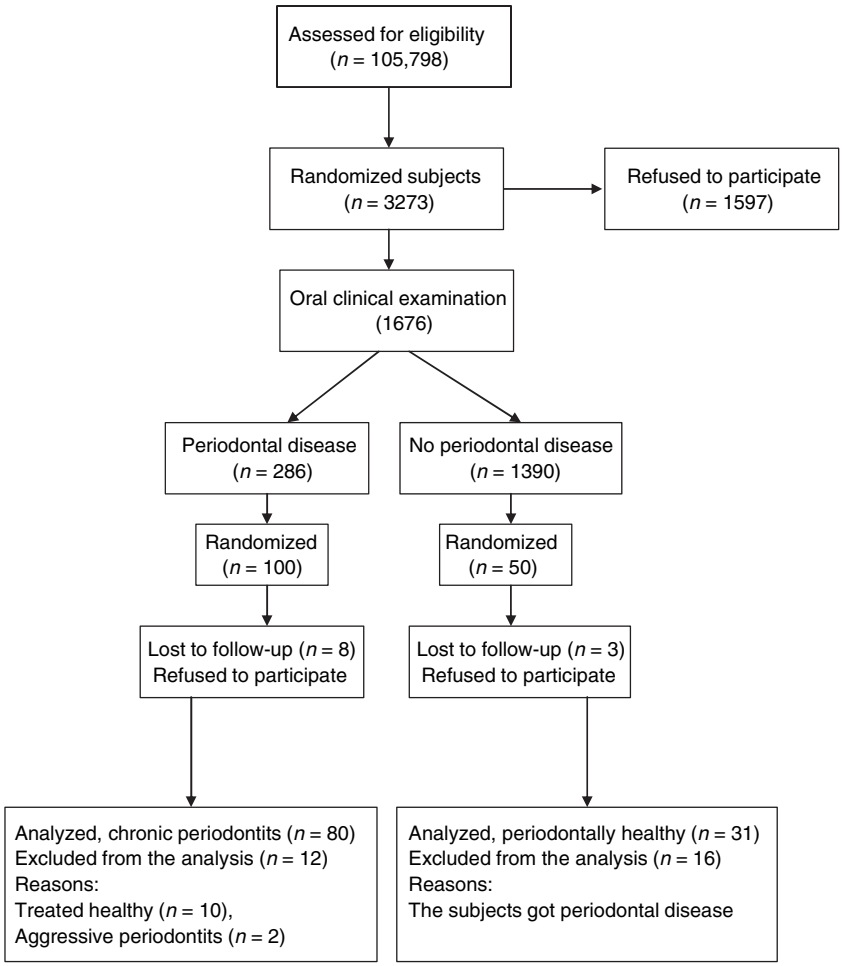

Fig. 1. Flow chart of selection of the subjects.

criterion was divided into smokers or former smokers and subjects who had never smoked. All subjects fulfilled the following inclusion criteria: no systemic disease, no systemic or local antibiotic therapy during the 6 months prior to the clinical examination and no periodontal treatment during the last 3 months. The Ethics Committee of the Karolinska University Hospital, Huddinge, Sweden had approved the study protocol. Subjects gave their informed written consent to participate.

Examination and analyses

In all subjects, the following clinical parameters of oral health were recorded at baseline in 1985 and at the end of the study in 2003: the number of remaining teeth excluding third molars, gingival inflammation around every tooth with bleeding on probing (BOP) and gingival index (18) as well as oral hygiene status using the dental plaque index (19) on six surfaces of all teeth excluding third molars. Periodontal pocket depth and tooth attachment level were determined with a periodontal probe and recorded to the nearest higher millimetre at six sites of each tooth on all teeth excluding third molars.

A full-mouth set of 14 periapical radiographs was obtained for each subject. At each measurable interproximal surface, except on third molars, alveolar bone height was determined as the percentage of the root length (20). Remaining bone height on radiographs was expressed as a percentage (BH%). Radiographs were obtained both at baseline and at the end of the study. Dental examinations were performed by one of the authors (B.S.) and the radiograms were evaluated by another author (P-Ö.S.), blinded to the results of the dental examination.

At the time of dental examination in 2003, blood pressure was measured and blood was collected for analysis of total plasma cholesterol after a 12 h overnight fast. Blood (20 mL) was drawn from the antecubital vein, and plasma was separated and stored at −70°C. Samples were later analyzed for the levels of total cholesterol fibrinogen at the Laboratory of Clinical Chemistry at Karolinska University Hospital, Huddinge, Sweden. Values of total cholesterol were expressed in millimoles per litre, and fibrinogen in grams per litre. Plasma was assayed for MMP-9 (both free and complexed) with enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Amersham Life Sciences Ltd, Amersham, England). The levels of

MMP-9 were expressed in nanograms per millilitre (21). The activity of human TIMP-1 was also measured in plasma using an immunoassay kit according to the recommendations of the manufacturer (Quantikine TIMP-1 immunoassay system, R&D systems Inc., Minneapolis, MN, USA).

Carotid B-mode ultrasonography

Carotid ultrasonography was performed at the time of re-examination in 2003. Carotid arteries were examined bilaterally with a duplex scanner (Aspen, Acuson, Mountain View, CA, USA) using a 7 MHz linear array transducer. The same trained sonographer carried out all recordings with the subjects in supine position, at the Department of Clinical Physiology, Karolinska University Hospital, Huddinge, Sweden. The method has previously been described in detail (8,22). 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. 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; 23) was calculated by using the formula:

$$3.14\,[(lumendiameter/2 + IMT)^2 - (lumendiameter/2)^2].$$

Study outcomes

The primary outcomes were plasma MMP-9 and TIMP-1 values in patients with or without a 16 year history of periodontal disease, and IMT verified by ultrasonography.

Statistical analysis

The study included 80 patients with chronic periodontitis. To reach a 0.05 significant difference between the chronic periodontitis and periodontally healthy subjects for MMP-9 with a power of 80% we needed over 30 periodontally healthy subjects according to Sample Power 2, SPSS 16.1 analysis (SPSS Inc., Chicago, IL, USA). The exact results from the analysis showed that at least 31 periodontally healthy subjects were needed to reach a significant difference of 0.05 between the subjects with chronic periodontitis and periodontally healthy subjects with a power of 82%.

Student's unpaired *t* test and analysis of variance (ANOVA) were used. Chi-square test with Fisher's exact *p*-value was used to determine the differences between categorical data. The Mann–Whitney *U*-test was used for non-parametric statistics on ordered categorical and continuous variables. Multiple logistic regression analysis was used to compare MMP-9, TIMP-1 and the MMP-9/TIMP-1 ratio in plasma while simultaneously controlling for several potential confounding variables. In the multiple logistic regression analysis models, we included the potentially confounding variables, e.g. age, sex, education, smoking habits and chronic periodontitis. The smoking habits criterion was divided into smokers (subjects who had ever smoked) and non-smokers (subjects who had never smoked). The model with these confounding variables was correlated with MMP-9, TIMP-1 and MMP-9/TIMP-1. 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 SPSS, version 16.0.

Results

All the 80 patients with a 16 year history of periodontal disease had developed early signs of carotid atherosclerosis as seen by the ultrasonographic examination in 2003. The 80 subjects with chronic periodontitis had significantly higher values of IMT than the 31 periodontally healthy subjects.

Figure 2 shows the MMP-9 and TIMP-1 values in plasma for the two groups. The concentrations of MMP-9 and TIMP-1 were significantly higher in the patients with than without periodontal disease. Education and heredity for atherosclerotic disease as well as smoking were also different between the groups (Table 1). Also, the clinical oral health parameters were significantly different between the groups, which confirmed the clinical diagnosis of chronic periodontitis in the diseased. The IMT and cIMA were significantly increased in subjects with chronic periodontitis when compared with subjects with no periodontitis.

In the multiple logistic regression analysis for IMT and cIMA, periodontal disease appeared to be the principal independent predictor, associated with 3.89 times the odds of increased IMT and 5.31 times the odds of increased cIMA, as shown in Table 2. Total cholesterol, age, sex (men) and body mass index were the other explanatory factors for carotid atherosclerosis in these subjects. Thus, smoking, education, heredity for atherosclerotic disease, hypertension and fibrinogen were not significant and omitted from Table 2.

Table 3 shows results of regression analysis for MMP-9, TIMP-1 and MMP-9/TIMP-1. Periodontal disease appeared to be the principal independent predictor, associated with 2.58 times the odds of increased MMP-9 and 5.53 times the odds of increased TIMP-1, as well as 3.41 times the odds of increased MMP-9/TIMP-1 ratio. The only other explanatory factors in this analysis were total cholesterol, sex and age.

Thus, smoking, education, body mass index, heredity for atherosclerotic disease, hypertension and fibrinogen were not significant and omitted from Table 3.

Discussion

This study addressed the issue of periodontal disease as a risk factor for atherosclerosis by evaluating the relationship between chronic periodontal disease, the occurrence of early changes in carotid arteries and plasma levels MMP-9 and its inhibitor TIMP-1 in patients without any symptoms of overt atherosclerotic disease at the baseline 16 years earlier. The present

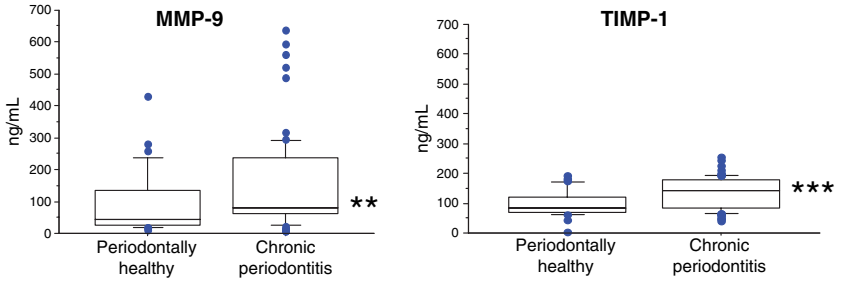

Fig. 2. Concentrations of MMP-9 and TIMP-1 in plasma from subjects with and without chronic periodontitis. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. subjects with no chronic periodontitis.

Table 2. The results of multiple logistic regression analysis of the relationship between common carotid artery IMT (bilaterally) and cIMA (bilaterally) as dependent variables and several independent variables (chronic periodontitis, age, sex, smoking, education, body mass index, heredity for atherosclerotic disease, hypertension, total cholesterol and fibrinogen)

Dependent variable	Explaining variable	β	χ^2	*p*-value	Odds ratio	95% confidence interval
Common carotid[a] artery IMT (bilaterally)	Chronic periodontitis	1.36	7.07	0.008	3.89	1.43–10.60
	Total cholesterol	0.96	4.71	0.030	2.61	1.10–6.19
Common carotid[b] artery cIMA (bilaterally)	Chronic periodontitis	1.67	9.15	0.002	5.31	1.80–15.68
	Age	1.10	5.25	0.022	2.99	1.17–7.63
	Sex (men)	1.33	7.63	0.006	3.79	1.47–9.77
	Body mass index	0.94	4.10	0.043	2.57	1.03–6.40

[a]Cox & Snell $r^2 = 0.175$, Nagelkerke $r^2 = 0.234$.
[b]Cox & Snell $r^2 = 0.26$, Nagelkerke $r^2 = 0.35$.

Table 3. The results of multiple logistic regression analysis of the relationship between MMP-9, TIMP-1 and MMP-9/TMP-1 ratio in plasma as dependent variables and several independent variables (chronic periodontitis, age, sex, smoking, education, body mass index, heredity for atherosclerotic disease, hypertension, total cholesterol and fibrinogen)

Dependent variable	Explaining variable	β	χ^2	*p*-value	Odds ratio	95% confidence interval
MMP-9[a]	Chronic periodontitis	0.95	3.88	0.049	2.58	1.01–6.64
	Total cholesterol	1.12	6.79	0.009	3.06	1.32–7.08
TIMP-1[b]	Chronic periodontitis	1.71	10.76	0.001	5.53	2.00–15.36
	Sex (women)	1.18	7.04	0.008	3.27	1.36–7.83
MMP-9/TIMP-1[c]	Chronic periodontitis	1.23	6.01	0.014	3.41	1.28–9.11
	Age	1.06	5.10	0.024	2.89	1.15–7.24
	Total cholesterol	1.14	6.066	0.014	3.13	1.26–7.52

[a]Cox & Snell $r^2 = 0.124$, Nagelkerke $r^2 = 0.165$.
[b]Cox & Snell $r^2 = 0.164$, Nagelkerke $r^2 = 0.219$.
[c]Cox & Snell $r^2 = 0.151$, Nagelkerke $r^2 = 0.201$.

results clearly identified periodontal disease as a principal independent predictor both for carotid atherosclerosis and for increased plasma MMP-9 and TIMP-1 as well as for an increased MMP-9/TIMP-1 ratio.

Some comments should be made concerning the reliability of our results. In this study the patients with or without periodontal disease were randomly chosen to avoid selection bias. Subsequently, even if the number of study participants appears small, a power calculation before the start of the study indicated that the number of subjects should be at least 31 in each group to reach 82% power for MMP-9, and this criterion was fulfilled 16 years later at the follow-up examination. Furthermore, the longitudinal prospective design of the study, with a cohort of patients, all of whom had suffered from documented chronic periodontal disease for at least 16 years at the time of re-examination, might show a temporal link when analyzing the association of periodontal disease with atherosclerosis of the carotid arteries.

The hypothesis that chronic inflammation, such as that caused by periodontal disease, represents a risk factor for cardiovascular disease has been

supported by results from several investigations (8–10). Previous studies have shown that serum MMP-9 is increased in subjects at risk of various forms of chronic inflammation and cardiovascular disease (24–26). The higher concentration of total cholesterol in subjects with periodontal disease, apart from high cholesterol being an atherosclerotic risk factor *per se*, may be the result of a periodontal disease-related alteration in lipid metabolism (27). Plasma levels of MMP-9 might therefore provide a useful risk marker for inflammation, since they are correlated with leukocyte count but are not associated with the lipid profile (28). A changed in the MMP-9/TIMP-1 ratio, with an excess of MMP-9, may break down the extracellular matrix and allow smooth muscle cells to invade the tissues and migrate; this contributes to pathological processes, including atherosclerosis (29). Circulating levels of MMPs and TIMPs have been examined in pathophysiological conditions (30). For example, Noji *et al*. (29) found that circulating levels of MMPs and TIMPs were altered in subjects with premature coronary atherosclerosis.

Herman *et al*. (31) have found that MMP-8 is capable of initiating the degradation of atherosclerotic lesions. Tuomainen *et al*. (32) found elevated serum concentrations of MMP-8 in men with subclinical atherosclerosis associated with cardiovascular disease or death.

In the present multiple logistic regression analyses of MMP-9 and TIMP-1 and of the MMP-9/ TIMP-1 ratio, as well as of IMT and cIMA, we adjusted for several demographic variables and established cardiovascular risk factors such as age, sex, education, heredity for atherosclerosis, body mass index, hypertension and smoking. The results of these analyses clearly showed that periodontal disease was a principal independent predictor of MMP-9/ TIMP-1 in blood, as well as a predictor for increased intima–media thickness and area in the common carotid arteries. Hence, the results of our study might strengthen the hypothesis that there may indeed be an association between periodontal disease and the atherosclerotic process in its early phase. The increased levels of MMP-9 and TIMP-1 and change in the MMP-9/TIMP-1 ratio may render arterial cell walls susceptible to damage, with subsequent development of atherosclerotic lesions. Consequently, the risk of future stroke and myocardial infarction may also increase.

The results of our study suggest that periodontal infections may potentially share a common inflammatory pathway and risk factors with atherosclerosis (33).

Even if the values of IMT in subjects with chronic periodontitis were significantly higher than in the periodontally healthy subjects, more data are needed to prove whether the effect of chronic periodontitis on IMT is causative or not. In this perspective, and by taking into account the high prevalence of both periodontal disease and cardiovascular diseases, together with their potential complications and the costs they incur to the society, new strategies for risk assessment need to be considered. We suggest that significantly increased plasma levels of MMP-9 and TIMP-1 and/or the MMP-9/TIMP-1 ratio in subjects with periodontal disease might be useful laboratory markers for increased IMT. This, in turn, may open new possibilities for prevention of atherosclerosis.

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