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The effect of smoking on the mRNA expression of MMPs and *TIMP-1* in untreated chronic periodontitis patients: a cross-sectional study

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Background and Objective: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are important for extracellular matrix. Expression of MMPs has been evaluated in gingiva without studying smoking. The aim of this study was to explore the effect of smoking on mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* in untreated chronic periodontitis and in periodontal health.

Material and Methods: Gingival samples were harvested from 30 subjects with untreated chronic periodontitis (15 nonsmokers and 15 smokers) and 30 periodontally healthy subjects (15 nonsmokers and 15 smokers). Full-mouth plaque score, gingival index, bleeding on probing, probing depth and clinical attachment level were recorded. Total RNA was isolated, and the mRNA expression of MMPs and *TIMP-1* was assessed by RT-PCR.

Results: Periodontitis groups were comparable in clinical measurements. Nonsmoker subjects with periodontitis had statistically significantly higher *MMP-1*, lower *MMP-9* and *TIMP-1* expression and higher *MMP-1*/*TIMP-1* ratio than smokers; and higher *MMP-8* expression and *MMP-8*/*TIMP-1* and *MMP-1*/*TIMP-1* ratios than healthy nonsmokers. Healthy nonsmokers had statistically significantly higher *MMP-13* expression than healthy smokers. Smoker periodontitis and healthy subjects had similar expression levels of MMPs and *TIMP-1* and MMPs/*TIMP-1* ratios. There was correlation among the MMPs only for smoker periodontitis subjects. Expression of *MMP-13* was correlated with mean clinical attachment level.

Conclusion: Within its limits, this study demonstrated that smoking affected mRNA expression of MMPs and *TIMP-1*, MMPs/*TIMP-1* ratios and relationships among MMPs in untreated chronic periodontitis and expression of MMPs in health. In the absence of smoking, chronic periodontitis affected expression of MMPs and MMPs/*TIMP-1* ratios.

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Periodontitis is an inflammatory disease of bacterial origin leading to progressive destruction of tooth-supporting tissues, specifically periodontal attachment loss and alveolar bone loss (1). Periodontitis is the result of complex interactions between periopathogenic bacteria, the host defence immune system and environmental factors. Periopathogenic bacteria and their products are the primary etiological agents for the initiation of periodontitis (2,3). Among host factors implicated in the destruction of periodontal supporting structures, MMPs are key enzymes for the degradation and remodeling of the extracellular matrix (4,5).

MMPs are a family of structurally related Zn^{2+}-dependent endopeptidases that are collectively capable of degrading extracellular matrix, composed of collagenous and noncollagenous proteins (6). They play an important role in various biological processes, including tissue remodeling, wound healing and pathological conditions such as periodontitis, arthritis, tumor cell invasion and metastasis (7). Human MMPs are classified into the following five groups in relation to their structure and function: collagenases, gelatinases, stromelysins, membrane-type (MT)-MMPs and other MMPs (8). The activity of MMPs is regulated by endogenous tissue inhibitors, especially TIMPs. TIMP-1, a 30 kDa glycoprotein, is the main inhibitor of MMPs synthesized by most cells. It forms high-affinity complexes with the active forms of MMPs and might be specially bound to proMMP-9 (9,10).

Metalloproteinases have mainly been studied in gingival tissues by immunochemistry at protein level and by RT-PCR at mRNA level (9). There are two distinct types of MMP-1, a 52 kDa glycoprotein and a 57 kDa protein. MMP-1 is expressed by fibroblasts, by keratinocytes during healing and by defence cells during inflammation (9,11). MMP-8, the neutrophil collagenase, had earlier been regarded to be synthesized by polymorphonuclear neutrophils only. The transcription of *MMP-8* occurs mainly in the maturing neutrophils in the bone marrow; MMP-8 is then stored in the neutrophil granules and released upon cell activation. Factors affecting the release of MMP-8 by degranulation regulate the activity of *MMP-8*. The expression of *MMP-8* mRNA has also been detected in tissues other than the bone marrow. Apart from the peripheral neutrophils, *MMP-8* mRNA is expressed in mononuclear fibroblast-like cells, in the rheumatoid synovial membrane and in human endothelial cells. There is MMP-8 activity in chronically inflamed tissues, such as in inflamed gingiva, chronic bronchiectasis and rheumatoid arthritis (12). Specifically, MMP-8 has been detected in sulcular epithelial and plasma cells in inflamed gingival connective tissues from untreated chronic periodontitis patients (12,13). Among the various types of MMP-8, the type originating from neutrophils is more glycated than the one originating from fibroblasts (12). Therefore, *MMP-8* expression is mainly regulated by protein degranulation of neutrophils and only less efficiently by *de novo* inducible mRNA levels (12,14). MMP-13 has been shown to be expressed by human sulcular epithelial cells (13,15), fibroblasts (15), macrophages (15) and macrophage-like cells (13) in inflamed gingival connective tissues in untreated periodontitis. The presence of MMP-13 in gingival tissues from untreated chronic periodontitis patients has also been confirmed in a more recent study (16). Furthermore, it has been demonstrated that during the progression of chronic periodontitis the MMP-13 level did not statistically differ between active and inactive sites (17).

Cigarette smoking has been identified as a risk factor for periodontitis (18,19). Smoking increases the susceptibility to periopathogenic bacteria and to periodontal tissue destruction (18,20,21). It remains unclear how smoking alters the immune response against the periopathogenic bacteria. Toxic components of tobacco smoke, especially nicotine, have local and systemic negative effects (22). Specific biomarkers, such as interleukin-1β and prostaglandin E_2, have been assessed in epidemiological studies (23–25) in an attempt to further explore the relationship between smoking and periodontitis. However, there is limited information concerning the effect of smoking on the degradation of extracellular matrix and especially the activation of MMPs (26).

The mRNA expression of MMPs in gingival tissues has been compared between periodontally healthy subjects and subjects with periodontitis (27–35) without exploring the possible effect of smoking. Altered mRNA expression of MMPs in gingival tissues has been found in periodontitis (28–33,35). The collagenases, namely MMP-1, MMP-8 and MMP-13, are mainly responsible for the destruction occurring in the inflamed connective tissue of the gingiva. Gelatinases and stromelycins have been implicated in the degradation of the extracellular matrix of the gingiva. Therefore, MMP-9, which is a gelatinase, and MMP-3, which is a stromelycin, were selected to be studied in the present experiment. Moreover, MMP-9 exerts a wide range of action in matrices and activates proMMP-1. MMP-3 might participate in the activation of other MMPs as well. TIMP-1 was selected to be studied among all TIMPs because it inhibits MMP-1, -3, -8, -9 and -13, and there are findings indicating its action in periodontitis, after periodontal treatment. The first aim of the present cross-sectional, controlled clinical study was to explore the possible effect of smoking on the mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* in gingival tissues from subjects with untreated chronic periodontitis and in gingival tissues from periodontally healthy subjects. The second aim of this study was to compare the mRNA expression of these MMPs and *TIMP-1* between untreated chronic periodontitis subjects and periodontally healthy subjects.

Material and methods

Study population

Sixty subjects from the patient pool of the Department of Periodontology, School of Dentistry, University of Athens were recruited from January 2007 to June 2007. Thirty of them were

periodontitis patients (experimental subjects; 15 smokers and 15 nonsmokers) with the following inclusion criteria: presence of generalized chronic severe periodontitis (1), presence of at least 20 teeth, presence of a periodontally hopeless posterior tooth with probing depth ≥5 mm, clinical attachment loss ≥5 mm and bleeding on probing (without suppuration), and presence of at least seven teeth with probing depth ≥5 mm.

For the remaining 30 subjects (controls; 15 smokers and 15 nonsmokers) the inclusion criteria were as follows: healthy and intact periodontium, presence of a tooth with indication for crown lengthening, no bleeding on probing, probing depth and clinical attachment loss or presence of a third molar with indication for extraction, no bleeding on probing, probing depth and clinical attachment loss.

For all subjects, the exclusion criteria were as follows: presence of known systemic disease or condition that could affect the periodontal tissues, indication for prophylactic antibiotics, presence of systemic medication or antibiotic treatment for the previous 6 mo, presence of pregnancy or lactation, or history of periodontal treatment for the previous 6 mo.

Subjects were classified into the four groups: nonsmoker periodontitis patients (CPN; five men and 10 women, mean age 51.7 ± 6.15 years, age range 43–65 years), smoker periodontitis patients (CPS; six men and nine women, mean age 49.3 ± 8.35 years, age range 39–69 years), nonsmoker periodontally healthy subjects (HN; six men and nine women, mean age 36.8 ± 9.15 years, age range 26–52 years) and smoker periodontally healthy subjects (HS; seven men, eight women, mean age 33.75 ± 6.67 years, age range 24–48 years). All smokers had smoked for at least 10 years, with daily cigarette consumption ≥20 cigarettes. Every participant signed an informed consent form prior to enrolment in the study. The study was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2000, and was approved by the Ethics and Research Committee of the School of Dentistry, University of Athens.

Patient management

Clinical examination— The clinical examination for each subject included a full-mouth plaque score (FMPS; 36), the simplified gingival index (GI-S; 37), bleeding on probing, clinical attachment level and probing depth. A 15 mm calibrated periodontal probe (PCPUNC-15; Hu-Friedy, Chicago, IL, USA) was used. Probing depth and clinical attachment level were measured to the nearest millimeter.

For periodontitis patients, additional data was documented, as follows: mean probing depth, mean clinical attachment level, number of teeth with probing depth ≥5 mm, number of sites with probing depth ≥5 mm, percentage of sites with probing depth 4–6 mm, percentage of sites with probing depth ≥7 mm, gingival tissue sampling site bleeding on probing, gingival tissue sampling site probing depth and gingival tissue sampling site clinical attachment level. All clinical measurements were performed by the same calibrated examiner (E.M.)

Collection of gingival tissue samples— Gingival tissue samples were harvested during extraction of the periodontally hopeless tooth for the periodontitis patients and during the surgical crown lengthening or third molar extraction for the healthy controls. External bevel gingivectomy incision and a fresh no. 15c blade were used for sample collection. The tissue sample weight ranged from 20 to 60 mg.

Isolation of total RNA and RT-PCR

Upon collection, each sample was submerged in 10 volumes of RNAlater solution (Qiagen, Valencia, CA, USA), stored for 1 d at 4°C and then at −20°C until usage. Homogenization and total RNA isolation were performed using RNeasy MiniKit (Qiagen) following the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) with SuperScript II Reverse Transcriptase (Invitrogen, Darmstadt, Germany) primed with oligo-dT in a final concentration of 20 pmol/μL. A ribonuclease inhibitor (RNaseOUT; Invitrogen) was also included in the reaction at a final concentration of 1 U/μL. All RT reactions were performed independently of the PCRs.

Gene-specific oligonucleotide primers were designed for *MMP-1* (38), *-3* (38), *-8*, *-9* (the primers for *MMP-8* and *MMP-9* were designed by Dr Gerassimos Voutsinas, Biologist, Institute of Biology, National Center of Scientific Research 'Demokritos', Athens) and *-13* (39), *TIMP-1* (40) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Invitrogen 41). The primer sequences are shown in Table 1. One microliter of cDNA was amplified with 10 pmol/μL primers at a final volume of 25 μL. Negative controls were used for each RT-PCR, omitting the template. The efficiency of reverse transcription was verified by the detection of the *GAPDH*. The PCR conditions are shown in Table 1. All products were resolved on a 2% agarose gel containing ethidium bromide and were visualized under ultraviolet light.

Densitometry

The band intensity on PCR photographs was quantified by densitometry. The intensity of the band representing each RT-PCR product for *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* was normalized to the intensity of the band for the housekeeping gene *GAPDH* and was expressed as a ratio of relative band intensity. All experiments were repeated once to ensure statistical accuracy.

Statistical analysis

Differences among the study groups (CPN, CPS, HN and HS) were compared using Student's unpaired *t*-test or the Mann–Whitney *U*-test for normally and non-normally distributed continuous variables, respectively, or by means of the chi-squared test for categorical variables. Relations between MMPs, or between the MMPs, *TIMP-1* and clinical parameters (bleeding on probing, clinical attachment level and probing depth) were studied using the Spearman correlation coefficient. Multivariate logistic regression analysis was used to assess associations of the expression of

Table 1. Primers and experimental conditions for PCR amplification

Gene	Primer	Sequence 5′ → 3′	Fragment size (bp)	PCR protocol
GAPDH	Forward	TGGTATCGTGGAAGGACTCATGAC	188	94°C (3 min); 32 cycles of 94°C (30 s),
	Reverse	ATGCCAGTGAGCTTCCCGTTCAGC		56°C (40 s), 72°C (30 s); 72°C (7 min)
MMP-1	Forward	AGCGACTCTAGAAACACA	246	94°C (3 min); 38 cycles of 94°C (30 s),
	Reverse	AGTGAGGACAAACTGAGC		48°C (30 s), 72°C (30 s); 72°C (7 min)
MMP-3	Forward	TCT TCC AAT CCT ACT GTT	211	94°C (3 min); 38 cycles of 94°C (30 s),
	Reverse	CCAATCCAAGGAACTTCT		48°C (30 s), 72°C (30 s); 72°C (7 min)
MMP-8	Forward	GAT GCT ATC ACC ACA CTC CGT	283	94°C (3 min); 38 cycles of 94°C (30 s),
	Reverse	GCT GCG TCA ATT GCT TGG A		52°C (30 s), 72°C (40 s); 72°C (7 min)
MMP-9	Forward	GCC TGC CAC TTC CCC TTC ATC	472	94°C (3 min); 38 cycles of 94°C (30 s),
	Reverse	CCC CAC TTC TTG TCG CTG TCA		60°C (40 s), 72°C (40 s); 72°C (7 min)
MMP-13	Forward	CTA TGG TCC AGG AGA TGA AG	389	94°C (3 min); 38 cycles of 94°C (30 s),
	Reverse	AGA GTC TTG CCT GTA TCC TC		54°C (30 s), 72°C (45 s); 72°C (7 min)
TIMP-1	Forward	AGTCAACCAGACCACCTTATACCA	385	94°C (3 min); 32 cycles of 94°C (30 s),
	Reverse	TTTCAGAGCCTTGGAGGAGCTGGTC		54°C (30 s), 72°C (50 s); 72°C (7 min)

MMPs and *TIMP-1* with potential confounders, such as age and sex. A commercially available statistical software program (STATA 9.0; Stata, College Station, TX, USA) was used for the statistical analysis. The level of statistical significance was set at 5% ($p = 0.05$).

Results

The two experimental groups (CPN and CPS) were statistically comparable for patient age, sex distribution, number of existing teeth, FMPS, GI-S, bleeding on probing, mean probing depth, mean clinical attachment level, number of teeth with probing depth ≥5 mm, number of sites with probing depth ≥5 mm, percentage of sites with probing depth 4–6 mm and percentage of sites with probing depth ≥7 mm, as well as in gingival probing depth and clinical attachment level at the tissue sampling site. All experimental tissue sampling sites presented bleeding on probing (Table 2). There was statistical comparability among all subject groups (CPN, CPS, HN and HS) in sex distribution, between the two groups of smokers (CPS and HS) in smoking habits, and between the two control groups (HN and HS) in patient age. The mean patient age was statistically significantly greater for the periodontitis than for the healthy control subjects (data not shown).

Not all MMPs and *TIMP-1* were detected in every gingival tissue sample, which indicated either complete absence of expression or expression at levels so low that they were not detectable with RT-PCR (Table 3).

Table 2. Descriptive analysis by periodontitis group

Parameter	CPN Mean ± SD	CPS Mean ± SD	Student's unpaired *t*-test *p*-Value
Sex (% men)	33.3%	40.0%	0.70*
Age (years)	51.7 ± 6.1	49.3 ± 8.4	0.38
Existing teeth	25.7 ± 4.8	24.1 ± 4.3	0.36
Sites with probing depth ≥5 mm	41(24–55)[a]	29(20–53)[a]	0.49[a]
Teeth with probing depth ≥5 mm	15 (9–21)[a]	13(8–20)[a]	0.53[a]
Percentage of sites with probing depth ≥5 mm	26.3 (16–38.2)[a]	28.8 (13.5–37.3)[a]	0.852[a]
% Teeth probing depth ≥5 mm	59 (42.3–82.1)[a]	65 (34.8–83.3)[a]	0.693[a]
FMPS	0.77 ± 0.16	0.72 ± 0.21	0.42
GI-S	0.76 ± 0.17	0.68 ± 0.19	0.22
Bleeding on probing	0.69 ± 0.16	0.69 ± 0.19	0.95
Mean probing depth (mm)	3.7 ± 0.8	3.6 ± 0.7	0.67
Mean clinical attachment level (mm)	3.5 (3.3–4.7)[a]	4 (3.1–4.3)[a]	0.98[a]
Percentage of sites with probing depth 4–6 mm	36.1 ± 13.9	41.3 ± 16.1	0.35
Percentage of sites with probing depth ≥7 mm	5.0 (1.6–9)[a]	3.0 (0.5–4.5)[a]	0.23[a]
Gingival probing depth (mm)	7.7 ± 2.9	6.6 ± 1.7	0.21
Gingival clinical attachment level (mm)	7.9 ± 2.9	7.1 ± 2.4	0.46
Gingival bleeding on probing (yes/total)	15/15	15/15	—[b]

*Values are percentages, and *p*-values were obtained by using the chi-squared test.
[a]Values are medians (Q_1–Q_3), and *p*-values were obtained by using the Mann–Whitney *U*-test.
[b]Variables did not vary.

The mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* was compared between the groups CPN and HN, CPS and HS, CPS and CPN, and HN and HS. Groups CPN and HN statistically significantly differed only in *MMP-8* expression, with higher expression in the CPN group ($p = 0.04$). However, there were non-significant indications that the *MMP-1* expression was higher in the CPN than in the HN group ($p = 0.06$). There were no statistically significant

Table 3. Number and percentage of gingival tissue samples with MMPs and *TIMP-1* expression by study group

Gene	CPN (%)	CPS (%)	HN (%)	HS (%)
MMP-1	15/15 (100)	7/15 (46)	10/15 (66)	6/15 (40)
MMP-3	12/15 (80)	12/15 (80)	13/15 (86)	10/15 (66)
MMP-8	11/15 (73)	14/15 (93)	5/15 (33)	10/15 (66)
MMP-9	9/15 (60)	12/15 (80)	9/15 (60)	7/15 (46)
MMP-13	0/15 (0)	4/15 (26)	11/15 (73)	1/15 (6)
TIMP-1	13/15 (86)	15/15 (100)	10/15 (66)	9/15 (60)

differences between the CPS and HS groups. The CPN and CPS groups were statistically significantly different in the expression of *MMP-1* and *-9* and *TIMP-1*, with higher *MMP-1* ($p = 0.0003$) and lower *MMP-9* and *TIMP-1* expression ($p = 0.02$ and $p = 0.01$, respectively) for the CPN group. Groups HN and HS were statistically significantly different only in the expression of *MMP-13*, with greater expression in the HN group ($p = 0.0003$; Table 4).

The *MMP-1*/*TIMP-1*, *MMP-3*/*TIMP-1*, *MMP-8*/*TIMP-1*, *MMP-9*/*TIMP-1* and *MMP-13*/*TIMP-1* ratios were compared between the groups CPN and HN, CPS and HS, CPN and CPS, and HN and HS. Groups CPN and HN statistically significantly differed in *MMP-8*/*TIMP-1* and *MMP-1*/*TIMP-1* ratios, with higher values for the CPN group ($p = 0.05$ and $p = 0.02$, respectively; Table 4). There were no statistically significant differences between CPS and HS groups and between HN and HS groups (Table 4). The CPN and CPS groups statistically significantly differed in *MMP-1*/*TIMP-1* ratio, with a higher value for the CPN group ($p = 0.0001$; Table 4). The *MMP-9*/*TIMP-1* ratio was lower for the CPN than for the CPS group, but the difference was at the borderline of statistical significance ($p = 0.06$; Table 4).

The possible existence of correlations between the mRNA expressions of the MMPs studied was explored for each subject group. Significant correlations were found only for the CPS group, specifically between *MMP-1* and *MMP-3* ($p = 0.02$), between *MMP-1* and *MMP-13* ($p = 0.04$), and between *MMP-3* and *MMP-13* ($p = 0.02$; Table 5).

There was no statistically significant correlation between age and expression of MMPs or *TIMP-1*. Comparison of expression of MMPs and *TIMP-1* between men and women revealed that for all subject groups women had statistically significantly higher *MMP-3* expression than men (data not shown).

The possible existence of correlation between the MMPs and *TIMP-1* expression and the clinical measurements (FMPS, GI-S, bleeding on probing, mean probing depth, mean clinical attachment level, ginvival site probing depth and gingival site clinical attachment level) was tested using the Spearman correlation coefficient. Statistically significant positive correlation was found only between *MMP-13* expression and mean clinical attachment level ($p = 0.048$; Table 6).

Discussion

The present cross-sectional, controlled clinical study compared the levels of mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* between smokers and nonsmokers with chronic periodontitis, between smokers with chronic periodontitis and smokers with healthy periodontium, between nonsmokers with chronic periodontitis and nonsmokers with healthy periodontium and between smokers and nonsmokers with healthy periodontium.

The greater *MMP-8* expression and the indicative data of greater *MMP-1* expression found for the nonsmoker periodontitis than nonsmoker healthy subjects could be partly attributed to their association with inflammation. MMP-8 is mainly expressed by neutrophils, which are highly recruited to the area due to the inflammation process (42). MMP-1, a collagenase,

Table 4. MMPs and *TIMP-1* expression and MMP/*TIMP-1* ratios: analysis by study group and comparison among the study groups

	CPN	CPS	HN	HS	CPN vs. HN	CPS vs. HS	CPN vs. CPS	HN vs. HS
Gene	Median (Q_1–Q_3)	Median (Q_1–Q_3)	Median (Q_1–Q_3)	Median (Q_1–Q_3)	*p*-Value*	*p*-Value*	*p*-Value*	*p*-Value*
MMP-1	0.55(0.34–0.81)	0 (0–0.37)	0.34 (0– 0.63)	0 (0– 0.63)	0.06	0.84	0.0003	0.27
MMP-3	0.35(0.16–0.86)	0.23(0.04–0.51)	0.58(0.35–0.66)	0.70(0–0.81)	0.52	0.62	0.38	0.69
MMP-8	0.78(0–1.08)	0.6(0.55–0.77)	0 (0–0.77)	0.55 (0–1.13)	0.04	0.71	0.19	0.09
MMP-9	0.21(0–0.46)	0.51(0.40–0.64)	0.36(0–0.71)	0 (0–0.54)	0.40	0.08	0.02	0.50
MMP-13	0[a]	0 (0–0.65)	0.54(0–0.83)	0 (0–0)	—	0.12	—	0.0003
TIMP-1	0.41(0.28–0.56)	0.56(0.50–0.59)	0.44(0–0.65)	0.37(0–0.60)	0.92	0.09	0.01	0.69
MMP-1/*TIMP-1*	1.20 (1.04–1.41)	0 (0–0.69)	0.64 (0–1.23)	0 (0–0.90)	0.05	0.63	0.0001	0.68
MMP-3/*TIMP-1*	0.80 (0.56–1.64)	0.42 (0.07–1.32)	0.98 (0.44–1.97)	1.06 (0.06–1.29)	0.88	0.53	0.12	0.62
MMP-8/*TIMP-1*	2.05 (0–2.36)	1.12 (0.84–1.47)	0(0–1.05)	1.32 (0–1.46)	0.02	0.86	0.16	0.07
MMP-9/*TIMP-1*	0.63 (0–0.92)	0.98 (0.70–1.50)	1.17(0–1.97)	0.77 (0–1.42)	0.09	0.51	0.06	0.59
MMP-13/*TIMP–1*	0[a]	0 (0–1.12)	1.50 (0.67–1.75)	0[a]	—	—	—	—

**p*-Values were obtained by using the Mann–Whitney *U*-test.
[a]Constant and equal to 0.

Table 5. Spearman correlation coefficients (*p*-value) between the expression of MMPs by study group

Genes	CPN	CPS	HN	HS
MMP-1 with *MMP-3*	0.33 (0.22)	0.61 (0.02)	0.17 (0.54)	0.21 (0.45)
MMP-1 with *MMP-8*	0.28 (0.31)	−0.05 (0.85)	0.18 (0.52)	005 (0.86)
MMP-1 with *MMP-9*	0.09 (0.75)	−0.17 (0.56)	−0.45 (0.09)	0.23 (0.41)
MMP-1 with *MMP-13*	—*	0.54 (0.04)	−0.31 (0.25)	0.42 (0.12)
MMP-3 with *MMP-8*	0.22 (0.43)	−0.30 (0.28)	0.18 (0.31)	0.5 (0.06)
MMP-3 with *MMP-9*	0.26 (0.35)	−0.30 (0.28)	0.16 (0.56)	0.19 (0.49)
MMP-3 with *MMP-13*	—*	0.60 (0.02)	0.26 (0.35)	0.06 (0.82)
MMP-8 with *MMP-13*	—*	0.22 (0.43)	−0.50 (0.06)	0.06 (0.82)
MMP-9 with *MMP-13*	—*	0 (1)	0.42 (0.12)	−0.23 (0.40)

*Constant and equal to 0.

degrades collagen, mainly type I, and is implicated in the extracellular matrix destruction of the inflammatory process (43).

For smokers, the presence of chronic periodontitis did not significantly affect the expression of MMPs and *TIMP-1*. The nonsignificant indications of greater *MMP-9* and *TIMP-1* expression seen for subjects with periodontitis than healthy subjects might be partly due to smoking. There are indicative data that smoking might modify the expression of MMPs (44), though there is not sufficient information on the cell types and MMPs that smoking could influence.

For periodontitis subjects, the differences in increased *MMP-1*, *MMP-9* and *TIMP-1* expression found between nonsmokers and smokers might be attributed to smoking, because the groups were comparable in age, sex distribution and clinical measurements. It seems that there is a dose-related pattern in the action of nicotine. Subjects who smoke for more than 10 years and/or smoke more than 10 cigarettes/d have worse periodontal condition, as assessed clinically, than subjects with less daily cigarette consumption or than former smokers (45–47). Smoking modulates destruction of the periodontium through various pathways. Changes in vascular formations and microcirculatory functions in the periodontal tissues of smokers may influence the inflammatory reaction in the gingival tissues. MMP-9 is involved in destruction of extracellular matrix and activation of cytokines. Smoking might help in cytokines overproduction, with a possible mechanism via MMP-9 (48). Moreover, smoking may suppress the activities of protease inhibitors. α2-Macroglobulin is an inhibitor for several proteases, such as MMPs. It has been shown that smokers have a significantly lower concentration of α2-macroglobulin in gingival crevicular fluid and total amounts of α2-macroglobulin and α1-antitrypsin than nonsmokers (22). Deleterious effects of smoking on the function of polymorphonuclear neutrophils, including reduced viability and phagocytosis, have been found in periodontally healthy smokers, in a dose–response manner (26). Smoking may alter neutrophil behavior in periodontal tissues. Neutrophils are the major source of MMP-8. Liu *et al*. (26). reported that MMP-8 expression in the gingival tissues was significantly higher in smokers than nonsmokers, whereas the salivary MMP-8 levels were significantly lower in current than former smokers.

It seems that the greater *MMP-13* expression demonstrated for nonsmoker than smoker healthy subjects is due to smoking, because the two groups were comparable in age, sex distribution and clinical inflammation.

Differences in MMP/*TIMP-1* ratios among the subject groups might be indicative of changes in MMPs and their inhibitor in the gingival tissues. The greater *MMP-1*/*TIMP-1* and *MMP-8*/*TIMP-1* ratios found for nonsmoker periodontitis than nonsmoker healthy subjects resulted from increased *MMP-8* and *TIMP-1* expression, respectively, in the former group. The greater *MMP-1*/*TIMP-1* ratio found for nonsmoker than smoker periodontitis subjects was caused by greater *MMP-1* and smaller *TIMP-1* expression in the former group.

Correlations among the MMPs studied were detected only for smoker periodontitis subjects. MMP-1 and MMP-13 belong to the same MMP group, the collagenases, which might partly explain their correlation. The ability of MMP-3 to activate proMMPs (10) might account for the correlation of *MMP-3* with *MMP-1* and *MMP-13*.

Previous studies on the mRNA expression of *MMP-1* (30,32,33,35), *-3* (27,30,32), *-8* (27,30, 32), *-9* (32,35) and *-13*, (32,35) the MMPs/*TIMP-1* ratios (32) and the correlations among the various MMPs (32) have compared periodontitis subjects after periodontal

Table 6. Spearman correlation coefficients (*p*-values) of the expression of the MMPs with the clinical measurements

Gene	FMPS	GI-S	Bleeding on probling	Mean probing depth	Mean clinical attachment level	Probing depth	Gingival site clinical attachment level
MMP-1	0.1134 (0.55)	0.1992 (0.29)	0.1075 (0.57)	−0.0294 (0.88)	−0.0175 (0.93)	−0.11 (0.57)	−0.11 (0.57)
MMP-3	0.2520 (0.18)	0.1897 (0.32)	−0.0828 (0.66)	−0.0937 (0.62)	−0.0101 (0.96)	−0.04 (0.83)	−0.09 (0.62)
MMP-8	−0.1648 (0.38)	−0.0765 (0.69)	0.2709 (0.15)	−0.3026 (0.10)	−0.3347 (0.07)	−0.14 (0.45)	−0.16 (0.39)
MMP-9	0.0311 (0.87)	−0.0567 (0.77)	0.0045 (0.98)	−0.1074 (0.57)	−0.0860 (0.65)	−0.03 (0.89)	0.00 (0.99)
MMP-13	0.1971 (0.30)	0.0424 (0.82)	0.1529 (0.42)	0.2961 (0.11)	0.3646 (0.048)	0.05 (0.75)	0.09 (0.91)
TIMP-1	0.0788 (0.68)	0.0763 (0.69)	0.1861 (0.32)	−0.1431 (0.45)	−0.0315 (0.87)	−0.11 (0.81)	−0.11 (0.64)

treatment with healthy subjects. Moreover, most of them do not specify the subjects' smoking habits. In the present study, gingival tissue samples were harvested from untreated periodontitis subjects, and subjects were classified as smokers (all heavy smokers) and nonsmokers. Therefore, comparison between the present findings and those of previous studies (27,30–32,35) is not feasible.

Concerning the *TIMP-1* expression, the present findings on the absence of a significant difference between untreated periodontitis subjects and healthy control subjects are in accordance with the findings of Aiba *et al*. (29); however, in the study by Aiba *et al*. the control group is not clearly defined and the number of experimental (4) and control subjects (5) is very limited.

A limitation of the present study is the gingival tissue sampling. For all healthy control subjects, the sampling site was clinically healthy, though the existence of slight, clinically undetectable inflammation cannot be excluded. Moreover, tissue samples might vary slightly in tissue type content (connective tissue, epithelium). Selecting real time RT-PCR rather than RT-PCR for the assessment of the mRNA expression would be preferable, though it has been found that both lead to comparable results (49).

Further research is required to evaluate the immunoexpression of MMPs in the gingival tissues, since the mRNA expression does not always reflect protein expression, owing to possible post-transcriptional modifications (50). Moreover, assessment of the precursor and active forms of MMPs in future studies seems challenging.

Conclusions

Within its limits, the present study demonstrates that smoking affects the mRNA expression of MMPs and *TIMP-1*, the MMPs/*TIMP-1* ratios (at transcriptional level), and relations among the MMPs in untreated chronic periodontitis patients, as well as it affects the expression of MMPs in healthy subjects. In the absence of smoking, untreated chronic periodontitis affects the mRNA expression of MMPs and the MMPs/*TIMP-1* ratios. In the presence of smoking, such an effect was not found. Expression of *MMP-13* is correlated with the mean clinical attachment loss.

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