Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 532–542

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01465.x

Expression of *MMPs* and *TIMP-1* in smoker and nonsmoker chronic periodontitis patients before and after periodontal treatment

E. Mouzakiti[1], E. Pepelassi[1], G. Fanourakis[2], C. Markopoulou[1], S. Tseleni-Balafouta[2], I. Vrotsos[1]

[1]Department of Periodontology, School of Dentistry, University of Athens, Athens, Greece and [2]Department of Pathology, School of Medicine, University of Athens, Athens, Greece

Mouzakiti E, Pepelassi E, Fanourakis G, Markopoulou C, Tseleni-Balafouta S, Vrotsos I. Expression of MMPs *and* TIMP-1 *in smoker and nonsmoker chronic periodontitis patients before and after periodontal treatment. J Periodont Res 2012; 47: 532–542. © 2012 John Wiley & Sons A/S*

Background and Objective: Nonsurgical periodontal treatment controls periodontal inflammation. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are implicated both in the destruction and in the healing of periodontal tissues. The aim of the present study was to compare the mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* in chronic periodontitis before and after initial periodontal treatment.

Material and Methods: Ninety gingival samples were harvested from 30 patients with chronic periodontitis (15 nonsmokers and 15 smokers) before and after nonsurgical treatment and from 30 periodontally healthy control subjects (15 nonsmokers and 15 smokers). Clinical parameters were assessed before and after treatment. Total RNA was isolated, and mRNA expression of *MMPs* and *TIMP-1* was assessed by RT-PCR.

Results: Periodontal treatment significantly increased *TIMP-1* expression and decreased the ratios of *MMPs*/*TIMP-1*. Post-treatment, nonsmokers with periodontitis had significantly higher *MMP-8* and *TIMP-1* expression than healthy nonsmokers, and smokers with periodontitis had significantly higher *MMP-13* and *TIMP-1* expressions than healthy smokers. Post-treatment, smokers had significantly higher *TIMP-1* expression and lower *MMP-8*/*TIMP-1* ratio than nonsmokers. Post-treatment, there was no correlation among *MMPs*, and the expression of *MMPs* and *TIMP-1* was not correlated with clinical measurements.

Conclusion: Periodontal treatment increased *TIMP-1* expression and decreased the ratios of *MMPs*/*TIMP-1* in chronic periodontitis. The post-treatment increase in *TIMP-1* expression was higher for smokers. The *TIMP-1* expression was higher post-treatment than in health. Post-treatment, *MMP-8* expression was higher in nonsmokers with periodontitis than in healthy nonsmokers, whereas *MMP-13* expression was higher in smokers with periodontitis than in healthy smokers.

Efterpi Mouzakiti, DDS, MS, Department of Periodontology, School of Dentistry, University of Athens, 2 Thivon Street, Athens 115 21, Greece
Tel: +30 210 7461203
Fax: +30 210 7461202
e-mail: bettymouzakiti@hotmail.com

Key words: chronic periodontitis; matrix metalloproteinase; reverse transcription–polymerase chain reaction; smoking; tissue inhibitor of matrix metalloproteinase-1

Accepted for publication December 19, 2011

Periodontitis develops as a result of an imbalance between dental plaque micro-organisms that act on periodontal tissues and host defense mechanisms (1,2). Nonsurgical periodontal treatment aims to control the periodontal inflammation by reducing the periopathogenic micro-organisms and their products and by giving the host the chance to heal. It results in a reduction in bleeding on probing and probing depth and a gain in clinical attachment (3,4). The improvement in clinical parameters following nonsurgical treatment has been sufficiently documented (5–7), but the possible association between clinical improvement achieved with treatment and changes in the expression of inflammatory and immunological genes has not yet been thoroughly analysed. Studies attempting to analyse the expression of inflammation-related genes following nonsurgical treatment have been limited to selected genes, such as interleukin (*IL*)-*1*β, interferon-γ, *IL-2*, *IL-4*, *IL-5*, *IL-6* and tumor necrosis factor-α (8,9). A more comprehensive analysis of gene expression related to immune and inflammatory processes is required to increase our understanding of the way in which specific genes are involved in tissue homeostasis following treatment.

Matrix metalloproteinases (MMPs) belong to a group of highly conserved endopeptidases that participate in many natural processes, such as tooth eruption, wound healing, angiogenesis and bone formation, as well as in inflammatory processes (10–12). Endogenous tissue inhibitors, especially tissue inhibitors of metalloproteinases (TIMPs), are important controlling factors in the actions of MMPs (13). A disturbed balance of MMPs and TIMPs is found in various pathological conditions (14), including periodontitis (15–17). Tissue destruction often correlates with an inbalance between MMPs and TIMPs (13,14,18). Tissue inhibitor of metalloproteinases-1 (TIMP-1), a 30 kDa glycoprotein, is the main inhibitor of MMPs and is synthesized by most cells. TIMP-1 precisely regulates MMP activity and function and inhibits MMP-1, -2, -3, -8, -9 and -13 (19). It forms high-affinity complexes with the active forms of MMPs and might be specially bound to proMMP-9 (13,18). In addition to MMP-inhibing activities, TIMP-1 has other biological functions that are distinct from MMP inhibition. It has erythroid-potentiating activity and antiapoptotic activity (18).

Matrix metalloproteinases have been detected in periodontally diseased gingival tissues from chronic (15,16,20–33) and aggressive periodontitis (16,32,34). Gingival tissues from chronic periodontitis patients subjected to nonsurgical treatment and from periodontally healthy subjects have been compared in terms of mRNA expression of *MMPs* and *TIMP-1* (16,17,20,21,26,31–33). Differences have been reported between periodontitis and health (16,17,21,25,26), but mRNA expression of *MMPs* and *TIMP-1* in gingival tissues has not yet been compared before and after periodontal treatment in the same subject population.

Of all MMPs, MMP-1, -3, -8, -9 and -13 were selected to be assessed in the present study because of their properties. The collagenases MMP-1, -8 and -13 play a key role in the destruction of the inflamed gingival connective tissue; specifically, they cleave interstitional collagens I, II and III at a specific site. Moreover, collagenases degrade a number of other extracellular matrix and nonextracellular matrix molecules (18). MMP-13 is involved in the initiation of bone resorption by degrading organic bone matrix and generating collagen fragments that could activate osteoclasts (35). MMP-13 induces proMMP-9 activation (36) and MMP-13 auto-activation (36,37). MMP-9, which is a gelatinase, degrades the denaturated collagen after being cleaved by collagenase and activates proMMP-1 (18). MMP-3, which is a stromelycin, degrades extracellular matrix components and activates various proMMPs, such as proMMP-1 (18). It seems that there are complex relationships among the MMPs.

When focusing on the progress of periodontitis, the changes in MMPs and TIMPs and the relationships between MMPs and TIMPs are important. Changes in the relationships between MMPs and their inhibitors might lead to lack of balance between them. This imbalance might enhance the progression of the inflammation or the healing of the tissues, accordingly. Exploring the relationship between each MMP studied and TIMP-1 seems important, because TIMP-1 inhibits all the MMPs studied. The MMP/TIMP-1 ratio reflects the relationship between a specific MMP and TIMP-1. Studying the possible relationship among the MMPs might reveal their role in periodontitis.

An impaired outcome of periodontal treatment has been documented for smokers (38–41). Upregulation of the inflammation of the periodontal tissues has been implicated in the impaired response to periodontal treatment in smokers (42). Smoker periodontitis patients have demonstrated a decrease in certain proinflammatory cytokines and chemokines and certain regulators of T cells in comparison with nonsmoker periodontitis patients (43). Decreased levels of regulators of tissue breakdown have been found in smokers compared with nonsmokers (44). Chemically modifying inflammation and tissue breakdown by using a subantimicrobial dose of doxycycline as an adjunct to nonsurgical periodontal treatment has been reported to be clinically beneficial in nonsmokers (45) but not in smokers (42). The deleterious effect of smoking on the immune system justifies studying the expression of MMPs and TIMP-1 in relation to periodontal treatment in smoker and nonsmoker periodontitis patients separately.

The main hypothesis of the present study was that the mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* is different post-treatment compared with pretreatment. The secondary hypothesis of the study was that the mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* differs between periodontitis patients subjected to periodontal treatment and periodontally healthy control subjects. Another secondary hypothesis was that the changes of the mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* obtained with periodontal treatment are different between smokers and nonsmokers. The first aim of the present interventional study was to compare the mRNA expression of

MMP-1, *-3*, *-8*, *-9* and *-13* and *TIMP-1* in gingival tissues from chronic periodontitis patients before and after nonsurgical periodontal treatment. The second aim of this study was to compare mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* between chronic periodontitis patients after nonsurgical treatment and periodontally healthy control subjects. Moreover, the possible role of smoking in the changes in mRNA expression of *MMPs* and *TIMP-1* obtained with nonsurgical treatment was assessed.

Material and methods

Subject characteristics

The subject population was recruited from the patient pool of the Department of Periodontology, School of Dentistry, University of Athens in the period from January 2007 to June 2007. Among them, 30 experimental subjects presented generalized chronic severe periodontitis (CPS group, 15 smokers and CPN group, 15 nonsmokers) and 30 control subjects presented healthy and intact periodontium (HS group, 15 smokers and HN group, 15 nonsmokers). Subject inclusion and exclusion criteria and characteristics by study group (CPS, CPN, HS and HN) have been analysed in an earlier study by Mouzakiti *et al*. (46) involving the same subjects. Briefly, each periodontitis patient was systemically healthy and had a hopeless posterior tooth with probing depth ≥ 5 mm, clinical attachment loss ≥ 5 mm and bleeding on probing without suppuration. In addition, the hopeless tooth had inadequate dental tissues to maintain the tooth and restore it and/or was not important for the treatment plan. Moreover, each periodontitis patient had at least 20 teeth and at least seven teeth with probing depth ≥ 5 mm. For each periodontally healthy subject there was indication either for crown lengthening at a tooth site with no bleeding on probing, probing depth and clinical attachment loss or for extraction of a third molar with no bleeding on probing, probing depth and clinical attachment loss. Third molars were extracted due to unfavorable tooth position and/or absence of opposing teeth. All smokers were heavy smokers (daily cigarette consumption ≥ 20 cigarettes for at least 10 years). Each subject had signed an informed consent form prior to inclusion in the study. The study was approved by the Ethics and Research Committee of the School of Dentistry (approval no. 68, 22/1), University of Athens and was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2000.

Clinical procedures

Clinical examination and periodontal treatment — All participants were subjected to clinical examination, including full-mouth plaque score (FMPS; 47), simplified gingival index (GI-S; 48), bleeding on probing, clinical attachment loss and probing depth (initial or pretreatment measurements). Each periodontitis patient was then subjected to nonsurgical treatment following the protocol of full-mouth disinfection (49–51) without using antibacterial products. Six weeks after, the same clinical parameters again were assessed, namely FMPS, GI-S, bleeding on probing, clinical attachment loss and probing depth (final or post-treatment measurements), and periodontal surgery was planned for sites with residual probing depth ≥ 5 mm and bleeding on probing. Additionally, the following parameters were assessed for each periodontitis patient: pretreatment and post-treatment mean probing depth (mPD) and clinical attachment loss (mCAL), 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 (gingival) bleeding on probing, gingival tissue sampling (gingival) probing depth and gingival tissue sampling (gingival) clinical attachment loss. All clinical measurements and periodontal treatment were performed by the same operator (E.M.).

Gingival tissue sampling — For each periodontitis patient, two gingival tissue samples were harvested. The first sample was harvested during extraction of the periodontally hopeless tooth before the periodontal treatment, while the second sample was harvested from another area during pocket elimination surgery. Caution was taken to select for each periodontitis patient sampling sites with similar pretreatment clinical measurements in terms of probing depth, clinical attachment loss and bleeding on probing. For each healthy control subject, one tissue sample was harvested during surgical crown lengthening or third molar extraction. The gingival tissue samples were harvested by using external bevel gingivectomy incision and a fresh no. 15c blade. The weight of each gingival tissue sample ranged from 20 to 60 mg. Upon tissue harvesting, the oral epithelium of the tissue sample was carefully excised from the underlying connective tissue, using a fresh no. 15c blade. Care was taken to remove the oral epithelium completely or almost completely. The sample consisted mainly of connective tissue and pocket epithelium, although tissue samples might vary slightly in contents of different tissue types (connective tissue and epithelium).

Isolation of total RNA and RT-PCR

Following the collection of each gingival tissue sample, the 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 use. 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 with SuperScript II Reverse Transcriptase (Invitrogen) primed with oligo-dT in a final concentration of 20 pmol/μL. A ribonuclease inhibitor (RNaseOUT; Invitrogen, Darmstadt, Germany) was also included in the reaction at a final concentration of 1 U/μL. All RT reactions were performed independently of the PCRs. An evaluation of RNA quality was performed for all samples. The quantity and purity of the isolated RNA was assessed using the NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Loughborough,

UK). Absorbance at 260 and 280 nm was measured, and in all cases the ratio was near or equal to two, thus indicating the purity of the samples. Moreover, another quality control was performed in order to evaluate the integrity of the RNA samples. Total RNA was resolved on a denaturating agarose gel containing ethidium bromide and visualized under ultraviolet light. All samples exhibited two discrete, thick 28S and 18S ribosomal RNA gel bands with no smear, thus indicating the integrity of the samples. Gene-specific oligonucleotide primers were designed for *MMP-1* (52–55), *-3* (52), *-8*, *-9* (the primers for *MMP-8* and *MMP-9* were designed by Dr Gerassimos Voutsinas, Institute of Biology, National Center of Scientific Research 'Demokritos', Athens) and *-13* (53), *TIMP-1* (54) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Invitrogen; 55). One microliter of complementary DNA 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 *GAPDH*. All products were resolved on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

Densitometry

Densitometry was used to quantify the band intensity on PCR photographs, using the ImageJ program (National Institutes of Health). Densitometry was used in order to ascribe a specific numerical unit to each PCR band so as to compare all bands in a computerized manner with no human interference. In order to calculate the intensity of each band, we used a specific outlined rectanglular for every PCR product, transferred to each lane. Lanes with negative PCR products or bands too weak to resolve on an ethidium bromide stained agarose gel, displayed only background density (BD) from the gel. On the contrary, positive PCR products displayed the sum (SD) of the background density plus the actual density of the product band (ADPB). For negative samples, the equation is SD = BD + ADPB,where ADPB = 0, and for positive samples the equation is SD = BD + ADPB, where ADPB > 0. 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 expressed as a ratio of relative band intensity. All experiments were repeated twice to ensure statistical accuracy.

Statistical analysis

Mean values and standard deviations or medians [Q_1–Q_3 (Q1 stands for the 1st quartile, Q3 stands for the 3rd quartile)] were calculated for normally and non-normally distributed continuous variables, respectively. Differences and treatment changes in clinical measurements among the study groups were compared using Student's unpaired *t*-test or Mann-Whitney *U*-test for normally and non-normally distributed continuous variables, respectively. The mRNA expression differences within groups were assessed using Student's paired *t*-test or Wilcoxon signed-rank test for paired data for normally and non-normally distributed continuous variables. Chi-squared or McNemar test was applied to test for differences in categorical variables.

Correlations of post-treatment *MMPs* and *TIMP-1* expression with clinical measurements were studied using Spearman correlation coefficient. Multivariate logistic regression analysis was used to assess associations of the expression of *MMPs* and *TIMP-1* with potential confounders, such as age. A commercially available statistical software program (STATA 9.0; Stata, College Station, TX, USA) was used for statistical analysis. Statistical significance was set at 5% ($p = 0.05$).

Results

Sex was equally distributed among the four subject groups. The number of men was: six, five, seven and six men for CPS, CPN, HS and HN groups, respectively. The mean age of the subjects was 49.3 ± 8.35, 51.7 ± 6.15, 33.75 ± 6.07 and 36.8 ± 9.15 years for CPS, CPN, HS and HN groups, respectively. The mean age did not statistically significantly differ between CPS and CPN groups or between HS and HN groups. The CPS and HS groups were similar in smoking habits.

Clinical measurements

The CPS and CPN groups did not statistically significantly differ in pretreatment clinical measurements. Periodontal treatment led to statistically significant reduction in all clinical measurements (FMPS, GI-S, bleeding on probing, mPD, mCAL, percentage of sites with probing depth 4–6 mm, percentage of sites with probing depth ≥ 7 mm) in both experimental groups (Table 1). Changes in all clinical measurements achieved with periodontal treatment were not statistically significantly different between the two groups.

For the CPS group, the first and second sampling sites did not statistically significantly differ in initial (pretreatment) probing depth (mean values 6.6 ± 1.7 and 5.9 ± 1.2 mm, respectively, $p = 0.22$), clinical attachment loss (mean values 7.1 ± 2.4 and 6.5 ± 1.5 mm, respectively, $p = 0.11$) and bleeding on probing (100 and 100%, respectively). For the CPN group, the first and second sampling sites did not statistically significantly differ in initial clinical attachment loss (mean values 7.9 ± 2.9 and 7.4 ± 2.4 mm, respectively, $p = 0.39$) and bleeding on probing (100 and 100%, respectively), but they did differ in initial probing depth (mean values 7.7 ± 2.9 and 7.1 ± 2.1 mm, respectively, $p = 0.02$; data not shown).

The CPN and CPS groups were not statistically significantly different in initial clinical measurements at the second sampling site (Table 1), but there were nonstatistically significant indications that the initial probing depth at the second sampling site was greater for CPN (7.1 ± 2.1 mm) than the CPS group (5.9 ± 1.2 mm, $p = 0.06$; Table 1). Initial and final (post-treatment) clinical measurements at the second sampling site were compared for each experimental group. For both CPN and CPS groups, the

Table 1. Comparison of initial and final clinical measurements by periodontitis group

Parameter	CPN			CPS		
	Initial	Final	*p*-Value*[a]	Initial	Final	*p*-Value*[b]
FMPS (%)	77.3 ± 16.1	16.3 ± 7.2	< 0.001	71.7 ± 21.0	14.7 ± 7.1	< 0.001
GI-S	76 (62–100)†	14 (9–21)†	0.001†	65 (52–81)†	8 (4–20)†	0.001†
Bleeding on probing	69.3 ± 15.9	23.9 ± 13.8	< 0.001	68.9 ± 19.0	17.1 ± 9.4	< 0.001
mPD (mm)	3.7 ± 0.8	3.0 ± 0.7	< 0.001	3.6 ± 0.7	2.9 ± 0.6	< 0.001
mCAL (mm)	3.5 (3.3–4.7)†	3.0 (2.7–3.9)†	0.001†	4 (3.1–4.3)†	3.2 (2.2–3.9)†	0.001†
% sites PD 4–6 mm	36.1 ± 13.9	21.4 ± 12.3	< 0.001	41.3 ± 16.1	20.0 ± 12.6	< 0.001
% sites PD ≥ 7 mm	5 (1.6–9)†	0 (0–1.6)†	0.002†	3 (0.5–4.5)†	0 (0–1.2)†	0.002†
Mean gingival probing depth (mm)	7.1 ± 2.1	5.6 ± 1.7	< 0.001	5.9 ± 1.2	4.5 ± 1.2	< 0.001
Mean gingival clinical attachment loss (mm)	7.4 ± 2.4	6.7 ± 2.4	0.05	6.5 ± 1.5	5.4 ± 1.8	< 0.001
Gingival bleeding on probing	15/15 (100%)‡	12/15 (80%)‡	0.25‡	15/15 (100%)‡	8/15 (53.33%)‡	0.01‡

Values are means ± SD. Abbreviations: FMPS, full mouth plaque score, GI-S, simplified gingival index; % sites PD 4–6 mm, % of sites with probing depth between 4 and 6 mm; and % sites PD ≥ 7 mm, % of sites with probing depth equal to or deeper than 7 mm.
[a] Final vs. initial measurements for CPN, nonsmoker periodontitis subjects group.
[b] Final vs. initial measurements for CPS, smoker periodontitis subjects group.
**p*-Values were obtained by using Student's paired *t*-test.
†Values are medians (Q_1–Q_3) and *p*-values were obtained by using the Wilcoxon signed-rank test.
‡Values are percentages and *p*-values were obtained by using McNemar's test.

mean final probing depth (5.6 ± 1.7 and 5.4 ± 1.8 mm, respectively) and clinical attachment loss (6.7 ± 2.4 and 5.4 ± 1.8 mm, respectively) at the second sampling site were statistically significantly lower compared with the initial measurements (Table 1). Bleeding on probing at the second sampling site was reduced for both groups, although this was statistically significant only for the CPS group. Final bleeding on probing at the second sampling site was not different between the CPN and CPS groups ($p = 0.12$). The changes in clinical measurements obtained with treatment at the second sampling sites were similar for both CPN (mean change in probing depth 1.5 ± 1.0 mm and for clinical attachment loss 0.7 ± 1.3 mm) and CPS groups (mean change in probing depth 1.4 ± 0.9 mm and mean change in clinical attachment loss 1.1 ± 0.8 mm; $p = 0.85$, 0.33 and 0.12 for probing depth, clinical attachment loss and bleeding on probing, respectively).

mRNA expression of *MMPs* and *TIMP-1*

The number of gingival tissue samples with *MMPs* and *TIMP-1* expression by study group are shown in Table 2.

The mRNA expression of *MMP-1*, *-3*, *-8*, *-9* and *-13* and *TIMP-1* and *MMP*/*TIMP-1* ratios were compared for each experimental group (CPN and CPS) pre- and post-treatment, between CPN and CPS post-treatment (Table 3), between CPN post-treatment and HN and between CPS post-treatment and HS (Table 4). For the CPN group, pre- and post-treatment values were statistically significantly different in *TIMP-1* expression and *MMP-1*/*TIMP-1* ratio, with higher *TIMP-1* expression ($p = 0.009$; Table 3) and lower *MMP-1*/*TIMP-1* ratio ($p = 0.01$; Table 5) post-treatment. For the CPS group, pre- and post-treatment values were statistically significantly different in *TIMP-1* expression and *MMP-8*/*TIMP-1* and *MMP-9*/*TIMP-1* ratios, with higher *TIMP-1* expression ($p = 0.009$; Table 3) and lower *MMP-8*/*TIMP-1* and *MMP-9*/*TIMP-1* ratios ($p = 0.07$ and 0.001, respectively; Table 5) post-treatment.

Table 2. Number of gingival tissue samples with *MMPs* and *TIMP-1* expression by study group

Gene	CPN_1	CPS_1	CPN_2	CPS_2	HN	HS
MMP-1	15/15	7/15	10/15	8/15	10/15	6/15
MMP-3	12/15	12/15	10/15	5/15	13/15	10/15
MMP-8	11/15	14/15	11/15	7/15	5/15	10/15
MMP-9	9/15	12/15	7/15	11/15	9/15	7/15
MMP-13	0/15	4/15	9/15	6/15	11/15	1/15
TIMP-1	13/15	15/15	14/15	14/15	10/15	9/15

Abbreviations: CPN_1 and CPS_1, initial samples from CPN and CPS groups, respectively; CPN_2 and CPS_2, final samples from CPN and CPS groups, respectively, HN, nonsmoker periodontally healthy subjects, HS, smoker periodontally healthy subjects.

The CPN group post-treatment and the HN group differed in *MMP-8* and *TIMP-1* expression, with higher values for the CPN group ($p = 0.04$ and 0.03, respectively; Table 4). The CPS group post-treatment and the HS group differed in *MMP-13* and *TIMP-1* expression, with higher values for the CPS group ($p = 0.02$ and 0.0004, respectively; Table 4), as well as in *MMP-3*/*TIMP-1* and *MMP-8*/*TIMP-1* ratios, which were lower ($p = 0.02$ and 0.03, respectively; Table 6) for the CPS group. The CPN and CPS groups post-treatment differed only in *TIMP-1* expression and *MMP-8*/*TIMP-1* ratio, with higher *TIMP-1* expression ($p = 0.0009$; Table 3) and lower *MMP-8*/*TIMP-1* ratio ($p = 0.03$; Table 5) in the CPS group.

Changes in *MMPs* and *TIMP-1* expression and in *MMPs*/*TIMP-1*

Table 3. Pre- and post-treatment *MMPs* and *TIMP-1* expression: analysis by periodontitis group and comparison among the periodontitis groups

	CPN_1	CPN_2		CPS_1	CPS_2		
Gene	Median (Q_1–Q_3)		p-Value*[a]	Median (Q_1–Q_3)		p-Value*[b]	p-Value†[c]
MMP-1	0.55 (0.34–0.81)	0.31 (0–0.54)	0.11	0 (0–0.37)	0.25 (0–0.51)	0.53	0.45
MMP-3	0.35 (0.16–0.86)	0.47 (0–0.76)	0.88	0.23 (0.04–0.51)	0 (0–0.76)	0.55	0.19
MMP-8	0.78 (0–1.08)	0.70 (0–1.13)	0.81	0.6 (0.55–0.77)	0 (0–0.81)	0.20	0.14
MMP-9	0.21 (0–0.46)	0 (0–0.96)	0.27	0.51 (0.40–0.64)	0.54 (0–0.64)	0.31	0.48
MMP-13	0‡	0.73 (0–1.16)	—	0 (0–0.65)	0 (0–0.78)	0.31	0.32
TIMP-1	0.41 (0.28–0.56)	0.75 (0.5–0.83)	0.009	0.56 (0.50–0.59)	0.94 (0.82–1.08)	0.009	0.009

Abbreviations: CPN_1 and CPS_1, pretreatment (initial) *MMP* or *TIMP-1* expression for CPN and CPS groups, respectively; CPN_2 and CPS_2, post-treatment (final) *MMP* or *TIMP-1* expression for CPN and CPS groups, respectively.
[a] CPN_2 vs. CPN_1, [b] CPS_2 vs. CPS_1 and [c] CPN_2 vs. CPS_2.
*p-Values were obtained by using the Wilcoxon signed rank test.
†p-Values were obtained by using the Mann–Whitney U-test.
‡Constant and equal to 0.

Table 4. *MMPs* and *TIMP-1* expression: comparison between the periodontitis groups post-treatment and the healthy control groups

	CPN_2	HN		CPS_2	HS	
Gene	Median (Q_1–Q_3)		p-Value*[a]	Median (Q_1–Q_3)		p-Value*[b]
MMP-1	0.31 (0–0.54)	0.34 (0–0.63)	0.90	0.25 (0–0.51)	0 (0–0.63)	0.86
MMP-3	0.47 (0–0.76)	0.58 (0.35–0.66)	0.60	0 (0–0.76)	0.70 (0–0.81)	0.13
MMP-8	0.70 (0–1.13)	0 (0–0.77)	0.04	0 (0–0.81)	0.55 (0–1.13)	0.29
MMP-9	0 (0–0.96)	0.36 (0–0.71)	0.74	0.54 (0–0.64)	0 (0–0.54)	0.21
MMP-13	0.73 (0–1.16)	0.54 (0–0.83)	0.95	0 (0–0.78)	0 (0–0)	0.02
TIMP-1	0.75 (0.5–0.83)	0.44 (0–0.65)	0.03	0.94 (0.82–1.08)	0.37 (0–0.60)	0.0004

Abbreviations: CPN_2 and CPS_2, post-treatment (final) *MMP* or *TIMP-1* expression for CPN and CPS groups, respectively.
[a] CPN_2 vs. HN and [b] CPS_2 vs. HS.
*p-Values were obtained by using the Mann–Whitney U-test.

Table 5. Pre- and post-treatment *MMPs*/*TIMP-1* ratios: analysis by periodontitis group and comparison among the periodontitis groups

	CPN_1	CPN_2		CPS_1	CPS_2		
Ratio	Median (Q_1–Q_3)		p-Value*[a]	Median (Q_1–Q_3)		p-Value*[b]	p-Value†[c]
MMP-1/*TIMP-1*	1.20 (1.04–1.41)	0.67 (0–0.92)	0.01	0 (0–0.69)	0.26 (0–0.62)	0.87	0.08
MMP-3/*TIMP-1*	0.80 (0.56–1.64)	0.63 (0–0.91)	0.51	0.42 (0.07–1.32)	0 (0–0.77)	0.21	0.06
MMP-8/*TIMP-1*	2.05 (0–2.36)	1.02 (0–1.24)	0.17	1.12 (0.84–1.47)	0 (0–0.85)	0.007	0.03
MMP-9/*TIMP-1*	0.63 (0–0.92)	0 (0–1.12)	0.91	0.98 (0.70–1.50)	0.56 (0–0.73)	0.01	0.53
MMP-13/*TIMP-1*	0‡	0.64 (0–1.89)	—	0 (0–1.12)	0 (0–92)	0.36	0.24

Abbreviations: CPN_1 and CPS_1: pretreatment (initial) *MMP*/*TIMP-1* ratio for CPN and CPS groups, respectively; CPN_2 and CPS_2, post-treatment (final) *MMP*/*TIMP-1* ratio for CPN and CPS groups, respectively.
[a] CPN_2 vs. CPN_1, [b] CPS_2 vs. CPS_1 and [c] CPN_2 vs. CPS_2.
*p-Values were obtained by using the Wilcoxon signed rank test.
†p-Values were obtained by using the Mann–Whitney U-test.
‡Constant and equal to 0.

ratios obtained with treatment were compared between the CPN and CPS groups. There were no statistically significant differences between them, except for the *MMP-1*/*TIMP-1* ratio change ($p = 0.02$), which was larger for the CPN group (data not shown).

There were no correlations among the *MMPs* expression for groups CPN and CPS post-treatment (data not shown).

The possible existence of an association between the post-treatment expression of *MMPs* and *TIMP-1* and the final clinical measurements (FMPS, GI-S, bleeding on probing, mPD and mCAL) as well as between changes in the expression of *MMPs* and *TIMP-1* and changes in clinical measurements obtained with treatment was studied (data not shown). No statistically significant associations were found.

Table 6. Pre- and post-treatment *MMPs/TIMP-1* ratios: analysis by periodontitis group and comparison among the periodontitis groups

	CPN_2	HN		CPS_2	HS	
Ratio	Median (Q_1–Q_3)		p-Value†[a]	Median (Q_1–Q_3)		p-Value†[b]
MMP-1/TIMP-1	0.67 (0–0.92)	0.64 (0–1.23)	1.00	0.26 (0–0.62)	0 (0–0.90)	0.69
MMP-3/TIMP-1	0.63 (0–0.91)	0.98 (0.44–1.97)	0.23	0 (0–0.77)	1.06 (0.06–1.29)	0.02
MMP-8/TIMP-1	1.02 (0–1.24)	0 (0–1.05)	0.09	0 (0–0.85)	1.32 (0–1.46)	0.03
MMP-9/TIMP-1	0 (0–1.12)	1.17 (0–1.97)	0.11	0.56 (0–0.73)	0.77 (0–1.42)	0.28
MMP-13/TIMP-1	0.64 (0–1.89)	1.50 (0.67–1.75)	0.22	0 (0–92)	0‡	—

Abbreviations: CPN_1 and CPS_1: pretreatment (initial) *MMP/TIMP-1* ratio for CPN and CPS groups, respectively; CPN_2 and CPS_2, post-treatment (final) *MMP/TIMP-1* ratio for CPN and CPS groups, respectively.
[a] CPN_2 vs. CPN_1, [b] CPS_2 vs. CPS_1 and [c] CPN_2 vs. CPS_2.
**p*-Values were obtained by using the Wilcoxon signed rank test.
†*p*-Values were obtained by using the Mann–Whitney *U*-test.
‡Constant and equal to 0.

Discussion

In the present study, all clinical parameters were significantly improved with nonsurgical periodontal treatment both for smokers and nonsmokers, with similar degrees of improvement. In most studies, smokers exhibit less favorable clinical response to nonsurgical periodontal treatment than nonsmokers (38–41), some of which are relatively long-term. In the present short-term study, the final evaluation was 6 wk after nonsurgical treatment. The findings of the present study on similar probing depth reduction and clinical attachment loss gain for smokers and nonsmokers at 6 wk post-treatment are in disagreement with the findings of Baumert Ah *et al*. (38) and Kadhal *et al*. (39) at 4 wk and with the findings of Apatzidou *et al*. (41) at 6 mo. The present similar reduction in bleeding on probing for smokers and nonsmokers is in accordance with the report by Kaldahl *et al*. (39). The present similar changes in FMPS, bleeding on probing and clinical attachment loss for smokers and nonsmokers at 6 wk post-treatment is in agreement with the results by Renvert *et al*. (40) at 6 mo post-treatment. The present similar reduction in probing depth with treatment for smokers and nonsmokers disagrees with the findings of Renvert *et al*. (40), who reported greater reduction for nonsmokers.

Having similar clinical measurements among the sampling sites was important in the present study. Smokers and nonsmokers had similar initial clinical measurements (probing depth, clinical attachment loss and bleeding on probing) at the second sampling site. Smokers and nonsmokers presented similar clinical measurement changes (probing depth, clinical attachment loss and bleeding on probing) with periodontal treatment at the second site. The two sampling sites (first and second) did not differ in initial probing depth, clinical attachment loss and bleeding on probing for smokers and in initial clinical attachment loss and bleeding on probing for nonsmokers. The mean initial probing depth was significantly deeper at the first (7.7 ± 2.9 mm) than at the second sampling site (7.1 ± 2.1 mm) for nonsmokers. The rationale for using two distinct tissue sampling sites for each periodontitis patient (one before and one after the periodontal treatment) was that a specific site can be used for tissue harvesting only once. Harvesting gingival tissues by using external gingivectomy incision is a minor surgical intervention, which would affect the clinical parameters, such as pocket depth. Therefore, if tissues were to be collected twice from the same site, the first tissue harvesting (before the periodontal treatment) might alter the findings from the second one.

Concerning the second sampling site, treatment significantly improved all clinical parameters in smokers, but it led to a significant probing depth reduction and clinical attachment loss gain and nonsignificant reduction in bleeding on probing in nonsmokers. Post-treatment, bleeding on probing remained in some sampling sites (12 for nonsmokers and eight for smokers), which could be attributed to the clinical characteristics. Sites with residual pockets and indication for pocket elimination surgery were selected as second sampling sites, which entails the risk of residual inflammation; however, the changes in probing depth, clinical attachment loss and bleeding on probing obtained with periodontal treatment at the second sampling site did not differ significantly between smokers and nonsmokers. The possibility of residual inflammation at the second sampling site after the nonsurgical periodontal treatment cannot be excluded. Nonsurgical treatment often results in reduction of the inflammation, not necessarily in elimination of the inflammation (3,4).

The pretreatment differences in *MMP-1* and *-3* expression between smokers and nonsmokers reported previously by Mouzakiti *et al*. (46) were eliminated post-treatment. *TIMP-1* expression was higher for smoker than nonsmoker periodontitis patients both before (46) and after periodontal treatment. This might partly be attributed to smoking, because both groups had similar initial and final clinical measurements and response to treatment. Concerning the *TIMP-1* expression, the nonsurgical treatment did not change the pretreatment relationship between smokers and nonsmokers.

Higher post-treatment *MMP-13* expression was found for smokers

with periodontitis than for healthy smokers. The higher pretreatment *MMP-8* expression reported previously (46) for nonsmoker periodontitis patients compared with healthy nonsmokers remained even after treatment. This might be explained by the possible residual inflammation at the second sampling sites and the association of *MMP-8* with inflammation. Even though all clinical parameters were improved following treatment, inflammation was not completely eliminated. MMP-8 is mainly expressed by neutrophils, which are highly recruited during inflammation (56). In smokers, the *MMP-8* expression was similar for periodontitis post-treatment and for periodontal health. Post-treatment, there were nonstatistically significant indications that the combination of periodontitis and smoking resulted in lower *MMP-8* expression than periodontitis alone. These findings may partly be explained by the following data. Smoking is related to upregulation of inflammation, endothelial cell dysfunction, impaired viability and function of the neutrophils (57), impaired macrophage function, lower gingival blood flow rate and gingival crevicular fluid volume (58) and lower gingival crevicular fluid IL-1α, IL-1rα (59,60) and IL-1β levels (61). MMP-8 is expressed by neutrophils, but by human endothelial cells as well (56). MMP-8 is upregulated by IL-1α,β and tumor necrosis factor-α (62,63). Peripheral neutrophil mRNA expression levels for *MMP-8* have been found to be lower for smokers than nonsmokers and increased 8 wk after smoking cessation (61).

Periodontal treatment reduced certain *MMP/TIMP-1* ratios, although this was different between smokers (*MMP-8/TIMP-1* and *MMP-9/TIMP-1*) and nonsmokers (*MMP-1/TIMP-1*). The mean changes in *MMP/TIMP-1* ratios obtained with treatment were similar for smokers and nonsmokers, except for *MMP-1/TIMP-1*, for which the changes were greater for nonsmokers. This might be explained by the higher *TIMP-1* expression in smokers than in nonsmokers. TIMP-1 has the widest range of actions among all metalloproteinase inhibitors (15) and binds to most MMPs, which makes the assessment of the *MMP/TIMP-1* ratios important.

TIMP-1 expression was increased post-treatment compared with pretreatment, for both smokers and nonsmokers, with both having a similar increase. The increased post-treatment *TIMP-1* expression might be attributed to tissue changes occurring during healing and to an attempt to overcome tissue destruction caused by MMPs. The increased *TIMP-1* expression might control the production of MMPs during healing and gradually help to restore the balance between MMPs and TIMP-1. It might be an attempt to suppress the production and activity of MMPs and restore the balance between MMPs and their inhibitors.

Post-treatment, *TIMP-1* expression was higher for chronic periodontitis than for periodontal health both for smokers and nonsmokers. Concerning *TIMP-1* expression, the present findings together with previous findings (46) on the same subject population showed that nonsurgical treatment did not change the pretreatment relationship between periodontitis and health, in both smokers and nonsmokers.

The present and previous findings (46) demonstrated that smokers had higher *TIMP-1* expression than nonsmokers both before and after treatment. The statistical significance of the difference was stronger after ($p = 0.009$) than before treatment ($p = 0.01$).

The present findings on the absence of a difference in *MMP-1* expression between periodontitis post-treatment and periodontal health are in accordance with findings by Dahan *et al.* (26) and Gonçalves *et al.* (31). Gonçalves *et al.* (31) studied only nonsmokers and found nonstatistically significant indications that *MMP-1* expression was higher in periodontitis. The present findings contradict results by Beikler *et al.* (33) and Kubota *et al.* (17) on higher post-treatment *MMP-1* expression in periodontitis than health. The studies by Beikler *et al.* (33) and Kubota *et al.* (17) evaluated periodontitis patients after the periodontal treatment and subjects with healthy periodontium, but the two studies are not really comparable to each other. Both Kubota *et al.* (17) and Beikler *et al.* (33) showed higher post-treatment *MMP-1* expression in periodontitis; however, these studies have differences in the study design that make comparisons difficult, such as differences in the procedure of the nonsurgical periodontal treatment, the timing of the gingival sampling, the mean probing depth at the gingival sampling sites and the method used to assess MMPs. A study by Aiba *et al.* (25) showed no difference in *MMP-1* expression between periodontitis patients and control subjects. In the study by Aiba *et al.* (25), it seems that the control group included periodontally affected subjects with residual pockets; therefore, the compared groups were periodontal patients before (experimental) and after nonsurgical treatment (control). The present findings, where the same subjects were compared before and after treatment, are in accordance with the results of Aiba *et al.* (25) on the absence of difference in *MMP-1* expression with periodontal treatment.

The absence of difference in post-treatment *MMP-3* expression between periodontitis and health found in the present study agrees with results by Tonetti *et al.* (20) and Kubota *et al.* (16). In an earlier study by Kubota *et al.* (17), where RT-PCR was used, there was higher *MMP-3* expression for periodontitis patients, but in a subsequent study by the same authors (16), where real-time RT-PCR was used, there were only nonstatistically significant indications of higher *MMP-3* expression in periodontitis.

Kubota *et al.* (17) reported higher post-treatment *MMP-8* expression in periodontitis than in health, though without reporting on smoking. Comparisons with their results are not feasible, because in the present study there was higher *MMP-8* expression only for nonsmokers. A later study by Kubota *et al.* (16) failed to assess *MMP-8* because of very low expression.

Similar post-treatment *MMP-9* expression between periodontitis and health was demonstrated by Kubota

et al. (16), without reporting on smoking, and by Gonçalves *et al.* (31) for nonsmokers. The present results are in accordance with these findings.

Concerning the post-treatment *MMP-9* expression, Kubota *et al.* (16) and Gonçalves *et al.* (31) reported no difference between periodontitis and health, although there were nonsignificant indications of higher expression in periodontitis. The present study showed similar expression for nonsmokers, which is in agreement with Gonçalves *et al.* (31). Comparisons with the results of Kubota *et al.* (16) are not feasible, because the absence of difference was restricted to nonsmokers in the present study.

The *MMP/TIMP-1* ratios have been studied by Kubota *et al.* (16), with similar findings for periodontitis and health. In the present study, there were differences only for smokers, which do not permit comparisons between the two studies.

Smoking modulates the destruction of the periodontium through various pathways, such as the microcirculatory system, the host immune inflammatory response systems, the connective tissue and the bone metabolism (57). Smoking might be implicated in overproduction of cytokines, with a possible mechanism via MMP-9 (64). MMP-9 has previously been described to activate proMMP-13 (36,65). MMP-13, in turn, might be implicated in the proMMP-9 activation (37). It has been suggested that MMP-13 and MMP-9 can potentially form an activation cascade overcoming the protective TIMP-1 shield (37). The higher *MMP-13* expression found for smoker periodontitis patients post-treatment than for healthy smokers in the present study might be linked to the relationship between MMP-13 and MMP-9. Furthermore, smoking may suppress the activities of protease inhibitors. α_2-Macroglobulin is an inhibitor for several proteases, such as MMPs. It has been reported that smokers have a significantly lower concentration of α_2-macroglobulin in gingival crevicular fluid and total amounts of α_2-macroglobulin and α_1-antitrypsin than nonsmokers (66). Nicotine has been found to downregulate IL-1β secretion by gingival mononuclear cells (67). TIMP-1 is differentially regulated by tumor necrosis factor-α, IL-1β and granulocyte–macrophage colony stimulating factor through prostaglandin-dependent and -independent mechanisms (68).

In the present study, RT-PCR was used for the assessment of the mRNA expression. The selection of real-time RT-PCR rather than RT-PCR would be preferable, though comparable results have been reported for both methods (69). Real-time PCR technology has revolutionized the potentials in detecting RNA and DNA, because efficiency and sensitivity are improved compared with conventional PCR. However, RT-PCR is still considered to be highly reliable in evaluating mRNA expression in a semi-quantitative approach, with great repeatability and sensitivity, and it is still used widely in experimental and diagnostic techniques (70). Different results when studying same molecules with real-time RT-PCR and RT-PCR may be attributed to the greater sensitivity of the former technique or to the experimental design, thus making it difficult to establish a reliable conclusion on the diversity of the results.

The administration of a subantimicrobial dose of doxycycline as an adjunct to nonsurgical periodontal treatment (41,45,71) enhances the clinical response to treatment. Gingival crevicular fluid MMP-8 has been further reduced with adjunctive use of a subantimicrobial dose of doxycycline to nonsurgical periodontal treatment (45,71–73). Modulation of the host response in addition to the elimination of the microbial load of the periodontal pockets might enhance the effectiveness of the periodontal treatment. Novel strategies aiming to increase the TIMP-1 expression further might enhance the outcome of periodontal treatment. Increasing the endogenous TIMP-1 expression or administrating engineered TIMP-1 might prove to be a beneficial adjunct to periodontal treatment. However, the overexpression of TIMP-1 could have drawbacks, because multiple MMPs might be inhibited. Of course, the increase in TIMP-1 expression may not be sufficient to compensate for the upregulation of all MMPs in periodontitis. Creating engineered TIMPs, which would have altered specificity, might help in targeting specific metalloproteinases.

Conclusions

Within its limits, this study demonstrated that periodontal treatment increased *TIMP-1* expression and decreased *MMPs/TIMP-1* ratios in nonsmoker and smoker chronic periodontitis patients. The post-treatment increase in *TIMP-1* expression was higher for smokers than for nonsmokers. *TIMP-1* expression was higher post-treatment than in health. Post-treatment, *MMP-8* expression was higher in nonsmoker periodontitis patients than in nonsmoker healthy subjects, whereas *MMP-13* expression was higher in smoker periodontitis patients than in smoker healthy subjects.

Acknowledgements

The present study was partially supported by a grant from the University of Athens, Greece. The authors thank Dr Gerassimos Voutsinas, Biologist, Institute of Biology, National Center for Scientific Research 'Demokritos', Athens, Greece for his scientific assistance in the RT-PCR procedure.

References

1. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000* 1994;**5:**78–111.
2. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
3. Cugini MA, Haffajee AD, Smith C, Kent RL Jr, Socransky SS. The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. *J Clin Periodontol* 2000;**27:**30–36.
4. Drisko CH. Nonsurgical periodontal therapy. *Periodontology 2000* 2001;**25:**77–88.
5. Badersten A, Nilvéus R, Egelberg J. Effect of nonsurgical periodontal therapy. VII. Bleeding, suppuration and probing depth in sites with probing attachment loss. *J Clin Periodontol* 1985;**12:**432–440.

6. Badersten A, Nilvéus R, Egelberg J. Effect of nonsurgical periodontal therapy (VIII). Probing attachment changes related to clinical characteristics. *J Clin Periodontol* 1987;**14:**425–432.
7. Greenstein G. Nonsurgical periodontal therapy in 2000: a literature review. *J Am Dent Assoc* 2000;**131:**1580–1592.
8. Renvert S, Lindahl C, Roos-Jansåker AM, Lessem J. Short-term effects of an anti-inflammatory treatment on clinical parameters and serum levels of C-reactive protein and proinflammatory cytokines in subjects with periodontitis. *J Periodontol* 2009;**80:**892–900.
9. Shimada Y, Komatsu Y, Ikezawa-Suzuki I, Tai H, Sugita N, Yoshie H. The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. *J Periodontol* 2010;**81**(8)**:**1118–1123.
10. Reynolds JJ, Hembry RM, Meikle MC. Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Adv Dent Res* 1994;**8:**312–319.
11. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 2003;**253:**269–285.
12. Sorsa T, Tjäderhane L, Konttinen YT *et al*. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 2006;**38:**306–321.
13. Reynolds JJ. Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. *Oral Dis* 1996;**2:**70–76.
14. Verstappen J, Von den Hoff JW. Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease. *J Dent Res* 2006;**85:**1074–1084.
15. Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *J Periodontol* 2003; **74:**188–195.
16. Kubota T, Itagaki M, Hoshino C *et al*. Altered gene expression levels of matrix metalloproteinases and their inhibitors in periodontitis-affected gingival tissue. *J Periodontol* 2008;**79:**166–173.
17. Kubota T, Nomura T, Takahashi T, Hara K. Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. *Arch Oral Biol* 1996;**41:**253–262.
18. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92:**827–839.
19. Goldberg GI, Strongin A, Collier IE, Genrich LT, Marmer BL. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J Biol Chem* 1992;**267:**4583–4891.
20. Tonetti MS, Freiburghaus K, Lang NP, Bickel M. Detection of interleukin-8 and matrix metalloproteinases transcripts in healthy and diseased gingival biopsies by RNA/PCR. *J Periodont Res* 1993;**28:**511–513.
21. Nomura T, Takahashi T, Hara K. Expression of TIMP-1, TIMP-2 and collagenase mRNA in periodontitis-affected human gingival tissue. *J Periodont Res* 1993;**28:**354–362.
22. Ingman T, Sorsa T, Michaelis J, Konttinen YT. Matrix metalloproteinases-1, -3, and -8 in adult periodontitis in situ. An immunohistochemical study. *Ann NY Acad Sci* 1994;**732:**459–461.
23. Ingman T, Sorsa T, Michaelis J, Konttinen YT. Immunohistochemical study of neutrophil- and fibroblast-type collagenases and stromelysin-1 in adult periodontitis. *Scand J Dent Res* 1994;**102:**342–349.
24. Meikle MC, Hembry RM, Holley J, Horton C, McFarlane CG, Reynolds JJ. Immunolocalization of matrix metalloproteinases and TIMP-1 (tissue inhibitor of metalloproteinases) in human gingival tissues from periodontitis patients. *J Periodont Res* 1994;**29:**118–126.
25. Aiba T, Akeno N, Kawane T, Okamoto H, Horiuchi N. Matrix metalloproteinases-1 and -8 and TIMP-1 mRNA levels in normal and diseased human gingivae. *Eur J Oral Sci* 1996;**104:**562–569.
26. Dahan M, Nawrocki B, Elkaïm R *et al*. Expression of matrix metalloproteinases in healthy and diseased human gingiva. *J Clin Periodontol* 2001;**28:**128–136.
27. Kiili M, Cox SW, Chen HW *et al*. Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *J Clin Periodontol* 2002;**29:**224–232.
28. Smith PC, Munõz VC, Collados L, Oyarzún AD. In situ detection of matrix metalloproteinase- 9 (MMP-9) in gingival epithelium in human periodontal disease. *J Periodont Res* 2004;**39:**87–92.
29. Hernandez M, Valenzuela MA, Lopez-Otin C *et al*. Matrix metalloproteinase-13 is highly expressed in destructive periodontal disease activity. *J Periodontol* 2006;**77:**1863–1870.
30. Bodineau A, Godeau G, Brousse N, Pellat B, Folliguet M, Séguier S. Langerhans cells express matrix metalloproteinases 9 and 2 and tissue inhibitors of metalloproteinases 1 and 2 in healthy human gingival tissue and in periodontitis. *Oral Microbiol Immunol* 2006;**21:**197–200.
31. Gonçalves LDR, Oliveira G, Hurtado PA *et al*. Expression of metalloproteinases and their tissue inhibitors in inflamed gingival biopsies. *J Periodont Res* 2008;**43:**570–577.
32. Kuula H, Salo T, Pirilä E *et al*. Human b-defensin-1 and -2 and matrix metalloproteinase-25 and -26 expression in chronic and aggressive periodontitis and in implantitis. *Arch Oral Biol* 2008;**53:**175–186.
33. Beikler T, Peters U, Prior K, Eisenacher M, Flemmig TF. Gene expression in periodontal tissues following treatment. *BMC Med Genomics* 2008;**1:**30–39.
34. Tervahartiala T, Pirilä E, Ceponis A *et al*. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000;**79:**1969–1977.
35. Holliday LS, Welgus HG, Fliszar CJ, Veith GM, Jeffrey JJ, Gluck SI. Initiation of osteoclast bone resorption by interstitial collagenase. *J Biol Chem* 1997;**272:**22053–22058.
36. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int J Dev Biol* 2004;**48:**411–424.
37. Hernández Rios M, Sorsa T, Obregón F *et al*. Proteolytic roles of matrix metalloproteinase (MMP)-13 during progression of chronic periodontitis: initial evidence for MMP-13/MMP-9 activation cascade. *J Clin Periodontol* 2009;**36:**1011–1017.
38. Baumert Ah MK, Johnson GK, Kaldahl WB, Patil KD, Kalkwarf KL. The effect of smoking on the responses to periodontal therapy. *J Clin Periodontol* 1994;**21:**91–97.
39. Kaldahl WD, Johnson GK, Patik KD *et al*. Levels of cigarette consumption and response to periodontal therapy. *J Periodontol* 1996;**67:**675–682.
40. Renvert S, Dahlén G, Wikström M. The clinical and microbiological effects of non-surgical periodontal therapy in smokers and non-smokers. *J Clin Periodontol* 1998;**25:**153–157.
41. Apatzidou DA, Riggio MP, Kinane DF. Impact of smoking on the clinical, microbiological and immunological parameters of adult patients with periodontitis. *J Clin Periodontol* 2005;**32:**973–983.
42. Needleman I, Suvan J, Gilthorpe MS *et al*. A randomised-controlled trial of low-dose doxycycline for periodontitis in smokers. *J Clin Periodontol* 2007;**34:**325–333.
43. Tymkiw K, Thuneli D, Johnson G *et al*. Influence of smoking on gingival crevicular fluid cytokines in severe chronic

periodontitis. *J Clin Periodontol* 2011;**38:** 219–228.
44. Kinane DF, Chestnut IG. Smoking and periodontal disease. *Crit Rev in Oral Biol* 2000;**11:**356–365.
45. Tüter G, Serdar M, Kurtiş B *et al*. Effects of scaling and root planing and subantimicrobial dose doxycycline on gingival crevicular fluid levels of matrix metalloproteinase-8, -13 and serum levels of HsCRP in patiens with chronic periodontitis. *J Periodontol* 2010;**81:**1132–1139.
46. Mouzakiti E, Pepelassi E, Fanourakis G, Markopoulou C, Tseleni-Balafouta S, Vrotsos I. The effect of smoking on the expression of MMPs and TIMP-1 in untreated chronic periodontitis patients: a cross-sectional study. *J Periodont Res* 2011;**46:**576–583.
47. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. *J Periodontol* 1972;**43:**38–42.
48. Lindhe J. *Textbook of Clinical Periodontology*. 1st edition. Copenhagen: Munskgaard, 1981: 327–352.
49. Quirynen M, Bollen CM, Vandekerckhove BN, Dekeyser C, Papaioannou W, Eyssen H. Full- vs. partial-mouth disinfection in the treatment of periodontal infections: short-term clinical and microbiological observations. *J Dent Res* 1995;**74:**1459–1467.
50. Koshy G, Corbet EF, Ishikawa I. A Full-mouth disinfection approach to nonsurgical periodontal therapy-prevention of reinfection from bacterial reservoirs. *Periodontology 2000* 2004;**36:**166–178.
51. Eberhard J, Jepsen S, Jervøe-Storm PM, Needleman I, Worthington HV. Full-mouth disinfection for the treatment of adult chronic periodontitis. *Cochrane Database Syst Rev* 2008;**1:**CD004622.
52. Gosiewska A, Yi CF, Brown LJ, Cullen B, Silcock D, Geesin JC. Differential expression and regulation of extracellular matrix-associated genes in fetal and neonatal fibroblasts. *Wound Repair Regen* 2001;**9:**213–222.
53. Sasaki K, Takagi M, Konttinen YT *et al*. Upregulation of matrix metalloproteinase (MMP)-1 and its activator MMP-3 of human osteoblast by uniaxial cyclic stimulation. *J Biomed Mater Res B Appl Biomater* 2007;**80:**491–498.
54. Palosaari H, Pennington CJ, Larmas M *et al*. Expression profile of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in mature human odontoblasts and pulp tissue. *Eur J Oral Sci* 2003;**111:**117–127.
55. Kano M, Shimada Y, Kaganoi J *et al*. Detection of lymph node metastasis of oesophageal cancer by RT-nested PCR for SCC antigen gene mRNA. *Br J Cancer* 2000;**82:**429–435.
56. Kuula H, Salo T, Pirilã E *et al*. Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis. *Infect Immun* 2009;**77:**850–859.
57. Ojima M, Hanioka T. Destructive effects of smoking on molecular and genetic factors or periodontal disease. *Tob Induc Dis* 2010;**8:**4–10.
58. Morozumi T, Kubota T, Sato T, Okuda K, Yoshie H. Smoking cessation increases gingival blood flow and gingival crevicular fluid. *J Clin Periodontol* 2004;**31:**267–272.
59. Rawlinson A, Grummitt JM, Walsh TF, Ian Douglas CW. Interleukin 1 and receptor antagonist levels in gingival crevicular fluid in heavy smokers versus non-smokers. *J Clin Periodontol* 2003;**30:** 42–48.
60. Petropoulos G, McKay IJ, Hughes FJ. The association between neutrophil numbers and interleukin-1alpha concentrations in gingival crevicular fluid of smokers and non-smokers with periodontal disease. *J Clin Periodontol* 2004;**31:**390–395.
61. Morozumi T, Kubota T, Sugita N, Itagaki M, Yoshie H. Alterations of gene expression in human neutrophils induced by smoking cessation. *J Clin Periodontol* 2004;**31:**1110–1116.
62. Koshy PJT, Lundy CJ, Rowan AD *et al*. The modulation of matrix metalloproteinas and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M. A time-course study using real-time quantitative reverse transcription-polymerase chain reaction. *Arthritis Rheum* 2002;**46:**961–967.
63. Hanemaaijer R, Sorsa T, Konttinen YT *et al*. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-α and doxycycline. *J Biol Chem* 1997;**272:**31504–31509.
64. Yu Q, Stamenkovic I. Cell surfacelocalized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000;**14:**163–176.
65. Overall CM. Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exocites. *Mol Biotechnol* 2002;**22:**51–86.
66. Persson L, Bergström J, Ito H, Gustafsson A. Tobacco smoking and neutrophil activity in patients with periodontal disease. *J Periodontol* 2001;**72:**90–95.
67. Bernzweig E, Payne JB, Reinhardt RA, Dyer JK, Patil KD. Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *J Clin Periodontol* 1998;**25:**246–252.
68. Zhang Y, McCluskey K, Fujii K, Wahl LM. Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-α, granulocyte-macrophage CSF, and IL-1β through prostaglandin-dependent and – independent mechanisms. *J Immunol* 1998;**161:**3071–3076.
69. Olesen LH, Nørgaard JM, Pallisgaard N, Bukh A, Hokland P. Validation and clinical implication of a quantitative real-time PCR determination of MDR1 gene expression: comparison with semi-quantitative PCR in 101 patients with acute myeloid leukemia. *Eur J Haematol* 2003;**70:**296–303.
70. Maan NS, Maan S, Nomikou K *et al*. RT-PCR assays for seven serotypes of epizootic haemorrhagic disease virus and their use to type strains from the Mediterranean region and North America. *PLoS ONE* 2010;**5**(9)**:**pii: e12782.
71. Lee HM, Ciancio SG, Tüter G, Ryan EM, Komaroff E, Golub LM. Sub antimicrobial dose doxycycline efficacy as a matrix metalloproteinase inhibitor in chronic periodontitis patients is enhanced when combined with a non-steroidal anti-inflammatory drug. *J Periodontol* 2004;**75:** 453–463.
72. Emingil G, Atilla G, Sorsa T, Luoto H, Kirilmaz L, Baylas H. The effect of adjunctive low-dose doxycycline therapy on clinical parameters and gingival crevicular fluid matrix metalloproteinase-8 levels in chronic periodontitis. *J Periodontol* 2004;**75:**106–115.
73. Choi DH, Moon IS, Choi BK *et al*. Effects of sub-antimicrobial dose doxycycline therapy on crevicular fluid MMP-8 and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis.. *J Periodont Res* 2004;**39:**20–26.