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ORIGINAL ARTICLE

Smoking and matrix metalloproteinases, neutrophil elastase and myeloperoxidase in chronic periodontitis

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OBJECTIVES: To investigate possible relationship between smoking and serum concentrations of matrix metalloproteinase-8,-9 (MMP-8, MMP-9), tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), neutrophil elastase (NE), myeloperoxidase (MPO) in chronic periodontitis (CP) patients relative to periodontally healthy subjects.

METHODS: Serum samples were obtained from 111 subjects before initiation of any periodontal intervention. Fifty-five CP patients (39 non-smokers, 16 smokers) and 56 periodontally healthy subjects (39 non-smokers, 17 smokers) were recruited. Serum concentrations of MMP-8 were determined by IFMA and MPO, MMP-9, TIMP-1, NE concentrations by ELISA. ANCOVA and Pearson correlation analysis was utilized for statistical analysis.

RESULTS: Serum MPO, NE concentrations were higher in smoker CP than non-smoker CP patients ($P = 0.002$ and $P < 0.001$, respectively), whereas these were similar in smoker, non-smoker periodontally healthy groups ($P > 0.05$). TIMP-1 concentration was higher in non-smoker CP than smoker CP group ($P < 0.05$). MMP-9/TIMP-1 ratios were higher in smoker CP than non-smoker CP group ($P = 0.01$). MMP-8 concentrations, MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios in CP group were not significantly different from those in periodontally healthy group ($P > 0.05$).

CONCLUSIONS: Our findings of significantly elevated serum MMP-9, MPO, NE together with decreased TIMP-1 in smoker CP patients than non-smokers support that smoking together with periodontal destruction may expose/predispose to cardiovascular diseases.

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Keywords: chronic periodontitis; MMP-8; MMP-9; myeloperoxidase; neutrophil elastase; serum; smoking; TIMP-1

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Introduction

Pathogens in microbial dental plaque are capable of stimulating host cells to increase their matrix metalloproteinase (MMP) release which is considered among the indirect mechanisms of tissue destruction seen during periodontitis (Sorsa *et al*, 2006). Periodontal tissues are infiltrated mainly by neutrophilic granulocytes and polymorphonuclear neutrophils (PMN) which play an important role in the development of inflammatory injury. MMPs represent a structurally related but genetically distinct superfamily of proteases acting not only in physiological development and tissue remodeling but also in pathological tissue destruction (Sorsa *et al*, 2004). MMPs can be divided into five major groups: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, -10, -11), membrane-type MMPs (MMP-14, -15, -16, -17) and others (Sorsa *et al*, 2004). MMPs can collectively degrade almost all components of extracellular matrix and basement membrane and their excess activity lead to periodontal tissue destruction. MMPs can also process bioactive non-matrix substrates such as cytokines, chemokines, growth factors and immune modulators thereby mediating anti-inflammatory and pro-inflammatory processes (Sorsa *et al*, 2006; Kuula *et al*, 2009). Upon bacterial insult triggered leukocytes migrate to the site of inflammation and release MMP-8 and MMP-9, which are activated locally (Sorsa *et al*, 2006). Tissue inhibitors of MMPs (TIMPs) regulate the activities of these enzymes and TIMP-1 is more effective on interstitial collagenases (Howard *et al*, 1991). An imbalance between MMPs and TIMPs results in the pathological tissue destruction observed in periodontitis (Aiba *et al*, 1996; Bıyıkoğlu *et al*, 2009). Various treatment modalities have been investigated so far in regards with their potential to control the activities of MMPs in periodontal tissue destruction (Buduneli *et al*, 2002, 2007; Golub *et al*, 2008; Vardar-Şengül *et al*, 2008).

Neutrophil elastase (NE) is one of the most destructive enzymes with the capability of degrading almost all

extracellular matrix components as well as plasma proteins and activating proMMPs and inactivating TIMP-1 (Sorsa *et al*, 2006; Geraghty *et al*, 2007). A high concentration of NE is stored in azurophilic granules of PMNs, providing an important step in host defense. When activated, NE can be released rapidly into the extracellular space and cause local tissue damage (Kawabata *et al*, 2002). Endogenous proteinase inhibitors are important to protect tissues from unregulated proteolysis. Once released in circulation, NE is rapidly inactivated by conjugating with protease inhibitors. Azurophilic granules of PMNs also contain the enzyme myeloperoxidase (MPO) which can generate a reactive oxidant species including hypochlorous acid (HOCl). MPO is produced by neutrophils whose oxidant products are capable of modifying low density lipoprotein cholesterol and has been shown to be present in human atheromas and instable plaques (Nicholls and Hazen, 2005). MPO is released into the extracellular environment following neutrophil stimulation and/or degranulation (Buchmann *et al*, 2002). MPO can oxidatively activate MMP-8 and -9 and inactivate TIMP-1 (Sorsa *et al*, 2006). Decreased plasma MPO levels following periodontal treatment of severe periodontitis patients suggests that MPO may play a major role in pathogenesis of destructive periodontal diseases (Behle *et al*, 2009). Thus, MPO and NE can potentiate the destructive MMP cascades and indeed MPO has been regarded as a promising marker of periodontal disease activity (Arbes *et al*, 1999; Yamalık *et al*, 2000; Wei *et al*, 2004). Periodontal diseases are associated with cardiovascular diseases and persistent and enhanced circulatory inflammation involving MMPs and their regulators (TIMP-1, MPO and NE) may form a link between these local and systemic inflammatory conditions (Arbes *et al*, 1999; Pussinen *et al*, 2007).

Risk factors including tobacco smoking modify the host response to the challenge of bacteria in microbial dental plaque. It has been shown that not only the number of leukocytes is increased but also leukocytes particularly PMNs are significantly activated in smokers, suggesting a systemic inflammatory state. Nicotine has been shown to activate PMNs. Smokers have been reported to be more susceptible to advanced and aggressive forms of periodontal disease than non-smokers (Calsina *et al*, 2002). Furthermore, smokers tend to respond less favorably to periodontal treatment procedures (Ah *et al*, 1994; Boström *et al*, 1999) although; smoker and non-smoker patients exhibit more or less the same periodontal pathogens (Renvert *et al*, 1998; Buduneli *et al*, 2006). It has been reported that host cytokine levels are affected by smoking (Boström *et al*, 1999; Rawlinson *et al*, 2003). However, the exact mechanisms by which smoking exerts detrimental effects on the periodontal tissues remain unclear. Our hypothesis was that smoking may affect MMPs and neutrophil degranulation products in the systemic level eventually leading more severe periodontal tissue destruction and systemic inflammation predisposing to cardiovascular diseases (Pussinen *et al*, 2007). Therefore, this study was undertaken to comparatively evaluate the serum concentrations of MMP-8, MMP-9, TIMP-1, NE, and MPO in smoker *vs* non-smoker chronic periodontitis patients as well as periodontally healthy subjects.

Materials and methods

Study population

A total of 111 subjects were included in this study from those patients seeking dental treatment at the School of Dentistry, Ege University. Fifty-five otherwise healthy chronic periodontitis (CP) patients (16 smokers, 39 non-smokers) and 56 systemically and periodontally healthy subjects (17 smokers, 39 non-smokers) were recruited for the study between February 2004 and December 2006. The study protocol was approved by the Ethics Committee of Ege University, School of Medicine. All participants gave written informed consent in accordance with the Helsinki Declaration and dental as well as medical histories were obtained. Patients with medical disorders such as diabetes mellitus, immunological disorders, hepatitis were excluded from the study. Smokers in both CP and healthy groups were smoking more than 10 cigarettes per day for more than 5 years.

The CP group consisted of 20 females and 35 males with an age range of 33–65 years (mean age 46.7 ± 8.8 years). CP patients were diagnosed in accordance with the clinical criteria stated in the consensus report of the World Workshop in Periodontitis (Armitage, 1999). All patients had at least 20 teeth present with four teeth in each jaw with a probing depth (PD) of ≥5 mm, clinical attachment level (CAL) of ≥4 mm, and ≥50% alveolar bone loss in at least two quadrants. Assessment of the extent and severity of alveolar bone loss was performed radiographically. Bitewing radiographs were evaluated for interproximal bone loss from the cemento–enamel junction (CEJ) of the tooth to the bone crest. These patients also had bleeding on probing (BOP) at >80% of the proximal sites. Moreover, a diagnosis of CP was made if the CAL was commensurate with the amount of supragingival plaque.

The healthy control group consisted of 32 female and 24 male volunteers from the staff and periodontally healthy patients who exhibited a mean age of 40.71 ± 8.26 years (ranging between 34 and 59 years). They had at least 20 teeth, ≥90% of the measured sited exhibited PD <3 mm and CAL ≤1 mm, and no BOP, no radiographic sign of alveolar bone loss (i.e., a distance of <3 mm between the CEJ and bone crest at >95% of the proximal tooth sites).

Determination of periodontal status

The clinical periodontal parameters were assessed at six sites/tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual locations) excluding third molars, and included PD, CAL, BOP, and visible plaque accumulation. CAL was assessed from the CEJ to the base of the probable pocket. BOP (deemed positive if it occurred within 15 s after periodontal probing) and visible plaque accumulation were recorded dichotomously by visual examination. All measurements were performed by a single calibrated

examiner (Ö. Özçaka) using a Williams periodontal probe and serum samples were obtained before initiation of any periodontal intervention.

MMP-8 analysis by immunofluorometric assay (IFMA)
MMP-8 levels in the serum samples were determined by a time-resolved immunofluorescence assay (IFMA) as described previously by Hanemaaijer *et al* (1997) and Tuomainen *et al* (2007). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica Oy Ab, Kauniainen, Finland) were used as a catching and tracer antibody respectively. The tracer antibody was labeled using europium-chelate. The assay buffer contained 20 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 mM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg l^{-1} diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with the tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using a fluoremeter (1234 Delfia Research Fluoremeter, Wallac, Turku, Finland). The specificity of the monoclonal antibodies (Hanemaaijer *et al*, 1997) against MMP-8 was the same as that of polyclonal MMP-8 antibodies (Sorsa *et al*, 1999). The serum concentrations of MMP-8 were expressed as ng ml^{-1}.

MMP-9 and TIMP-1 analysis by ELISA
MMP-9 and TIMP-1 analyses were carried out by ELISA as described earlier (Rautelin *et al*, 2009). MMP-9 (Biotrak ELISA Systems, Amersham Biosciences Ltd, Buckinghamshire, UK) and TIMP-1 (Duoste ELISA Development Systems, R & D Systems, MN, USA) concentrations were determined using commercially available ELISA kits. All samples were analyzed in duplicate. As stated by the manufacturer, the present MMP-9 and TIMP-1 ELISAs detect the active, pro-, complexed, and fragmented forms of the studied MMPs and TIMP-1. The serum concentrations of the proteins are expressed as ng ml^{-1}.

MPO and NE analysis
MPO (Immunodiagnostic AG, Bensheim, Germany) and NE (Bender MedSystems mbH, Vienna, Austria) levels were measured according to the manufacturers' instructions. The secondary antibody in each kit was conjugated with horseradish peroxidase, and tetramethyl benzidine was used as a substrate (Rautelin *et al*, 2009). The serum concentrations are expressed as ng ml^{-1}.

Statistical analysis
A pilot experiment, where MPO levels measured and a 50% difference obtained, was utilized for statistical power calculations. With a power of 80% and $\alpha = 0.05$ the minimum number of patients required for the comparisons was 47 for each group.

Numerical variables were tested for normality by Shapiro–Wilk test to check whether they are normally distributed. The numerical variables that were not normally distributed were transformed ($\log_{10}$) and controlled again for normality and then analyzed by factorial ANCOVA. First, ANCOVA was used with group (healthy/CP), sex (male/female), smoking (smoker/non-smoker) and age (as a covariate) factors. Sex and age were treated as confounding factors. As the main effects and the interactions of these factors with others were not significant, they were then removed from the model. So, we performed 2×2 factorial ANOVA for two factors; group and smoking. When the interaction between group and smoking factors was found to be significant, independent sample *t* test was used for homogenous subgroups (smokers/non-smokers). Bleeding on probing and plaque index measurements were obtained in terms of scores at six sites of each tooth present and then the full mouth percentages were calculated for BOP as well as plaque accumulation. BOP and plaque index values were compared between smoker and non-smoker groups by Mann–Whitney *U*-test separately for chronic periodontitis patients and healthy controls. The possible correlations between the biochemical variables and clinical periodontal measurements were computed by the Pearson correlation coefficient or Spearmen rho rank test. All tests were performed at $\alpha = 0.05$ significance level. All the statistical calculations were performed using the SPSS version 17.0 statistical software package.

Results

Clinical periodontal analyses
The mean values of clinical periodontal measurements are outlined in Table 1. The healthy control group exhibited significantly lower values in all clinical

Table 1 Clinical periodontal measurements (mean ± s.d.) of smoker and non-smoker chronic periodontitis patients and periodontally healthy subjects

	Chronic periodontitis n = *55*		*Periodontally healthy* n = *56*	
Clinical variable	*Smokers* n = *16*	*Non-smokers* n = *39*	*Smokers* n = *17*	*Non-smokers* n = *39*
Age (years)	45.66 ± 8.45	47.53 ± 8.26	39.68 ± 8.30	41.10 ± 8.26
Female/male (*n*)	7/9	13/26	10/7	22/17
PD (mm)	4.33 ± 0.58	4.08 ± 0.14	1.95 ± 0.14	1.23 ± 0.20
CAL (mm)	5.38 ± 1.28	5.06 ± 0.87	0.00 ± 0.00	0.00 ± 0.00
Plaque (%)[a]	100.00 (41–100)	100.00 (31–100)	18.00 (00–31)	13.00 (00–31)
BOP (%)[a]	83.00 (13–100)	78.00 (17–100)	19.00 (00–25)	14.00 (00–25)

The healthy control groups exhibited significantly lower values in all clinical periodontal measurements than the chronic periodontitis groups ($P < 0.05$).
[a]Plaque index and bleeding on probing values are given as median (min–max).

Table 2 Log concentrations of biochemical parameters

Biochemical variable	*Chronic periodontitis* (n = *55*)		*Periodontally healthy* (n = *56*)	
	Smokers n = *16*	*Non-smokers* n = *39*	*Smokers* n = *17*	*Non-smokers* n = *39*
MPO	2.16 ± 0.45[a]	1.53 ± 0.26	1.68 ± 0.30	1.70 ± 0.30
NE	2.41 ± 0.35[b]	1.86 ± 0.42	2.02 ± 0.60	2.12 ± 0.45
MMP-9	2.49 ± 0.35[a]	2.41 ± 0.28	2.60 ± 0.35[c]	2.35 ± 0.29
TIMP-1	1.83 ± 0.18[d]	2.08 ± 0.14	1.95 ± 0.14	1.90 ± 0.20
MMP-9/TIMP-1	1.38 ± 0.28[b]	1.16 ± 0.18	1.34 ± 0.25[e]	1.24 ± 0.21
MMP-8	1.31 ± 0.31	1.22 ± 0.33	1.36 ± 0.35	1.37 ± 0.40
MMP-8/TIMP-1	0.72 ± 0.13	0.59 ± 0.17	0.70 ± 0.22	0.73 ± 0.24

[a]Significantly higher than the non-smoker chronic periodontitis group ($P < 0.05$).
[b]Significantly higher than the non-smoker chronic periodontitis group ($P < 0.01$).
[c]Significantly higher than the non-smoker healthy control group ($P < 0.05$).
[d]Significantly lower than the non-smoker chronic periodontitis group ($P < 0.05$).
[e]Significantly higher than the non-smoker healthy control group ($P < 0.05$).

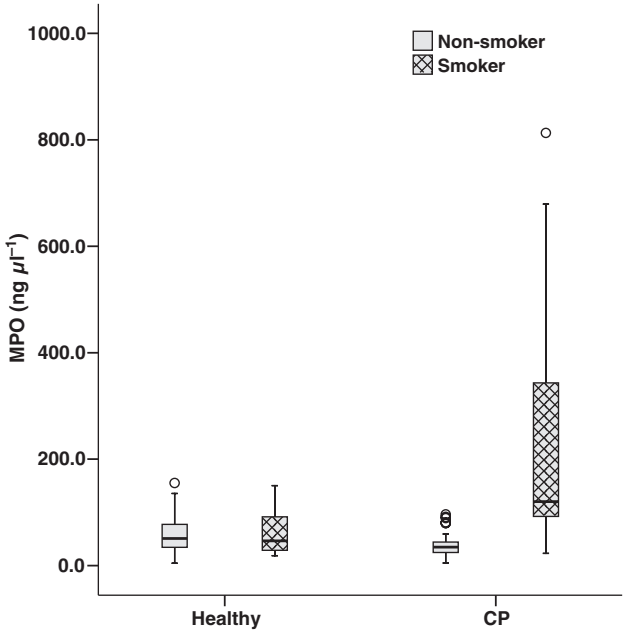

Figure 1 Serum myeloperoxidase (MPO) concentrations of smoker *vs* non-smoker chronic periodontitis (CP) patients and periodontally healthy subjects

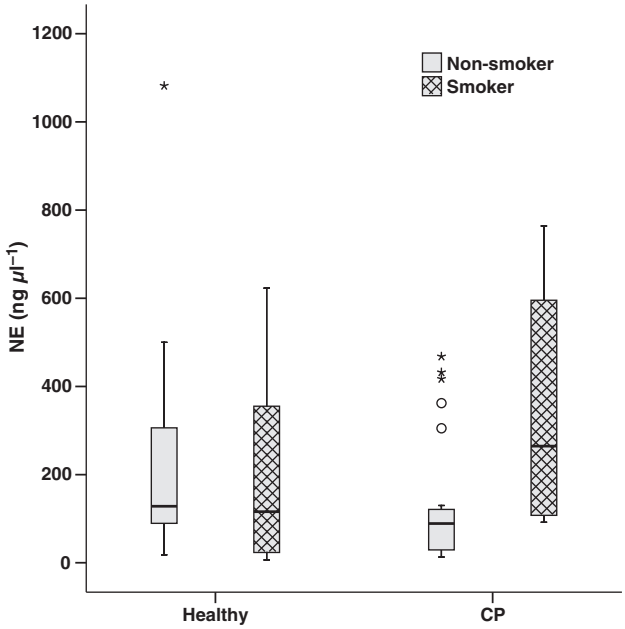

Figure 2 Serum neutrophil elastase (NE) concentrations of smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

periodontal measurements than the CP group ($P < 0.05$). There were no significant differences between the non-smoker CP and smoker CP groups in the clinical periodontal parameters ($P > 0.05$).

Biochemical analyses

The results of biochemical analysis are outlined in Table 2. The serum concentrations of MPO, NE, MMP-8, MMP-9, TIMP-1, MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios of the study groups are presented in Figures 1–7. The interaction between smoking and periodontal diagnosis was significant for MPO and NE analyses. Therefore, comparison of the smoker and non-smoker chronic periodontitis groups was performed by two-group *t* test and the same comparison was applied to the clinically healthy control groups. Serum MPO and NE concentrations of the smoker CP group were significantly higher than those of the non-smoker CP group ($P < 0.001$), whereas the smoker and non-smoker healthy control groups exhibited similar serum MPO, NE concentrations ($P > 0.05$).

There was no interaction between smoking and periodontal diagnosis for MMP-8, MMP-9 and TIMP-1 analyses. According to the variance analysis, there were no significant differences in MMP-8, MMP-9, TIMP-1, MMP-8/TIMP-1 ratio, and MMP-9/TIMP-1 ratio between the CP and healthy control groups ($P > 0.05$). Both the smoker CP and smoker healthy control group exhibited significantly higher serum concentrations of MMP-8 and MMP-9 than the non-smoker counterparts ($P < 0.001$). The serum TIMP-1 concentrations of the non-smoker CP group were significantly higher than those of the smoker CP group ($P = 0.009$). MMP-9/TIMP-1 ratio was significantly

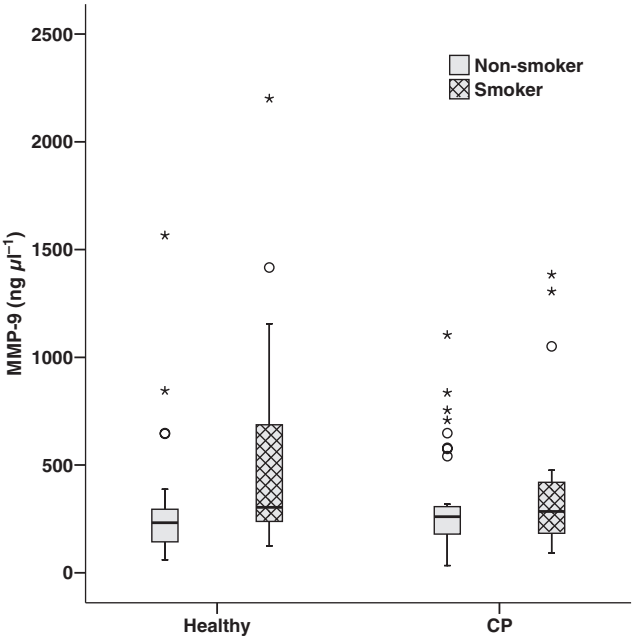

Figure 3 Serum concentrations of matrix metalloproteinase-9 (MMP-9) in smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

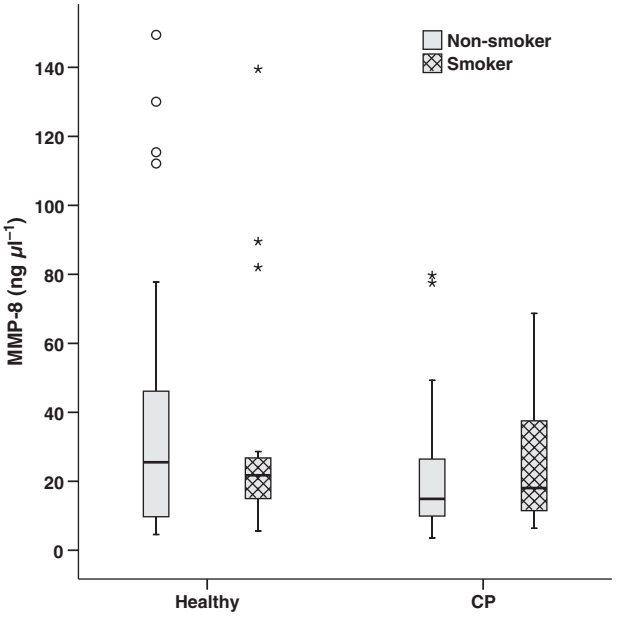

Figure 5 Serum concentrations of matrix metalloproteinase-8 (MMP-8) in smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

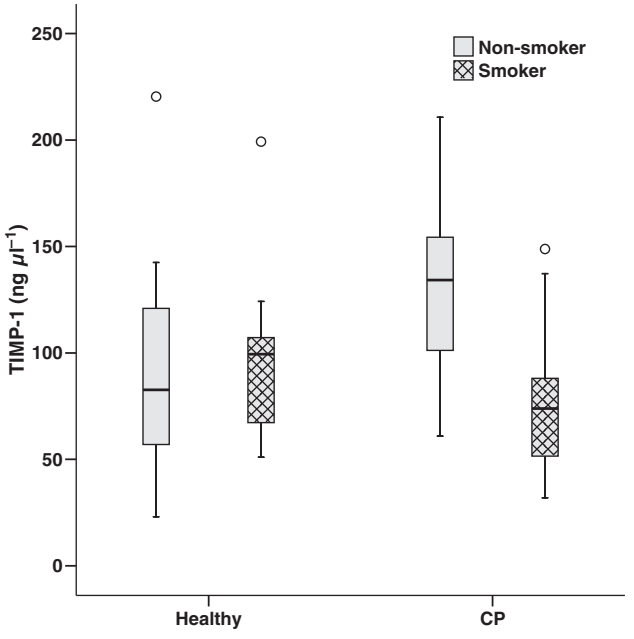

Figure 4 Serum concentrations of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

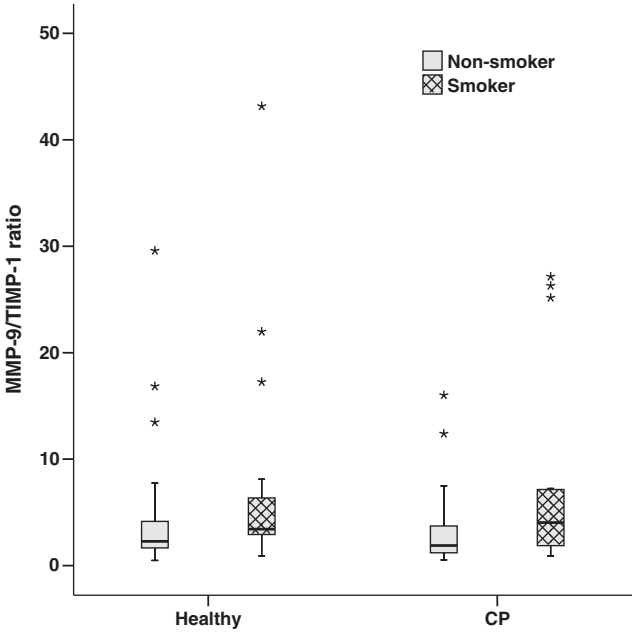

Figure 6 Ratios of serum concentrations of MMP-9/TIMP-1 in smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

higher in the smoker CP group than the non-smoker CP group ($P = 0.01$). There were no significant differences between the study groups in serum MMP-8 concentrations ($P > 0.05$).

Pearson correlation analysis revealed significant positive correlations between MPO and NE concentrations ($r = 0.503$, $P = 0.001$), MMP-9/TIMP-1 ratio ($r = 0.533$, $P = 0.000$) and MMP-8/TIMP-1 ratio ($r = 0.637$, $P = 0.000$) in the non-smoker CP group. In the same group, MPO concentrations correlated negatively with CAL ($r = 0.376$, $P = 0.018$). In the smoker CP group, MPO concentrations correlated positively with NE concentrations ($r = 0.665$, $P = 0.005$), MMP-9/TIMP-1 ($r = 0.710$, $P = 0.002$)

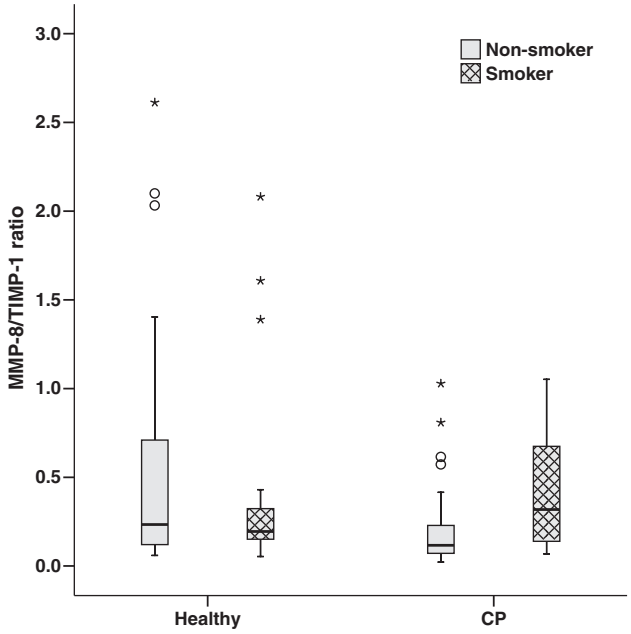

Figure 7 Ratios of serum concentrations of MMP-8/TIMP-1 in smoker *vs* non-smoker chronic periodontitis (CP) patients and smoker *vs* non-smoker periodontally healthy subjects

and MMP-8/TIMP-1 ($r = 0.561$, $P = 0.024$). There were significant positive correlations between NE concentrations and MMP-9/TIMP-1 ratio in the smoker and non-smoker CP groups ($r = 0.792$, $P = 0.000$; and $r = 0.525$, $P = 0.001$, respectively). In the periodontally healthy non-smoker group, MPO and NE concentrations correlated with MMP-9/TIMP-1 ratio ($r = 0.401$, $P = 0.011$ and $r = 0.497$, $P = 0.001$, respectively) and also with MMP-8/TIMP-1 ratio ($r = 0.617$, $P = 0.000$ and $r = 0.370$, $P = 0.021$, respectively). MPO concentrations were positively correlated with NE concentrations ($r = 0.582$, $P = 0.000$). MMP-9/TIMP-1 ratios correlated positively with MPO concentration and MMP-8/TIMP-1 ratio ($r = 0.780$, $P = 0.000$ and $r = 0.864$, $P = 0.000$, respectively) in the smoker periodontally healthy group.

Discussion

In this exploratory study, we analyzed the serum levels of MMP-8, -9, MPO, NE, and TIMP-1 in smoker *vs* non-smoker chronic periodontitis patient groups in an attempt to at least partly explain possible mechanisms of the detrimental effects of smoking on periodontal tissues and systemic inflammation. The clinical periodontal measurements were recorded and serum samples were analyzed in 55 chronic periodontitis patients (16 smoker and 39 non-smoker) and 56 periodontally healthy subjects (17 smoker and 39 non-smoker). Tobacco smokers are reported to exhibit an increased susceptibility for development and progression of periodontal diseases (Ah *et al*, 1994; Grossi *et al*, 1995; Tonetti *et al*, 1998; Bergstrom *et al*, 2000; Mullally, 2004). Accordingly, we hypothesized that the significantly increased susceptibility of tobacco smokers to the development and progression of periodontitis may be due, in part, to tobacco-induced increases in circulating levels of MMP-8, and -9, TIMP-1 and their regulators MPO and NE. However, our findings did not reveal significant differences in clinical periodontal measurements between smokers and non-smokers. This may be explained by the relatively small number of smoker chronic periodontitis patients as well as smoker healthy controls compared with the non-smoker counterparts. Indeed, this may be one limitation of our study and increasing the number of smokers might indicate different findings. The power analysis suggested including 47 subjects in each group, however, because of the time limits and the difficulty we experienced in finding smoker subjects fulfilling the inclusion criteria, we had to stop data collection when significant differences were found in the biochemical parameters between the smoker and non-smoker CP groups.

Apart from the relatively small number of smokers included in this study, it should be kept in mind that self-reports of non-smoking people can sometimes be unreliable (Buduneli *et al*, 2006). Therefore, confirmation of the smoking status by serum and/or salivary cotinine analysis may result in changing the accurate group of individual subjects which may eventually affect the results. Thus, lack of cotinine analysis in this study may be regarded as a limitation.

MMP-8 is considered to be a key mediator of the irreversible tissue destruction associated with periodontitis (Sorsa *et al*, 2004, 2006). Neutrophils are major cellular sources of MMP-8, and a large and persistent neutrophil influx is a hallmark of inflammatory periodontal diseases (Sorsa *et al*, 2004). Additionally, MMP-8 expression by several non-PMN-lineage cells found in the periodontium can be induced by pro-inflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Cole *et al*, 1996; Hanemaaijer *et al*, 1997). Furthermore, specific bacterial proteinases present in microbial dental plaque can activate the PMN-type MMP-8 (Sorsa *et al*, 2004). Therefore, tobacco-induced degranulation events in neutrophils, tobacco-induced alterations to the microbial flora, and tobacco-induced increases in pro-inflammatory cytokine burden could each, theoretically, influence MMP-8 levels in the periodontal tissues of smokers.

Increased MMP-8 expression is associated with remodeling of extracellular matrix components and the basement membrane components, including collagen destruction in the periodontal tissues and other tissues (Sorsa *et al*, 1999; Said *et al*, 1999; Bıyıkoğlu *et al*, 2009). Furthermore, physiological MMP-8 levels have recently been shown to exert protective and anti-inflammatory properties in alveolar bone loss during periodontal infection by *Porphyromonas gingivalis*, and MMP-8 deficiency in knockout mice alters the response to serum cholesterol and triglycerides participating to the early development of artherosclerosis (Kuula *et al*, 2009). MMP-8 activity has been found to be modified in various organs and body fluids in tobacco smokers.

Knuutinen *et al* (2002) noted an increased MMP-8 concentration in the resulting fluid infiltrate in smokers compared with that in the non-smokers following the induction of suction blisters on the upper arm. Furthermore, Betsuyaku *et al* (1999) have shown increased MMP-8 and MMP-9 activity in the bronchial alveolar lavage fluids of smokers with emphysema compared with those without emphysema. A significant correlation between increased MMP-8 levels and periodontal disease severity has been suggested. Liu *et al* (2006) have reported increased local MMP-8 expression in the periodontal tissues of smokers compared with the non-smokers and a slight increase in the serum MMP-8 concentration, although the difference between the smoker and non-smoker groups did not reach the level of significance.

On the contrary, Soder *et al* (2002) found a positive correlation between elastase complexed to α_1-anti-trypsin and MMP-8 concentrations in the gingival crevicular fluid (GCF) of smokers in individuals with various persistent periodontal diseases. However, they did not observe any difference in GCF MMP-8 levels between smokers and non-smokers. Persson *et al* (2003) reported that GCF MMP-8 levels remained unchanged in the smokers following surgical treatment for periodontitis, whereas decreased levels were observed in the non-smokers, suggesting a tobacco-induced MMP-8 burden. Liede *et al* (1999) examined salivary MMP-8 concentrations in 327 smokers and 82 quitters and found lower MMP-8 levels in the current smokers than the ex-smokers. According to our present data, serum MMP-8 concentrations did not differ significantly between the smokers and non-smokers.

Myeloperoxidase is an antimicrobial enzyme of PMNs released to the extracellular environment following neutrophil stimulation (Buchmann *et al*, 2002). MPO can activate latent MMPs and inhibit antiproteases thereby acting in connective tissue injury oxidatively (Yamalık *et al*, 2000). MPO was suggested as an early marker of systemic inflammation in smokers without severe airway symptoms in a study aiming to relate smoking and chronic obstructive pulmonary disease (Andelid *et al*, 2007). The authors reported significant increases in serum MPO concentrations in smokers than never smokers at sixth year of follow up. Enhanced serum levels of MPO indicate increased degranulation of specific granules of neutrophils (Rautelin *et al*, 2009). Accordingly, our present data indicated significant increases in serum concentrations of smoker chronic periodontitis patients, although the clinical periodontal measurements did not differ from those of the non-smoker counterparts. This increase in serum MPO concentration may be regarded as an indicator of increased risk for local and systemic inflammation such as periodontal tissue destruction or an early sign of atherosclerosis.

Neutrophil elastase, which is a serine protease, can also accelerate MMP-cascades by activating latent proMMPs and inactivating TIMP-1 (Sorsa *et al*, 2006). NE was suggested to be involved in the degradation of non-collagenous protein-covered collagen fibrils in the early destructive stages of periodontal diseases (Ujiie *et al*, 2007). To our best knowledge, serum NE levels as a systemic inflammatory parameter reflecting effects of smoking on chronic periodontitis has not been evaluated before. Very recently, Wohlfeil *et al* (2009) investigated serum levels of NE in 40 systemically and periodontally healthy individuals comparing males with females. They reported that serum NE correlated positively with probing depth even in subjects with clinically healthy periodontium and negatively with BOP. The authors reported no significant difference between the study groups (males *vs* females) and therefore, no effect of smoking on serum NE levels. In this study, we compared smoker chronic periodontitis patients and clinically healthy subjects with non-smoker counterparts, where we found that serum NE concentrations of smoker chronic periodontitis patients were significantly higher than those of non-smokers.

Our present findings of significantly increased serum concentrations of MMP-9 together with significant decreases in TIMP-1 concentrations in smoker chronic periodontitis patients deserve further investigation and suggest that chronic periodontitis together with smoking can predispose the development of cardiovascular diseases (Pussinen *et al*, 2007). Persistent smoking and periodontal inflammation predispose the patients for enhanced systemic inflammation in addition to enhanced periodontal destruction. This is reflected as elevated serum MMP-9, MPO, and NE as well as MMP-9/TIMP-1 ratio eventually leading increased suspected risk for the development of cardiovascular diseases. In a very recent study, Marcaccini *et al* (2009) reported increased plasma levels of MMP-8 and MMP-9 in chronic periodontitis patients and emphasized the importance of periodontal treatment to avoid elevated MMP-8 and -9 levels which are associated with many systemic diseases, particularly cardiovascular disorders.

As a conclusion, our present finding of significantly higher serum concentrations of MPO, NE, MMP-9, and significantly lower concentrations of TIMP-1 in smoker chronic periodontitis patients than their non-smoker counterparts provides further support for the smoking-associated risk for development of cardiovascular diseases in periodontitis patients.

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Author Contributions

All authors contributed extensively to the work presented in this paper. In particular, N Buduneli, N Bıçakçı, T Sorsa were responsible for study conception and design; Ö Özçaka was responsible for clinical work and data collection; P Pussinen and T Sorsa were responsible for laboratory analysis; T. Köse was responsible for statistical analysis and preparation of figures, all authors were extensively contributed in manuscript preparation; N Buduneli was responsible for study coordination.

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