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Periodontal health and serum, saliva matrix metalloproteinases in patients with mild chronic obstructive pulmonary disease

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Background and Objectives: The present case–control study aimed to evaluate comparatively the salivary and serum levels of matrix metalloproteinases (MMP)-8 and- 13 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in patients with mild chronic obstructive pulmonary disease (COPD) and non-COPD controls.

Material and Methods: Clinical periodontal measurements were recorded before any periodontal intervention in 36 patients with mild COPD and 20 non-COPD controls admitted to Ege University Department of Chest Diseases COPD out-patient clinic (İzmir, Turkey). Salivary and serum levels of MMP-8, MMP-13, and TIMP-1 were determined by immunofluorometric assay (IFMA) and enzyme-linked immunosorbent assay (ELISA). Data were analyzed with non-parametric statistical tests.

Results: Patients with COPD were significantly older than the control group ($p < 0.05$). The COPD group showed significantly higher serum levels of MMP-8 IFMA, MMP-8/TIMP-1 IFMA than the control group ($p < 0.005$). By ELISA, serum MMP-8, MMP-8/TIMP-1, TIMP-1, and MMP-13 levels were similar in both groups ($p > 0.05$). Salivary MMP-8, MMP-13, and TIMP-1 levels were similar in both groups ($p > 0.05$).

Conclusions: The present findings suggest that immunodetection of MMP-8 is dependent on the selected techniques and even with mild COPD some systemic inflammatory markers such as MMP-8 tend to increase. However, the present clinical periodontal and biochemical findings do not provide support for the previously proposed interaction between COPD and periodontal diseases.

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In inflammatory periodontal diseases, both local and systemic immuno-inflammatory responses are triggered by an oral infection (1). Periodontal diseases may induce or perpetuate an elevated systemic chronic inflammatory state. On the other hand, chronic obstructive pulmonary disease (COPD) has traditionally been considered as a

disease of the lungs mostly secondary to cigarette smoking and characterized by airflow obstruction due to abnormalities of both airway (bronchitis) and lung parenchyma (emphysema). It is now well known that COPD is associated with comorbidities such as cardiovascular diseases, lung cancer, malnutrition, muscle wasting, osteoporosis, depression and anemia, which have been attributed to an increased level of systemic inflammation. However, it is still unclear whether they represent consequences of the pulmonary disorder, or whether COPD should also be considered as a systemic disease.

In recent years, there is increasing evidence to show a possible association between periodontal disease and COPD. Didilescu *et al.* (2) indicated that dental plaque in patients with chronic lung diseases often serves as a reservoir of bacteria known to cause nosocomial pneumonia. Numerous cross-sectional studies suggest an association between periodontitis and COPD (3–5). In the study by Leuckfeld *et al.* (6), the prevalence of periodontitis was reported to be 44% in patients with COPD vs. 7.3% in the non-COPD group. The authors stated that chronic marginal periodontitis is common in patients with severe COPD.

It is quite clear that matrix metalloproteinases (MMPs) play a significant role in periodontal tissue destruction (7,8). Pathogens in microbial dental plaque are capable of stimulating host cells to increase their MMP release, which is considered among the indirect mechanisms of tissue destruction seen during periodontitis (9). 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 (9,10). Neutrophil elastase, MMPs, and their inhibitors, which are markers of systemic inflammation, are also regarded to play an important role in the development of COPD, resulting in extensive tissue damage and malfunctioning of the airways (11). Vernooy *et al.* (12) reported increased MMP-8 and MMP-9 activity in the airway compartment of patients with mild-to-moderate COPD and suggested an impaired proteinase–antiproteinase balance in COPD. In the same study, MMP-2 and MMP-13 levels were below the detection threshold of the assays. However, it is not determined yet how consistently or inconsistently specific MMPs are elevated in COPD of various phenotypes.

Although the high prevalence of periodontitis in severe COPD has been documented, there is not enough information on the inflammatory markers and periodontal status in patients with mild COPD. It is hypothesized that clinical periodontal status may be worse in patients with mild COPD and salivary and/or serum levels of MMPs and their inhibitors may be involved in a possible interaction between periodontal disease and mild COPD via proinflammatory mechanisms. Therefore, the aim of the present case–control study was to comparatively evaluate the salivary and serum levels MMP-8, MMP-13 and tissue inhibitors of MMPs-1 (TIMP-1) in patients with mild COPD and non-COPD controls. Furthermore, we aimed to compare the applicability of two MMP-8 immunodetection methods, time-resolved immunofluorometric assay (IFMA) and enzyme-linked immunoassay (ELISA) in serum and saliva.

Materials and methods

Study population

Sixty-five patients were screened between November 2009 and June 2010 and 56 of them were enrolled in this case–control study. The study population included two groups: patients with mild COPD ($n = 36$) and non-COPD individuals with normal pulmonary function ($n = 20$). Diagnosis of COPD was based on previous smoking history with at least 10 pack years of cigarette smoking and if they fulfilled the criteria for COPD according to the Global Strategy for Diagnosis, Management, and Prevention of COPD guideline (13). The individuals in the control group had been referred to the same outpatient clinic with respiratory symptoms but were finally assessed as not having COPD. All individuals were recruited from the COPD outpatient clinic of the Department of Chest Diseases, School of Medicine, Ege University following routine visits. Age, gender, smoking, history of comorbidities, and frequency of dental visits were recorded from all patients. The study was conducted in accordance with ethical principles, including the World Medical Association Declaration of Helsinki as revised in 2000. The study protocol was explained and written informed consent was received from each individual before clinical periodontal examination and biofluid sampling.

Spirometry

Spirometry was performed pre- and post-bronchodilator (15–30 min after 400 μg salbutamol administered via a spacer device) using a spirometer (Sensor Medics 2400, Homestead, FL, USA) to achieve three acceptable expiratory maneuvers according to American Thoracic Society/European Respiratory Society criteria, of which at least two forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) measurements were reproducible within 200 mL. The criterion for confirmation of a diagnosis of COPD was a post-bronchodilator FEV_1/FVC ratio of < 0.7. The severity of airway obstruction was graded according to Global Initiation for Chronic Obstructive Lung Disease (13) criteria for predicted FEV_1 (mild: $FEV_1 \geq 80\%$; moderate: $50\% \leq FEV_1 < 80\%$; severe: $30\% \leq FEV_1 < 50\%$; very severe: $FEV_1 < 30\%$ or $FEV_1 < 50\%$ + chronic respiratory failure). Pre-bronchodilator spirometry but not post-bronchodilator spirometry was performed in the control group as their lung volumes were in the normal range.

Saliva and serum sampling

Saliva and serum samples of each individual were obtained on the same day. Whole saliva samples were obtained before clinical periodontal measurements and any periodontal intervention by having patients expectorate into polypropylene tubes in the morning following an overnight fast during which subjects were requested not to drink (except water) or chew gum. The saliva samples were clarified by centrifugation (800 ***g***) for 10 min at +4°C, immediately frozen and stored for 2–6 mo at −40°C until the sample collection period was completed and thawed immediately before assays.

Venous blood samples were taken in a standardized fashion by nurses in the hospital. Serum samples were separated and frozen within 2 h of blood drawn and stored at −40°C until the biochemical analyses.

Clinical periodontal measurements

Full-mouth clinical periodontal measurements were recorded at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual, mesio-lingual), including probing depth (PD), clinical attachment level (CAL), and dichotomous bleeding on probing (BOP) (±). BOP (deemed positive if it occurred within 15 s after periodontal probing) and visible plaque accumulation were recorded dichotomously as present or absent by visual examination. CAL was assessed from the cement enamel junction to the base of the probable pocket. Number of pockets with PD > 4 mm was counted for each subject. A Williams' periodontal probe was used for clinical periodontal measurements, which were all performed by one researcher (EY) who was blinded to the systemic status of the patient.

Matrix metalloproteinase-8 analysis by immunofluorometric assay and enzyme-linked immunosorbent assay in saliva and serum samples

MMP-8 levels in the serum samples were determined by a time-resolved IFMA as described previously by Hanemaaijer *et al.* (14) and Tuomainen *et al.* (15). The monoclonal MMP-8 specific antibodies 8708 (Medix Biochemica Oy Ab, Kauniainen, Finland) 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 diethylenetriaminepentaacetic acid. 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 fluorometer (1234 Delfia Research Fluorometer, Wallac, Turku, Finland). The specificity of the monoclonal antibodies (14) against MMP-8 was the same as that of polyclonal MMP-8 antibodies (16). A commercial ELISA-kit (Quantikine human MMP-8 total immunoassay; R&D Systems Europe Ltd., Abingdon, UK) was used according to the manufacturer's instructions. All samples were analyzed in duplicate and serum concentrations of MMP-8 were expressed as ng/mL.

Matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 analysis in saliva and serum samples

MMP-13 and TIMP-1 analyses were carried out by ELISA as described earlier (17). MMP-9 (Biotrak ELISA Systems, Amersham Biosciences Ltd., Buckinghamshire, UK) and TIMP-1 (Duoste ELISA Development Systems, R & D Systems, Minneapolis, MN, USA) concentrations were determined using commercially available ELISA kits. All samples were analyzed in duplicate. As stated by the manufacturer, the present MMP-13 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.

Statistical analysis

Wilcoxon *W* and Mann–Whitney *U* tests were used when appropriate for the group comparisons of the biochemical data as well as the clinical periodontal measurements. Pearson chi-square and Fisher exact tests were used to compare gender and smoking habits between the groups. Correlation between clinical and biochemical data were evaluated with non-parametric Spearman rho rank correlation analysis. All tests were performed at $\alpha = 0.05$ significance level. All the statistical calculations were performed using a statistical software package (SPSS TURKEY, v.17.0; Maltepe, Istanbul, Turkey).

Results

Characteristics of the study groups are presented in Table 1. The study groups were similar in gender distribution ($p > 0.05$). The control group was significantly younger than the patients with COPD were (mean age 59.5 vs. 68.9 years, respectively) ($p < 0.05$). There were significantly more former smokers in the COPD group than the control group ($p < 0.005$).

The data from lung function tests are presented in Table 2. All parameters [FVC (%), FVC (L), FEV_1 (%), FEV_1 (L), FEV_1/FVC (%)] showed significantly lower values in the COPD group than the control group ($p < 0.005$).

Clinical periodontal parameters are given in Table 3. The mean PD was significantly higher in the control group (2.9 mm) than the COPD group (2.5 mm) ($p = 0.01$). There were no significant differences between the study groups in CAL, PI, and BOP mean values ($p > 0.05$).

Serum and salivary levels of MMP-8, MMP-13 and TIMP-1 are presented in Table 4. The COPD group showed significantly higher serum levels of MMP-8 IFMA and MMP-8/TIMP-1 IFMA than the control group ($p < 0.005$). Serum MMP-8 ELISA, MMP-8/TIMP-1 ELISA, TIMP-1 and MMP-13 levels were similar in the study groups ($p > 0.05$). The salivary MMP-8, MMP-13 and

Table 1. Characteristics of the study groups

Variable	COPD group (n = 36)	Non-COPD group (n = 20)
Age (mean ± SD) (years)	68.9 ± 8.8*	59.5 ± 9.1
Age (range) (years)	47–80	46–74
Male/female (n)	34/2	19/1
Never smoker (n)	4[a]	9
Former smoker (n)	32*	8
Current smoker (n)	0	3
BMI (kg/m^2) (mean ± SD)	27.4 ± 3.8	27.8 ± 3.7

BMI, body mass index; COPD, chronic obstructive pulmonary disease; SD, standard deviation.
*Significantly higher than the control group ($p < 0.005$).
[a]These patients had a history of passive smoking.

Table 2. Lung functions in the study groups

Parameters	COPD group (n = 36)	Non-COPD group (n = 20)
FVC (%)	79.1 ± 11.6	94.3 ± 11.9*
FVC (mL)	2.74 ± 0.72	3.56 ± 1.02*
FEV_1 (%)	81.5 ± 13.3	94.5 ± 9.6*
FEV_1 (mL)	1.73 ± 0.57	2.67 ± 0.89*
FEV_1/FVC (%)	62.9 ± 9.7	74.7 ± 10.5*

COPD, chronic obstructive pulmonary disease; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s. In the COPD group all presented data are post-bronchodilator values.
*Significantly higher than the COPD group ($p < 0.005$).

TIMP-1 levels were also similar in both groups ($p > 0.05$).

Correlations between clinical and biochemical parameters in the study groups are presented in Table 5. In the COPD group, a positive correlation was present between serum MMP-8 (IFMA) level and FEV_1, FVC (L) ($p < 0.05$). In the control group, serum MMP-8 (IFMA) level correlated with FEV_1 (%) ($p < 0.05$).

Table 3. Clinical periodontal parameters (mean ± SD) of the study groups

Clinical periodontal variable	COPD group (n = 36)	Non-COPD group (n = 20)
PD (mm)	2.5 ± 0.9	2.9 ± 0.8*
Sites with PD > 4 mm (n)	5.3 ± 7.6	10.5 ± 13.1
CAL (mm)	3.2 ± 1.1	3.7 ± 1.1
PI (%)	93 ± 1.1	92 ± 1.9
BOP (%)	57.8 ± 2.3	51.0 ± 2.7

PD, probing depth; CAL, clinical attachment level; COPD, chronic obstructive pulmonary disease; PI, plaque index; BOP, bleeding on probing.
*Significantly higher than COPD group ($p = 0.010$).

Discussion

In the present case–control study, serum and salivary levels of MMP-8, MMP-13 and TIMP-1 were analyzed comparatively in mild COPD and non-COPD control groups. To the best of our knowledge, this is the first study investigating serum and salivary levels of MMPs and TIMP-1 in mild COPD cases and non-COPD controls in relation with clinical periodontal status. Our clinical and biochemical data indicated no relationship between periodontal disease and mild COPD. By ELISA, the serum MMP-8, MMP-8/TIMP-1, TIMP-1 and MMP-13 levels were all similar in the study groups. However, by IFMA, serum levels of MMP-8 and MMP-8/TIMP-1 were higher in the COPD group. The salivary MMP-8, MMP-13 and TIMP-1 levels were similar in both groups. Clinical periodontal findings were similar in the study groups apart from the higher mean PD in the control group.

Whole saliva represents a pooled sample with contributions from all periodontal sites and analysis of biomarkers in saliva may provide an overall assessment of disease status (18). We used two different immunoassay techniques to determine MMP-8 levels in the biofluid samples. IFMA utilizing two monoclonal anti-MMP-8 antibodies exerts higher accuracy than ELISA, which uses a single antibody. Eventually, this can explain the differences in sensitivities between the IFMA and ELISA assays. It should be kept in mind that the antibody used in IFMA identifies the neutrophil- and fibroblast-type MMP-8 isoforms and particularly their active forms (14,19), whereas the ELISA method detects all forms of MMP-8. Moreover, differences in manufacturers of ELISA kits may be another factor underlying the discrepancies between different studies. It is well known that ELISA kits produced by different manufacturers may vary in their sensitivities as well as specifities.

The possible relationship between COPD and oral health or periodontal condition has been investigated in a number of studies. Hayes *et al.* (3) suggested that periodontal status was associated with increased risk for COPD. The risk for COPD has been proposed to significantly increase with severe attachment loss (20). Significantly higher prevalence of chronic periodontitis in the COPD group was reported and suggested to be independent of known risk factors for periodontitis such as age and smoking (6). Wang *et al.* (21) indicated that patients with COPD had fewer teeth and higher plaque index scores than controls. The same research group stated that presence of fewer teeth, higher plaque index scores and lower tooth brushing times correlated with COPD exacerbations (22). The same Chinese group also suggested that plaque index might be a major periodontal factor for predicting COPD (23). Our present findings differ from those of the Chinese group as clinical attachment loss, plaque index, bleeding index and number of sites with PD > 4 mm were all similar in patients with mild COPD and non-COPD individuals. Moreover, the non-COPD group revealed statistically higher mean PD values than the COPD group, although the difference was clinically insignificant. This higher PD in a younger group is rather unexpected but may be just an incidental finding and is likely not to be confirmed in larger scale study groups. Moreover, this difference is significant neither from the clinical

Table 4. MMP-8, MMP-13 and TIMP-1 levels in the serum and saliva samples (ng/mL)

	Serum samples		Saliva samples	
Inflammatory marker	COPD group (n = 32)	Non-COPD group (n = 19)	COPD group (n = 21)	Non-COPD group (n = 11)
MMP-8 IFMA	82.09 ± 70.79*	42.02 ± 43.10	1427.89 ± 856.67	1420.02 ± 1252.53
MMP-8 /TIMP-1 IFMA	0.61 ± 0.55*	0.28 ± 0.31	3.62 ± 5.11	3.54 ± 3.46
MMP-8 ELISA	29.59 ± 26.33	18.19 ± 18.91	854.24 ± 881.53	838.19 ± 724.83
MMP-8/ TIMP-1 ELISA	0.23 ± 0.16	0.19 ± 0.10	2.64 ± 4.37	2.17 ± 2.68
TIMP-1 ELISA	143.40 ± 42.48	166.19 ± 58.48	828.76 ± 656.99	670.77 ± 595.65
MMP-13 ELISA	0.033 ± 0.006	0.0013 ± 0.0026	0.53 ± 1.36	0.70 ± 1.06

COPD, chronic obstructive pulmonary disease; MMP, matrix metalloproteinase; TIMP, MMP tissue inhibitor.
*Significantly higher than the control group ($p < 0.05$).

Table 5. Significant correlations found in the study groups

Study group	Spearman's rho correlation coefficient sig. (2-tailed)	FVC (%)	FVC (L)	FEV_1 (%)	FEV_1 (L)
COPD group	MMP-8 IFMA (serum)	$p > 0.05$	$r = 0.438$ $p = 0.012$	$p > 0.05$	$r = 0.446$ $p = 0.011$
	Bleeding on probing (mean)	$r = -0.454$ $p = 0.005$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Control group	MMP-8 IFMA (serum)	$p > 0.05$	$p > 0.05$	$r = 0.524$ $p = 0.021$	$p > 0.05$
	MMP-8 IFMA (saliva)	$p > 0.05$	$p > 0.05$	$p > 0.05$	$r = 0.645$ $p = 0.032$
	Clinical attachment level (mean)	$p > 0.05$	$r = -0.58$ $p = 0.042$	$p > 0.05$	$r = -0.529$ $p = 0.16$

COPD, chronic obstructive pulmonary disease; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; IFMA, immunofluorometric assay; MMP, matrix metalloproteinase.

point of view nor for the scope of the study. Another likely explanation for this inconsistency may be the genetic differences between the study populations having different genetic background. Hyman and Reid (24) reported no relationship between periodontal disease and COPD when PD was used as the measure of periodontal disease. Our findings are in accordance with their study and we consider our study groups were similar in clinical periodontal status. The difference between our findings and those of the Chinese group may be explained by the difference in patient numbers. Moreover, possible differences in severity of COPD and periodontal disease in the study groups may explain differences in findings.

Recently, the same group from China reported that poor periodontal health as reflected by missing teeth and plaque index was significantly associated with lower quality of life in patients with COPD (25). The authors suggested that promoting dental health could improve the quality of life in patients with COPD. In accordance with the findings of Hyman and Reid (24), the present findings do not provide support for this claim as the clinical periodontal parameters were similar in our study groups.

Various mechanisms have been suggested to explain the possible relationship between oral diseases and respiratory diseases. First, severe anaerobic lung infections are likely to occur following aspiration of salivary secretions, especially in patients with periodontal disease (26). Aspiration of pathogenic bacteria from the oropharynx into the lungs has been proposed to result in respiratory infections such as bacterial pneumonia and chronic pulmonary diseases (27–29). Moreover, it was suggested that lower serum 25-hydroxyvitamin D concentrations might be associated with poor periodontal health and a higher risk for COPD (30).

Vernooy *et al*. (12) reported that MMP-8 and MMP-9 were significantly increased in the COPD group, whereas MMP-2, MMP-13 remained below the detection limit of the assays. A positive correlation was found between MMP-8, MMP-9 activity in sputum and the degree of airflow limitation (12). TIMP-1 level in sputum of patients with chronic bronchitis correlated with the decrease in lung function (31,32). Russell *et al*. (33) reported that alveolar macrophages from non-smokers released more TIMP-1 than cells from healthy smoker subjects and subjects with COPD. In the present COPD group, FEV_1 and FVC (L) correlated positively with serum MMP-8 levels. The increased serum MMP-8 and MMP-8/TIMP-1, but not levels of TIMP-1 or MMP-13, in the COPD group suggests that COPD may have a systemic effect on MMP-8. The present observations are in agreement and further extend previous studies (34) suggesting that COPD in addition to its local surrogate effects exerts systemic proinflammatory effects. There is also associative evidence from human genetic

and animal studies suggesting a pathological link between COPD and both MMP-8 and MMP-9 (35).

This study has a number of limitations. One of them is the rather low numbers of individuals in each study group. The other is the rather narrow scope as only MMP-8, MMP-13 and TIMP-1 levels were analyzed in serum and saliva samples.

It is quite clear that inflammation increases with increasing severity of COPD. Proteases therefore, may be responsible for additional pathogenic mechanisms in COPD other than tissue degradation. Overall, MMPs deserve intense *in vivo* investigation in patients with COPD going forward and it seems to be essential to develop significant therapies for this disease (36). Serum C-reactive protein and MMP-9 levels have been related to the FEV_1 decline in patients with COPD (37). These biomarkers were beyond the scope of our study. de Torres *et al*. (38) reported that in patients with stable COPD, there were gender differences in circulating biomarker levels as well as in the association between biomarker levels and important clinical or physiological variables. In the present study, almost all individuals were males and, therefore, we consider our findings were free of gender effects. Indeed, this could be another explanation for the difference in our findings and those of the Chinese group, as they have included many more females.

In conclusion, the present findings suggest that immunodetection of MMP-8 is dependent on the selected technique and even with mild COPD some systemic inflammatory markers such as MMP-8 tend to increase. Within the limits of the present study, we found no relationship between COPD and periodontal disease. Larger scale studies, including patients with different COPD severity categories as well as periodontal disease are required to better clarify this issue.

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