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Evaluation of Serum RANKL and OPG Concentrations in Patients with Periodontitis in Saudi Arabia

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ABSTRACT

Objective: To investigate RANKL and OPG serum concentrations and the RANKL/OPG ratio in patients diagnosed with chronic periodontitis and attending dental school clinics in Makkah, Saudi Arabia. Methods : Patients seeking dental treatment at the Faculty of Dentistry Clinic, Umm Al Qura University were divided into periodontitis and control groups based on their clinical findings. Periodontitis was diagnosed in patients with a PD >4mm or CAL >3mm at more than 30% of the examined sites, and the radiographic evidence of alveolar bone loss was > 30% of teeth. RANKL and OPG serum concentrations (pg/ml) and RANKL/OPG ratio were determined. Results: Forty-three patients, including 32 females and 11 males with a mean age of 30 +/- 8.48, were studied. Thirty patients were diagnosed with periodontitis, and 13 were in the control group. The patients with periodontitis had significantly higher RANKL (31.8 +/- 27.5 vs. 18.7 +/- 14.2 pg/ml, p=0.04) and OPG concentrations (58.0 +/- 23.2 vs. 45.3 +/- 10.0 pg/ml, p=0.03) than the control patients. The patients with periodontitis that smoked had significantly lower serum RANKL concentrations than those that did not smoke (37.8 +/- 10.0 vs. 14.8 +/- 10.0, p=0.0057 pg/ml). The RANKL/OPG serum ratio was higher in non-smokers than in smokers (0.6 vs. 0.3, p=0.017). The patients with generalized advanced periodontitis had significantly higher serum RANKL concentrations than those with the localized disease (56.8 +/- 42.0 vs. 22.0 +/- 12.8 pg/ml, p=0.03). Serum RANKL/OPG ratio was increased in patients with generalized disease (0.96 vs. 0.39, p=0.036). Serum RANKL/OPG ratio was also correlated with the amount of CAL (r = 0.459, p=0.04). Conclusions: Serum RANKL concentrations were increased in patients with periodontitis, in non-smokers, and in patients with the generalized advanced disease.

Key words: *Periodontal Disease, Serum, RANKL, OPG, Biomarker, Alveolar Bone Loss*

INTRODUCTION

Periodontitis is characterized by the loss of connective tissue attachment and alveolar bone and has been the most common form of the inflammatory diseases in humans.[1]. Bone mass is determined by a careful balance between osteoblastic and osteoclastic activity, a process which is tightly regulated by the interaction of matrix metalloproteinases, prostaglandins and inflammatory cytokines [1]. Receptor activator of nuclear factor-κb (RANK), osteoprotegerin (OPG), and RANK ligand (RANKL) have been the key regulators of osteoclastic activity and recruitment, and therefore, bone resorption in periodontal diseases [2, 3]. Several factors play a role in the initiation of periodontal disease, however, the host response is the major determinant of the severity and the progression of the tissue destruction [4].

RANKL is a 314 AA polypeptide member of the TNF-α superfamily that has been secreted by a variety of inflammatory cells including lymphocytes, stromal cells and periodontal ligament cells [5]. RANKL binds to RANK receptors on the pre-osteoclast cells leading to their activation. OPG is a secreted tumor necrosis factor receptor homologue that increases the bone mineral density by acting as a decoy receptor for RANKL [6]. The altered systemic concentrations of RANKL and OPG have been reported in a number of diseases associated

with the active inflammation and tissue damage including rheumatoid arthritis, [7] cardiovascular diseases, [8] and the complications of type 2 Diabetes [9].

RANKL has been reported to be up-regulated, and OPG to be down-regulated in patients with the periodontal diseases [10-12]. Local RANKL secretion has been greater in patients with active periodontal diseases and continuous periodontal destructions than in inactive diseases. However, Bostanci et al. reported that RANKL and OPG levels were not different among the chronic and generalized aggressive periodontitis patients [12].

In addition, RANKL and OPG can be detected in human gingival crevicular fluid (GCF) during orthodontic tooth movement [13] or in peri-implant crevicular fluid [14]. Gingival tissues from the patients with periodontal diseases expressed higher concentrations of RANK and RANKL than those from the patients with normal gingival tissues [15, 16]. Local RANKL mRNA expression has also been shown to be higher in patients with advanced periodontal diseases than in patients with little or no diseases [5], and greater local RANKL/OPG tissue ratio expression has been reported in patients with periodontitis than in the healthy patients [17].

However, the systemic concentrations of these factors in this disease have not been well defined, and although substantial works have been published on RANKL and OPG, no data has been available on these mediators in Saudi Arabian population

The aim of this study was to investigate RANKL and OPG serum concentrations and the RANKL/OPG ratio in patients diagnosed with chronic periodontitis and attending dental school clinics in Makkah, Saudi Arabia.

MATERIAL AND METHODS

Ethics and consent

This cross-sectional study evaluated the patients seeking dental treatment at the Faculty of Dentistry Clinic, Umm Al Qura University. The study protocol was approved by The Ethics Committee for Clinical Research at Umm Al Qura University Faculty of Dentistry, and a written informed consent was obtained from all the subjects before the enrolment in the study.

Inclusion and exclusion criteria

Healthy subjects who did not receive any periodontal treatment during the last 3 months were included in the study. Patients who received periodontal treatment or antibiotic, anticonvulsant or immunosuppressive drugs in the last 3 months, or were pregnant were excluded.

Clinical examination

All subjects were examined by one periodontist. Smoking history and dental radiographs were reviewed from the patients' records. The clinical examination included the assessment of plaque index (PI), gingival index (GI), periodontal probing depth (PD), and clinical attachment loss (CAL).

PI values were defined as: 0-no plaque, 1- a film of plaque present and adhering to the free gingival margin, and was only seen by disclosing tablets, 2- when moderate accumulation of deposits was seen on the teeth, and 3- when abundant soft material along the gingival margin was detected. GI was used to evaluate the degree of inflammation. Normal gingiva had a value of 0, 1- if the mild inflammation was present but there was no bleeding on probing (BOP), 2- when there was moderate inflammation and BOP, and 3- when there was gingiva with severe inflammation, and a tendency to bleed spontaneously. The PD was defined as the distance between the gingival margins and the base of the sulcus. CAL was determined from the cementoenamel junction to the base of the sulcus using a manual periodontal probe at the mesial, distal, buccal, and palatal/lingual aspects of each tooth, excluding third molars.

The patients were divided into a periodontitis or control group. Periodontitis group included patients with a PD $\geq$4mm or CAL $\geq$3mm at more than 30% of the examined sites, and radiographic evidence of alveolar bone loss in $\geq$ 30% of teeth. The control group had a PD $\leq$ 3mm or CAL $\leq$2mm, and the radiographic evidence of normal alveolar bone level in > 90% of teeth. The generalized disease was defined as >30% of sites examined which had a PD > 5mm.

Mild periodontitis on radiographic exam was defined as bone loss up to 15% of the root length in $\geq$ 30% of teeth, and moderate to severe periodontitis with any bone loss >16 % of root length in > 30% of teeth.

RANKL and OPG determination

Five ml of venous blood was collected in a red vacutainer tube at the beginning of the dental appointment, before starting any dental treatment. The samples were centrifuged at 4000 rpm for 5 min and the serum were stored at -80^{o} C until analysis. RANKL and OPG concentrations were determined using a commercial ELISA

kit following manufacturer instructions (Abcam, Cambridge, UK). The results were expressed as picograms per milliliter (pg/ml).

Data analysis

The data was analyzed using the SPSS statistical package software, for Windows version 17.0 (SPSS Inc., Chicago, USA). The quantitative data was presented as the mean and standard deviation. A Mann–Whitney U test was carried out to evaluate the differences between different groups. A Spearman's correlation test was used to analyze the relationship between the serum concentrations of RANKL and OPG and RANKL/OPG ratio and clinical periodontal parameters. The P value less than 0.05 was considered statistically significant. All the statistical tests were 2-sided.

RESULTS

Clinical evaluation

Forty-three patients were evaluated, including 32 (74.4 %) females and 11(25.5%) males with a mean age of 30 ± 8.48 (**Table 1**). Thirty patients (70%) were diagnosed with periodontitis (**Table 2**). All the control subjects had all their teeth. Sixteen of the patients with periodontitis had missing teeth; 3 missing one tooth, 6 missing two teeth, and 7 missing more than three teeth. Periodontitis patients had significantly greater PD, CAL, % of teeth with PD > 5 mm, and % of teeth with CAL > 3mm than the control patients (**Table 2**).

The only 6 patients with a smoking history (10.0 ± 2.0 pack-years) had periodontitis. The mean smoker age was 33.0 ± 12.0 years, similar to that of the entire group. The smokers and non-smokers with periodontitis had a similar PD (smokers, 3.55 ± 1.4mm vs. non-smokers, 4.4 ± 1.04mm p>0.05) and CAL (smokers, 2.8 ± 0.15mm vs. non-smokers, 2.6 ± 1.4mm, p>0.05).

The mean PD in patients with periodontitis and mild bone loss was lower to that of patients with periodontitis and moderate to severe bone loss (mild, 4.02 ± 0.56mm vs. moderate to severe, 4.86 ± 1.44mm, p > 0.05). The mean CAL in patients with periodontitis and mild bone loss was significantly less than that of periodontitis patients with moderate to severe bone loss (mild, 2.2 ± 1.2mm vs. moderate to severe, 4.15 ± 1.2mm, p = 0.002).

Table 1. Patient demographic – Periodontitis and Healthy Control groups.

	Periodontitis	**Control**
Mean age (years)	31.7 ± 11.0	27.8 ±7.0
Gender (female: male)	19:11	13:0
Smoking history pack years	10.0 ± 2.0	0

Table 2. Clinical parameters in Control and Periodontitis Groups.

	Periodontitis	**Control**
PD, mm*	4.3 ± 1.0	3.08 ± 0.52
CAL, mm†	2.76 ± 1.49	0
% of teeth with PD >5mm‡	18%	0%
% of teeth with CAL >3mm§	30%	0%
Plaque index	2.0 ± 0.80	1.9 ± 0.21
Gingival index	2.4 ± 0.80	2.1 ± 0.31

PD = periodontal probing depth

CAL = clinical attachment loss

*Mean probing depth, periodontitis versus control groups, P = 0.0001

† Mean attachment level, periodontitis versus control groups, P = 0.001

‡ % of teeth with PD >5mm, periodontitis versus control groups, P =0001

§ % of teeth with CAL>3mm, periodontitis versus control groups, P = 0.0001

Serum RANKL and OPG concentration analysis

Serum RANKL concentration was greater in patients with periodontitis than in control patients (31.8 ± 27.5 pg/ml) vs. 18.7 ± 14.2 pg/ml), p = 0.04). Serum OPG concentration was greater in patients with periodontitis than in control patients (58.0 ± 23.2 pg/ml (n = 30) vs. 45.3 ±10.3 pg/ml (n = 13), p = 0.03). There was no difference in the RANKL/OPG ratio of the two groups (periodontitis, 0.6 ± 0.30) vs. 0.45 ± 0.35, p = 0.3) **(Figure 1).**

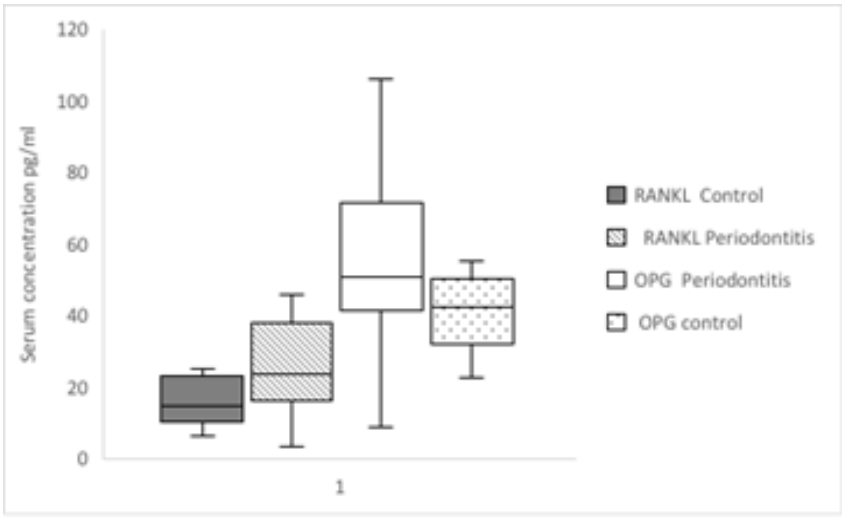

Figure 1. Serum RANKL and OPG concentration in subjects with periodontitis and controls. The horizontal line represents the median value and 75% and 25% quartiles are represented by the upper and lower edges of boxes. RANKL and OPG levels in periodontitis versus control group were different at statistically significant level P<0.05.

None of the control patients had a history of smoking. Of the patients with periodontitis, 6 were smokers, and 24 were non-smokers. Serum RANKL concentration was greater in patients with periodontitis that did not smoke than in those that smoked (37.8 ± 10.0 pg/ml vs. 14.8 ± 10.2 pg/ml, p = 0.0057). There was no difference in the serum OPG concentration of these 2 groups (non-smokers, 57.8 ± 22.7 pg/ml vs. Smokers, 51.8 ± 16 (p =0.4). The RANKL/OPG ratio was greater in non-smokers than in smokers (0.67 ± 0.42 vs. 0.3 ± 0.13 p = 0.017) **(Table 3)**.

Periodontal disease severity was evaluated based on radiographic bone loss. Serum RANKL and OPG levels and the serum RANKL/OPG serum level ratio of the two groups were not significantly different **(Table 3)**. However, when disease extent was considered, the patients with generalized disease (PD> 5mm in >30% of sites, n = 7) had greater serum RANKL concentration than those with the localized disease (PD> 5mm in <30% of sites, n = 23) (56.8 ± 42.0 pg/ml vs. 22.0 ± 12.8 pg/ml, p = 0.03). There was no difference in the serum OPG concentration of the two groups (generalized disease, 56.6 ±10.4 pg/ml vs. localized disease, 55.6 ± 26.2 pg/ml, p = 0.44). The RANKL/OPG ratio was greater in patients with generalized disease (0.96 ± 0.60 pg/ml than in those with localized disease (0.39 ± 0.34, p = 0.036) **(Table 3)**.

Spearman's rank correlation was performed to identify the associations between serum concentrations of RANKL and OPG and clinical parameters (**Table 4).** Serum RANKL and OPG concentrations were not correlated with any of the parameters evaluated (PD, CAL, % of deep PD, and % of advanced CAL). RANKL/OPG ratio was correlated with the amount of CAL.

Table 3. Serum RANKL, OPG concentrations and RANKL/OPG ratio; comparison in Periodontitis group, Smoker vs. Non-smoker groups, Mild vs. Severe periodontal disease groups and localized vs, generalized periodontal disease groups. • P<0.05

Periodontitis patients	RANKL (pg/ml)	OPG (pg/ml)	RANKL/OPG ratio
Non-Smokers (n = 6)	37.8 ±10.0	57.8 ±22.7	0.67± 0.42
Smokers (n = 24)	14.8 ± 10.2•	51.8 ± 16	0.3 ± 0.13•
Mild bone loss (n = 20)	30.6 ± 27.0	56.2 ± 28.0	0.58 ±0.45
Moderate to Severe bone loss (n= 10)	34.1 ± 32.0	58.7 ± 17.5	0.53± 0.37
Localized disease (PD> 5mm in <30% of sites) (N=23)	22.0 ± 12.8	55.6 ± 26.2	0.39 ± 0.34
Generalized disease (PD> 5mm in >30% of sites) (N= 7)	56.8 ± 42.0•	56.6 ±10.4	0.96 ± 0.60•

Table 4. Spearman's Rank correlation between PD, CAL, % of deep PD and % of advanced CAL with serum concentrations of RANKL and OPG and RANKL/OPG ratio.

	Serum RANKL		Serum OPG		RANKL/OPG ratio	
	r	**p-value**	**r**	**p-value**	**r**	**p-value**
PD	0.34	0.08	0.055	0.78	0.07	0.85
CAL	0.12	0.15	0.08	0.66	0.459	**0.04**
% of deep PD	0.19	0.37	0.1	0.66	0.165	0.438
% CAL	0.03	0.88	0.017	0.93	0.106	0.62

DISCUSSION

Periodontitis has been associated with increased local expression of RANKL, decreased local expression of OPG, and increased RANKL/OPG ratio, factors associated with increased bone resorption. [11, 12, 17, 18]. The systemic expression of these factors in periodontitis has not been well defined. In this study, the serum concentrations of these factors in otherwise healthy, untreated, Saudi patients seen at the Umm Al Qura University dental clinics were examined to better characterize this expression.

The evaluation of all the patients tested showed that the serum concentrations of RANKL and OPG were significantly higher in patients with chronic periodontitis than in those without periodontitis. The ratio of these concentrations (RANKL/OPG) was greater in patients with chronic periodontitis than in the control, although this finding was not significant. This was in agreement with previous studies which reported a higher systemic RANKL level in periodontitis patients as compared to the subjects without periodontitis [19-21]. While OPG level showed more contradictory results, and some studies demonstrated OPG serum level was not significantly higher in healthy as compared to periodontitis subjects [20, 21]. In addition to its protective role against bone loss, OPG has been associated with the progression of chronic disease as rheumatoid arthritis [7], cardiovascular disease, [8] and the complications of type 2 Diabetes [9].

The effect of smoking on systemic RANKL and OPG concentrations was evaluated. Serum RANKL concentration was significantly lower in smokers with periodontitis than in non-smokers with periodontitis, and the serum OPG concentration of smokers was lower, but not significantly less than that of the control group. These findings were consistent with the inhibitory effect of nicotine on osteoclast activity in *in vitro* studies [22]. The examined patients were generally younger in age and did not have a heavy, long term exposure to smoking, which might have lessened the smoking effect in the patients. Tang *et al* speculated that an increased life time exposure to smoking could negatively affect the local OPG production and RANKL/OPG ratio with a threshold of 20 pack years, greater than what was found in the patients of this study [19].

Lappin *et al* [20] found lower serum OPG concentrations in patients that smoked than in non-smokers and similar RANKL concentrations in both groups. Serum RANKL/OPG ratio was greater in smokers than in non-smokers. While both the present study and those reported by Lappin included healthy and periodontitis patients, Lappin also included patients with treated periodontitis. Ozcaka *et al* [22] performed a similar analysis and, in contrast to the findings of this study, observed no difference in the plasma RANKL and OPG concentrations of smokers and non-smokers with periodontitis. In contrast, the patients with periodontitis that smoked had significantly lower plasma OPG concentrations and a higher RANKL/OPG ratio than the smokers without periodontitis [23]. Similar to the patients of this study, RANKL/OPG ratio was higher in non-smoking periodontitis patients than in smoking ones. The evaluation of testing methods has shown that serum concentrations of RANKL and OPG were markedly lower than plasma concentrations, and that time of sample storage could have a strong negative impact on the test outcomes [24]. The larger number of patients was evaluated, the use of plasma concentrations, and differences in storage findings time and RANKL-OPG stability during the storage might have contributed to those different findings [25].

The previous reports have found a negative correlation, [12] positive correlation, [11, 26] or no correlation [27] between the local fluid concentrations of RANKL and RANKL/OPG ratio and the severity of periodontal disease. Some evidence of a correlation between serum findings and the extent and severity of the disease was identified. The patients evaluated with generalized disease (as defined by >30% of sites had 5mm or more probing depth) had a significantly greater serum RANKL concentration and RANKL/OPG ratio than the patients with localized disease. A significant correlation was found between serum RANKL concentration, and the percentage of PD sites >5mm and RANKL/OPG ratio was correlated to the amount of CAL. As far as it has been known, this is the first report that found this relation. And at the present time, the interrelationship between

OPG and RANKL has not been completely understood, it is important to remember that systemic RANKL and OPG levels may reflect a systemic inflammatory process that is not related to the periodontal diseases alone.
Although the use of biomarkers for the diagnosis and monitoring of periodontal disease progression is promising, it is important to remember that in multifactorial diseases like periodontal diseases, a complex array of biomarkers is involved. All of which, need to be investigated and validated with the existing criteria of the diseases, like clinical attachment and alveolar bone loss. It is unlikely that a single molecule will be reliable to diagnose and monitor the disease alone.
There were several limitations to this study. The small number of patients limited the power of this study to detect differences in study populations. Smoking history was based on self-reporting which may have led to underestimating the patient exposure to nicotine. Also, the groups compared were not matched for demographic and disease factors, which could have introduced a bias in the findings.

CONCLUSIONS

Serum RANKL concentrations were increased in patients with periodontitis and in non-smokers. Serum RANKL and RANKL/OPG ratio were increased in patients with more generalized advanced diseases. Longitudinal studies are needed in larger groups of patients in order to better understand the effect of periodontitis and smoking together on serum RANKL and OPG concentrations and RANKL/OPG ratio.

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Conflict of interest:
The author reported no conflict of interest in this work.

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