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Differential expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin mRNA in periodontal diseases

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Background and Objective: Receptor activator of nuclear factor-κB ligand (RANKL) is responsible for the induction of osteoclastogenesis and bone resorption, whereas its decoy receptor, osteoprotegerin, can directly block this action. Because this dyad of cytokines is crucial for regulating the bone remodelling process, imbalances in their expression may cause a switch from the physiological state to enhanced bone resorption or formation. This study investigated the mRNA expression of RANKL and osteoprotegerin, as well as their relative ratio, in the gingival tissues of patients with various forms of periodontal diseases.

Material and Methods: Gingival tissue was obtained from nine healthy subjects and 41 patients, who had gingivitis, chronic periodontitis, generalized aggressive periodontitis, and chronic periodontitis and were receiving immunosuppressant therapy. Quantitative real-time polymerase chain reaction was employed to evaluate the mRNA expression of RANKL and osteoprotegerin in these tissues.

Results: Compared with healthy individuals, patients in all periodontitis groups, but not those with gingivitis, exhibited stronger RANKL expression and a higher relative RANKL/osteoprotegerin ratio. In addition, osteoprotegerin expression was weaker in patients with chronic periodontitis. When patients with generalized aggressive periodontitis and chronic periodontitis were compared, the former exhibited stronger RANKL expression, whereas the latter exhibited weaker osteoprotegerin expression, and there was no difference in their relative ratio. When chronic periodontitis patients were compared with chronic periodontitis patients receiving immunosuppressant therapy, osteoprotegerin, but not RANKL, expression was stronger in the latter.

Conclusion: This study demonstrates that RANKL and osteoprotegerin expression are differentially regulated in various forms of periodontitis, and the relative RANKL/osteoprotegerin ratio appears to be indicative of disease occurrence. This information may confer diagnostic and therapeutic value in periodontitis.

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Bone resorption is regulated by the molecular interplay of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin, which are members of the tumor necrosis factor ligand and receptor families, respectively. RANKL is a predominantly membrane-bound ligand that is expressed by osteoblasts, fibroblasts and activated T- and B cells, and triggers the differentiation and activation of osteoclasts; the action of RANKL can be blocked by its soluble decoy receptor, osteoprotegerin (1). The expression of RANKL and osteoprotegerin is tightly regulated by systemic and local stimuli, including hormones, inflammatory mediators, and bacterial products (2). The balanced regulation of the RANKL–osteoprotegerin expression system can determine health from disease, as demonstrated in a number of bone destructive diseases, including bacterial arthritis, rheumatoid arthritis, and periodontitis (3–9). Changes in the relative RANKL/osteoprotegerin expression ratio affect the bone remodeling process, and an increase in this ratio is a preamble for bone resorption. Compared with healthy individuals, periodontitis patients exhibit increased RANKL expression in their periodontal tissues (10,11) or gingival crevicular fluid (12), reflecting an increased RANKL/osteoprotegerin ratio (13). Nevertheless, little is known about the differences of expression of RANKL and osteoprotegerin in tissue in various forms of periodontal diseases.

Treatment with immunosuppressive drugs has been shown to inhibit gingival inflammation and progression of periodontitis, resulting in a significant reduction of alveolar bone loss (14–18). As immunosuppressive drugs have been implicated in the progression of periodontal disease, chronic periodontitis patients under immunosuppressive medication, but with a potentially pathogenic bacterial load, may be a useful model for using to study periodontal disease mechanisms. To date there is no information regarding the expression of RANKL and osteoprotegerin in gingival tissues of chronic periodontitis patients under immunosuppressive treatment.

Therefore, this study aimed to compare RANKL and osteoprotegerin mRNA expression levels, as well as their relative expression ratio, in the gingival tissues of patients with gingivitis, chronic periodontitis and generalized aggressive periodontitis, in chronic periodontitis patients under immunosuppressive treatment, and in healthy subjects.

Material and methods

Study population and clinical examination

A total of 50 subjects were included in this study. All subjects were recruited from the Department of Periodontology, School of Dentistry (Ege University, Ýzmir). Written and informed consent was obtained from each subject prior to enrollment in the study. Complete medical and dental histories were taken from all subjects. None of the subjects had taken medication (such as antibiotics or contraceptives), which could affect their periodontal status, for at least 3 mo prior to the study. The immunosuppressant group consisted of renal transplant patients with chronic periodontitis who had been followed by the Nephrology Department at Ege University. These patients had been receiving cyclosporine A therapy for a minimum of 6 mo, and the cyclosporine A dose had been adjusted to maintain stable serum levels of 80–300 ng/mL. Cyclosporine A-treated patients also received azathioprine and prednisolone. Patients taking any other drugs, such as calcium channel blockers, reported to cause drug-induced gingival overgrowth, were excluded. No sign of graft rejection was detected in these renal transplant patients. Selection of the patients was made according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (19). To determine the clinical periodontal status, all subjects had a clinical periodontal examination, including the measurement of probing depth and clinical attachment loss. Dichotomous measurement of supragingival plaque accumulation and bleeding on probing were also recorded. Measurements were performed at six sites per tooth for the whole mouth.

Healthy group

The healthy group consisted of four women and five men ranging in age from 16 to 38 years with a mean age of 21.4 ± 7.6 years. They had no clinical signs of gingival inflammation (no bleeding on probing), exhibited a probing depth of < 3 mm, and had no radiographic evidence of alveolar bone loss.

Gingivitis group

The gingivitis group included four women and four men with varying degrees of gingival inflammation (bleeding on probing), but with no radiographic evidence of alveolar bone loss. These patients ranged in age from 16 to 59 years (mean age 34 ± 16.3 years).

Chronic periodontitis group

The chronic periodontitis group included five women and six men, ranging in age from 42 to 61 years, with a mean age of 50.1 ± 5.5 years. They had at least four sites with a probing depth of ≥ 6 mm and clinical attachment loss of ≥4 mm at the same site. A diagnosis of chronic periodontitis was made if the clinical attachment loss was commensurate with the amount of local factors of the patients.

Immunosuppressive drug-receiving chronic periodontitis group

The chronic periodontitis patients receiving immunosuppressant therapy included seven women and three men ranging in age from 16 to 54 years with a mean age of 34.8 ± 12.5 years. They had severe gingival overgrowth (Hyerplasia Index = 3), and exhibited at least four sites with a probing depth of ≥6 mm and clinical attachment loss of ≥4 mm at the same site.

Generalized aggressive periodontitis group

The generalized aggressive periodontitis group consisted of six women and six men between the ages of 18 and 39 years (mean of 28.9 ± 5.3 years). These patients demonstrated a generalized pattern of severe periodontal destruction and clinical attachment loss of ≥4 mm on eight or more teeth; at least three of those were other than central incisors or first molars. These patients showed severe periodontal tissue destruction and loss of periodontal support that were inconsistent with age and plaque levels.

Collection of gingival tissue samples

Gingival tissue samples, including both epithelium and connective tissue, were taken from the approximal sites of single rooted teeth prior to nonsurgical periodontal therapy in the instance of diseased subjects, and during tooth extractions for orthodontic reasons or crown-lengthening procedures in healthy subjects. One tissue sample from each subject was obtained and immediately submerged in a sterile tube containing RNAlater solution (Ambion, Inc., Huntington, UK) and stored at +4°C overnight, before long-term storage at −40°C until further laboratory analysis.

RNA extraction and reverse transcription

Total RNA from gingival tissue biopsies was extracted using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Briefly, RNA Lysis Buffer (175 μL per 30 mg of tissue) was added to the biopsy, inverted three or four times, and 350 μL of RNA Dilution Buffer was added to the lysate. The mixture was then heated at +70°C for 3 min, and then centrifuged at 12,000 ***g*** for 10 min. The nucleic acids were then precipitated with ethanol and bound to the silica surface of the glass fibers on the membrane. RNAse-free DNAseI (Promega) was directly applied to the silica membrane to digest contaminating genomic DNA. The bound RNA was further purified by subsequent washing steps (162.8 mM potassium acetate, 21.1 mM Tris–HCl). The total RNA was finally eluted from the membrane by the addition of nuclease-free water (Promega). The quality of RNA was assessed by agarose gel electrophoresis, and its concentration and purity were assayed by ultraviolet spectrophotometry. One microgram of RNA was used for the reverse transcription (RT) reaction, using the Protoscript First Strand cDNA Synthesis Kit (New England Bio-Laboratories Inc., Ipswich, MA, USA), according to the manufacturer's instructions. The cDNA was then stored at −20°C until the polymerase chain reaction (PCR) amplifications were carried out.

Quantitative real-time PCR

Quantitative real-time RT-PCR analyses for RANKL, osteoprotegerin and 18S rRNA were performed using an ABI Prism 7900HT Sequence Detection System and software (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used as an endogenous RNA control in the samples. The probes and the primers were synthesized by Applied Biosystems (Assay IDs RANKL: Hs00243522-m1, osteoprotegerin: Hs00171068-m1, and 18S rRNA: Hs99999901-s1) and the amplification reactions were performed with qPCR Master Mix (Abgene, Epsom, UK). The standard PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. The expression levels of RANKL and osteoprotegerin transcripts were calculated by using the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to the 18S rRNA.

Statistical analysis

Statistical analysis was performed using nonparametric techniques. Comparisons between all groups were performed using the Kruskal–Wallis test. When there were significant differences ($p < 0.008$), two-group comparisons were assessed with Mann–Whitney *U*-tests, and a *p*-value of < 0.05 was considered to be statistically significant. In order to analyze the correlations between RANKL and osteoprotegerin levels and clinical parameters, Spearman's rank correlation analysis was used and a *p*-value of < 0.01 was considered significant. All data analyses were performed using the SPSS 12.0 software.

Results

Clinical findings

The mean clinical data for the sampling areas are shown in Table 1. Chronic periodontitis and generalized aggressive periodontitis groups had significantly higher mean probing depth and clinical attachment loss scores of sampling sites than the healthy and gingivitis groups ($p < 0.05$). The mean probing depth and clinical attachment loss scores of sampling sites in chronic periodontitis patients receiving immunosuppressant therapy and in patients of the chronic periodontitis group were similar. All patient groups had a significantly higher percentage of sites with bleeding on probing and plaque compared with the healthy group. The generalized aggressive periodontitis, chronic periodontitis, chronic periodontitis patients receiving immunosuppressant therapy and gingivitis groups had a similar percentage of sites with bleeding on probing and plaque.

RANKL and osteoprotegerin expression analysis by real-time quantitative PCR

To determine differences in RANKL and osteoprotegerin gene expression in different clinical forms of periodontal diseases, quantitative real time RT-PCR was employed in nine healthy and 41 periodontally diseased gingival tissues. All of the gingival tissue samples studied expressed osteoprotegerin mRNA, but not all expressed detectable levels of RANKL mRNA (Figs 1B, 2B). RANKL was not detected in any samples of healthy tissue (0/9), but was detectable in 25% (2/8) of tissue

Table 1. Clinical parameters of the sampling areas in the study groups (mean ± SD)

	Healthy	Gingivitis	G-AgP	CP	IS-CP
PD (mm)	2 ± 0.26	2.75 ± 0.46[b]	7.45 ± 1.10[a]	6.36 ± 1.18[a]	5.8 ± 0.94[a]
CAL (mm)	0	0	8 ± 1.39[a]	8 ± 1.5[a]	6.3 ± 1.39[a]
Plaque (% of sites)	0	100[b]	100[b]	100[b]	100[b]
BOP (% of sites)	0	100[b]	100[b]	100[b]	100[b]
HI	–	–	–	–	3

[a]Significant difference from gingivitis and healthy groups.
[b]Significant difference from the healthy group (Kruskal–Wallis test, $p < 0.008$, Mann–Whitney *U*-test, $p < 0.05$).
CAL, clinical attachment loss; CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; HI, hyperplasia index; IS-CP, chronic periodontitis patients receiving immunosuppressant therapy; PD, probing pocket depth.

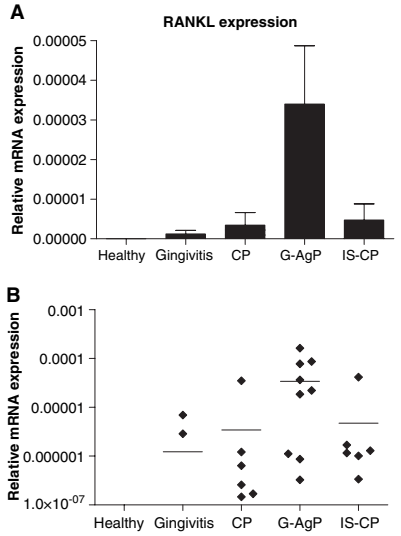

Fig. 1. (A) Receptor activator of nuclear factor-κB ligand (RANKL) gene expression in the gingival tissues of healthy subjects ($n = 9$) and of subjects with gingivitis ($n = 8$), chronic periodontitis ($n = 11$), generalized aggressive periodontitis ($n = 12$), and chronic periodontitis patients receiving immunosuppressant therapy ($n = 10$). The bars represent average values expressed as RANKL mRNA levels calibrated to housekeeping gene mRNA levels (RANKL/18S RNA). The error bars represent the standard error of the mean. *$p < 0.05$. (B) Distribution of RANKL mRNA expression among subjects. The graph is plotted on a logarithmic scale. CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; IS-CP, chronic periodontitis patients receiving immunosuppressant therapy.

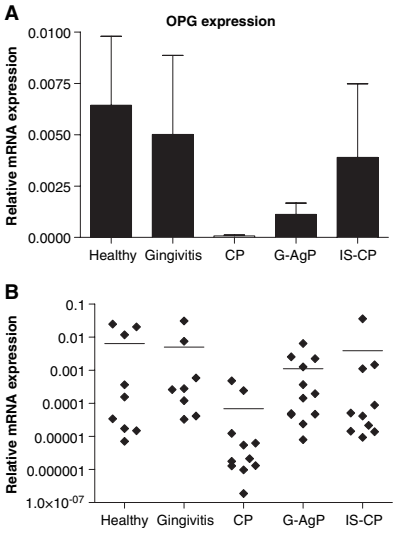

Fig. 2. (A) Osteoprotegerin gene expression in the gingival tissues of healthy subjects ($n = 9$) and of subjects with gingivitis ($n = 8$), chronic periodontitis ($n = 11$), generalized aggressive periodontitis ($n = 12$) and chronic periodontitis patients receiving immunosuppressant therapy ($n = 10$). The bars represent average values expressed as osteoprotegerin mRNA levels calibrated to housekeeping gene mRNA levels (OPG/18SRNA). The error bars represent the standard error of the mean. *$p < 0.05$. (B) Distribution of receptor activator of nuclear factor-κB ligand (RANKL) mRNA expression among subjects. The graph is plotted on a logarithmic scale. CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; IS-CP, chronic periodontitis patients receiving immunosuppressant therapy.

samples from patients with gingivitis, in 54% (6/11) of tissue samples from patients with chronic periodontitis, in 75% (9/12) of tissue samples from patients with generalized aggressive periodontitis, and in 60% (6/10) of tissue samples from patients with chronic periodontitis receiving immunosuppressant therapy (Fig. 1A). In contrast, all of the tissue samples expressed detectable levels of osteoprotegerin. In comparison with healthy tissues, RANKL expression was significantly ($p < 0.05$) induced in all periodontitis groups (chronic periodontitis, generalized aggressive periodontitis, chronic periodontitis patients receiving immunosuppressant therapy), but not in gingivitis (Fig. 1B). In addition, RANKL levels in generalized aggressive periodontitis were 10-fold higher than in chronic periodontitis ($p = 0.048$). Compared with healthy tissues, osteoprotegerin expression was decreased in all disease groups (Fig. 2A), but this was statistically significant only in chronic periodontitis ($p = 0.004$). Moreover, osteoprotegerin levels in chronic periodontitis were 16-fold lower than in generalized aggressive periodontitis ($p = 0.002$).

As the relative RANKL/osteoprotegerin expression ratio is considered indicative of bone resorption, we investigated if changes in RANKL and osteoprotegerin expression reflected changes in this ratio. In comparison with healthy tissues, this ratio was significantly higher in all periodontitis groups ($p < 0.05$), but not in gingivitis (Fig. 3A). There was no significant difference between generalized aggressive periodontitis and chronic periodontitis.

To investigate if administration of immunosuppressive medication has any effect on RANKL and osteoprotegerin expression, their expression levels were compared in gingival tissues of chronic periodontitis and chronic periodontitis patients receiving immunosuppressant therapy. Osteoprotegerin expression was significantly higher in chronic periodontitis patients receiving immunosuppressant therapy than in patients with chronic periodontitis ($p = 0.005$) by 56-fold. However, there was no difference in RANKL expression, and the relative RANKL/osteoprotegerin ratio was not significantly different between these groups ($p > 0.05$).

To test whether there was an association of RANKL and osteoprotegerin expression levels with clinical parameters, further analysis

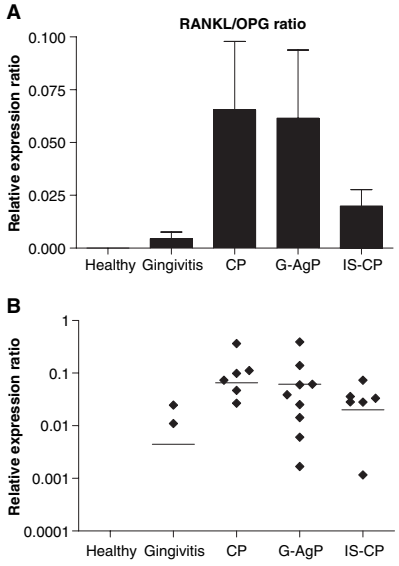

Fig. 3. (A) Relative Receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin gene expression ratio in the gingival tissues of healthy subjects ($n = 9$) and of subjects with gingivitis ($n = 8$), chronic periodontitis ($n = 11$), generalized aggressive periodontitis ($n = 12$), and chronic periodontitis patients receiving immunosuppressant therapy ($n = 11$). The bars represent average values of the relative RANKL/osteoprotegerin mRNA levels, calibrated to a housekeeping gene (18S RNA). The error bars represent the standard error of the mean. *$p < 0.05$. (B) Distribution of RANKL mRNA expression among subjects. The graph is plotted on a logarithmic scale. CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; IS-CP, chronic periodontitis patients receiving immunosuppressant therapy.

was performed. Spearman's rank correlation analysis (Table 2) indicated that the expression of RANKL was positively correlated with clinical attachment loss, probing depth, bleeding on probing, and the presence of plaque ($R = 0.464$, 0.508, 0.407, respectively, $p < 0.01$). Moreover, the relative RANKL/osteoprotegerin ratio was significantly correlated with clinical attachment loss, probing depth, and bleeding on probing ($R = 0.499$, 0.474, 0.407, respectively, $p < 0.01$). However, the expression of osteoprotegerin did not correlate with any of these clinical parameters (Table 2).

Table 2. Correlations between receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), and RANKL/OPG and clinical parameters

Clinical parameters	RANKL	OPG	RANKL/OPG
PD	0.508[a]	0.11	0.474[a]
CAL	0.464[a]	0.167	0.499[a]
BOP (%)	0.407[a]	0.004	0.407[a]
Plaque (%)	0.387[a]	0.004	0.387[a]

Spearman's rank correlation analysis was used.
[a]Significant at $p < 0.01$.
BOP, bleeding on probing; CAL, clinical attachment loss; PD, probing pocket depth.

Discussion

The regulation of bone resorption is orchestrated by RANKL and its cognate inhibitor, osteoprotegerin. Disruption of their balanced expression leads to bone loss, as demonstrated in experimental models of bone destructive diseases, including periodontitis (4,20). The present study investigated the levels of RANKL and osteoprotegerin mRNA expression in gingival tissue samples from healthy subjects and patients with periodontal diseases, by using quantitative real-time PCR. RANKL was expressed in more than 50% of periodontitis tissues, but only in 25% of gingivitis tissues. RANKL levels were significantly induced in all three forms of periodontitis studied, but not in gingivitis, and the most prominent increase was observed in generalized aggressive periodontitis. On the contrary, osteoprotegerin was expressed by all tissues, and its levels markedly decreased in chronic periodontitis, but not in the other forms of periodontal disease. The present findings corroborate previous reports demonstrating higher frequency and expression levels of RANKL (10,11,13,21), and lower expression levels of osteoprotegerin (11,13), in the gingival tissues of patients with periodontitis compared with healthy patients. Enhanced RANKL and decreased osteoprotegerin protein levels have also been demonstrated in the gingival crevicular fluid of chronic periodontitis patients (12). However, when generalized aggressive periodontitis and chronic periodontitis were compared, the present findings contrast a previous report showing no differences in RANKL levels between the two forms of the disease, and higher osteoprotegerin levels in chronic periodontitis than in generalized aggressive periodontitis (10). This discrepancy may arise from different protocols employed for the collection of the gingival tissues, or potential differences in the disease progression stage at the sampling time-point.

The differences in RANKL and osteoprotegerin expression reflected changes in their relative ratio, which, compared with health was significantly increased in all periodontitis groups. However, in the gingivitis group this ratio was low and not statistically significant from the healthy group. Moreover, this ratio was similar in generalized aggressive periodontitis and chronic periodontitis patients. Taken together, these findings imply that an increased RANKL/osteoprotegerin ratio is indicative of periodontitis occurrence, in agreement with previous reports (12,13). In addition, gingival tissue RANKL and osteoprotegerin expression appear to be differentially regulated, depending on the form of periodontitis. One possible explanation is that the prolonged and slow progression rate of chronic periodontitis may allow ample periods of infection establishment, to which periodontal tissues may adapt by down-regulating osteoprotegerin. On the other hand, the high incidence and expression levels of RANKL in the studied tissue samples may reflect the frequent and severe destruction bursts observed in generalized aggressive periodontitis. Alternatively, because differences in oral microbial compositions are correlated with different innate responses (22), it should not be excluded that the differential regulation of RANKL and osteoprotegerin expression in chronic periodontitis and

generalized aggressive periodontitis may account for their bacterial specificities. To this extent, *Actinobacillus actiomycetemcomitans*, which is highly associated with localized aggressive periodontitis, is shown to be a strong inducer of RANKL in T cells (3,4) and periodontal fibroblasts (23).

In the present study, RANKL and osteoprotegerin were also expressed in chronic periodontitis patients receiving immunosuppressive medication. Immunosuppression has been implicated in the progression of periodontal disease (14–17,24). Gingival specimens were taken from renal transplant recipients, under immunosuppressive medication regimen comprising cyclosporine A, prednisone and azathioprine, all of which have been shown to impair immune responses (25), such as inhibition of osteoclast formation (26–29) and bone resorption (16,18), as well as regulation of RANKL and osteoprotegerin expression (30–34). The present study demonstrated a significant enhancement of osteoprotegerin expression in chronic periodontitis patients receiving this combined immunosuppressant regime, compared with otherwise healthy patients with chronic periodontitis. Although it is difficult to evaluate the contribution of each drug individually in the overall effect, this regulation could be a putative mechanism involved in the inhibition of alveolar bone destruction in chronic periodontitis patients receiving immunosuppressant therapy. As T cells are a major source of RANKL in rheumatoid and adjuvant arthritis, as well as in periodontitis (4,7,35), and because chronic periodontitis patients receiving immunosuppressant therapy have impaired T-cell functions, it is possible that RANKL expression in the gingival tissues of chronic periodontitis patients receiving immunosuppressant therapy may derive from resident connective tissue and bone cells. This may not be surprising, as localization of RANKL in gingival tissues with periodontitis has also been identified in the resident periodontal tissue cells, apart from leukocyte infiltrates (11,13).

In the present study, the expression levels of RANKL, as well as the relative RANKL/osteoprotegerin ratio, were significantly correlated with probing depth, clinical attachment loss, and bleeding on probing. On the contrary, osteoprotegerin expression levels were not correlated with these clinical parameters. Although previous studies failed to correlate RANKL expression levels with these clinical parameters, there is evidence that RANKL, but not osteoprotegerin, expression correlates with the severity of chronic periodontitis (13).

In conclusion, the present study strengthens the involvement of RANKL and osteoprotegerin in periodontal diseases and demonstrates that the respective genes may be differentially regulated in the various forms of the disease. This information may provide adjunct diagnostic markers for periodontitis, but further studies are required to confirm this potential. It may also indicate the necessity for individual treatment approaches, based on controlling the osteolytic cytokines preferentially regulated in the different forms of the disease.

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