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Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio

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Abstract

Aim: Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) are a system of molecules that regulate bone resorption. This study aims to compare the levels of RANKL, OPG and their relative ratio in gingival crevicular fluid (GCF) of healthy and periodontal disease subjects.

Material and Methods: GCF was obtained from healthy ($n = 21$), gingivitis ($n = 22$), chronic periodontitis ($n = 28$), generalized aggressive periodontitis ($n = 25$) and chronic periodontitis subjects under immunosuppressant therapy ($n = 11$). RANKL and OPG concentrations in GCF were measured by enzyme-linked immunosorbent assays.

Results: RANKL levels were low in health and gingivitis groups, but increased in all three forms of periodontitis. OPG levels were higher in health than all three periodontitis, or gingivitis groups. There were no differences in RANKL and OPG levels between chronic and generalized aggressive periodontitis groups, whereas these were lower in the immunosuppressed chronic periodontitis group. The RANKL/OPG ratio was significantly elevated in all three periodontitis forms, compared with health or gingivitis, and positively correlated to probing pocket depth and clinical attachment level.

Conclusion: GCF RANKL and OPG levels were oppositely regulated in periodontitis, but not gingivitis, resulting in an enhanced RANKL/OPG ratio. This ratio was similar in all three periodontitis groups and may therefore predict disease occurrence.

Key words: bone resorption; ELISA; gingival crevicular fluid; OPG; RANKL

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Gingival crevicular fluid (GCF) is regarded as a window for non-invasive analysis of periodontal conditions, including markers of connective tissue and bone destruction (Uitto 2003). The resorption of bone is regulated by the molecular interplay of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), a system of two molecules belonging to the tumour necrosis factor ligand and receptor families, respectively (Lerner 2004). RANKL is expressed predominantly as a membrane-bound ligand on osteoblasts, fibroblasts and activated T and B cells, and its osteoclastogenic action can be blocked by its soluble decoy receptor OPG (Teitelbaum 2000, Lerner 2006, Teng 2006). The expression of RANKL and OPG are tightly regulated by systemic and local stimuli, including hormones, inflammatory mediators, bacterial products, and immunosuppressive drugs (Lerner 2004).

The key roles of RANKL and OPG expression in the regulation of bone destruction has been demonstrated in several in vivo disease models, including bacterial arthritis, rheumatoid arthritis, and periodontitis (Horwood et al. 1999, Kong et al. 1999, Teng et al. 2000, Firestein 2003, Sakurai et al. 2003, Taubman et al. 2005). Recent clinical studies have confirmed that both RANKL and OPG can be detected in

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human GCF, and indicate that RANKL is elevated, whereas OPG is decreased in periodontitis, or during orthodontic tooth movement (Mogi et al. 2004, Vernal et al. 2004, Kawasaki et al. 2006, Lu et al. 2006, Nishijima et al. 2006). Another line of studies have focused on the expression of these molecules in diseased periodontal tissues, rather than in GCF (Crotti et al. 2003, Liu et al. 2003, Garlet et al. 2004, Kawai et al. 2006, Lu et al. 2006). We have recently shown that RANKL and OPG gene expressions are differentially regulated in gingival tissues depending on the form of periodontal disease, and that an increase in the RANKL/OPG expression ratio in the tissues may indicate the occurrence of periodontitis (Bostanci et al. 2007). Nevertheless, at present there is no information on putative differences in RANKL and OPG levels in GCF among patients with various forms of periodontal diseases.

Immunosuppressive drugs have been shown to inhibit the progression of periodontitis resulting in reduction of alveolar bone loss (Tollefsen & Johansen 1985, Fischer et al. 1996, Saether et al. 1998, Nassar et al. 2004, Spolidorio et al. 2004). To date there is no information regarding the RANKL and OPG levels in GCF of chronic periodontitis patients under immunosuppressive treatment.

Therefore, the aim of this study was to investigate RANKL and OPG levels, as well as their relative ratio in GCF of patients with gingivitis, chronic periodontitis, generalized aggressive periodontitis, and chronic periodontitis under immunosuppressive treatment, as well as healthy subjects.

Material and Methods

Study population and clinical examination

A total of 107 subjects were included in this study. All subjects were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir. Written and informed consent was obtained from each subject before enrolment 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 that could affect their periodontal status for at least 3 months before the study. The immunosuppressant group consisted of renal transplant patients with chronic periodontitis who had been followed by the Nephrology Department at the Ege University. They were on cyclosporin-A (CsA) therapy for a minimum of 6 months and the CsA dose was adjusted to maintain stable serum levels between 80 and 300 ng/ml. CsA-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 signs of graft rejection were detected in these renal transplant patients. The 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 (Armitage 1999). To determine the clinical periodontal status, all subjects had a clinical periodontal examination including the measurement of probing pocket depth (PPD) and clinical attachment level (CAL) at six sites around each tooth with a manual probe (Williams probe). The full-mouth papilla bleeding index (PBI) and plaque index (PI) were also recorded. The degree of gingival overgrowth was classified based on the criteria of Angelopoulos and Goaz (1972).

Healthy group

The healthy group consisted of 11 females and 10 males ranging in age from 16 to 59 years with a mean age of 29.62 ± 15.07 years. They had no clinical signs of gingival inflammation (no bleeding on probing), exhibited PPD < 3 mm, and no radiographic evidence of alveolar bone loss.

Gingivitis group

The gingivitis group included nine females and 13 males with varying degrees of gingival inflammation, but with no radiographic evidence of alveolar bone loss. These patients ranged in age from 14 to 63 years (mean age 31.05 ± 15.07 years).

Generalized aggressive periodontitis (G-AgP) group

The G-AgP group consisted of 17 females and nine males between the ages of 18 and 38 years (mean of 29.48 ± 4.98 years). These patients demonstrated a generalized pattern of severe periodontal destruction and CAL $\geqslant 4$ 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 inconsistent with age and plaque levels.

Chronic periodontitis (CP) group

The CP group included 13 females and 15 males ranging in age from 39 to 64 years, with a mean age of 51 ± 6.65 years. They had at least four sites with a PPD $\geqslant 6$ mm and CAL $\geqslant 4$ mm at the same site. Diagnosis of CP was made if the CAL was commensurate with the amount of local factors of the patients.

Immunosuppressive drug receiving chronic periodontitis (IS-CP) group

The IS-CP group included eight females and three males ranging in age from 16 to 54 years with a mean age of 34.45 ± 11.68 years. They had severe gingival overgrowth (HPI = 3), and exhibited at least four sites with a PPD $\geqslant 6$ mm and CAL $\geqslant 4$ mm at the same site.

Collection of GCF

After recruitment to the study, subjects were recalled for GCF sampling. GCF samples were collected from the mesiobuccal aspect of a single-rooted tooth exhibiting PPD of 6–8 mm. In the gingivitis group, GCF samples were collected from the mesiobuccal aspect of single-rooted teeth with $\leqslant 3$ mm PPD and with bleeding on probing, but without CAL. In the healthy group, GCF samples were collected from the mesiobuccal aspect of single-rooted teeth exhibiting PPD up to 3 mm without bleeding on probing. The selected sites were cleared of supragingival plaque, isolated with cotton rolls and dried with a gentle stream of air to prevent saliva contamination. A sterile Periopaper strip (ProFlow Inc., Amityville, NY, USA) was gently inserted into the periodontal pocket and left in place for 30 s. Mechanical irritation was avoided and strips contaminated with blood were discarded.

The GCF sample volume was measured with a calibrated Periotron 8000 (Periotron 8000, Proflow Inc., Amityville, NY, USA) and then the readings were converted to an actual volume (μl)

by reference to the standard curve. All the samples were lyophilized and stored at −80°C before laboratory analysis. For analysis, 200 μl of phosphate-buffered saline (PBS, pH 7.2) was used to re-elute the samples. The tubes were shaken gently for 1 min and then centrifuged at 2000 × *g* for 15 min at 4°C, before being processed on the enzyme-linked immunosorbent assays (ELISA) plate.

RANKL and OPG analysis in GCF

The amount of RANKL and OPG in the GCF samples was determined using commercially available human-specific ELISA in accordance with the manufacturer's instructions (total sRANKL ELISA kit: Immundiagnostik AG, Bensheim, Germany, and Osteoprotegerin ELISA kit: Biomedica, Vienna, Austria). These assays measure the total levels of RANKL or OPG present in the GCF, including both their unbound-free forms, and their RANKL-OPG complex form. Calculation of the RANKL and OPG concentration in each GCF sample was performed by dividing the total amount of RANKL or OPG by the volume of the sample [RANKL or OPG concentration (pg/μl) = total RANKL or OPG (pg)/volume (μl)].

Statistical analysis

Statistical analysis was performed using non-parametric methods. 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 $p<0.05$ was considered to be statistically significant. In order to analyse the correlations between GCF RANKL and OPG levels and clinical parameters, Spearman's rank correlation analysis was used and $p<0.01$ was considered as significant. All data analyses were performed using the SPSS 12.0 software.

Results

Clinical findings of sampling sites

The demographic and clinical data are shown in Table 1. CP and G-AgP groups had significantly higher mean PPD and CAL scores of sampling sites than the healthy and gingivitis groups ($p<0.05$). The mean PPD and CAL scores of sampling sites in IS-CP and CP groups were similar. No significant differences were detected between gingivitis and periodontitis groups regarding the PBI and PI of sampling sites ($p>0.05$).

Analysis of RANKL and OPG concentrations in GCF

Healthy and gingivitis subjects demonstrated equally low levels of RANKL, which were 6.8 ± 3.8 and 9 ± 5.7 pg/μl, respectively (Fig. 1). RANKL was detected in only seven of 21 healthy subjects and nine of 22 gingivitis patients. However, RANKL was detected in GCF from of all periodontitis sites, and its levels were significantly elevated in all three periodontitis groups (CP: 370.7 ± 54.7 pg/μl, G-AgP 387.5 ± 42.8 pg/μl, IS-CP 168.1 ± 36 pg/μl), compared with both healthy or gingivitis groups ($p<0.05$) (Fig. 1). There was no difference in RANKL levels between CP and G-AgP groups, although this was approximately 2.2-fold lower in the IS-CP group, compared with the CP group.

In contrast to RANKL, OPG was detected in all GCF samples (Fig. 2). Among all groups, OPG levels were highest in healthy subjects (408.1 ±

Table 1. Demographic parameters of subjects and clinical parameters of the sampling areas in the study groups (mean ± SD)

	Healthy	Gingivitis	G-AgP	CP	IS-CP
Gender F:M	11:10	9:13	17:9	13:15	8:3
Age	29.6 ± 15.0	31.0 ± 15.1	29.4 ± 4.9	51.0 ± 6.6	34.4 ± 11.6
PPD (mm)	1.9 ± 0.3	2.86 ± 0.34*	7.64 ± 1.72†	6.33 ± 1.30†	5.77 ± 0.8†
CAL (mm)	0	0	8.4 ± 2.16†	7.8 ± 1.27†	6.2 ± 1.31†
PI	0	1.82 ± 0.72*	1.2 ± 0.5*	1 ± 0.38*	2 ± 0.97*
PBI	0	2.36 ± 0.83*	2.20 ± 1.49*	2.03 ± 1.42*	2.45 ± 1.59*
HI	–	–	–	–	3
GCF (μl)	0.11 ± 0.05	0.30 ± 0.15*	0.43 ± 0.24†	0.38 ± 0.17*	0.74 ± 0.24†

*Significant difference from healthy group (Kruskal–Wallis test, $p<0.008$, Mann–Whitney *U*-test, $p<0.05$).

†Significant difference from gingivitis and healthy groups.

PPD, probing pocket depth; CAL, clinical attachment loss; PI, plaque index; PBI, papilla bleeding index; CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; IS-CP, immunosuppressive drug receiving chronic periodontitis group; HI, hyperplasia index.

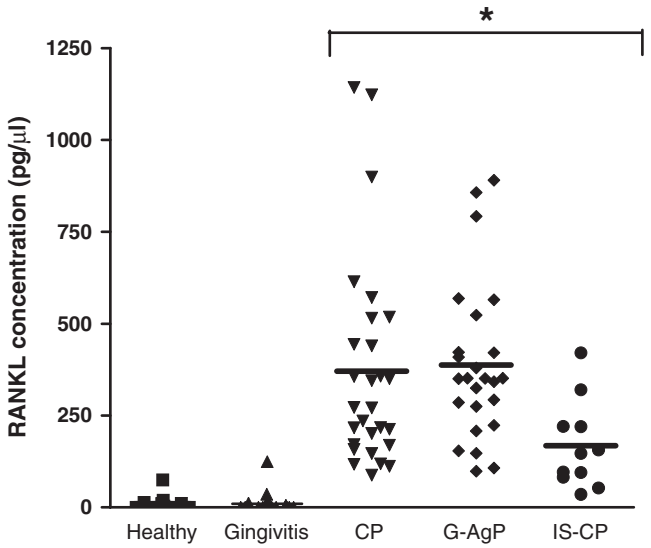

Fig. 1. Distribution of receptor activator of NF-κB ligand (RANKL) levels in gingival crevicular fluid (GCF) from healthy ($n=21$), gingivitis ($n=22$), chronic periodontitis (CP) ($n=28$), generalized aggressive periodontitis (G-AgP) ($n=25$) and immunosuppressive drug receiving chronic periodontitis (IS-CP) ($n=11$) subjects. The individual values represent the GCF concentration of RANKL [total RANKL (pg)/volume (μl)] in each subject. *Groups that are significantly different to the healthy group.

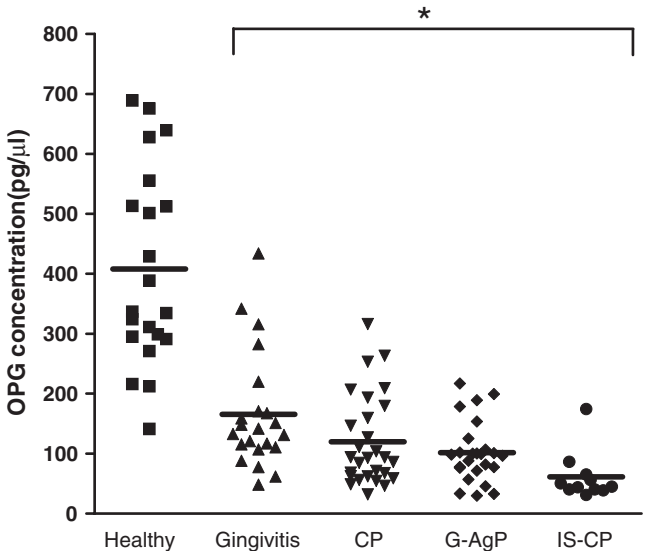

Fig. 2. Distribution osteoprotegerin (OPG) levels in gingival crevicular fluid (GCF) from healthy ($n = 21$), gingivitis ($n = 22$), CP ($n = 28$), generalized aggressive periodontitis (G-AgP) ($n = 25$) and immunosuppressive drug receiving chronic periodontitis (IS-CP) ($n = 11$) subjects. The individual values represent the GCF concentration of OPG [total OPG (pg)/volume (μl)] in each subject. *Groups that are significantly different to the healthy group.

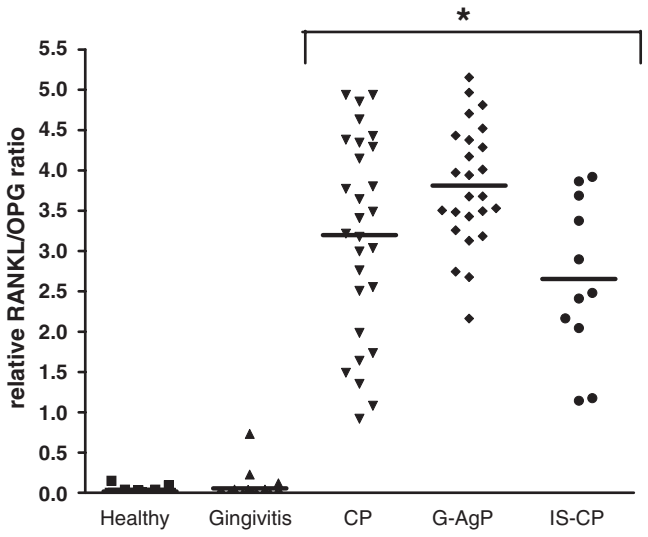

Fig. 3. Distribution of relative receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio levels in gingival crevicular fluid (GCF) from healthy ($n = 21$), gingivitis ($n = 22$), CP ($n = 28$), generalized aggressive periodontitis (G-AgP) ($n = 25$) and immunosuppressive drug receiving chronic periodontitis (IS-CP) ($n = 11$) subjects. The individual values represent the GCF values for RANKL/OPG ratios [total RANKL (pg)/volume (μl)/ total OPG (pg)/volume (μl)] in each subject. *Groups that are significantly different to the healthy group.

36.6 pg/μl), but this was significantly decreased in all four diseased groups (gingivitis: 167.6 ± 20.6 pg/μl, CP: 119.6 ± 14.4 pg/μl, G-AgP 101.5 ± 10.2 pg/μl, and IS-CP: 61.2 ± 12.3 pg/μl) (Fig. 2). There was no difference in GCF OPG levels between CP and G-AgP groups, but in the IS-CP group this was significantly lower compared with CP.

The relative RANKL/OPG ratio was further investigated. Healthy subjects, as well as gingivitis patients exhibited a low RANKL/OPG ratio, which was 0.017 ± 0.008 and 0.053 ± 0.034, respectively (Fig. 3). However, this ratio was significantly higher in all periodontitis groups (CP: 3.19 ± 0.23, G-AgP 3.81 ± 0.15, and IS-CP 2.65 ± 0.3), but there were no significant differences among these groups (Fig. 3).

Correlation of RANKL and OPG and relative ratio with clinical parameters

The correlation of RANKL, OPG and their relative ratio with clinical parameters was investigated by Spearman's rank correlation analysis (Table 2). RANKL was positively correlated with PPD, CAL ($p < 0.01$), and PI ($p < 0.05$), but not with PBI. In contrast, OPG was negatively correlated with PPD, CAL, PBI, and PI ($p < 0.01$). Finally, the relative RANKL/OPG ratio was positively correlated with PPD, CAL ($p < 0.01$), and PI ($p < 0.05$), but not with PBI.

Discussion

Local deregulation of the RANKL-OPG interplay may lead to alveolar bone resorption as has been demonstrated in experimental periodontitis models (Teng et al. 2000, Taubman et al. 2005). In the present study, we analysed the levels of RANKL, OPG, as well as their relative ratio, in GCF from healthy and periodontally diseased subjects. RANKL levels were significantly elevated in all three forms of periodontitis studied, but not in gingivitis. These results are in agreement with previous studies demonstrating that compared with healthy subjects, periodontitis patients exhibit higher RANKL expression in GCF (Mogi et al. 2004, Vernal et al. 2004, Lu et al. 2006), or gingival tissues (Nagasawa et al. 2002, Crotti et al. 2003, Liu et al. 2003, Garlet et al. 2004, Bostanci et al. 2007). In contrast, OPG concentration in GCF was significantly decreased in all periodontal disease groups, including gingivitis. The demonstrated reduction of OPG levels in CP compared with health is in line with earlier studies performed in either GCF (Mogi et al. 2004), or gingival tissues (Crotti et al. 2003, Liu et al. 2003). Nonetheless, a recent study failed to detect OPG in any of the GCF samples from healthy subjects (Lu et al. 2006). Such differences may account for variant levels of sub-clinical inflammation among healthy subjects, or for differences in the sensitivity of various ELISA kits employed in each study.

Table 2. Correlations between RANKL, OPG, and their ratio with clinical parameters

Clinical parameters	RANKL	OPG	RANKL/OPG
PPD	0.771**	− 0.588**	0.809**
CAL	0.796**	− 0.534**	0.841**
PBI	0.131	− 0.407**	0.155
PI	0.212*	− 0.446**	0.207*

Spearman's rank correlation analysis was used.
**$p < 0.01$, *$p < 0.05$.
PPD, probing pocket depth; CAL, clinical attachment loss; PI, plaque index; PBI, papilla bleeding index.

The GCF levels of both RANKL and OPG were similar in CP and G-AgP. This finding could imply that the RANKL-OPG system is equivocally regulated in these two forms of periodontitis. However, we have recently shown that both RANKL and OPG mRNA expression are higher in gingival tissues of G-AgP compared with CP patients, but this resulted in a similar RANKL/OPG ratio between these two groups (Bostanci et al. 2007). Moreover, a previous study demonstrated that OPG gene expression is higher in gingival tissues of CP, compared with G-AgP patients, whereas there were no differences in RANKL expression (Garlet et al. 2004). These inconsistencies between tissue expression and GCF levels of these cytokines highlight the complexity of clinical data interpretation, and may relate to a combination of factors such as variations in disease progression stage. In addition, there could be a lag time between the production of these molecules in the tissue and their subsequent release into the periodontal pocket microenvironment.

Immunosuppression has been implicated in the progression of periodontal diseases (Tollefsen & Johansen 1985, Seymour et al. 1987, Saether et al. 1998, Nassar et al. 2004). In the present study, RANKL and OPG levels in GCF of IS-CP patients were also investigated. The medication regime comprised of CsA, prednisone, and azathioprine, all of which have been shown to impair immune responses (Hayes 1993), including inhibition of osteoclast formation (Orcel et al. 1991, Awumey et al. 1999, Ishida et al. 2002, Fuller et al. 2006) and bone resorption (Nassar et al. 2004, Spolidorio et al. 2004). The present study demonstrates a significant reduction of both OPG and RANKL GCF levels in IS-CP patients, compared with CP patients. Although it is difficult to evaluate the relative contribution of each drug individually, these medications have been previously shown to regulate RANKL and OPG expression (Hofbauer et al. 2001, Malyszko et al. 2003, Humphrey et al. 2006, Miyazaki et al. 2006). Patients under immunosuppressive medication have impaired T-cell responses, and since these are a crucial source of RANKL in periodontal disease (Taubman & Kawai 2001, Taubman et al. 2005, Kawai et al. 2006, Teng 2006), a reduction of RANKL levels in the GCF of IS-CP patients would be expected.

Alterations of RANKL and OPG levels reflected changes in their relative RANKL/OPG concentration ratio in GCF. Compared with health, this ratio was significantly higher in all periodontitis groups, which is in agreement with previous reports (Liu et al. 2003, Mogi et al. 2004), but was notably not altered in the gingivitis group. No significant differences in RANKL/OPG ratio were detected among the periodontitis groups. Importantly, these RANKL/OPG concentration ratio trends are in direct agreement with our recent findings of RANKL/OPG mRNA expression ratios in gingival tissues (Bostanci et al. 2007). Collectively, these data indicate that gingival tissue and GCF RANKL/OPG ratios are regulated in a similar manner in periodontal diseases, and suggest that an increase in this ratio may denote the occurrence of periodontitis.

Moreover, the present study demonstrates a positive correlation of the RANKL/OPG ratio in GCF with PPD and CAL, but not with PBI. Accordingly, we have recently demonstrated a positive correlation between the RANKL/OPG mRNA expression ratio in gingival tissues and these clinical parameters (Bostanci et al. 2007). In agreement with our results, a previous study has demonstrated that RANKL GCF levels are positively correlated with PPD and CAL in chronic periodontitis patients (Vernal et al. 2004). These findings strengthen further the diagnostic value of the RANKL/OPG ratio, as they indicate that it may associate with the progression of periodontal destruction, rather than the state of periodontal inflammation.

In conclusion, this study provides further evidence of a possible role of RANKL upregulation and OPG downregulation in periodontal diseases. However, monitoring RANKL or OPG regulation individually may not provide adequate information on the disease state. It is suggested that RANKL and OPG regulation should be studied concomitantly as their relative ratio, as this is consistently elevated in both GCF (present study) and gingival tissues (Bostanci et al. 2007) with the occurrence of periodontitis. Therefore, defining the RANKL/OPG concentration ratio in GCF may prove to be an important indicator of periodontitis, as it mirrors the relative expression ratio in the tissue, and especially as GCF collection is a non-invasive approach. Moreover, as adjunctive host response modulation therapies may be an advantageous approach to periodontal disease management (Salvi & Lang 2005), targeting RANKL and OPG regulation by the host may prove to have therapeutic value in this respect.

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Clinical Relevance

Scientific rationale for the study: RANKL induces, whereas OPG inhibits bone resorption, and their local molecular interplay regulates bone remodelling. Increase in the RANKL/OPG ratio may indicate bone destruction. It is hypothesized that this ratio may be increased in the GCF of periodontitis patients.

Principal findings: GCF levels of RANKL were increased, whereas OPG were decreased in periodontitis compared with health, resulting in an increase of their relative ratio. This was also correlated with PPD and CAL.

Practical implications: Increase in the relative RANKL/OPG ratio in GCF is indicative of the occurrence of periodontitis. This may confer a clinical diagnostic potential.