

## Relation of soluble RANKL and osteoprotegerin levels in blood and gingival crevicular fluid to the degree of root resorption after orthodontic tooth movement

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**Abstract:** The aim of the present study was the determination of the levels of osteoprotegerin and soluble RANKL in blood serum and in gingival crevicular fluid relative to the degree of orthodontic root resorption in a rat model. Blood samples and gingival crevicular fluid were collected from fourteen 6-month-old male Wistar rats weighing 350-500 g. A 25-g closed orthodontic coil spring was inserted between each upper right first molar and the upper incisors. After 21 days of loading, both upper first molars (treated and control) were extracted and studied under microcomputed tomography scanning. Statistical analysis demonstrated a positive linear correlation between the initial concentration of RANKL in blood serum and the degree of root resorption. The ratio of the initial concentrations of osteoprotegerin to RANKL in blood serum proved to be an independent prognostic factor of the degree of root resorption. The initial concentration of RANKL in gingival crevicular fluid showed a negative correlation to the initial concentration of RANKL in blood serum and for a finite range of initial concentrations of osteoprotegerin in gingival crevicular fluid, the dental root seemed protected against extreme external root resorption. Finally, the concentration of osteoprotegerin in blood serum decreased significantly in cases of severe root resorption.

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### Introduction

Osteoprotegerin (OPG), receptor activator of nuclear factor-(KB) ligand (RANKL), and its cognate receptor RANK, are protein ligands. They share homologies with members of the tumor necrosis factor receptor superfamily and function as paracrine regulators of osteoclastogenesis and bone metabolism (1-5). Osteoprotegerin lacks transmembrane and cytoplasmic domains and is secreted as a soluble protein, mainly by osteoblastic lineage cells (6-8). The primary biologic actions of OPG are inhibition of osteoclast differentiation, inhibition of osteoclast resorptive function, and stimulation of osteoclast apoptosis (9).

RANK is a 616-amino-acid peptide on the cell surface of osteoclast precursors (2). RANKL is a 317-amino-acid peptide produced by osteoblastic lineage cells and activated T-cells. When RANKL is expressed by osteoblastic lineage cells, it is cell-bound and when it is expressed by T-lymphocytes, it is soluble (sRANKL) (1). The role of RANKL, together with another very important protein ligand, M-CSF (which binds to its receptor c-fms), is to promote osteoclast formation, fusion, differentiation, activation, and survival, thus enhancing bone resorption (10-19). The biological effects of RANKL are produced when it binds to RANK. The biological effects of OPG

are opposite to the RANKL-mediated effects, because OPG acts as a soluble receptor antagonist that neutralizes RANKL and thereby prevents RANKL-RANK interaction (20).

These ligands also appear to be key regulators of bone remodeling during orthodontic tooth movement (9). During orthodontic tooth movement, on the compressed side of the tooth, RANKL expression is induced (9,21). In contrast, on the tensile side of the tooth, there is an increase in OPG synthesis (8,22-24). The relative expressions of OPG and RANKL on the tensile and compressed sides of the tooth during orthodontic tooth movement regulate bone remodeling.

The cellular mechanisms of osteoclastic bone resorption appear to be quite similar to those of root resorption (25-37). Thus, the functional coordination of the OPG/RANKL/RANK system seems to contribute, not only to alveolar remodeling, but also to physiological root resorption and root resorption during orthodontic tooth movement. During physiological root resorption, in the dental follicle environment, the ratio of OPG to RANKL supports, rather than inhibits, osteoclastinogenesis. Cytotrophic factors released from the dental follicle and/or the stellate reticulum, such as parathyroid hormone-related peptide (PTHrP), interleukin- $1\alpha$ , and transforming growth factor- $\beta$ 1, stimulate the expression of RANKL during permanent tooth eruption (26). During orthodontic tooth movement, this RANKL to OPG ratio in periodontal ligament cells also contributes to root resorption. The compressed periodontal ligament cells, in cases of severe external apical root resorption, may produce a large amount of RANKL and up-regulate osteoclastogenesis. This explains the greater increase of RANKL and decrease of OPG in cases of severe root resorption (37-39).

RANKL and OPG in periodontal tissues are important determinants for the regulation of bone remodeling as well as root resorption during orthodontic tooth movement. Determination of serum OPG and sRANKL levels can give insight into the regulation of bone homeostasis by the OPG/RANKL/RANK system and their concentrations might be useful for predicting the rate of bone remodeling during orthodontic tooth movement, the net effect between bone remodeling and root resorption, and the degree of root resorption. Although circulating OPG and sRANKL originate from several sources and their concentrations may be altered by different coexisting pathological processes (40,41), it would be of great interest to investigate whether serum and gingival crevicular fluid (GCF) concentrations of RANKL and OPG can offer valuable information related to the degree of root resorption induced by orthodontic therapy.

The aim of the present study was to investigate, in a rat model, the hypothesis that the levels of OPG and sRANKL in blood serum and in GCF relate to the degree of root resorption developed during orthodontic tooth movement and if they relate, whether the initial concentrations of sRANKL and OPG can help us in predicting the degree of root resorption induced by orthodontic treatment.

## Materials and Methods

### Experimental animals

Fourteen 6-month-old male Wistar rats with a body weight of 350-500 g were used. They were housed in individual cages, at room temperature, in a room with 12:12-h artificial light cycle. The experimental protocol was approved by the Veterinary Service of the Athens Prefecture, according to Greek law, and in accordance with the European Directive 86/609/EEC.

### Blood and gingival crevicular fluid collection

Under general anaesthesia, blood samples were collected from the eye area of each rat. Blood collection was performed with the use of a thin sterile laboratory pipette, which was inserted in the eye area, behind the eyeball. Blood samples were consecutively deposited in laboratory vials for evaluation. In addition, gingival crevicular fluid (GCF) was collected at the frontal cervical margin of each upper right incisor (Fig. 1). Collecting GCF from the cervical margin of each upper right first molar of a rat is technically difficult. Therefore, it was decided to collect GCF from the cervical margin of each upper right incisor only on Day 1 of the experiment, so as to have an indication of each animal's initial concentrations of sRANKL and OPG in GCF. This is also why GCF was not collected at Day 21, because our purpose was not to measure the difference of sRANKL and OPG concentrations in GCF in the resorption region but to evaluate the initial concentrations of each animal in relation to the degree of root resorption developed after orthodontic tooth movement. Gingival crevicular fluid was collected according to the protocol described by Nishijima et al. (38). First, the upper right incisor of each animal was isolated. It was gently washed with physiologic serum and gently dried with an air syringe across the crevice. Then a paper strip (Periopaper, Harco, Tustin, CA, USA) was carefully inserted with cotton pliers 1 mm into the gingival crevice, until the paper strip reached the base of the crevice. Following the insertion of the strip, the grip of the pliers on the strip was released and the paper strip was allowed to remain *in situ* for 1-2 min (Fig. 1). Next, the paper strip was removed by grasping the orange handle of the paper strip with the cotton pliers and transferred to the Periotron



Fig. 1 Collection of gingival crevicular fluid at the frontal cervical margin of each upper right incisor.

meter for GCF volume measurement. In particular, the volume of the GCF on the paper strip was measured with a Periotron 8000 meter (Harco) that had been calibrated first with human serum. In this way, GCF collection was standardized so that the experimental animals could be compared. Then the paper strips were stored at  $-20^{\circ}\text{C}$ . For evaluation, the paper strips were placed individually in 100  $\mu\text{l}$  of Tris buffer and then vortexed three times over a 30-min period. The evaluation of the paper strips and the estimation of sRANKL and OPG concentrations in the extract were made according to the laboratory procedure described by Nishijima et al. (38).

### Experimental design

After blood and GCF collection, a 25-g Sentalloy closed orthodontic coil spring (Dentsply GAC International, Bohemia, NY, USA) was applied between each upper right first molar and the upper incisors (Fig. 2), according to the method used by Verna et al. (42,43), to generate orthodontic tooth movement and provoke root resorption. The coil spring was left in place for 3 weeks to generate mesial movement of the first molar. A 3-week application of force influences the metabolic state of hard tissues for at least one remodeling cycle in 6-month-old rats. The untreated left first molar served as the control tooth. Composite resin was placed over the ligature wire ends anteriorly to prevent mucosal trauma. During the experiment, all rats were fed with the same ground laboratory chow and provided with water.

At the end of the treatment period, blood samples were collected again. The animals were then killed and the



Fig. 2 Orthodontic coil spring inserted to generate orthodontic tooth movement.

maxillae were excised; all upper right and left molars were extracted carefully, under a strict protocol. After removal, the teeth were immersed completely in physiologic serum and placed in an ultrasonic bath for 10 min to remove all traces of residual periodontal ligament as described by Harris et al. (44). After the ultrasonic bath, the teeth were rubbed with damp gauze, disinfected in 70% alcohol for 30 min, and stored in physiologic serum at room temperature.

Subsequent analysis of the teeth was carried out using a desktop microcomputed tomography X-ray system (Skyscan 1072, Skyscan, Aartselaar, Belgium). Prior to the analysis, the teeth were left to dry at least for 2 days. The teeth were scanned individually by the same operator and under the same scanning operation data. Viewbox 4 software (45) was used for the three-dimensional reconstruction of the tooth images and for the measurement of root resorption areas.

### Estimation of the root resorption ratio

The upper first molar of the rat presents five roots, the mesial being the longest and largest (Fig. 3). Following microcomputed tomography scanning, the image of the longest and largest mesial root of each upper first molar was selected (Fig. 4). The coronal level of the roots undergoes the greatest change after the application of the aforementioned type of force (tipping force) and the amount of root resorption is increased at the coronal level (42,43). Therefore, for each upper first molar (treated or



Fig. 3 The upper first molar of the rat after microcomputed tomography scanning.

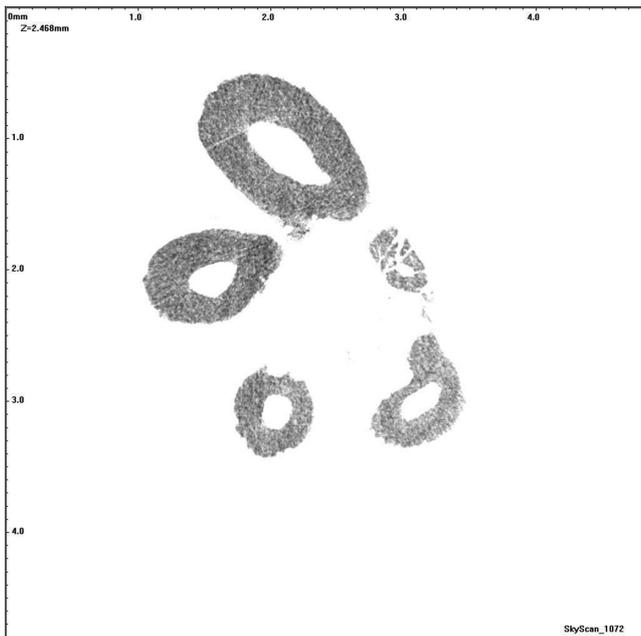


Fig. 4 Tomographic image of the five roots of the upper first molar of the rat. The largest mesial root was selected for the estimation of root resorption.

control), the percentage of root resorption was measured at the coronal level of each mesial root as follows.

As a first step, for each mesial root of the control teeth, we measured the mean number of tomographic cuts that corresponded to one-third of the root length, starting from

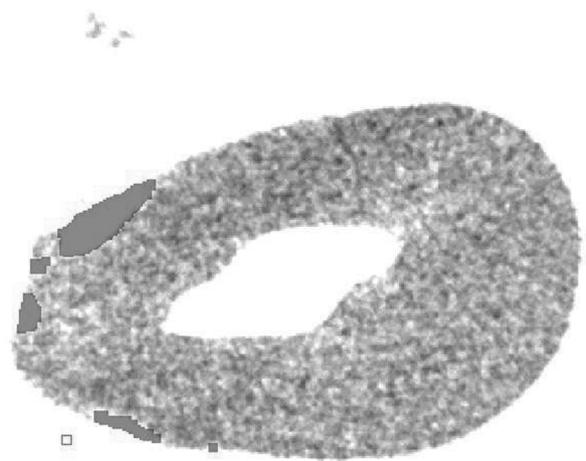


Fig. 5 Estimation of the number of voxels (black area) that corresponded to resorption lacunae in the tomographic cut of the mesial root.

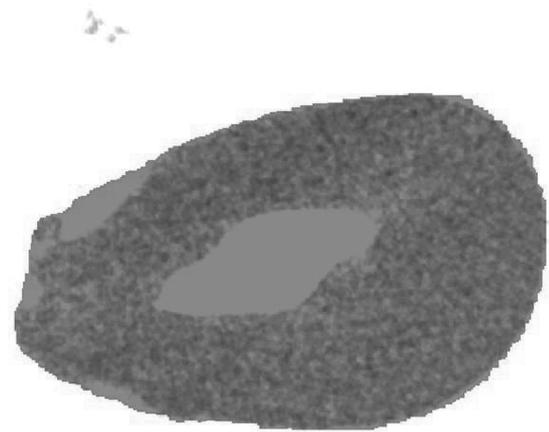


Fig. 6 Estimation of the number of voxels (black area) that corresponded to the full surface of the tomographic cut of the mesial root.

the first tomographic cut at which the five roots of the molar were fully divided (Fig. 4). It was found that the mean number of coronal tomographic cuts of the mesial root of each upper first molar was 180. Of the 180 coronal tomographic cuts, for all teeth (treated and control), we studied 18 tomographic cuts of the mesial root (one out of every consecutive group of ten) starting from the first tomographic cut at which the five roots of the molar were fully divided, as mentioned. At each tomographic cut, root resorption was estimated with the help of Viewbox 4 software (45) as the ratio of the number of voxels that corresponded to small, wide, shallow or deep resorption lacunae (Fig. 5) to the number of voxels corresponding to

the full surface of the tomographic cut (Fig. 6). The degree of root resorption of each tooth (treated upper first molar or control upper first molar) was defined as the mean ratio of voxels of the 18 coronal tomographic cuts studied; this mean ratio was used in our statistical analysis. The mean ratio of resorption voxels of each treated tooth (upper right first molar) corresponded practically to the degree of root resorption of each treated animal.

### Statistical analysis

Continuous variables are reported as mean  $\pm$  SD. The normality of the distribution was assessed by the Kolmogorov-Smirnov test and graphs. Comparisons between unpaired continuous variables were performed with the unpaired *t*-test and Mann-Whitney *U*-test, where appropriate. Comparisons between correlated quantitative variables were performed with the paired *t*-test and Wilcoxon's signed rank test.

Linear correlations between variables were assessed with Pearson's correlation coefficient in cases of continuous normally distributed variables and with the estimation of nonparametric Spearman's rho.

Multiple linear regression was performed by inserting basic parameters into the model. The selection of the best predictive parameters was performed with a stepwise insertion model.

A nonlinear regression model was selected by graphing methods initially and afterwards by selection of the best fitting curve, in regards to maximal  $R^2$  and minimum residuals.

All performed tests were two-sided and all values with  $P < 0.05$  were considered statistically significant.

## Results

First, we examined the ratio of root resorption on the treated teeth to that on the control teeth. As expected, root resorption was significantly greater ( $P < 0.001$ ) on the treated teeth (ratio of root resorption  $0.0488 \pm 0.01156$ ) than on the control teeth (ratio of root resorption  $0.0157 \pm 0.00331$ ) (Fig. 7, Table 1). We also examined the ratio of root resorption among the treated molars (which corresponded practically to the ratio of root resorption among the treated animals) and observed that four treated upper first molars (from four treated animals) exhibited a higher mean root resorption ratio ( $0.062 \pm 0.005$ ) comparative to the rest of the treated upper molars (from the rest of the treated animals) ( $0.044 \pm 0.009$ ). This difference was found to be statistically significant ( $P < 0.05$ ) (Fig. 8, Table 2).

Accordingly, we statistically evaluated the two groups of treated animals (animals with a higher root resorption

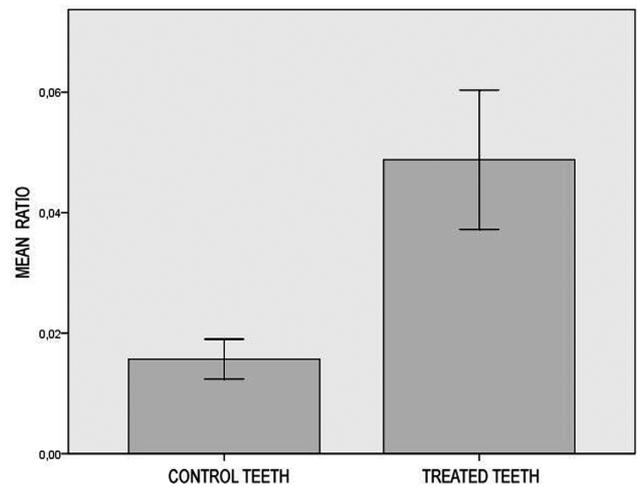


Fig. 7 Columns correspond to  $\pm$  SD. Ratio of root resorption on the treated and on the control teeth. Root resorption was greater on the treated teeth ( $P < 0.001$ ).

Table 1 Root resorption in treated and control teeth

	Group	Mean voxels	Std. Deviation voxels
Root resorption	Control	12023.60	2285.56
	Treated	37233.18	9848.12
Total surface	Control	770450.90	73196.34
	Treated	762572.32	77391.97
Ratio	Control	0.0157	0.00331
	Treated	0.0488	0.01156

ratio in treated teeth and animals with a lower root resorption ratio in treated teeth) regarding OPG and RANKL concentrations in blood serum (before and after treatment) (Table 3) and in GCF (before treatment). It was found that animals with higher root resorption ratios in treated teeth had statistically significantly higher ( $P < 0.05$ ) initial RANKL concentrations in blood serum (mean  $59.0 \pm 2.3$  pg/ml) in relation to animals with lower root resorption ratios in treated teeth (mean  $50.8 \pm 5.1$  pg/ml). Regarding the final concentrations of RANKL in blood serum (at Day 21), although a difference in mean concentrations of RANKL still existed (mean  $51.0 \pm 6.0$  pg/ml in the lower resorption group and mean  $54.9 \pm 1.8$  pg/ml in the higher resorption group) (Table 3), no statistically significant difference was detected between the two groups, which can be attributed to the change in equilibrium of sRANKL and OPG after tooth movement and the initiation of resorption. Initial mean concentrations of RANKL in GCF exhibited a strong tendency ( $P = 0.059$ ) to be lower in the higher root resorption group

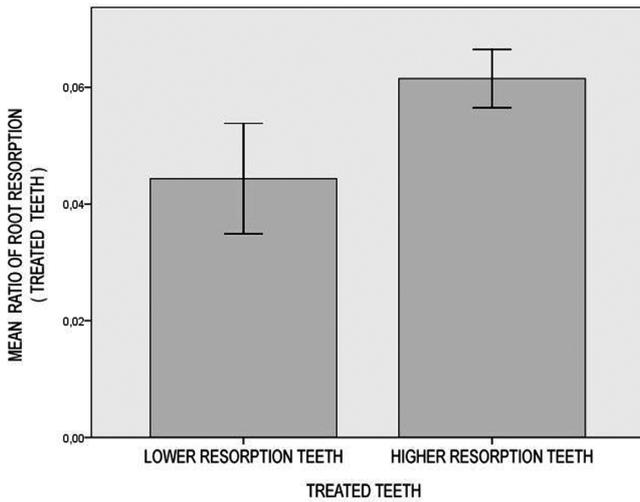


Fig. 8 Columns correspond to  $\pm$  SD. Ratio of root resorption between treated teeth. A group of four treated teeth (from four treated animals) exhibited a higher mean root resorption ratio ( $P < 0.05$ ).

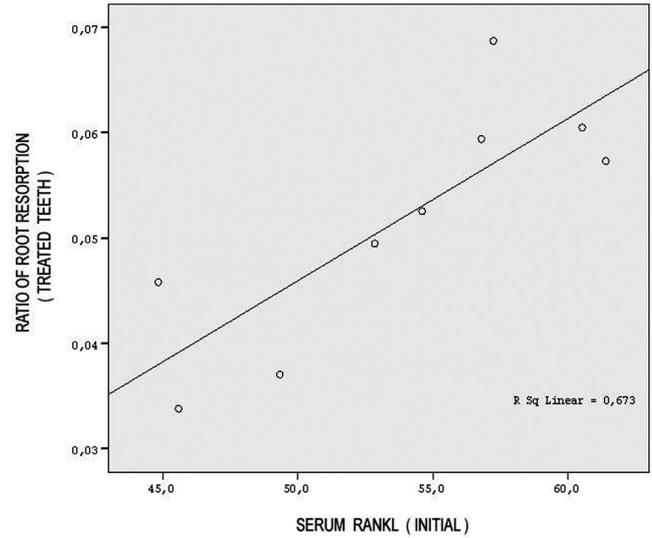


Fig. 9 Positive linear correlation between the ratio of root resorption and the initial concentration (pg/ml) of RANKL in blood serum ( $r = 0.833$ ,  $P < 0.01$ ).

Table 2 Root resorption between treated teeth (treated animals)

Variable (treated teeth-treated animals)	Lower resorption teeth (animals)	Higher resorption teeth (animals)	<i>p</i>
Root resorption (voxels)	33224.4 $\pm$ 7816.1	47255.3 $\pm$ 7036.1	$P < 0.05$
Total surface (voxels)	760298.8 $\pm$ 73311.4	768256.1 $\pm$ 98846.2	NS
Ratio	0.044 $\pm$ 0.009	0.062 $\pm$ 0.005	$P < 0.05$

Table 3 Evaluation of the two groups of treated teeth (treated animals) regarding OPG and RANKL concentrations in blood serum

Variable (treated teeth-treated animals)	Lower resorption teeth (animals)	Higher resorption teeth (animals)	<i>p</i>
OPG ( $t_0$ ) pg/ml	282.0 $\pm$ 24.3	292.5 $\pm$ 25.4	NS
OPG ( $t_1$ ) pg/ml	300.3 $\pm$ 35.8	263.4 $\pm$ 4.6	$P < 0.05$
sRANKL( $t_0$ ) pg/ml	50.8 $\pm$ 5.1	59.0 $\pm$ 2.3	$P < 0.05$
sRANKL( $t_1$ ) pg/ml	51.0 $\pm$ 6.0	54.9 $\pm$ 1.8	NS
Difference of OPG pg/ml	18.3 $\pm$ 39.8	-29.1 $\pm$ 24.9	$P < 0.05$
% Difference OPG	7.0 $\pm$ 14.4	-9.5 $\pm$ 7.5	$P < 0.05$
Difference of sRANKL pg/ml	0.2 $\pm$ 5.7	-4.1 $\pm$ 1.6	NS

(48.08  $\pm$  1.05 pg/ml) in relation to the lower root resorption group (49.98  $\pm$  1.67 pg/ml).

Also apparent from the statistical analysis was the significant decrease of the final (after the end of orthodontic treatment, at Day 21) concentration of OPG in blood serum ( $P < 0.05$ ) in the animals with a higher ratio of root resorption in treated teeth (mean difference -29.1  $\pm$  24.9 pg/ml), while the initial concentrations of OPG in blood serum (mean 282.0  $\pm$  24.3 pg/ml for the lower resorption group and mean 292.5  $\pm$  25.4 pg/ml for the higher resorption group) did not demonstrate any statistically significant

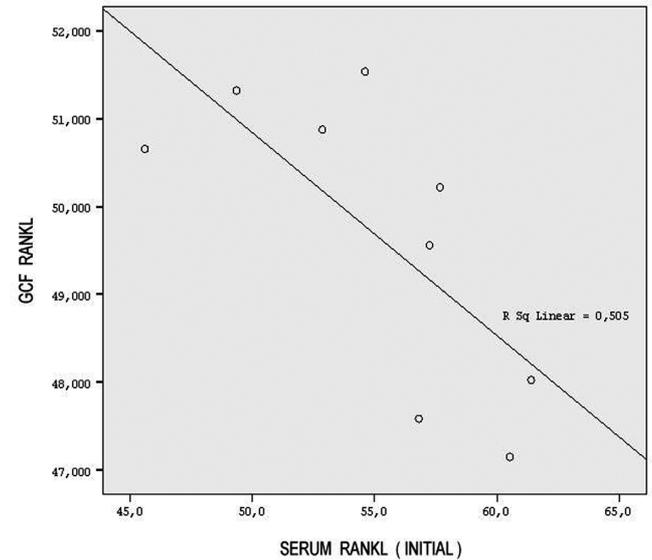


Fig. 10 Negative linear correlation between the initial concentration of RANKL (pg/ml) in blood serum and the concentration of RANKL (pg/ml) in GCF ( $r = -0.7$ ,  $P < 0.05$ ).

difference in the two groups (Table 3). Regarding the initial mean concentrations of OPG in GCF in the two groups, no statistically significant difference was detected (937.38 pg/ml for the higher root resorption group and 804.69 pg/ml for the lower root resorption group).

The above correlations were also linear correlations. In particular, a positive linear correlation was found between the ratio of root resorption and the initial concentration of

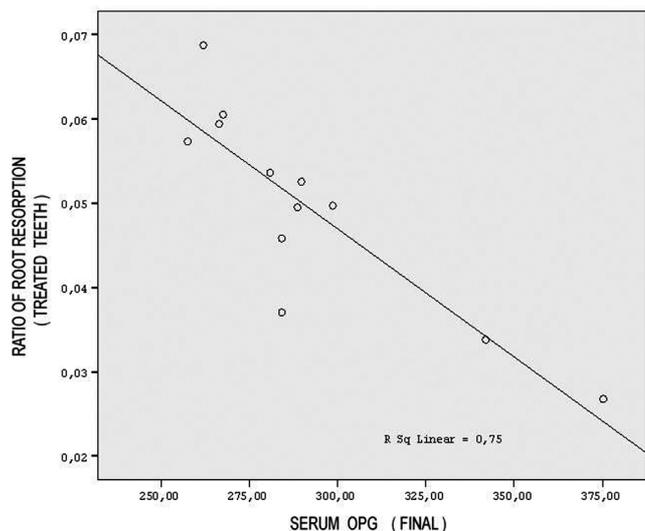


Fig. 11 Negative linear correlation between the ratio of root resorption and the final concentration of OPG (pg/ml) in blood serum ( $r = -0.823$ ,  $P = 0.001$ ).

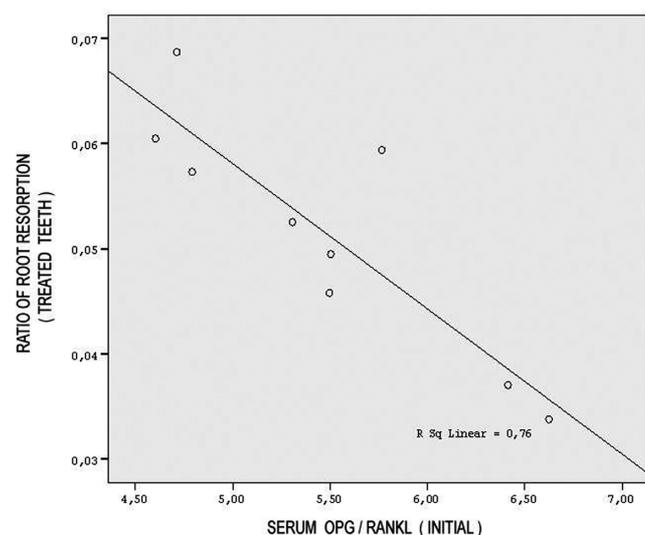


Fig. 12 Negative linear correlation between the ratio of root resorption and the ratio of initial OPG/RANKL concentrations in blood serum ( $r = -0.8$ ,  $P < 0.05$ ).

RANKL in blood serum ( $r = 0.833$ ,  $P < 0.01$ ) (Fig. 9). A negative linear correlation was found between the initial concentration of RANKL in blood serum and the concentration of RANKL in GCF ( $r = -0.7$ ,  $P < 0.05$ ) (Fig. 10). A negative linear correlation was also found between the ratio of root resorption and the final concentration of OPG in blood serum ( $r = -0.823$ ,  $P = 0.001$ ) (Fig. 11) and finally, as expected, there was a negative linear correlation between the ratio of root resorption and the ratio of the initial OPG/RANKL concentrations in blood serum

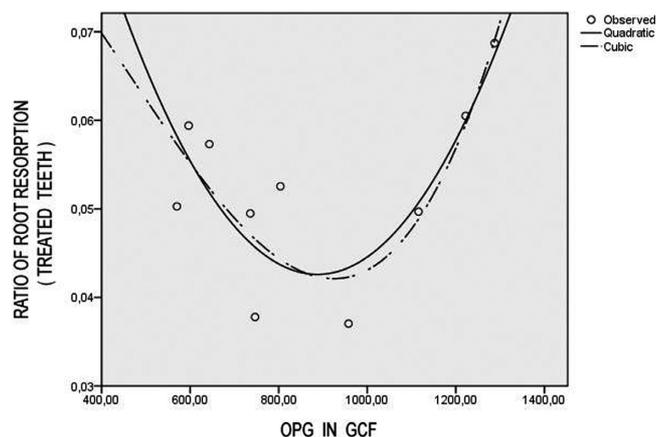


Fig. 13 Nonlinear correlation between root resorption ratio and the concentration of OPG (pg/ml) in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $x^2$  and  $R = 0.83$ ,  $P < 0.05$  for  $x^3$ ).

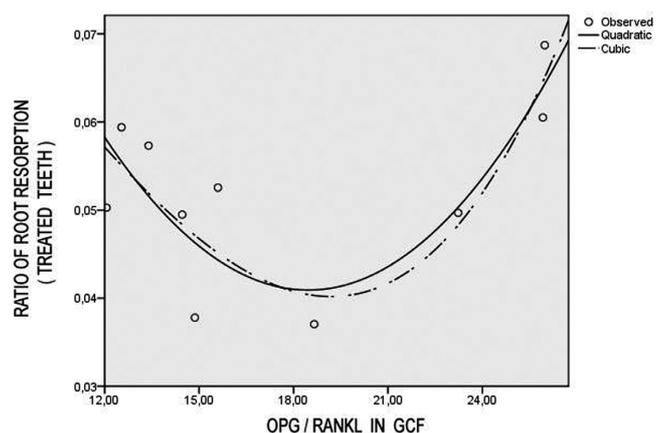


Fig. 14 Nonlinear correlation between root resorption ratio and the ratio of OPG/RANKL concentrations in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $x^2$  and  $R = 0.83$ ,  $P < 0.05$  for  $x^3$ ).

( $r = -0.8$ ,  $P < 0.05$ ) (Fig. 12).

From all of the above variables with linear correlation to the ratio of root resorption, the ratio of initial OPG/RANKL concentrations in blood serum proved to be the most powerful independent prognostic factor of the root resorption ratio ( $R^2 = 0.87$ ,  $\text{Adj } R^2 = 0.73$ ,  $\text{Beta} = -0.872$ ,  $P < 0.01$ ).

Finally, two statistically significant nonlinear correlations were also detected: one between the root resorption ratio and the concentration of OPG in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $x^2$  and  $R = 0.83$ ,  $P < 0.05$  for  $x^3$ ) (Fig. 13) and one between the root resorption ratio and the ratio of OPG/RANKL concentrations in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $x^2$  and  $R = 0.83$ ,  $P < 0.05$  for  $x^3$ ) (Fig. 14).

Results regarding pure concentrations of RANKL and OPG in blood and in GCF have been presented. Future investigation describing changes in expression of RANKL mRNA and OPG mRNA in blood and in GCF detected by the reverse transcriptase polymerase chain reaction during root resorption may further enable us to better understand the mechanisms involved in root resorption.

### Discussion

The results of this study showed a strong influence of the proteinic system OPG/RANKL/RANK on the amount of root resorption after orthodontic tooth movement. The greater ratio of root resorption was found in animals of higher initial sRANKL concentrations in blood serum ( $P < 0.05$ ). This positive correlation proved to be linear ( $r = 0.833$ ,  $P < 0.01$ ). The aforementioned result is consistent with the logical sequence of the biological effects that sRANKL exerts on osteoclast and odontoclast biology (10-19). The major role of RANKL in hard tissues is the stimulation of osteoclast-odontoclast differentiation and the inhibition of osteoclast-odontoclast apoptosis. The cellular mechanisms of root resorption appear to be quite similar to those of osteoclastic bone resorption. Thus, the increased degree of root resorption in animals with higher initial serum sRANKL levels is explained by the enhanced root-resorbing activity of odontoclasts by RANKL (25,30-37). Regarding the final concentration of RANKL in blood serum (at Day 21), no statistically significant difference was detected between the two groups of treated teeth (treated animals), which can be attributed to the change in equilibrium of sRANKL and OPG in blood serum after tooth movement and the initiation of resorption.

The present investigation demonstrated that the initial levels of serum sRANKL showed differentiation in relation to the degree of root resorption after orthodontic tooth movement. This result is considered to be original, because all previous investigations studied the change in OPG and sRANKL levels in the periodontal ligament during orthodontic tooth movement. Nishijima et al. (38) determined the levels of RANKL and OPG in the GCF 0, 1, 24, and 168 h after the application of retracting force and investigated the effect of compression force on RANKL and OPG production from human periodontal ligament cells. Yamaguchi et al. (39) supported the hypothesis that in cases of severe external apical root resorption, the compressed periodontal ligament cells may produce a large amount of RANKL and up-regulate osteoclastogenesis. Low et al. (46) described the changes in expression of RANKL and OPG mRNA in tissues subjected to heavy orthodontic forces and experiencing root resorption. Tang et al. (8) investigated how different

magnitudes of cyclic tensile strain affect osteoblasts, OPG synthesis, and sRANKL release. In the present study, changes in the initial levels of serum sRANKL were detected in relation to the degree of root resorption after the application of heavy orthodontic forces. From the statistical analysis, the ratio of initial OPG/RANKL concentrations in blood serum proved to be the most powerful independent prognostic factor at the beginning of the orthodontic tooth movement for the prediction of the degree of root resorption after the orthodontic treatment ( $R^2 = 0.87$ ,  $\text{Adj } R^2 = 0.73$ ,  $\text{Beta} = -0.872$ ,  $P < 0.01$ )

In medicine, serum OPG or RANKL levels or their ratio are strongly predictive of several pathologic conditions. For example, OPG serum level is considered to be a stable and reliable indicator of the overall activity of the OPG/RANKL/RANK system and may find application as a biomarker of vascular disease risk and prognosis (47). Lower serum levels of RANKL and RANKL/OPG ratio may serve to predict remission of rheumatoid arthritis after treatment (48). In addition, serum OPG is strongly predictive of long-term mortality and heart failure development in patients with acute coronary syndromes (49). The OPG/RANKL ratio is a strong biomarker of several pathologic conditions and our present investigation provides indications that this OPG/RANKL ratio could also be a strong biomarker for the prediction of the degree of root resorption after orthodontic treatment.

Regarding serum OPG levels, our results are consistent with changes in cases demonstrating higher root resorption. In particular, the levels of OPG in blood serum decreased significantly in rats that exhibited a higher ratio of root resorption ( $P < 0.05$ ). Before the orthodontic treatment, no difference was found in OPG serum levels between the different groups of animals. We attribute this decrease to the decrease in OPG levels induced in the periodontal ligament after the application of heavy orthodontic forces (8,25,26). More specifically, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  increase during orthodontic tooth movement and these cytokines enhance RANKL mRNA and inhibit OPG mRNA (46,50,51). This decrease in OPG in compressed periodontal ligament cells is greater in cases of severe root resorption (37-39). Nishijima et al. (38), using an *in vitro* model, demonstrated that compression force significantly increases the secretion of RANKL and decreases the secretion of OPG in human periodontal ligament cells in a time- and force magnitude-dependent manner. In addition, Spyropoulos et al. (41) refer to distal and remote alveolar bone responses in relation to orthodontic tooth movement; the decrease in serum OPG levels could represent the result of the decrease in OPG in the periodontal ligament.

The results related to the concentrations of RANKL and

OPG in GCF before orthodontic treatment are especially interesting. The concentrations of RANKL in GCF were inversely related to the initial concentrations of RANKL in blood serum and this inverse relation proved to be linear ( $r = -0.7$ ,  $P < 0.05$ ) (Fig. 10). This result is confusing because GCF is practically modified blood serum and its composition is almost identical to blood serum. Consequently, a positive linear correlation would be expected instead of a negative linear one. A possible explanation of this result may be the protection of the cementum by the cementoblasts. Under nonresorbing conditions, cementoblasts seem to secrete large amounts of OPG and this is considered one mechanism by which cementum is protected more than bone from resorption (26). In addition, apart from cementoblasts, other periodontal ligament cells were found to express OPG and not RANKL. This preferential expression inhibits osteoclast formation and thus protects the root from resorption (26).

Gingival crevicular fluid collection was performed at the frontal cervical margin of each upper right incisor (Fig. 1) and not from the cervical margin of each upper right first molar. For technical reasons, we were not able to collect GCF from the crevice of experimental teeth, which undoubtedly constitutes the optimal experimental design. However, in our study, our interest was focused on the initial concentrations of RANKL and OPG in the periodontal ligament, regardless of any tooth movement. Furthermore, the periodontal ligament of each upper right incisor may represent the periodontal ligament of each animal in nonresorbing conditions and provide us with indications regarding the initial concentrations of RANKL and OPG in GCF. Recent literature does not give information regarding the effect of root resorption procedures in the periodontal ligament of teeth, other than those that have been moved orthodontically. However, Spyropoulos et al. (41) refer to distal and remote alveolar bone response in relation to orthodontic tooth movement; taking into consideration the decrease in serum OPG levels as a result of root resorption in the molar region, it may be possible that changes in OPG and RANKL concentrations in the periodontal ligament of other teeth, than those treated orthodontically, may appear. Further investigation may help us answer this intriguing question.

Regarding the initial concentrations of OPG in GCF, no linear statistically significant correlation was detected in relation to root resorption ratios. However, the statistical analysis revealed two powerful statistically significant nonlinear correlations: a) between the ratio of root resorption and the concentrations of OPG in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $\chi^2$  or  $R = 0.83$ ,  $P < 0.05$  for  $\chi^3$ ) and b) between the ratio of root resorption and the ratio of

OPG/RANKL concentrations in GCF ( $R = 0.81$ ,  $P < 0.05$  for  $\chi^2$  and  $R = 0.83$ ,  $P < 0.05$  for  $\chi^3$ ). These correlations are better depicted in Figs. 13 and 14. In Fig. 13, there is an area of OPG concentration in GCF in which it seems that the root is protected against extreme resorption. Outside of this area of OPG concentration, a greater ratio of root resorption is observed. Similarly, in Fig. 14, there is an area of OPG/RANKL ratio in GCF in which it seems that the root is protected against extreme resorption, while outside of this area, greater root resorption ratios were detected.

Because the ratio of OPG/RANKL concentrations in periodontal ligament is strongly correlated to the strain accumulated in the periodontal ligament due to the orthodontic force (compression or tension), Figs. 13 and 14 could represent the biological mechanism by which the 'mechanostat theory' developed by Frost (52) can be interpreted. The 'mechanostat theory' is one of the major theories that relates mechanical load to biological reaction. Frost observed that in the case of low strain values, a net loss of bone occurs. With increasing strain, a positive balance is achieved. Even larger strains result in a negative balance (53). The cellular mechanisms of root resorption appear to be quite similar to those of osteoclastic bone resorption (54) and Melsen (55) pointed out that the 'mechanostat theory' can be used as a model for understanding tissue reaction during orthodontic tooth movement.

As expected, root resorption was greater on the treated than on the control side. The mean ratio of root resorption proved to be statistically significantly higher ( $P < 0.001$ ) in the treated teeth than in the control teeth. This result is in accordance with the results of other studies, during which the same experimental design was used to provoke root resorption in orthodontically moving teeth (42,43,46).

The study of the 18 tomographic cuts at the coronal level of the mesial root of each upper first molar of the rat is an original method for the estimation of root resorption, which practically constitutes a combination of the methods used by Verna et al. (42,43) and Harris et al. (44). Verna et al. (42,43) studied two histological sections at the coronal level and two histological sections at the apical level of each molar. They projected each histological section onto a randomly positioned reticular grid and concluded that the coronal level is the area that undergoes the largest changes after the application of a tipping force and presents the greater degree of root resorption. For this reason, we limited our study to the coronal level of each mesial root. Harris et al. (44), on the other hand, made a direct volumetric measurement of the craters of each root using the same microcomputed tomography X-ray system

that we used in our study (Skyscan 1072, Skyscan, Aartselaar, Belgium) in combination with convex hull software (CHULL2D) specifically developed for this project. However, they studied the roots of human premolars, the size of which is significantly greater than the mesial root of a rat molar. Therefore, we decided to use the same microcomputed tomography X-ray system and study 18 instead of 2 tomographic cuts at the coronal level of each mesial root of the rat upper first molars. The increased number of tomographic cuts gave us the potential to make a detailed observation of resorption craters at the coronal level of each mesial root and increase the credibility of our results.

The aforementioned results may have high clinical significance, taking into consideration that some patients have a high degree of root resorption during orthodontic therapy. The prognosis of the probability of extreme root resorption can lead to the modification of the therapeutic design to avoid this development. Future clinical investigation is needed for the application of these results in humans. The determination of the 'safe' ratio of OPG/RANKL concentrations in blood serum in humans may be the first step for the application of these results in humans.

Conclusively,

- 1) There is a positive correlation between the initial concentration of RANKL in blood serum and the degree of root resorption after orthodontic treatment.
- 2) The ratio of the initial OPG/RANKL concentrations in blood serum proved to be the most powerful independent prognostic factor of the degree of root resorption after orthodontic therapy.
- 3) The initial concentration of RANKL in GCF showed a negative linear correlation to the initial concentration of RANKL in blood serum.
- 4) The concentration of OPG in blood serum decreased significantly in cases of severe root resorption after the orthodontic treatment.
- 5) The ratios of initial OPG/RANKL concentrations in GCF suggest whether the dental root will be protected against extreme external root resorption.

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