

# Evolution of Periodontal Disease: Immune Response and RANK/RANKL/OPG System

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The aim of this study was to evaluate markers of bone loss and immune response present in evolution of periodontal disease. One hundred and two Wistar rats were divided into three animals groups: PDO, without ligation and PD15 days and PD60 days, submitted to ligation placement with a sterile 3-0 silk cord in the cervical region of the upper first molar on both sides. Samples were obtained from the gingival tissue for histomorphometric analysis, immunohistochemical analysis of RANK, RANKL, OPG, characterization of the inflammatory infiltrate, quantification of nitric oxide, MCP-1, RANTES, IP10 chemokines, and expression of the TGF- $\beta$ 1, VEG, and bFGF. The number of inflammatory cells in gingival tissue was higher in PD60 samples. The collagen content and the area occupied by birefringent collagen fibers were lower for PD60. Differential leukocyte counting showed that there was a significantly higher polymorphonuclear influx in group PD15, while PD60 showed a greater number of lymphocytes. PD60 showed higher RANTES, IP-10, MCP-1 gene transcripts, as well as a higher nitric oxide concentration. Clinical evaluation revealed that the PD60 group presented an increase in furcal area. In conclusion, in this animal model the increase of RANK/RANKL and HGF markers is related to a specific immune response, and probably contributed to the evolution of periodontal disease. Investigating the effect of these biomarkers can help in targeted therapy for bone resorption, since blocking these can inhibit bone loss.

Key Words: periodontal disease, cytokines, chemokines, leucocytes, RANK/RANKL/OPG.

## Introduction

Periodontal disease is characterized by inflammation, affecting the tissues that support the teeth. Bone loss is closely associated with inflammation and can be influenced by other infections and hosts/bacteria interactions. However, the immune response developed by the host organism against microorganisms of the dental biofilm can either provide protection or cause bone loss, accounting for the wide variety of tissue alterations observed in periodontal disease (1). The alterations can lead to gingivitis and degeneration of the periodontal ligament, as well as alveolar bone loss and periodontitis (2). The periodontitis is related to the development of an altered inflammatory response. However, to study the relationship between periodontal disease and inflammatory response in patients is very complicated, because they vary in time pathology, patients who are already undergoing treatment, and patients who have recently discovered periodontitis. Therefore, the use of animals for the development of periodontal disease has become timely (3).

In periodontitis, the inflammatory progression response involves a wide variety of inflammatory and immunological mediators, including nitric oxide and inflammatory cytokines and chemokines. The chemokines are responsible for the migration and activation of subpopulations of

leukocytes in inflamed periodontal tissues (4). One of the chemokine subfamilies includes monocyte chemotactic proteins (MCP-1) that can attract T cells, monocytes, and eosinophils, known as RANTES (regulated on activation, normal T cell expressed and secreted), as well as gamma-interferon inducible protein 10 (IP-10) (5). Nitric oxide influences vascular tone, microvascular permeability, leukocyte migration and oxidant activity, directly contributing to the death of microorganisms (6).

In periodontitis, particularly, the infiltration of inflammatory cells causes endothelial damage and microcirculatory failure, resulting in decreased oxygen tension (4). This condition, as well as the activity of cytokines and growth factors such as TGF- $\alpha$ , TGF- $\beta$ , PDGF, FGF, PGE, IL-1, IL-6 and IL-8 and endotoxins, stimulates the production of vascular endothelial growth factor (VEGF), which is one of the most important proangiogenic factors (5).

Several proteins are involved during the development of an inflammatory process. The presence of transforming growth factor (TGF- $\beta$ ) and vascular endothelial growth factor (VEGF) during periodontal disease is a consequence of endothelial damage and microcirculatory failure caused by inflammatory infiltrate (7). The basic fibroblast growth factor (bFGF) presents pro-angiogenic activity and its role is fundamental for satisfactory tissue repair (8). It also acts

as a chemotactic agent in different cell types, influencing proliferation and differentiation. Acute effects in normal tissue include improvement of the recruitment of PMN cells, monocytes, and T cells in response to inflammatory cytokines, independent of angiogenesis. Hepatocyte growth factor (HGF) is an important growth factor involved in the repair and regeneration of various organs and tissues (9).

The development of the inflammatory condition can influence osteoclast formation and activation, which are fundamental in bone loss. This process is regulated by expression of the RANK receptor found in osteoclasts, dendritic cells, fibroblasts, and T lymphocytes, by increases of the RANKL cytokine present in osteoblasts and T lymphocytes, and/or by decreased expression of the soluble OPG receptor found in different osteoblast and endothelial immune cells (10).

The initial proposal of the study was to evaluate the signaling initiated in soft tissue that reflects in the bone resorption frame. Investigating the involvement of these molecules can lead to the future development of drugs that can act in the modulation of these mediators and thus contribute to the improvement of the clinical picture.

## Material and Methods

### Animals

This work was conducted in accordance with established experimental norms and animal welfare practices (11,12). It was approved by the Committee for Research Ethics and Value of the Herminio Ometto University Center, UNIARARAS (protocol 054/2013). One hundred and two adult male Wistar rats (*Rattus norvegicus*), 90 days old and weighing an average of 300 g, were obtained from the Prof. Dr. Luiz Edmundo de Magalhães Center of Animal Experimentation, Herminio Ometto University Center (UNIARARAS) (Araras, SP, Brazil). The animals were housed in polycarbonate cages (n=5), with a 12 h light/dark cycle and free access to water and food. The rats were divided into three groups, each with 34 individuals: PDO, no submitted to periodontal disease, PD15, submitted to periodontal ligature for 15 days, and PD60, submitted to periodontal ligature for 60 days.

### Ligature-Induced Periodontal Disease

After intraperitoneal anesthesia (ketamine chlorohydrate, 0.08 mL/100g body weight; xylazine chlorohydrate, 0.04 mL/100g), a thread of sterile 3-0 black silk (Johnson and Johnson) was placed (on both sides) around the cervix of each upper first molar and knotted mesially (13). The thread remained fully attached in a subgingival position in the cervical region. The animals were healthy and the procedure did not promote stress.

### Animal Sacrifice and Analysis

The 34 animals of each group randomly divided (PDO, PD15 and PD60) were euthanized by anesthetic excess (1 mL/100 g). The gingival tissue around the first molars was excised for extraction of protein and determination of nitric oxide (n=6), Western blotting (n=5), reverse-transcription semi-quantitative polymerase chain reaction (RT-PCR) (n=12), and cellular profile analysis (n=6). After dissection of the gingival tissues, the blocks were fixed in 4% paraformaldehyde for 48 h, stored in 70% ethanol and embedded in paraffin. Serial 6- $\mu$ m longitudinal sections were used for histomorphometric analysis and 4- $\mu$ m-thick sections were mounted on silanized slides for immunohistochemical analysis (n=5). Experimental development was conducted in the laboratory of protein and cell analysis.

### Histomorphometry

For the number determination of inflammatory cells, neoformed blood vessels, epithelium thickness, and area occupied by birefringent collagen fibers, longitudinal tissue sections from the groups studied were treated using the hematoxylin-eosin, Picrosirius Red and Masson's trichrome techniques. Three fields were quantified for each of five sections obtained from five animals of each group. The images were captured, digitized and measured using Sigma Scan Pro 5.0 software.

### Western Blotting

The protein concentration measurement in the samples was performed by the Biuret method. Samples containing 50  $\mu$ g protein in Laemmly were boiled for 5 min and submitted to 10% (VEGF, 40 kDa), 12% (TGF- $\beta$ 1, 25 kDa) and 12% (bFGF, 21 kDa) SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Next, the protein bands were transferred from the gel to a PVDF membrane (BioRad, Hercules, CA, USA). The membranes were incubated in blocking solution (basal solution plus 5 % Molico® skim milk) for 2 h to reduce nonspecific protein binding. After the membranes were incubated overnight at 4 °C with specific antibodies Anti-TGF- $\beta$ 1 (TB21, Santa Cruz Biotechnology, Dallas, TX, USA), Anti-VEGF (VG-1, Santa Cruz Biotechnology, USA) and Anti-bFGF (C18, Santa Cruz Biotechnology). Next, the membranes were incubated with the specific secondary. The reaction was developed using a chemoluminescence kit (SuperSignal® West Pico Chemiluminescent Substrate 34080; ThermoScientific, Rockford, IL, USA). The membranes were exposed in Syngene G: Box and the bands intensity was evaluated by densitometry using the Scion Image 4.0.3.2 software (Scion Co., Frederick, MD, USA) (14). The densitometric values of VEGF, TGF and bFGF signals are expressed relative to proteins

stained with Ponceau S, which were taken as 100 %.

### RNA Extraction and Semi Quantitative RT-PCR

Total RNA was isolated from gingival tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and digested with amplification grade DNase I (Invitrogen). The conversion of total RNA (2  $\mu$ L) to cDNA was performed using random primers in the presence of the reverse transcriptase (RT) enzyme (SuperScript II, Invitrogen), in a final volume of 20  $\mu$ L. Semi-quantitative analysis of mRNA expression of RANTES, MCP-1, IP-10, and HGF was performed by RT-PCR in a final volume of 25  $\mu$ L containing 1  $\mu$ L of cDNA, 200  $\mu$ M of each dNTP, 0.2  $\mu$ M of each primer, and 0.04 U of Taq DNA polymerase (Invitrogen). The sequences of the primers are provided in Table 1, together with the concentrations of  $MgCl_2$  and the amplification conditions used for the mRNA analysis.

The amplified products were separated on 1.5% agarose gel stained with ethidium bromide. The gel was photographed using Syngene G:Box<sup>®</sup> (Gurgaon, Haryana 122 002 India), and the signal intensities of the bands were measured densitometrically using Scion Image software (Scion). The results were expressed as average ratios of the

Table 1. Morphometric parameters assessed in the gingival tissue of rats after the 0 (PD0), 2 (PD15) and 8 (PD60) weeks of induction of experimental periodontitis

Parameter	Time	Mean $\pm$ SD
N <sup>o</sup> of inflammatory cells (n/10 <sup>4</sup> $\mu$ m <sup>2</sup> )	PD0	9.8 $\pm$ 3.2
	PD15	49.8 $\pm$ 3.9
	PD60	87.8 $\pm$ 4.1*
Blood vessels (n/10 <sup>4</sup> $\mu$ m <sup>2</sup> )	PD0	29.3 $\pm$ 4.8
	PD15	39.9 $\pm$ 2.5
	PD60	40.6 $\pm$ 2.7
Birefringent collagen fiber area (%)	PD0	91.6 $\pm$ 3.6
	PD15	88.4 $\pm$ 4.3
	PD60	72.4 $\pm$ 3.6*
Collagen (%)	PD0	92.2 $\pm$ 4.8
	PD15	88.4 $\pm$ 3.3*
	PD60	75.4 $\pm$ 3.5
Thickness of epithelium ( $\mu$ m)	PD0	89.7 $\pm$ 4.1
	PD15	93.7 $\pm$ 6.9
	PD60	96.2 $\pm$ 5.5

Values are expressed as the mean and standard deviation of each sample and were compared by ANOVA One-way and Tukey post-test (5% level of significance). (p<0.05) Significant difference compared between groups. SD: standard deviation.

relative expression of transcripts normalized with  $\beta$ -actin as the control housekeeping gene.

### Evaluation of the Leukocyte Subpopulation

In the 0, 15th and 60th, day gingival tissues were excised, washed in PBS, minced and digested enzymatically for 1 h in 1 mL of digestion buffer (RPMI, 5% fetal calf serum, 2.5 mg/mL liberase [Roche, Basel, Switzerland] and tissue fragments were pressed through a 70- $\mu$ m cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). After erythrocyte lysis using  $NH_4Cl$  buffer, cells were washed, re-suspended in complete media and centrifuged for 30 min at 1,200  $\times$  g in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Total leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was always higher than 85%. The absolute number of a leukocyte subset was equal to the percentage of that cell subset multiplied by the total number of leukocytes recovered from the digested organ, divided by 100.

### Nitric Oxide Determination

Nitric oxide (NO) production was quantified by the accumulation of nitrite in the gingival tissue homogenates by a standard Griess reaction. Briefly, 50  $\mu$ L of homogenates were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylene diamine dihydrochloride, 2.5%  $H_3PO_4$ ) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. The conversion of absorbance to mmol/L NO was deduced from a standard curve by using a known concentration of  $NaNO_2$  diluted in RPMI medium. All determinations were performed in duplicate and expressed as micromolar NO.

### Immunohistochemical Analysis of RANK, RANK-L, and OPG

Longitudinal sections with thickness of 4  $\mu$ m were incubated with 1 mg/mL Pronase E in PBS for 15 min at room temperature. Endogenous peroxidase was blocked for 30 min with methanol containing 0.3% (v/v) hydrogen peroxide. After washing with PBS, the sections were treated with fetal bovine serum for 20 min and incubated for 18 h with primary antibody (anti-RANK-sc9072, RANKL-7628, and monoclonal OPG-sc11383, 1:200, Santa Cruz Biotechnology) diluted in 0.01 M PBS with 1% bovine albumin, at 4  $^{\circ}C$  in a humid chamber. Sections without the addition of primary antibodies were used as negative controls. After incubation, the sections were washed with PBS and treated with secondary antibody (anti-mouse IgG and peroxidase, 1:500, Sigma, USA) for 30 min. The sections were then washed with 0.05 M Tris-HCl buffer (pH 7.4),

treated for 5 min with 0.05% (v/v) diaminobenzidine in 0.05 M Tris-HCl (pH 7.4) containing 0.03% (v/v) hydrogen peroxide and prepared for permanent mounting in resin.

### Clinical Evaluation

Clinical evaluation was determined by quantifying the furcal area between the mesiobuccal and mesiopalatine roots. The bone area was determined between the mesiobuccal and mesiopalatine roots and below to the bone level found, through the use of ImageJ software. The image was obtained through the EOS Rebel t4i (Canon) equipment at an equivalent distance between all the teeth aiming at a standardization between the measurements made.

### Statistical Analysis

The results were analyzed by ANOVA and the Tukey post-test ( $p < 0.05$ ) using the GraphPad Prism® 5.0 Software.

## Results

### Histomorphometry of the Inflammatory Process

Figure 1 shows the distribution of the gingival tissue elements. The organization of the epithelium showed no changes in the number of layers in the two experimental periods. No areas of folding or integrity loss were observed in the PD0 group. However, the PD15 group showed more regions with projections from the deep layers into the interior of the connective tissue. For both experimental periods, the latter was intact, with all the typical elements

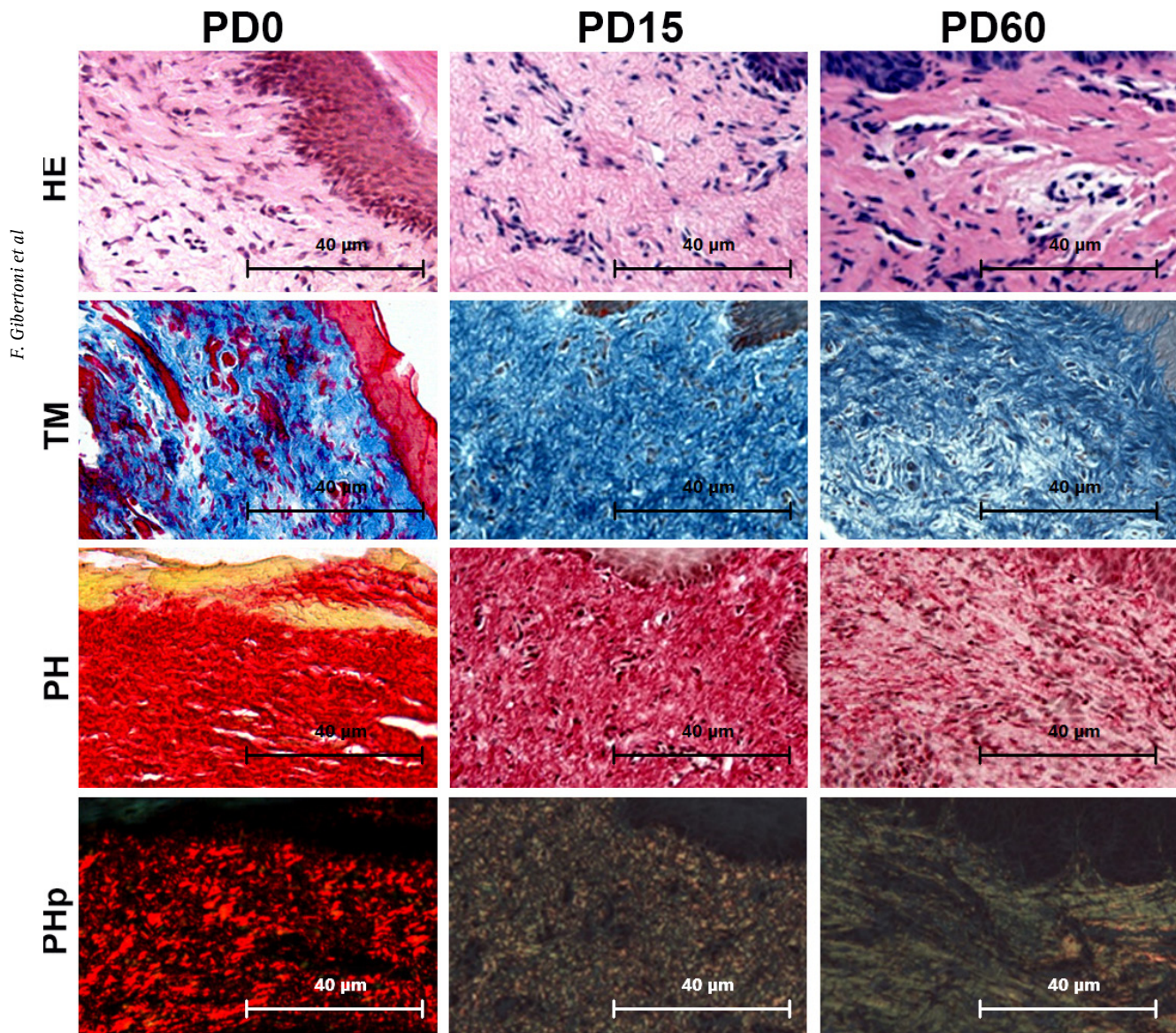


Figure 1. Sagittal sections of gingival tissue after 0 (PD0), 2 (PD15) and 8 (PD60) weeks after induction of experimental periodontitis. The sections were stained with hematoxylin-eosin (HE), Masson trichrome (TM) and Picrosirius-hematoxylin (PH). The sections were documented in bright-field and under polarized light (PHP). Bar: 100 µm (400).

of this tissue present, although a greater quantity of inflammatory infiltrate was found for the PD60 group. In the samples from the PD15 group, the collagen fibers presented similar thickness and homogeneous packing in all regions of the connective tissue, while in the PD60 group the fibers presented different thicknesses, with thicker and more compact fibers in regions closer to the epithelium, compared to deeper regions. The data obtained for the morphometric parameters are summarized in Table 1. The average thickness of the epidermis did not differ significantly between the experimental periods. The number of inflammatory cells observed in the connective tissue was significantly higher in the PD60 group ( $p < 0.0001$ ). On the other hand, the connective tissue collagen content and the area occupied by birefringent collagen fibers were higher than in the PD60 samples ( $p < 0.05$ ).

### Western Blotting Analysis

Densitometric analysis showed that there was significantly greater VEGF expression in the PD60 group, compared to the PD0 and PD15 groups. On the other hand, FGF expression was lower for the PD0 and PD60 groups. Expression of TGF- $\beta$  was similar for groups PD15 and PD60 and lower than in the PD0 group ( $p < 0.05$ ; Fig. 2).

### RT-PCR Analysis

The gene expressions of the chemokines involved in the migration of immune cell MCP-1, RANTES and IP-10 are shown in Figure 3A. All the chemokines studied showed ( $p < 0.001$ ) increases in the expression of genes in the PD60 group, compared to the PD0 and PD15 groups. The same behavior was observed for the gene expression of HGF, with higher values for animals of the PD60 group, compared to the PD0 and PD15 groups. The expression of the analyzed markers in PD0 was lower in all parameters analyzed in relation to PD15 and PD60.

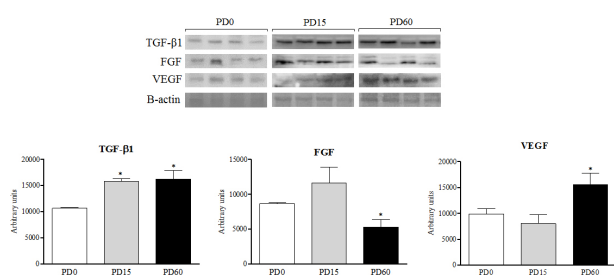


Figure 2. Analysis of TGF- $\beta$ 1, VEGF and bFGF protein expressions by Western blotting in the gingival tissue of the periodontitis at PD0, PD15 and PD60. In the graph, values are expressed as the mean  $\pm$  standard deviation. \* Significant difference compared to PD15 and PD0 ( $p < 0.05$ ).

### Leukocyte Counts and Nitric Oxide

About the gingival tissue homogenate of the PD15 group, animals showed a significantly higher PMN cell influx, compared to the PD0 and PD60 groups. Conversely, the influx of lymphocytes was greater in the PD60 group than in the PD0 and PD15 groups ( $p < 0.05$ ; Fig. 3B). The influx of macrophages was similar in the three studied groups. In terms of NO levels found in the gingival tissue, animals in the PD0 and PD15 groups showed lower ( $p < 0.05$ ) amounts of this mediator, compared to the PD60 group (Fig. 3C).

### Immunohistochemical Analysis of RANK, RANKL and OPG

The results of the immunohistochemical analysis are shown in Figures 4A and 4B. The immunostaining observed in the connective tissue revealed a greater number of cells positive for RANK and RANKL in the PD60 group, compared

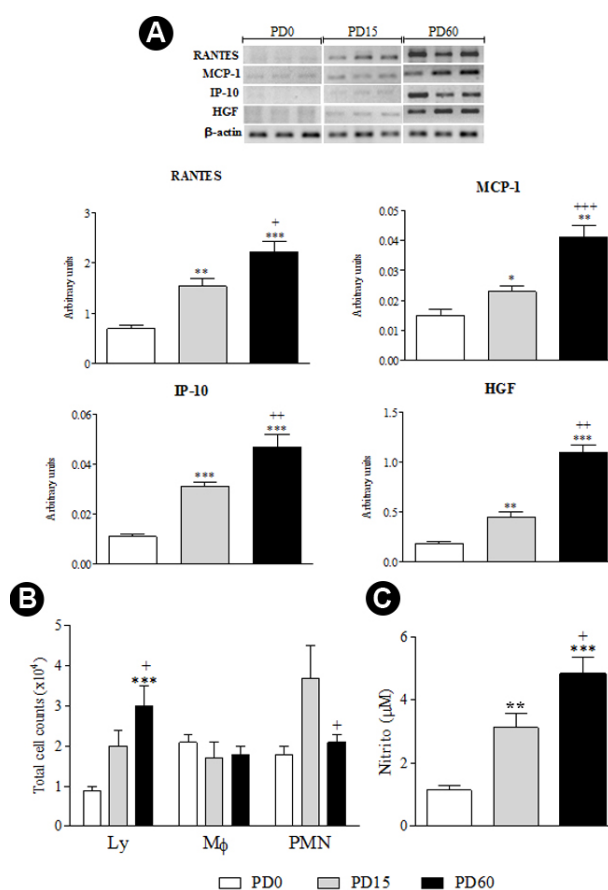


Figure 3. Analysis of RANTES, MCP-1 and IP-10 of mRNA expression by semi-quantitative RT-PCR (A), number of leukocytes (B) and levels of nitric oxide in the gingival tissue (C) of the PD0, PD15 and PD60 days. In the graph, values are expressed as the mean  $\pm$  standard deviation. \* Significant difference between PD15 and PD60 when compared to PD0 + Significant difference PD60 when compared to PD15. (\*/+  $p < 0.05$ , \*\*/+  $p < 0.005$ , \*\*\*/+++  $p < 0.0001$ ).

to the PD0 and PD15 groups ( $p < 0.05$ ). In terms of the number of cells positive for OPG, there were no significant differences among the three groups.

**Macroscopic Evaluation**

Figure 4C indicates in PD60 group a process of bone loss during the progression of periodontal disease, since there was an increase of the exposed bone area in the furcal

region in relation to groups PD0 and PD15.

**Discussion**

The initial proposal of the study was to evaluate the signaling initiated in soft tissue that reflects in the bone resorption frame. The evaluation of periodontal disease through the alteration in the expression of the biomarkers found in the soft tissue is of great importance since the

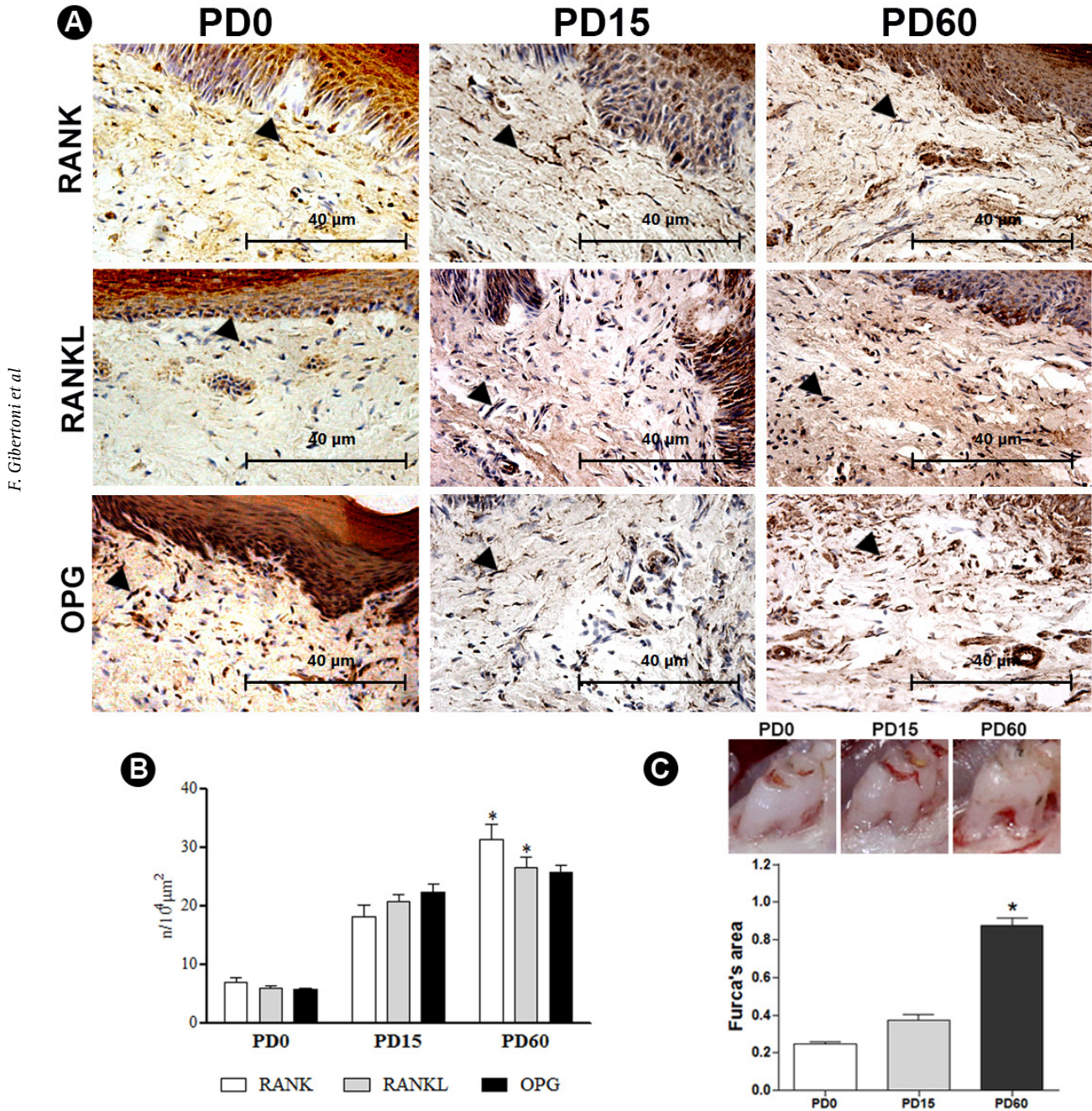


Figure 4. A: Immunohistochemical expression of RANK, RANKL and OPG in the connective tissue of the periodontitis area in PD15 and PD60. Bar: 50 μm. Ep: epidermis. Ds: Dermis. Arrow head: positive cells. B: Immunohistochemical analysis. Means and standard deviations of the number of RANK-positive cells, RANKL-positive cells and OPG-positive cells. \* Significant difference compared to PD15 and PD0 ( $p < 0.05$ ). C: Clinical evaluation determined by furcation area.

analysis of these allows the correlation with the clinical signs characteristic of this pathology (4). It appears that a number of mediators are involved in periodontal inflammation, including the generation of chemokines by several different cell types and the presumed upregulation of molecules adhesion on leukocytes and endothelial cells, which allow extravasation of leukocytes from the vascular lumen.

The development of periodontal disease is associated with the progressive infiltration of inflammatory cells into the deeper periodontal tissues, given that the blocking of inflammation reduces the evolution of the disease, while the persistence of inflammation leads to the destruction of the periodontal connective tissue (4).

Chemokines are among the inflammatory mediators that play an important role in assisting infiltration of selective subsets of leukocytes at the site of the inflammation. These substances, including MCP-1, are produced locally in the inflamed tissues, and the interaction of the chemokine with its receptor regulates the infiltration of inflammatory cells (5). In addition, MCPs, IP-10 and RANTES are chemotactic factors for monocytes/macrophages and T helper cells. IP-10 is a ligand for the CXCR3 receptor present in Th1 cells (15). Here, the expression of the MCP-1, RANTES and IP-10 chemokines were detected in gingival tissue in all periods studied, although all the chemokines expression was significantly higher in the PD60 group. In a review of the effects caused by the presence of these chemokines, Kayal (16) reported several studies showing association between the persistence of these mediators and the severity of periodontal disease.

Another feature that indirectly reflected the inflammatory process was increased VEGF expression. This is one of the most important pro-angiogenic factors, playing an important role during the remodeling phase, as well as being a strong stimulator of the migration, proliferation and survival of endothelial cells. VEGF expression can be induced when the cells are subjected to hypoxia. In periodontitis, the infiltration of inflammatory cells leads to endothelial damage and microcirculation failure, resulting in lower oxygen saturation (16). This could explain the increased expression of this growth factor in the PD60 group at the end of the experimental period, since at this stage the number of vessels was similar to the number found for the PD0 and PD15 groups. VEGF is an important factor in physiological and pathological neovascularization, and increased expression of this protein has been found in patients with periodontal disease (17).

Differential analysis of inflammatory influx revealed that in the PD15 group there was a predominance of polymorphonuclear cells, while the PD60 group showed a predominance of lymphocytes. Lymphocytes are present

in the periodontal tissue. The lesion infiltrate consists of lymphocytes, macrophages, neutrophils and mastocytes that migrate to the tissue due to the presence of different concentrations of chemokines and cytokines. IFN- $\gamma$  produced by T lymphocytes stimulates the phagocytic activity of macrophages and neutrophils (4), which can subsequently govern the destruction of gingival tissue, as observed here.

We suggest that the increased inflammatory influx observed in the PD60 group was directly related to increased expression of the MCP-1, RANTES and IP-10 chemokines, since angiogenesis was not responsible for the observed increase in cellular influx.

The results showed that cell influxes and nitric oxide levels were higher in the PD60 group. Excess production of NO during inflammation can be harmful, leading to the destruction of the host tissue, as observed during the experimental period. It is produced via iNOS by macrophages and polymorphonuclear leukocytes during the progression of periodontal lesions, resulting in worsening of the pathology. Several studies have investigated the role of NO in the progression of periodontal diseases in humans, as well as in ligature-induced periodontitis in rats, demonstrating its increasing participation during the progression of periodontal disease. The nitric oxide concentration can therefore be used as a marker of inflammation, indicating the status of the pathology (18,19). Elevated HGF levels have been found in patients suffering chronic diseases including periodontitis (9). The HGF concentration in gingival crevicular fluid (GCF) and saliva, proportionally increases with the progression of periodontitis and HGF can be considered a biomarker of the severity of this disease (20). These features help to explain the results obtained in the present experimental model, where the PD60 group showed significantly higher levels of this protein, compared to the PD0 and PD15 groups, reflecting increased severity of the disease in later stages. Hepatocyte Growth Factor (HGF) is synthesized and secreted by various cell types (osteoclasts (21), osteoblasts, chondrocytes and synovial cells), regulates the growth and differentiation of many tissues and is considered a regulator of Bone remodeling (22). It also has a significant effect on the differentiation and proliferation of osteoclast precursors and the activity and survival of these cells. (23).

The addition of HGF in osteoclast cultures inhibited bone resorption (24) suggesting its modulatory role between reabsorption and migration. Therefore, the presence of HGF is a determining factor for the prevention of bone loss. HGF induces biological responses in osteoclasts and osteoblasts by promoting the migration of important cells in the early stages of bone resorption and also balances the proliferation of these cells. Its important role in angiogenesis has also

been demonstrated (23). It is known that in the model of periodontitis the concentrations of HGF are increased, however, this indicator is not related to its metabolic activity (25,26). This fact may explain the increase in HGF in our model. In addition, the observed increase of this growth factor during the marked inflammation in late stages of periodontitis may indicate the need for angiogenesis and bone remodeling once it has its role in these two processes.

The bFGF growth factor may be an important positive regulator for the recruitment of leukocytes during acute and chronic inflammation. These considerations provide an explanation for the results obtained in the present experimental model, where the expression of bFGF was greater in the PD15 group than in the PDO and PD60 groups, because during the early stage (PD15) the inflammatory process initiated the recruitment of inflammatory cells. Expression corresponding to bFGF was observed in the immunohistochemical analysis of the periodontal tissues including the gingiva and the periodontal ligaments. The data showed that bFGF expression could be detected in immature granulation tissue after 7 days of the experimental procedure, with a decrease on day 14, indicating that bFGF was induced during the initial stages of regeneration of the periodontal tissue (27).

The TGF- $\beta$ 1 cytokine plays a crucial role in connective tissue regeneration, bone remodeling, recruitment of leukocytes and stimulation of the inflammatory phase (28). In the present work, there was persistence of an inflammatory condition throughout the trial period, independent of the stage of progression of periodontal disease, as shown by the constant levels of TGF- $\beta$ 1. On the other hand, in the PDO group TGF- $\beta$ 1 expression was decreased in relation to the other groups, indicating tissue integrity. Other studies have described increased TGF- $\beta$  expression associated to periodontal disease, which could be related to its role in the remodeling of gingival tissue (29).

The increased expressions of the inflammatory markers (TGF, HGF, VEGF and chemokines) observed in this study are indicators of an aggressive inflammatory process and explain a picture of red edema-like gingiva and bleeding probing that characterize the clinical signs of periodontitis.

An important mechanism influencing the development of periodontal disease involves the RANK, RANKL and OPG system. RANK, the activation receptor of NF- $\kappa$ B, is found on the surfaces of osteoclasts. Its ligand, RANKL, is a transmembrane protein expressed in different cell types, especially osteoblasts and activated T cells. RANKL forces the cell to interact physically with the precursors of osteoclasts and cementoclasts and binds to its receptor (RANK) to induce the resorption of hard tissue. Osteoprotegerin (OPG) is a secreted member of the TNF family, expressed by osteoblasts, cementoblasts, fibroblasts and T lymphocytes.

It inhibits bone or tooth resorption by strongly binding to its ligand (RANKL), hence preventing the latter from binding to its receptor (RANK) (30).

The results showed that the development of periodontal disease was associated with increased levels of cells that are positive markers of bone resorption (RANK and RANKL) and decrease of the RANKL ligand (OPG), possibly due to the observed increase in lymphocyte infiltrate during the trial period. The production of RANKL occurs by activation of T lymphocytes present in the tissue, modulating osteoclastogenesis and bone remodeling. This would explain the bone loss in periodontitis (PD60). Increases of lymphocytes and the persistence of inflammatory cells in the PD60 group could have been responsible for the increased expression of markers indicators of bone destruction. RANKL expression was greater in an advanced stage of periodontitis, compared to gingivitis. This suggests that RANKL could play an important role in periodontal resorption and that its inhibition might decrease resorption of periodontal bone (31). This fact can be reinforced by the decrease in OPG (RANKL ligand and osteoclastic modulator) observed in periodontitis (PD60). However, this fact was not observed in periodontal disease (PD15) where we can observe a balance in RANKL / OPG concentrations indicating osteoclastogenesis modulation by non-activation of RANK. In the PDO group the results showed decreased levels of these immunomodulators when compared to the experimental groups. These results are corroborated with the macroscopic evaluation and furcal area in which the increase in this region was higher in PD60 than in the other groups.

In conclusion, in this animal model the increase of RANK/RANKL and HGF markers is related to a specific immune response and probably contributed to the evolution of periodontal disease. Investigating the effect of these biomarkers can help in targeted therapy for bone resorption, since blocking these can inhibit bone loss.

## Resumo

Este estudo avaliou marcadores de perda óssea e da resposta imune presentes na evolução da doença periodontal. Cento e dois ratos Wistar foram divididos em três grupos de animais: PDO, sem ligadura e PD15 dias e PD60 dias, submetidos a colocação de ligadura com um fio de seda estéril 3-0 na região cervical do primeiro molar superior em ambos os lados. Foram obtidas amostras de tecido gengival para análise histomorfológica, análises imunohistoquímicas de RANK, RANKL, OPG, caracterização do infiltrado inflamatório, quantificação de óxido nítrico, expressão de quimiocinas MCP-1, RANTES, IP10 e do TGF- $\beta$ 1, VEGF e bFGF. O número de células inflamatórias no tecido gengival foi maior nas amostras PD60. O teor de colágeno na área ocupada pelas fibras de colágeno birrefringentes foram menores para PD60. A contagem diferencial de leucócitos mostrou que houve um influxo polimorfonuclear significativamente maior no grupo PD15, enquanto que PD60 mostrou número maior de linfócitos. PD60 apresentou transcritos de genes RANTES, IP-10, MCP-1 mais elevados, bem como uma maior concentração de óxido nítrico. A avaliação clínica revelou que o grupo PD60 apresentou aumento da área óssea exposta



na região da furca. Em conclusão, neste modelo animal o aumento dos marcadores RANK/RANKL e HGF está relacionado a uma resposta imunológica específica e provavelmente contribuiu para a evolução da doença periodontal. Investigar o efeito destes biomarcadores pode ajudar na terapia dirigida para a reabsorção óssea, uma vez que bloquear estes pode inibir a perda óssea.

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