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Effects of periodontal therapy on white blood cell count and levels of transforming growth factor beta in serum of subjects with severe periodontitis

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Abstract

This study aimed to investigate the effects of nonsurgical periodontal therapy on white blood cell (WBC) count and levels of transforming growth factor beta (TGF-β) in serum from subjects with severe periodontitis. Serum from 28 subjects with periodontitis (mean age: 34.36±6.24; 32% men) and 27 healthy controls (mean age: 33.18±6.42; 33% men) were collected prior to therapy. Blood samples were obtained from 23 subjects who completed therapy (9-12 months). A well-controlled periodontal treatment protocol was established in three stages: mechanical periodontal therapy (scaling and root planning), reinstrumentation of dental sites, and supportive periodontal therapy. Periodontal and systemic parameters such as the total number of WBCs and TGF-β levels, accessed by enzyme-linked immunosorbent assay (ELISA), were included. After therapy, all clinical periodontal parameters decreased ($p<0.0001$). There were no statistical differences in WBC count between experimental and control groups before or after therapy. However, after therapy, the mean value of lymphocytes in patients with localized aggressive periodontitis (LAgP) was statistically higher than that of patients with generalized chronic periodontitis (GCP) ($p<0.0357$). Additionally, TGF-β levels in LAgP and GCP patients were higher compared to controls before therapy ($p<0.05$ and $p<0.01$, respectively). In LAgP patients, periodontal therapy was associated with increased number of lymphocytes.

Key words: Inflammation; periodontitis; leukocyte count; transforming growth factor beta.

Introduction

Periodontal diseases are characterized as the pathological manifestation of the host immune response to the bacterial infection at the tooth/gingival interface. These are mainly caused by Gram-negative bacteria, including *Porphyromonas gingivalis* (*Pg*), *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* (*Aa*), and *Tannerella forsythia*. Individual response variations can be explained by a combination of factors that confer upon them a complex nature. Among these factors, susceptibility becomes prominent, with special attribution given to genetic polymorphisms, environmental factors and pathogen virulence factors (1, 2). Individuals affected by the disease share common polymorphisms in specific genes that are important for the regulation of the inflammatory response (3, 4). However, the determinants for the susceptibilty of periodontitis are not well defined (5).

Severe periodontitis affects up to 15% of most populations (6). In Brazil, the most recent National Survey of Oral Health reported that the distribution of the most severe forms of periodontal disease is more significant in adults between 35-44 years of age, with a prevalence of 19.4% (7).

The term periodontitis generically comprises of chronic forms of periodontal disease, which is the result of a polymicrobial infection and is characterized by the loss of collagen fibers and insertion in the cementum surface (mineralized tissue that covers the root surface), apical migration of junctional epithelium (epithelium continuous with the oral epithelium that promotes the insertion of the gum to the tooth), periodontal pocket formation (cementum surface devoid of periodontal fibers) and alveolar bone reabsorption. Such damage compromises the function of the periodontal tissues and may result in tooth loss (1).

More than 700 bacterial species can occupy periodontial pockets (8), and a combination of aerobic and anaerobic microbiota is typically seen in infection. Substantial tissue destruction in patients with severe periodontitis is characterized, in many cases, by the presence of deep periodontal pockets around many or all the teeth. The aggregated epithelial lesions equal in size to an ulcerated wound with an area of 8 to 20 cm^2, in accordance with clinical estimations (9). However, the disease can remain asymptomatic for decades, during which time its detection is performed only by clinical examination with periodontal probe and/or intraoral x-rays (9). Thus, the chronic and cyclical nature of periodontal disease provides an opportunity for continuous hematogenous dissemination of periodontal pathogens, bacterial antigens and inflammatory mediators (5, 10).

Since the beginning of the 1990s, evidence has pointed to periodontitis as a risk factor for systemic conditions, such as cardiovascular disease (CVD), adverse outcomes of pregnancy, diabetes and lung disease (11). Recently, a review of the evidence on associations between periodontitis and systemic diseases and conditions, particularly respiratory disease, chronic kidney disease, rheumatoid arthritis, cognitive impairment, obesity, metabolic syndrome and cancer was also published. There was strong evidence that improving oral hygiene has positive effects on the prevention of

nosocomial pneumonia. The published evidence supports modest correlations between periodontitis and chronic kidney disease and obesity and weak correlations between periodontitis and cognitive impairment, rheumatoid arthritis and metabolic syndrome (5). In this context, the increase in the number of white blood cells (WBC) is associated with the increased risk of coronary heart disease, CVD, atherosclerosis, thrombosis and myocardial ischemia. This increase may be caused by the inflammatory nature of chronic infections such as periodontitis (12).

The T cells CD4+ CD25+ that express transcription factor Foxp3 are regulatory T cells (Tregs) that prevent the overreactive adaptive immune response and may be associated with periodontal disease and its alleviation. These specifically regulate the activation, proliferation and effector function of activated T cells and the restoration of homeostasis after infection (13). In the presence of Tregs, a reduction in the levels of interferon gamma (IFN-γ), interleukin (IL) -17, tumor necrosis factor (TNF) and IL-1β is observed at murine and canine periodontal disease sites (14). Cytokines produced by Tregs are IL-10, transforming growth factor beta (TGF-β) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which, in general, subregulate inflammation. IL-10, TGF-β1 and CTLA-4 are reported to slow the progression of periodontal disease (5). In addition to the protective role in periodontal tissue damage, Tregs also play an important role in immune tolerance (15). Also, TGF-β has been studied as a form of treatment in the context of the healing and regeneration of periodontal bone (14). For example, Tregs negatively modulates proinflammatory cytokines responsible for the inhibition of various processes during the regeneration of tissue.

Nonsurgical periodontal therapy acts to reduce inflammation markers in the blood (C-reactive protein [CRP], IL-6, etc.) and reveals some important aspects of the systemic reach of periodontal inflammation (4, 15-17). Thus, based on the concept of the resolution of disease, it was established in this study that periodontal therapy would be considered complete only after confirming the remission of clinical signs of inflammation such as the absence of bleeding on probing (BOP) and residual periodontal pockets. However, it is emphasized that the mechanical removal of the biofilm by scaling and root planning, with or without another supporting modality, does not mean that, at any magnitude, inflammation / infection does not persist. Sometimes the removal of local factors is only achieved after repeated specific actuations principally at sites in which there still exist clinical signs of inflammation. Sites with residual periodontal pockets that continue bleeding on probing undoubtedly require new instrumentation (scaling and root planning) and reinforcement of oral hygiene instruction. Moreover, the need for reinstrumentation and clinical response are unique to each individual patient and for this reason, there is no way to generically establish the completion of therapy for all patients at the same time.

The goal of this study was to investigate the effects of severe periodontitis as well as the contributions of nonsurgical periodontal therapy on the systemic inflammatory response regarding the serum levels of TGF-β and WBC count. Furthermore, the relationship between the number of lymphocytes, serum levels of TGF-β, and subgroups of severe periodontal disease (generalized aggressive periodontitis [GAgP], localized aggressive periodontitis [LAgP] and generalized chronic periodontitis [GCP]) was explored. For this, the individual response of each patient to therapy was considered, in respect to the time needed to resolve inflammation in each individual and, from this, the establishment of supportive periodontal therapy (SPT), also known as periodontal maintenance.

Materials and methods

Subjects and study groups

The study protocol was approved by the Ethics Committee of the Faculty of Health Sciences - University of Brasília, Brazil (045/2008). All subjects were informed about the purpose of the study, both orally and written, and an informed consent document prior to participation was also signed. The clinical trial was registered at http://www.clinicaltrials.gov.br/, No. RBR-24T799.

The total sample (convenience sampling) consisted of 55 systemically healthy subjects. The periodontitis group (PG) consisted of 19 women (68%) and nine men (age range 20-45; mean age: 34.36±6.24), with ≥18 teeth. The classification of periodontal disease was according to Armitage and Cullinan (18). Chronic periodontitis (CP), with a higher prevalence in adults, can also occur in children and adolescents. In this, there is correlation between the severity of bone destruction and quantity of dental biofilm and calculus, in addition to the moderate growth rate in the majority of cases. CP is classified as localized when less than 30% of dental sites (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) are affected, and as generalized (GCP) when it exceeds this limit. LAgP is characterized by the destruction of the periodontal tissues located in the first molar / incisor with interproximal insertion loss in at least two permanent teeth, one of which is the first molar, and involving no more than two teeth beyond the first molars and incisors. This is normally detected and diagnosed during puberty in systemically healthy individuals. The GAgP usually affects individuals under age 30, but can affect older individuals. The loss of interproximal insertion is characterized as generalized when it affects at least three permanent teeth, in addition to the first molars and incisors. In addition, the AgP is characterized by a rapid loss of clinical attachment and alveolar bone.

The control group (CG) consisted of 18 women (67%) and nine men (age range 21-44; mean age: 33.18±6.42) with clinical probing depth (PD) ≤3mm and clinical attachment level (CAL) ≤ 3mm, ≤ 10% of sites with BOP, and no radiographic evidence of bone loss (**Figure 1**). Exclusion criteria were history of smoking, pregnancy or lactation, periodontal therapy, antimicrobial therapy for systemic conditions or use of topical oral antibiotics in the last twelve months, diabetes, autoimmune disease, acute infections, severe allergies, gastrointestinal and renal diseases, cancer, morbid obesity (body mass index [BMI] >40kg/m^2) or underweight (malnourished BMI <18.5kg/m^2) (19), and use of medications that alter the levels of inflammatory mediators.

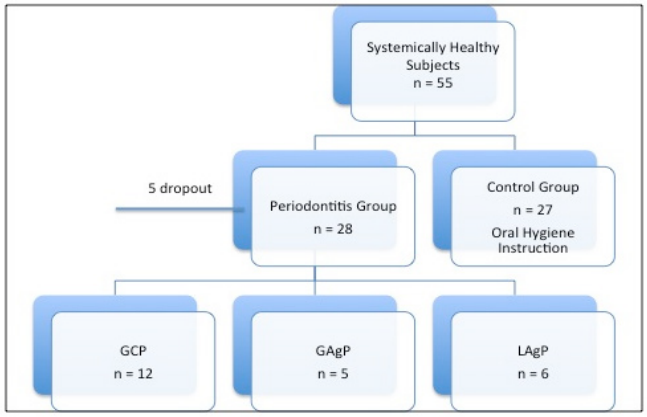

Figure 1. Study design. GCP - generalized chronic periodontitis; GAgP - generalized aggressive periodontitis; LAgP - localized aggressive periodontitis.

Clinical examination

The clinical examination performed at baseline and after six months of SPT included detection of visible plaque accumulation described as plaque index (PI) (20), BOP (20), PD and CAL. Measurements were assessed at four sites around each tooth, buccal, lingual and both proximal sites using a manual probe (probe Michigan O with Williams markings) excluding third molars. Clinical examination was performed by a single experienced examiner. The calibration and measurements for PD and CAL were repeated within 24 h and demonstrated a concordance rate above 80%. The BOP was calculated by Kappa coefficient and the intra-examiner agreement was > 0.85.

Treatment protocol and laboratory analysis

PG subjects were treated in three stages: mechanical periodontal therapy (scaling and root planning) with Gracey curettes (5/6, 7/8, 11/12, 13/14), reinstrumentation of sites and SPT. The first stage was carried out in ≤14 days. One month later, in stage 2, new mechanical instrumentation was performed in patients who persisted with deep pockets, BOP and calculus. At this stage, meticulous scaling and root planning were performed until the following periodontal conditions were reached: PD greater than 4mm in at least three or fewer sites, PD greater than 5mm in two places at most, PI ≤ 15% and BOP ≤ 10%. In Stage 3, subjects were scheduled biweekly or monthly, according to the need to control biofilm formation. SPT was performed for six months. CG subjects received oral hygiene instruction at baseline.

Out of the 28 subjects in the PG (14 patients diagnosed with GCP; 8 GAgP patients; 6 LAgP patients), five did not complete the treatment. Twenty-three completed the three stages of periodontal protocol (12 GCP patients, 5 GAgP patients and 6 LAgP patients). Among these, ten subjects (43%) completed the treatment in nine months, ten subjects (43%) in ten months, and three subjects (14%) in 12 months.

Blood samples were collected for biochemical analysis at baseline for all 28 PG subjects and 27 CG subjects. Subsequent blood samples were collected from 23 PG subjects who completed treatment in their respective time periods after the beginning of treatment: 9 months (ten individuals), 10 months (ten individuals) and twelve months (three individuals). Blood was collected in gel separator tubes between 7 and 8 h. Each EDTA tube was analyzed within three hours in order to perform blood counts with standard measurements for the number of neutrophils, lymphocytes, monocytes, basophils and eosinophils. Plasma and serum samples were immediately placed on ice and stored at -80°C.

Serum levels of TGF-β were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems). Briefly, 96-well plates were coated with anti-human TGF-β. After blocking for 2 h to avoid non-specific binding, the standard cytokine provided with the kit or serum samples were inserted. The cytokine was detected by horseradish peroxidase-labeled monoclonal antibody specific to the target after anti-human biotinylated antibodies were placed in each well and incubated for 2 h at room temperature. The microplate was washed to remove unbound enzyme-labeled antibodies. The amount of horseradish peroxidase bound to each well was determined by the addition of a substrate solution. The reaction was stopped by the addition of 1 M sulfuric acid, and the plates were read at 492 nm in a microplate reader (Thermo Scientific). The concentrations of TGF-β in each sample were determined by interpolation from a standard curve and presented as picograms per milliliter (± standard deviation). Serum levels of IL-6 were also investigated but were non-detectable in the samples.

Statistical analysis

The clinical periodontal parameters PI, PD (≤3, 5 and 6mm) and BOP prior to and post-therapy in the PG were compared by paired Student's *t*-test, as well as lymphocytes, monocytes, neutrophils and total white blood cells. In both periods evaluated for this group, a nonparametric Wilcoxon test was used for the following variables: number of teeth (NT), PD = 4 and ≥7 mm, eosinophils and basophils. The measurements NT, PI, BOP and hematological characteristics were compared between both groups before and after therapy by Student's *t*-test for variables that showed the Gaussian distribution. In cases where normality was not observed for both groups, the nonparametric Mann-Whitney test was used. Data were analyzed by SAS 9.2 for Windows. For purposes of analysis, a significance level of 5% was used ($p<0.05$).

To avoid interference of periodontal disease classification in the interpretation of the results, comparisons of the mean values of the variables studied in post-treatment between the three types of periodontitis (GCP, GAgP, LAgP) from the periodontitis group were made. Thus, comparisons of all tested measures cited above were made using the analysis of covariance model (ANCOVA). The value of the measurement after therapy was considered as the dependent variable and the value of the measurement at baseline was the auxiliary variable. Tukey's adjustment was used to adjust for three multiple comparisons and pre-specified contrasts were used to test three hypotheses: first, the mean values in GAgP subgroup do not significantly differ from the mean values in LAgP subgroup; second, the mean values in GAgP subgroup do not significantly differ from the mean values in the GCP subgroup; and third, the mean values in LAgP subgroup do not significantly differ from the mean values in the GCP subgroup. For the age variable, comparisons were made of the mean

Table 1. Clinical oral parameters before and after supportive periodontal therapy.

Parameters*	Control (n=27)	Pre-therapy (n=28)	Post-therapy (n=23)	p value pre x post	p value control x pre	p value control x post
NT	28.78±2.01	27.25±4.84	24.70±5.79	0.0001[1]	0.5317[3]	0.0294[3]
PI (%)	4.74±2.30	63.61±33.64	4.83±6.73	<0.0001[2]	<0.0001[3]	0.0679[3]
BOP (%)	2.67±1.49	44.46±29.35	1.63±3.35	<0.0001[2]	<0.0001[3]	<0.0001[3]
PD (mm)						
≤3 mm	100.00±0.00	68.71±14.38	98.32±1.79	<0.0001[2]	NA	NA
4 mm	0.00±0.00	4.02±4.02	0.63±0.96	<0.0001[1]	NA	NA
5-6 mm	0.00±0.00	17.08±8.85	0.85±1.22	<0.0001[2]	NA	NA
≥7 mm	0.00±0.00	10.45±8.89	0.17±0.61	<0.0001[1]	NA	NA
CAL (mm)						
≤3 mm	100.00±0.00	62.56±18.20	NA	NA	NA	NA
4 mm	0.00±0.00	4.97±4.77	NA	NA	NA	NA
5-6 mm	0.00±0.00	18.74±8.96	NA	NA	NA	NA
≥7 mm	0.00±0.00	13.73±11.49	NA	NA	NA	NA

*Results shown as mean ± standard deviation (SD).

[1]Wilcoxon test; [2]pair T test; [3]Mann-Whitney test; NA – not apllicable.

NT - number of teeth, PI - plaque index, BOP - bleeding on probing, PD - probing depth, CAL - clinical attachment level.

Table 2. Hematologic parameters before and after supportive periodontal therapy.

Parameters*	Control (n=27)	Pre-therapy (n=28)	Post-therapy (n=23)	p value pre x post	p value control x pre	p value control x post
Eosinophils (mm^3) references 45 a 400	143.74±102.00	227.96±179.80	178.26±95.26	0.1666[1]	0.0549[3]	0.2548[3]
Basophils (mm^3) references 0 a 100	14.19±31.35	8.71±21.97	13.30±21.30	0.2188[1]	0.5765[3]	0.6033[3]
Lymphocytes (mm^3) references 900 a 3300	2238.52±520.45	2136.75±508.12	2075.22±550.65	0.3018[2]	0.4663[4]	0.2870[4]
Monocytes (mm^3) references 90 a 800	428.22±141.26	368.75±128.59	366.65±138.50	0.5187[2]	0.1082[4]	0.1277[4]
Neutrophils (mm^3) references 2295 a 6500	3208.56±865.86	3548.64±1279.78	3104.59±1496.55	0.0871[2]	0.3279[4]	0.8049[4]
Total Leukocytes (mm^3) references 4500 a 10000	6171.48±1260.51	6297.50±1515.88	5970.00±1734.72	0.1808[2]	0.7393[4]	0.6373[4]

*Results shown as mean ± standard deviation (SD).

[1]Wilcoxon test; [2]pair T test; [3]Mann-Whitney test; [4]T test.

values after therapy among the three groups using the analysis of variance model (ANOVA). Tukey's adjustment was used to adjust for three multiple comparisons, and pre-specified contrasts were used to test the same above-mentioned three hypotheses. A comparison of the proportion of males, as well as females, between groups was tested using the chi-square test.

Finally, serological data, pre- and post-therapy, in reference to the quantification of TGF-β by ELISA were analyzed by ANOVA following a criterion of Tukey's post-test in GraphPad Prism (v5.0). For purposes of analysis, a significance level of 5% was used ($p<0.05$).

Results

Demographic, hematological characteristics and clinical oral parameters of the study population, before and after nonsurgical periodontal therapy

Initially, 28 patients with periodontitis were involved in this study (32% men; mean age: 34.36±6.24) and 27 healthy controls (33% men; mean age: 33.18±6.42). There was no difference between PG and CG for age ($p=0.4955$) and gender ($p=0.9251$). Of all the patients in the PG, 23 were followed until remission of periodontal disease. Clinical oral parameters prior to and post-SPT are shown in Table 1.

In the PG, nonsurgical periodontal therapy led to a significant decrease in all clinical periodontal parameters ($p<0.0001$). At baseline, the CG was significantly different from the PG for PI and BOP ($p<0.0001$). The mean values for PI and BOP in the PG were greatly reduced after the therapy.

As shown in Table 2, there were no statistical differences in hematological parameters between the PG and the CG before or after therapy. In addition, these parameters for the PG were not modified after nonsurgical periodontal therapy.

Among the 23 individuals who completed treatment, 12 presented GCP (8 women and 4 men; mean age: 37.17±1.63), five, GAgP (4 women and 1 man; mean age: 31.60±2.53) and six LAgP (4 women and 2 men; mean age: 30.67±2.31). The values for the age, as well as the proportion of males and females in the PG after therapy did not differ among the three periodontitis subgroups ($p=0.553$ and $p=0.8485$, respectively). Since the PG in this study consisted of three subgroups of severe periodontitis, comparisons were made of the mean values post-treatment between these subgroups for the above-mentioned measures (Tables 3 and 4). It was observed that except for lymphocytes cells, the

mean values did not significantly differ among the three subgroups of severe periodontitis (GCP; GAgP; LAgP). However, the mean value corresponding to the lymphocyte cells in the LAgP subgroup was statistically higher than the mean value in the GCP subgroup ($p<0.0357$), with no statistically significant difference between the other subgroups. The mean values for lymphocyte cells in three subgroups, in descending order, were LAgP > GCP > GAgP.

ELISA before and after nonsurgical periodontal therapy

In the PG, samples of the disease groups (LAgP, GAgP, GCP) showed a higher amount of TGF-β before nonsurgical periodontal therapy, however, there was no significant difference when the TGF-β levels were compared before and after therapy for all groups (**Figure 2)**. In addition, TGF-β levels were significantly higher in patients with LAgP ($p<0.05$) and GCP ($p<0.01$) compared to controls prior to nonsurgical periodontal therapy. IL-6 was not detectable in any disease group and analyzed time.

Discussion

In individuals susceptible to periodontitis, unnatural resolution of periodontal inflammation results in chronic inflammation, which may have a systemic impact (5, 21-25). The acute inflammatory response is protective, but a failure to remove the inflammatory cells, especially neutrophils, characterize chronic, pathological and destructive lesions (5). Therefore, it is evident that the three aspects of the pathogenesis of periodontitis (infection, inflammation and adaptive immunity) have a potential role and impact on the systemic immunoinflammatory response, which either initiate or mediate a wide range of systemic diseases (5).

In a previous study by our group, we found levels of high-sensitivity C-reactive protein (hs-CRP) > 0.3 mg/dL in individuals with severe periodontitis compared to controls (60.87 versus 23.08, respectively; $p=0.0216$) (7) and periodontal therapy was associated with a decrease in hs-CRP levels circulating in serum and an increase in high density lipoprotein.

This study investigated the WBC count in peripheral

Table 3. Clinical oral parameters before and after supportive periodontal therapy in periodontitis subgroups.

Variables	periodontitis subgroups*			p value			
	GAgP	LAgP	GCP	Comparison between subgroups – F test†	GAgP vs LAgP§	GAgP vs GCP§	LAgP vs GCP§
NT	22.81 ± 1.09	24.79 ± 1.01	25.43 ± 0.72	0.1722	0.3736	0.1551	0.8729
PI (%)	9.71 ± 2.18	3.26 ± 2.02	3.57 ± 1.41	0.0624	0.1035	0.0701	0.9916
BOP (%)	3.22 ± 1.26	1.61 ± 1.16	0.98 ± 0.77	0.3304	0.6541	0.3012	0.8946
PD (mm)							
≤3 mm	98.16 ± 0.77	98.66 ± 0.75	98.21 ± 0.47	0.8742	0.9029	0.9984	0.8702
4 mm	0.89 ± 0.45	0.43 ± 0.42	0.63 ± 0.29	0.7568	0.7364	0.8791	0.9204
5-6 mm	1.63 ± 0.54	0.07 ± 0.53	0.91 ± 0.35	0.1719	0.1480	0.5138	0.4220
≥7 mm	0.06 ± 0.30	-0.05 ± 0.27	0.33 ± 0.18	0.4406	0.9664	0.7131	0.4794

***** Values plus or minus are the adjusted mean ± standard error.

† p-values calculated using ANCOVA; § p-values between subgroups calculated using ANCOVA. Multiple comparisons were adjusted by Tukey's test.

GAgP - generalized aggressive periodontitis, LAgP - localized aggressive periodontitis, GCP - generalized chronic periodontitis.

NT - number of teeth, PI - plaque index, BOP - bleeding on probing, PD - probing depth.

Table 4. Hematologic parameters before and after supportive periodontal therapy in periodontitis subgroups.

Variables	periodontitis subgroups*			p value			
	GAgP	LAgP	GCP	Comparison between subgroups – F test†	GAgP vs LAgP§	GAgP vs GCP§	LAgP vs GCP§
Eosinophils (mm^3) references 45 a 400	143.65 ± 41.92	214.29 ± 38.38	174.67 ± 27.30	0.4625	0.4374	0.8143	0.6887
Basophils (mm^3) references 0 a 100	16.62 ± 8.64	10.92 ± 7.92	13.11 ± 5.68	0.8840	0.8741	0.9411	0.9740
Lymphocytes (mm^3) references 900 a 3300	1939.53 ± 153.83	2397.50 ± 132.14	1970.61 ± 89.95	**0.0372**	0.1100	0.9840	**0.0357**
Monocytes (mm^3) references 90 a 800	384.91 ± 57.47	353.58 ± 54.42	365.58 ± 36.34	0.9282	0.9234	0.9556	0.9823
Neutrophils (mm^3) references 2295 a 6500	3397.12 ± 903.39	2890.54 ± 542.42	3153.11 ± 391.02	0.8672	0.8785	0.9684	0.9205
Total Leukocytes (mm^3) references 4500 a 10000	6028.84 ± 628.26	6124.48 ± 593.06	5868.24 ± 411.66	0.9360	0.9933	0.9751	0.9357

***** Values plus or minus are the adjusted mean ± standard error.

† p-values calculated using ANCOVA; § p-values between subgroups calculated using ANCOVA. Multiple comparisons were adjusted by Tukey's test.

GAgP - generalized aggressive periodontitis, LAgP - localized aggressive periodontitis, GCP - generalized chronic periodontitis.

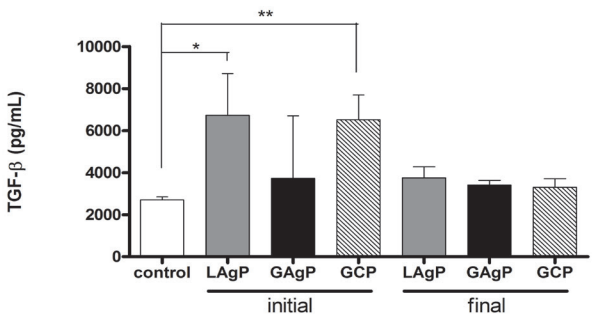

Figure 2. Serum levels of TGF-β pre and post-treatment. TGF-β - transforming growth factor beta.

blood and TGF-β levels in the three severe periodontitis groups (GAgP, LAgP e GCP) as well as the effects of nonsurgical periodontal therapy on them. It was noted that the chronic lesions of human periodontal disease showed an increased expression of regulatory cytokines such as TGF-β (26). Although Tregs and Th17 cells perform different roles in the pathogenesis of infection, it has been shown that there is a reciprocal path of development. Naive T cells exposed to TGF-β upregulate Foxp3 and become induced Tregs, but when cultured with TGF-β and IL-6, the naive T cells generate Th17 cells with pathological activities (osteoclastogenesis). Thus, when the immune response is not activated, TGF-β promotes the generation of induced Tregs that suppress inflammation. However, when an infection is established, for example, during the gingivitis or early stage of periodontitis, IL-6 is synthesized during the innate immune response by inhibiting the generation of Tregs and inducing the differentiation of Th17 cells that are pro-inflammatory and pro-resorptive in the presence of TGF-β (27). It is also known that the absence of Tregs, it is an indication for a great variety of disorders, such as autoimmunity, dermatitis, periodontitis and even transplant rejection. A potential treatment option for these disorders revolves around increasing the number of Tregs in local sites (28, 29).

This study demonstrated, prior to the nonsurgical periodontal therapy, an increase in TGF-β levels in the serum of patients with LAgP and GCP compared to controls (**Figure 2)**. This suggests a systemic effect of the disease, where TGF-β in periodontitis patients appears as a molecular component associated with the immunosuppression of the inflammatory response, as TGF-β levels were higher in patients with LAgP and GCP than in the controls. IL-6 levels are not shown here in the results, since this cytokine was not detectable in the samples. Since the prevalence of LAgP is low (18, 30), the adopted grouping criterion also allowed the obtainment of a larger number of individuals with severe disease in the periodontitis group (Tables 1 and 2). However, the analysis of these individuals, also in periodontitis subgroups (LAgP, GAgP e GCP), becomes justified by previous studies (30-32) having shown a biological distinction in relation to systemic inflammatory markers between the AgP and CP.

WBC count is characterized as a crude marker of systemic inflammation and correlates to the host response with respect to a variety of stimuli. This marker has also been associated with a significant prediction of future cardiovascular events and glucose intolerance in different populations (33, 34).

The number of eosinophils, basophils, lymphocytes, monocytes, neutrophils and total leukocytes found in patients with severe periodontitis in this study (Table 2) did not set any leukocytosis frame, indicating that systemic inflammation raised by periodontal infection was not well pronounced. This also suggests that in general, participants in the study were systemically healthy, and nonsurgical periodontal therapy produced no change in leukocyte differential counts.

These data confirmed the results of earlier studies (35, 36) showing that peripheral blood mononuclear cells from patients with periodontitis did not proliferate in response to bacterial antigens. However, other studies (37, submitted paper in *J. Investig. Clin. Dent.* 2014, Oct 6) have confirmed an increased number of WBCs and neutrophils in the peripheral blood of patients with moderate to severe periodontitis, with a positive correlation between disease severity and number of WBCs in the blood. It was recently demonstrated a significant increase in the number of neutrophils in the peripheral blood of patients with GAgP (submitted paper in *J. Investig. Clin. Dent.* 2014, Oct 6). In the present study, after nonsurgical periodontal therapy, the number of lymphocytes in patients with LAgP was statistically higher than patients with GCP (p <0.0357) (Table 4). Among the other subgroups (GAgP versus LAgP and GAgP versus GCP) there was no statistically significant difference (Table 4). The lymphocyte numbers in the three subgroups were, in descending order, LAgP > GCP > GAgP, similar to that reported by a previous study (38). These authors compared the composition of gingival lesions of 21 patients with CP and six LAgP children. Reviews of the stereological and histochemical analysis showed that lymphocytes and B cells, in particular, occupied fractions of volume significantly greater in the LAgP than in the CP. Also, Cairo *et al.* (2010) (31) found a strong antibody response in the serum for infectious agents in LAgP and poor antibody response in the serum for GAgP.

As for the effects of nonsurgical periodontal therapy on clinical periodontal parameters, a significant decrease became clear in all of them (p <0.0001) (Table 1). It should be noted that there was a significant reduction of PI from 63.61±33.64 to 4.83±6.73 and of BOP from 44.46±29.35 to 1.63±3.35. After therapy, the NT was statistically fewer due to the extraction of periodontally condemned teeth (p =0.0001) (Table 1). This set of measures relating to active periodontal therapy and SPT ensured adequate clinical response to all patients. Furthermore, such conduct allowed for a more accurate verification of the effects of therapy on TGF-β levels and WBC count and thus confirm the role of severe periodontitis in systemic inflammation.

Additionally, rigorous therapeutic protocols and periodontal maintenance contributed to the restoration of phagocytic function in peripheral blood neutrophils (39) and reduction of hs-CRP levels in patients with severe periodontitis (7). These findings also support the conclusions of D'Aiuto *et al.* (2004) (16, 17) and Kamil *et al.* (2011) (40) when reporting a greater reduction in the levels of systemic inflammatory markers among those with better clinical responses to periodontal therapy.

It should be noted that an interpretation of the results of related studies becomes difficult due to some

methodological differences between them. Small sample sizes, non-comparable study populations in terms of age, ethnicity, geographic location, as well as different measures and definitions of periodontitis are aspects that make it difficult to compare results (41). Likewise, parameters used to assess the effects of periodontal treatment and improvement in the levels of circulating biomarkers differ among studies (22, 42).

A unique feature of the biofilm of the oral cavity, particularly the subgingival biofilm, is its close proximity to highly vascularized tissues. Any disruption of natural integrity of the subgingival epithelium, whose thickness is at most 10 layers, can lead to bacteremia (43). Likewise, in periodontitis the periodontal pocket epithelium is typically thin and ulcerated and, therefore, often opens, allowing access of pathogens to the connective tissue and blood vessels. In patients with moderate to severe periodontitis, the total area of the pocket epithelium in direct contact with the subgingival biofilm is surprisingly large, reaching to about the size of the palm of the human hand or much larger in advanced cases (44). Therefore, both the access of microorganisms to the bloodstream and the onset of chronic inflammation with area and intensity sufficient to elicit a significant host response provides the basis for the study of the inter-relationship between periodontitis and CVD and obesity, among others. Thus, the complexity of the processes involved in the immunological host response leads to differences in the manifestation and progression of periodontitis (45), which leads to the need of different therapeutic approaches.

Since periodontitis is characterized as a polymicrobial infection, pathogens can modulate the T cell response to promote its own adaptability, and the immune response itself becomes a mixture of immunological responses mediated by all the microorganisms represented in the biofilm (45, 46). Therefore, it may not be possible to reliably dissect patterns of dominant activities of Th1, Th2, Th17 or Tregs among the diseased periodontal tissue samples collected. Thus, it may be simpler and more productive to consider the roles of each cytokine (Th1, Th2, Th17 or Tregs) in periodontal infection, since the performance of T cells in the maintenance of homeostasis between the biofilm microorganisms and host becomes remarkable.

It is unclear which specific signaling pathways need to be blocked or enhanced to mitigate the disease in order to promote host defense. Moreover, the modulation of the host immune system through drugs may result in adverse side effects, requiring close monitoring of this approach (28). Therefore, additional studies are needed to understand the onset and progression of periodontitis and develop therapeutic interventions to control them.

In conclusion, this study demonstrated in systemically healthy patients with severe periodontitis (LAgP, GAgP and GCP) a decrease of all local clinical signs of inflammation after periodontal therapy. Moreover, periodontal therapy was associated with lower levels of TGF-β in comparison to those before the treatment, although not statistically significant for all disease groups. Also, after the treatment, the number of lymphocytes in patients with LAgP was statistically greater than the number of lymphocytes in patients with GCP. Nonsurgical periodontal therapy based on the reduction and remission of the clinical signs of inflammation in the local sites of disease and significant improvements in the biological markers of inflammation in the peripheral blood requires efficient control of the formation of dental biofilm which can only be monitored through rigorous SPT with frequent and individualized therapeutic interventions for each patient in treatment and maintenance.

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