

RESEARCH ARTICLE

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Levels of Pro and Anti-inflammatory Cytokines and C-Reactive Protein in Patients with Chronic Periodontitis Submitted to Nonsurgical Periodontal Treatment

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Abstract

Aim: to compare the levels of IFN- γ , TGF- β and C-reactive protein (CRP) in healthy patients (HP) and chronic periodontitis patients (CP) before and seven days after the last session of Non-Surgical Periodontal Treatment (NSPT). **Materials and Methods:** 40 subjects were divided into two groups: healthy (n= 20), and with chronic periodontitis (n = 20). Serum and gingival crevicular fluid (GCF) were collected from each patient and quantified for IFN- γ , TGF- β and CRP using the enzyme-linked immunosorbent assay (ELISA). **Results:** IFN- γ was found to be higher in the GCF of the CP group before NSPT in relation to the HP group (p<0.05), and it had significant higher levels after seven days of NSPT (p<0.05). The levels of TGF- β in the GCF of CP patients before NSPT were significantly higher when compared to HP (p<0.05), but they decreased after seven days of NSPT (p>0.05). Serum CRP levels did not show statistical difference between CP and HP before or after NSPT. **Conclusion:** Therefore, our results demonstrated for the first time that NSPT causes early exacerbation of the immune response at the local level represented by increased levels of IFN- γ and decreased levels of TGF- β in the gingival crevicular fluid after seven days of treatment.

Keywords: C-reactive protein- cytokines- immune system- inflammation- periodontitis

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Introduction

Periodontitis is a disease associated with the host immune response that is caused by Gram-negative bacteria, and which affects much of the world's population (Yilmaz et al., 2005; Emingil et al., 2006; Gokyu et al., 2014). The interactions between the bacteria and the host trigger immune mechanisms that cause periodontal tissue damage (Pradeep et al., 2014; Park et al., 2015). There is also evidence based on microbiological, immunological and clinical studies supporting that a type of periodontitis can remain stable for several years, whereas the other forms, despite extensive treatment, continue to progress, ultimately leading to tooth loss. Despite the fact that periodontal bacteria are the main etiological agents, host susceptibility is also of great relevance to the development of periodontitis (Latha et al., 2015).

The local production of inflammatory mediators and the accumulation of such mediators are one of the mechanisms responsible for bone loss (Tonetti, 2009). Moreover, studies have suggested that T cells that produce Th1 cytokines are

the main modulators of inflammation in initial or stable lesions (Tsai et al., 2007). Th1 cells produce Interferon gamma (IFN- γ) and are more prominent in cell-mediated inflammatory reactions. On the other hand, Th2 cells produce IL-4, which increases the production of antibodies and plays a prominent role in anthelmintic and allergic responses (Suárez et al., 2004). More recently, research on a new lineage of T cells, regulatory T cells (Tregs) has gained prominence. Those cells produce cytokines, such as Transforming Growth Factor β (TGF- β), that regulate the induction and activity of effector T cells by controlling exacerbated immune responses (Dutzan et al., 2009).

The traditional treatment modality of non-surgical periodontal treatment (NSPT) remains the primary choice for the management of chronic periodontitis. Its purpose is to remove the bacterial colonies and the mechanical and chemical irritants that cause inflammation, aiming to eradicate disease. Some studies suggest that periodontitis increases C-reactive protein (CRP) and other markers of systemic inflammation, such as cytokines. However, effective periodontal

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therapy might be reducing those levels. Although CRP and other acute-phase molecules are usually present at relatively low levels in the plasma, they could increase dramatically within 72 h of tissue injury or after infection. CRP, IL-1 β , IL-6, and TNF- α have been associated with the presence of various bacterial infections, including periodontitis (Gani et al., 2009).

Previous studies showed increased levels of IFN- γ in chronic periodontitis patients, as well as a significant decrease of those levels after non-surgical periodontal treatment (Ebersole and Taubman, 1994; Tsai et al., 2007). More specifically, it has been demonstrated that there is no significant difference between the total levels of IFN- γ in the gingival crevicular fluid (GCF) when sites with clinical attachment level (CAL) \geq 6 mm and sites with CAL < 6 mm were compared before NSPT (Tsai et al., 2007). Furthermore, Skaleric et al., (1997) demonstrated increased TGF- β levels in sites with higher CAL. However, in those studies, the GCF was collected at baseline and one or two months after the last session of NSPT. The inflammatory response is initiated immediately after tissue aggression, but there are few data in literature comparing the levels of cytokines TGF- β and IFN- γ , and of CRP in a short-term evaluation after NSPT in patients with generalized severe periodontitis and localized severe periodontitis, which would indicate an early response of the organism.

The aim of this study was to assess the levels of CRP and cytokines TGF- β and IFN- γ in patients with severe chronic periodontitis (localized and generalized) and in healthy patients and, finally, to assess the levels of these markers seven days after NSPT.

Materials and Methods

Patient selection

Twenty patients with moderate to advanced chronic periodontitis (CP) were consecutively enrolled at the Basic Health Center Dr. Randolph Borges, which is a public facility for dental treatment that belongs to the Health Secretary of Uberaba, in Minas Gerais state, Brazil. Detailed medical and dental records were obtained and subjects who fulfilled the following inclusion criteria were invited to participate in the study. All eligible subjects were thoroughly informed of the nature, potential risks and benefits of their participation in the study, and signed an informed consent form. This study protocol was previously approved by the Ethics Committee of Clinical Research of the University of Uberaba, under protocol n $^{\circ}$ 033/06. The criteria for patient selection were as follows: (a) a minimum of 15 natural teeth, excluding third molars and including at least 10 posterior teeth, (b) no previous periodontal therapy, (c) absence of relevant systemic diseases, such as diabetes mellitus and inflammatory or infection diseases, (d) no intake of antibiotics or non-steroid anti-inflammatory drugs in the previous 6 months, (e) no current or former smokers or tobacco users.

A control group of 20 patients was enrolled from the community, and differences regarding gender, age and ethnics, and all exclusion criteria described above were

taken into account. Clinical examination was performed and the subjects that did not show any symptoms of gingival inflammation, such as bleeding on probing or periodontal pocket with attachment loss, were included in control group. Patients in the periodontitis group and in the control group were between 35-45 years. All demographic data were assessed so as to obtain a homogeneous distribution between both groups.

Clinical measurement and diagnosis of moderate to advanced chronic periodontitis

At baseline, all selected patients underwent a periodontal clinical examination by a single calibrated examiner who registered probing pocket depth (PPD), clinical attachment level (CAL) at six periodontal sites in all teeth, excluding third molars. The diagnosis of moderate to advanced chronic periodontitis was established when up to 30% of the sites had CAL \geq 5mm (localized severe chronic periodontitis), or when more than 30% of the sites had CAL \geq 5mm (generalized severe chronic periodontitis), associated with radiographic bone loss, according to a classification system of periodontal diseases and conditions (Armitage, 2002). The percentage of bleeding on probing (BoP) was calculated by dividing the number of teeth that showed bleeding on probing by the total number of teeth in each patient.

Collection of Gingival Crevicular Fluid (GCF)

GCF was sampled one week after clinical examination so as not to change the nature of the GCF. Four non-contiguous sites per individual with PD, CAL \geq 5mm, BoP, and no furcation involvement were chosen for sampling. After removing the supragingival biofilm with sterile cotton pellets, the sites were isolated with cotton rolls and gently dried with an air syringe in order to eliminate the possibility of contamination with saliva. The GCF was collected by inserting standard paper strips (Periopaper, Oraflow Inc., Smithtown, NY, USA) approximately 2 mm into the sulcus/pocket for 30s. Strips visually contaminated with blood were discarded. The strips of the four selected sites were immediately placed in separate microcentrifuge tubes containing 250 μ l phosphate-buffered saline and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). The GCF samples were also taken from the same sites seven days after NSPT. The samples were then stored at -20 $^{\circ}$ C for subsequent assays.

Blood collection

Blood sample was collected from HP and CP patients before NSPT. Seven days after the last session of NSPT, another blood sample was collected from CP patients for ELISA and CRP measurements. The blood was collected in a vial, and anticoagulant was later centrifuged in order to collect the serum. The serum was stored at -70 $^{\circ}$ C for further analysis.

Non-Surgical Periodontal Treatment (NSPT)

Prior to the clinical intervention, the patients were instructed to rinse their mouths with 0.12% chlorhexidine digluconate (Periogard) (Colgate, New York City, NY, USA)

for 1 min. Patients with CP received NSPT, which consisted of approximately three sessions of scaling and root planing with an electronic scaler device (Profi-II Ceramic) (Dabi Atlante, Ribeirão Preto, SP, Brazil) with intervals of seven days between sessions. Treatment was concluded in a maximum of 21 days by the same examiner without use of antibiotics or local antimicrobials. Seven days after the last day of the treatment period, new samples of GCF and peripheral blood.

Quantification of cytokines

Blood samples were centrifuged at 5,000 rpm for 15 min. at 4 °C, and the serum was transferred to another tube and stored at -70°C for further analysis. The individual tubes with paper strips used to collect the GCF were centrifuged at 10,000 rpm for 15 min. at 4°C. Aliquots of each GCF or serum samples were assayed by ELISA so as to determine the levels of IFN- γ and TGF- β according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA). Briefly, 100 μ l of detection antibody was added to all wells, except blank wells, covered with strips of plastic film (Parafilm), and it was gently mixed and incubated overnight (16-24 h) at 4°C. Plates were washed five times and standards, GCF and serum samples were added in duplicate to the respective wells. After the incubation time, the plates were washed again and incubated with 200 μ l of conjugate for 60 min. at room temperature. Plates were washed 5 times again and 200 μ l of substrate was added and incubated for 15 min. at room temperature (18-26 °C) in the dark. The reaction was stopped by adding 50 μ l of stop solution, and color was measured at a wavelength of 405nm in an automated microplate spectrophotometer (Microplate Reader/Model 3550 Bio Rad, GMI, Montreal, KC, Canada). The total amounts of cytokines were determined as picograms/ml (pg/ml). Results were calculated using the standard curves created in each assay. The ELISA assay was performed in a blinded fashion.

C-reactive protein assay

The serum was processed in a blinded fashion by staff at the end of the trial for CRP levels by ultrasensitive turbidimetric immunoassay (Cobas Integra, Roche AG Diagnostics, Mannheim, Germany; detection limit, 0.25 mg/L). CRP was not measured in the GCF because studies show that CRP levels in the GCF do not seem to

function as a systemic marker because not all patients with periodontitis have detectable levels of CRP in the GCF and some non-periodontitis patients have detectable levels of CRP in the GCF (Megson et al., 2010).

Statistical analyzes

Multiple comparisons between groups were employed using Kruskal-Wallis test followed by Dunn test. Student's t test and Fisher's exact test were used for pairing. Mann-Whitney test was used to compare two groups that showed non-normal distribution. Significance was accepted when the p value \leq 0.05.

Results

No statistical significant differences in age, gender or ethnics were observed between chronic periodontitis patients (CP) and healthy patients (HP) (Table 1), hence both groups were homogeneous. Significantly higher PPD, CAL and BoP were observed in the periodontal conditions of individuals with CP ($p < 0.001$) in comparison with HP (Table 1). Ten CP patients were diagnosed with generalized severe CP, and 10 were diagnosed with localized severe CP.

Patients with chronic periodontitis showed significantly higher levels of IFN- γ in the GCF ($p < 0.05$) than healthy patients (Figure 1A). After NSPT, an increase in the levels of IFN- γ was observed in the GCF ($p < 0.05$) (Figure 1A). Nevertheless, CP patients had a slightly higher amount of IFN- γ in the serum; however, this difference is not statistically significant when compared to HP ($p > 0.05$) (Figure 1B), and there was no change in the cytokine levels ($p > 0.05$) after NSPT (Figure 1B).

Moreover, CP patients showed significant higher levels of TGF- β in the GCF than HP before NSPT ($p < 0.05$) (Figure 2). Nonetheless, after NSPT, TGF- β levels decreased to similar levels as those found in HP ($p > 0.05$) (Figure 2). Furthermore, the TGF- β was not detected in serum.

In order to compare the cytokine profiles in the different periodontal statuses, CP patients were grouped according to the classification of periodontitis type (localized and generalized). The results demonstrated that both types of periodontitis had increased IFN- γ levels in comparison with HP ($p < 0.05$). Importantly, statistically higher levels of IFN- γ ($p < 0.05$) were observed in the GCF of

Table 1. Distribution of Age, Gender, Ethnicity, Probing Pocket Depth (PPD), Clinical Attachment Level (CAL), and Bleeding on Probing (BOP) between Chronic Periodontitis Patients (CP) and Healthy Patients (HP)

	Chronic periodontitis (CP) (n=20)	Healthy patients (HP) (n=20)
Age (years) ^a	42.00 \pm 12.88	35.70 \pm 10.32
Gender ^b (M:F)	09:11	07:13
Ethnicity ^c (C:NC)	19:01	20:00
PPD (mm) ^d	2.93 (3.29-2.42)	1.66 (1.82-1.54)
CAL (mm) ^e	3.06 (3.29-2.61)	1.00 (1.13-1.00)
BOP (%) ^f	0.10 (0.25-0.01)	0.00 (0.00-0.00)

Note, The values are presented as media \pm standard deviation. M, Male; F, Female; C, caucasian; NC, Non- caucasian. ^a Student t test; t,-1,707; p, 0,096; ^b Fisher's exact test; P,1; ^c Fisher's exact test; P,1; ^d Mann-Whitney test; p<0,001; ^e Mann-Whitney test; p<0,001; ^f Mann-Whitney test; p<0,001.

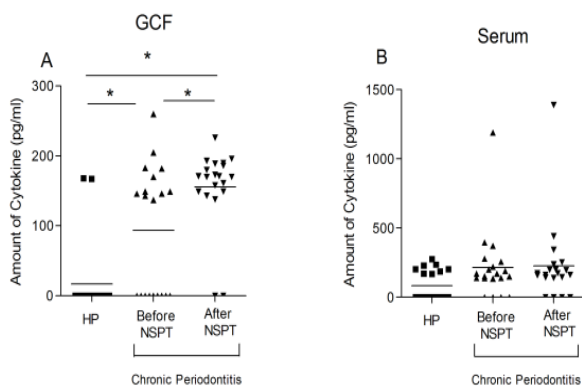


Figure 1. Levels of IFN- γ from the Gingival Crevicular Fluid (A) and Serum (B) of Healthy Patients (HP) and Chronic Periodontitis Patients (CP) before and after NSPT. * Indicates a Significant Statistical Difference between the Groups. Kruskal-Wallis followed by Dunn test. The values are presented as median.

patients with generalized severe CP in comparison with localized severe CP patients before NSPT (Figure 3A). Interestingly, seven days after NSPT, the IFN- γ levels in the GCF of the patients with localized severe CP increased ($p > 0.05$) to levels similar to those of generalized severe CP (Figure 3B), thus suggesting a local transitory inflammatory response.

Furthermore, TGF- β levels were found to be statistically higher ($p < 0.05$) in the GCF of the generalized severe CP patients than in localized severe CP patients and HP before NSPT (Figure 4A). However, seven days after NSPT, TGF- β levels decreased in generalized severe CP patients to levels similar to those of localized severe CP patients (Figure 4B; $p > 0.05$).

Patients with severe chronic periodontitis have a moderate systemic inflammatory response with increased levels of CRP, which is associated with the extent and the severity of periodontitis. An increased amount of CRP was observed in the serum of CP patients in comparison with HP in this study, however, a median of both groups within normal values (< 8 mg/L), although there was no statistical difference ($p > 0.05$) (Figure 5). Furthermore, when both serum CRPs were compared in CP patients before and seven days after NSPT (Figure 5),

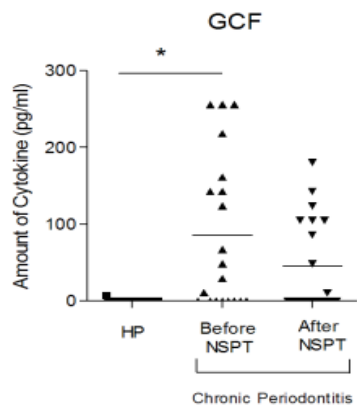


Figure 2. Amounts of TGF- β from Gingival Crevicular Fluid of Healthy Patients (HP) and Chronic Periodontitis (CP) Patients before and after NSPT. * Indicates statistical significant difference between the groups. Kruskal-Wallis followed by Dunn test. The values are presented as median.

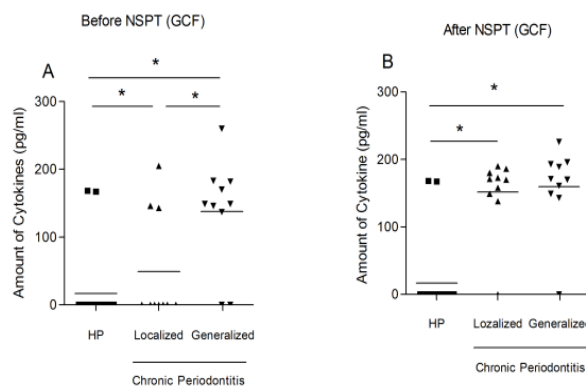


Figure 3. Comparison between the levels of IFN- γ in the GCF of Healthy Patients with Localized Periodontal Disease and with Generalized Periodontal Disease before NSPT. (B) Comparison between the levels of IFN- γ in the GCF of healthy patients with localized periodontal disease and with generalized periodontal disease after NSPT. * Indicates a significant statistical difference between the groups. Kruskal-Wallis followed by Dunn test. The values are presented as median.

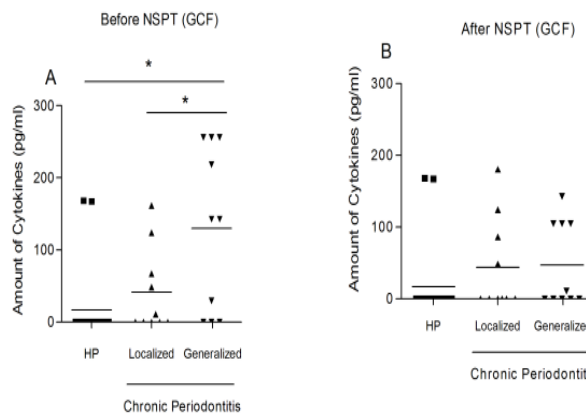


Figure 4. Comparison between the levels of TGF- β in the GCF of Healthy Patients with Localized Periodontal Disease and with Generalized Periodontal Disease before NSPT. * Indicates significant statistical difference between the groups. Kruskal-Wallis followed by Dunn test. The values are presented as median.

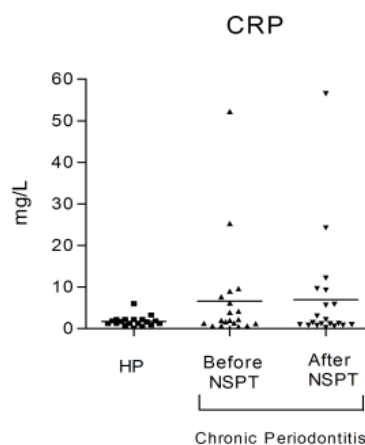


Figure 5. Levels of C-reactive Protein (CRP) in Healthy Patients and in CP Patients before and after NSPT. Kruskal-Wallis followed by Dunn test. The values are presented as median.

there was also no statistical difference, which led to the assumption that there were no systemic repercussions seven days after NSPT.

Discussion

The therapeutic goals of periodontal therapy are to decrease or to eliminate pathogens and their metabolites, thereby arresting the progression of the disease and maintaining oral health, comfort and function with appropriate esthetics, ultimately preventing the recurrence of periodontitis. Nonsurgical periodontal therapy aims to reduce the number of periodontal pathogens thereby reduces the inflammation (Siddeshappa et al., 2016).

In the present study, significant higher levels of IFN- γ were found in the GCF of patients with CP before NSPT in comparison with HP. IFN- γ is a cytokine produced mainly by effector CD8+ T cells and by CD4 Th1 cells, whose main role is macrophage activation (Suárez et al., 2004). This cytokine plays a major protective role against aggressive agents, as it stimulates macrophages to produce toxic metabolites against bacteria (Bahia-Oliveira et al., 2000). It also contributes to sustain inflammatory reactions through up-regulation of the production of inflammatory cytokines and chemokines (Sedgwick et al., 2000; Vaday et al., 2001; Garlet et al., 2007). In spite of this, the persistence of bacterial aggression leads to constant release of IFN- γ by the host, contributing to the persistence of the inflammatory state (Dutzan et al., 2009). In accordance with the results described herein, several studies also have demonstrated higher levels of IFN- γ in the GCF of patients with CP than in HP (Tsai et al., 2007; Suárez et al., 2004; Johnson and Serio, 2007; Dutzan et al., 2009; Gani et al., 2009; Souto et al., 2014; Zekeridou et al., 2017). Interestingly, the increased levels of IFN- γ observed in the GCF of CP patients in relation to HP after NSPT contrasts with some reports in the literature, in which a decrease in the total amount of IFN- γ in the GCF was reported after NSPT (Ebersole and Taubman, 2000; Tsai et al., 2007), in other hand, as concerning as the results of Zekeridou et al (2017), that after NSPT the level of IFN- γ in periodontitis group was higher than healthy group. However, in those studies the quantification of IFN- γ levels in the GCF was done at baseline and after at least a month or later of the last session of NSPT, whereas in this study the total amount of IFN- γ was assessed at baseline and seven days after NSPT. The increased total amount of IFN- γ in the GCF of patients with CP seven days after NSPT suggests a early local transitory exacerbation of immune response, which could be important for the destruction of periodontal pathogens, leading to resolution of CP as reported in previous studies (Dutzan et al., 2009). In this sense of evaluating the short-term therapeutic effects on chronic periodontitis, da Cruz Andrade et al., (2017), using Antimicrobial photodynamic therapy (aPDT), found that seven days after therapy there was an increase of IFN- γ in the GCF in subjects with chronic periodontitis. In this context, the present study is pioneer in reporting that NSPT modifies the expression of IFN- γ in GCF in a short

posttreatment period, since few studies were found that evaluated this association.

Even though there was no statistical difference regarding serum levels of IFN- γ between CP patients and HP in this study, some studies reported increased serum levels of IFN- γ in patients with CP (Zong et al., 2005; Tsai et al., 2007). Furthermore, decreased serum levels of IFN- γ were reported after NSPT (Zong et al., 2005; Tsai et al., 2007; Wright et al., 2008). However, there was no difference between IFN- γ serum levels in CP patients before and after NSPT in this study, which is in agreement with previous studies (Gorska et al., 2003; Lalla et al., 2007). Only local levels of IFN- γ were found to have increased seven days after NSPT, without any change in plasmatic levels, this shows that the levels of IFN- γ in the GCF after the NSPT are higher than the plasma levels and therefore more easily detectable.

TGF- β is a pleiotropic cytokine produced by regulatory T cells (Tregs) that regulates the induction and the activity of effector T cells, modulating exacerbated immune responses (Dutzan et al., 2009), and which also plays an important role in the metabolism of connective tissue during CP (Babel et al., 2006). TGF- β plays a modulator role, increasing healing and connective tissue remodeling, and inducing angiogenesis. The absence of TGF- β may contribute to periodontal destruction, since decreased levels of TGF- β were reported in the GCF of patients with CP (Babel et al., 2006; Sabarish et al., 2016; Naufel et al., 2017). Nonetheless, CP patients in this study were observed to have significantly higher levels of TGF- β in the GCF than in HP before NSPT. Therefore, not only would these increased TGF- β levels in the GCF of CP patients be acting as an inflammatory response modulator against persistent bacterial aggression, but they would also minimize the extent of damaged tissue during the development of CP in order to counterbalance the inflammatory response (Babel et al., 2006). After NSPT, TGF- β levels decreased in the GCF, but not significantly. Given the fact that TGF- β is a modulatory cytokine in the immune-inflammatory response (Dutzan et al., 2009), the decreasing trend in TGF- β levels in the GCF after NSPT suggests a transient exacerbation of local inflammatory response due to NSPT, which is in agreement with the increased amount of IFN- γ (Figure. 2B). Seven days after NSPT, this possible exacerbation would lead to an enhancement of local immune response so that the chronic disease could be managed.

There is little evidence that patients with different degrees or types of periodontitis show varied local immune response. Tsai et al., (2007) did not find a significant difference between the total amount of IFN- γ in the GCF when comparing sites with CAL \geq 6 mm and with CAL < 6mm before NSPT. Despite this, the levels of both cytokines in generalized and localized severe CP were assessed in this study. In generalized severe CP patients, increased IFN- γ and TGF- β levels were found in comparison with localized severe CP patients. Accordingly, Skaleric et al. (1997) also demonstrated increased TGF- β levels in sites with greater CAL. Nevertheless, Gurkan

et al., (2005) reported no significant differences between TGF- β levels in the GCF of severe generalized CP patients and HP. Interestingly, in this study, TGF- β levels tended to decrease in generalized severe CP patients seven days after NSPT, thus reflecting the exacerbation of inflammatory response as a way to solve CP. On the other hand, Gurkan et al., (2005) reported increased TGF- β levels in the GCF three months after NSPT. These data suggest that NSPT may temporarily reduce TGF- β levels in the GCF, as well as sustain IFN- γ levels in an attempt to transiently up-regulate local immune response, whereas TGF- β levels in the GCF decrease three months after NSPT, hence recovering the ability to repair and modulate CP (Dutzan et al., 2009). However, naive T cells exposed to TGF- β have been reported to differentiate into Treg cells; however, when cultured with TGF- β and IL-6, naive T cells transform into Th17 cells (Bettelli et al., 2006; Yamazaki et al., 2007). Thus, when the immune response is not activated, TGF- β favors the generation of Treg cells, hence suppressing the inflammation.

Another important inflammatory marker is the C-reactive protein (CRP), also known as an acute-phase marker, represents one of the most sensitive makers used to evaluate the inflammatory status of an individual.

Some studies have reported higher levels of serum CRP in patients with CP than in healthy patients (Paraskevas et al., 2008; Podzimek et al., 2015, Moghadam et al., 2017) and these levels decreased after NSPT (D'aiuto et al., 2004a; D'aiuto et al., 2004b; Ertugrul et al., 2017). In the present study, as there was no significant difference in serum CRP levels between CP and HP patients, we suggested that the inflammatory response was only local and exacerbated seven days after NSPT since IFN- γ levels were increased.

Therefore, the inflammatory response was only local observed in these patients, and it was exacerbated after NSPT, when IFN- γ levels increased. Therefore, it is hypothesized that NSPT could transiently exacerbate the local inflammatory response even though there are no systemic repercussions seven days afterwards.

In conclusion, our results demonstrated for the first time that NSPT causes early exacerbation of the immune response at the local level represented by increased levels of IFN- γ and decreased levels of TGF- β in the gingival crevicular fluid after seven days of treatment.

Conflict of interest

None

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